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A high throughput passive dosing format for the Fish Embryo Acute Toxicity test

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ABSTRACT

High throughput testing according to the Fish Embryo Acute Toxicity (FET) Test (OECD Testing Guideline 236) is usually conducted in well plates. In the case of hydrophobic test substances, sorptive and evaporative losses often result in declining and poorly controlled exposure conditions. Therefore, our objective was to improve exposure conditions in FET tests by evaluating a passive dosing format using silicone O-rings in standard 24-well polystyrene plates. We exposed zebrafish embryos to a series of phenanthrene concentrations until 120 hours post fertilization (hpf), and obtained a linear dilution series. We report effect values for both mortality and sublethal

morphological effects based on (1) measured exposure concentrations, (2) (lipid normalized) body residues and (3) chemical activity. The LC_{50} for 120 hpf was 310 µg/L, CBR_{50} (critical body residue) was 2.72 mmol/kg fresh wt and La_{50} (lethal chemical activity) was 0.047. All values were within ranges expected for baseline toxicity. Impaired swim bladder inflation was the most pronounced morphological effect and swimming activity was reduced in all exposure concentrations. Further analysis showed that the effect on swimming activity was not attributed to impaired swim bladder inflation, but rather to baseline toxicity. We conclude that silicone O-rings (1) produce a linear dilution series of phenanthrene in the 120 hpf FET test, (2) generate and maintain aqueous concentrations for reliable determination of effect concentrations, and allow for obtaining mechanistic toxicity information, and (3) cause no toxicity, demonstrating its potential as an extension of the FET test when testing hydrophobic chemicals.

Keywords

Phenanthrene; polycyclic aromatic hydrocarbon; zebrafish embryo; fish early life stages; acute toxicity; critical body residue

Abbreviations

AhR: aryl hydrocarbon receptor

AOP: adverse outcome pathway

C: (regular) control

$CBR_{(LIP)50}$: (lipid normalized) critical body residue

Ea_{50}: effective chemical activity

$EBR_{(LIP)50}$: (lipid normalized) effective body residue

48 ED_{50}: effective dose

49 EI: electron ionization mode

50 EURL ECVAM: European Union Reference Laboratory for alternatives to animal testing - European
51 Centre for the Validation of Alternative Methods

52 FET: Fish Embryo Acute Toxicity

53 HOC: hydrophobic organic compound

54 Hpf: hours post fertilization

55 La_{50}: lethal chemical activity

56 LC: loading control

57 LD_{50}: lethal dose

58 LOQ: limit of quantification

59 MS-222: tricaine methanesulfonate

60 PAH: polycyclic aromatic hydrocarbon

61 PDMS: polydimethylsiloxane

62 RC: O-ring control

63 SIM: selected ion monitoring

64 TG: Test Guideline

1. Introduction

The REACH legislation requires the collection of ecotoxicological information on chemicals, including aquatic toxicity information, while at the same time advocating reduced animal use. This has resulted in an increased attention for the development of alternative, non-animal test methods to assess chemical toxicity. In Europe, fish in general are non-protected animals until the stage of free-feeding (EU Directive 2010/63/EU). For zebrafish, this limit was set at 120 hours post fertilization (hpf) when kept at approximately 28 °C (Commission Implementing Decision, 2012/707/EU; Straehle et al., 2012). Therefore, the Fish Embryo Acute Toxicity (FET) Test (OECD Test Guideline (TG) 236; OECD, 2013) up to 96 hpf (or extended up to 120 hpf), which is specifically developed for zebrafish embryos, is considered an alternative testing method. In a validation study by EURL ECVAM (European Union Reference Laboratory for alternatives to animal testing - European Centre for the Validation of Alternative Methods) on behalf of the OECD, intra- and interlaboratory reproducibility of the FET test was assessed and found to be within acceptance criteria (Busquet et al., 2014). Additionally, Lammer et al. (2009) and Belanger et al. (2013) showed a high predictive capacity of the FET test for acute juvenile/adult fish toxicity. The FET test is the first ever accepted alternative for an animal testing method in ecotoxicology. However, a consensus about the predictive value of FET data for adult acute toxicity (as measured by e.g. OECD TG 203 [OECD, 1992]) for regulatory purposes has not yet been reached (Wim De Coen, ECHA, personal communication).

There is an ongoing international effort to further refine the FET test in terms of increasing its ability to inform on the mode of action (Hagenaars et al., 2014), as well as to solve practical issues such as dealing with poorly water-soluble chemicals (Butler et al., 2013; Seiler et al., 2014). Although the FET test performed well in the validation study by ECVAM, analytical measurement of the chemicals' concentration in the test medium showed important losses for some chemicals even though the medium was refreshed daily, probably due to sorption to the test chamber walls, volatilization and degradation (OECD, 2011, 2012a, b; Busquet et al., 2014). For the FET test, similar to other aquatic

toxicity tests, hydrophobic organic compounds (HOCs) pose a challenge because of poorly controlled exposure conditions, and consequently poor estimation of toxicity.

Passive dosing is a method developed to overcome some of these challenges (Mayer et al., 1999). A polymer reservoir is loaded with the test chemical(s) and placed inside the test chamber during exposure. Continuous equilibrium partitioning of the test compound(s) between polymer and test medium ensures stable and controlled exposure concentrations, and the system thereby compensates loss processes. Also, the use of solvents, which can cause or modify toxicity, is avoided in this method.

Several passive dosing formats have recently been developed for different purposes. Smith et al. (2010a) used glass test vessels cast with a layer of PDMS as a passive dosing reservoir for exposure of *Daphnia magna*. Kramer et al. (2010) placed PDMS sheets on the bottoms of the wells of a 24-well plate, for exposure of fish cell lines that were grown on membranes of well plate inserts, and modelled free medium concentrations. Recently, two studies applied passive dosing for exposing zebrafish early life stages to HOCs. Butler et al. (2013) exposed zebrafish to three concentrations of phenanthrene, from fertilization up to 30 days. During the first 5 days, embryos were exposed individually in PDMS coated glass vials. After 5 days, surviving eleutheroembryos (developmental phase starting with hatching and ending with free-feeding) were transferred to a recirculating flow-through setup applying silicone O-rings. Seiler et al. (2014) tested the suitability of a passive dosing format for exposing zebrafish embryos to 10 polycyclic aromatic hydrocarbons (PAHs) until 48 hpf using glass jars with cast PDMS. They exposed 5 to 10 embryos in each glass jar and transferred them to plastic multi-well plates for microscopic observation at 24 and 48 hpf. Applying passive dosing in standard polystyrene 24-well plates, which are routinely used in the FET test, would combine the advantages of the passive dosing method with easy individual microscopic follow-up and high throughput. This would also allow for the application of individual automated behaviour analysis directly in the well plates. By combining these measurements with measures of growth,

morphology and body residue, vital mechanistic toxicity information can be obtained. Smith et al. (2010b) developed a suitable passive dosing format for use in polystyrene 24-well plates in *in vitro* toxicity tests. Silicone O-rings proved to be practical and showed excellent equilibrium partitioning characteristics for HOCs with log $K_{ow} \geq 3$. In this study, we used this methodology for exposing zebrafish embryos up to 120 hpf to a HOC in standard 24-well plates. We used phenanthrene (log K_{ow}: 4.46) as a model compound, and loaded silicone O-rings by equilibrium partitioning from a phenanthrene dilution series in methanol.

In summary, our objective was to evaluate passive dosing with silicone O-rings for use in a 120 hpf FET test. We wanted to assess whether (1) silicone O-rings are able to produce a linear dilution series of phenanthrene in the 120 hpf FET test, (2) whether controlled exposure concentrations facilitate precise determination of effect concentrations, and allow for obtaining mechanistic toxicity information, and (3) whether the passive dosing format itself causes toxicity in the zebrafish embryos.

2. Material and Methods

2.1. Experimental design

Zebrafish embryos were exposed in a 120 hpf FET test to six phenanthrene concentrations (Table 1) using silicone O-rings (outer diameter: 14.4 mm; cross section: 2.4 mm; average weight: 0.22 g; product no. ORS-0096-24, Altec, Cornwall, UK). Additionally, losses from a conventionally spiked medium without O-rings were assessed. A series of controls was used to assess potential effects of the O-rings: a regular reconstituted freshwater control without O-rings (control, C), a control with O-ring that was not subjected to the loading procedure (O-ring control, RC), and a control with O-ring that was subjected to the loading procedure with clean methanol (loading control, LC). For each of the ten conditions (Table 1), one 24-well plate was used. 20 wells were used to test each respective condition (i.e. 20 embryos per condition) and four wells were used as internal negative control (reconstituted freshwater). One 48-well plate contained four concentrations of 3,4-dichloroaniline (CAS 95-76-1, purity of 98%, Sigma-Aldrich, Saint Louis, US), 0.5, 1, 2 and 4 mg/L as positive control (OECD, 2013).

2.2. Passive dosing

After an initial cleaning, silicone O-rings were loaded from methanol solutions by equilibrium partitioning (Smith et al., 2010a). Loading from a saturated methanol solution results in saturation of the silicone and subsequently saturation of the test medium during exposure. Additionally, loading from dilutions of the saturated methanol solution results in corresponding dilutions of the test medium proportional to the maximum soluble concentration. However, this only holds true when loading and exposure are performed at the same temperature. A saturated methanol solution at 28.5 °C immediately precipitates when the temperature drops even slightly, for example due to handling with a pipette. Therefore, loading was carried out at room temperature (20 °C) while equilibration and exposure were performed at 28.5 °C. Estimated concentrations are needed as a reference for assessing the actual exposure concentrations in the test, and were calculated applying

a temperature correction. O-rings were equilibrated with reconstituted freshwater for 40 h prior to the start of the exposure (see Appendix for more details on the passive dosing method and calculations).

2.3. Preparation of spiked phenanthrene exposure

The appropriate quantity of phenanthrene was dissolved in medium through sonication. The test wells were pre-equilibrated with 2 mL of the test solution at 28.5 °C 24 h prior to the start of the exposure. Immediately prior to the start of the exposure, the test solution in the wells was replaced.

2.4. Experimental procedure

2.4.1. Collection of eggs and exposure

Non-exposed adult wildtype zebrafish (*Danio rerio*) were maintained and used for egg production (Appendix). Reconstituted freshwater (45 mg/L $CaCO_3$) was prepared by adding Instant Ocean® Sea Salt (Blacksburg, VA, US) to reverse osmosis water (Werner, Leverkusen, Germany) up to a conductivity of 500 µS/cm and adjusting the pH to 7.5 using $NaHCO_3$, and was used to prepare all test solutions as well as to keep adult fish. Embryos were transferred to exposure plates within 2 hpf and placed in an incubator at 28.5 °C with a 14 h/10 h light/dark cycle. Mortality, using the four endpoints described in OECD TG 236 (OECD, 2013), and hatching were recorded every 24 h, by observing the embryos directly in the wells using a S8APO stereomicroscope (Leica Microsystems GmbH, Germany), and the chorion was removed from the test chamber after hatching.

Exposure medium and O-rings in all treatments were replaced at 72 hpf, in order to maintain adequate water quality and because preliminary experiments showed depletion of the O-rings after 120 h. The spiked phenanthrene solution, as well as the DCA positive control solution, were replaced every 48 h.

2.4.2. Sublethal effect assessment

2.4.2.1. Automated swimming behaviour analysis

At 120 hpf, the swimming activity of all eleutheroembryos was determined in the 24-well plates using a Zebrabox 3.0 video tracking device (ViewPoint, Lyon, France). One hour before analysis, O-rings were removed because they interfere with image detection. Additionally, eleutheroembryos were allowed to acclimate for 20 min to the Zebrabox environment before analysis. Data were analysed using the ZebraLab software version 3.20.5.104. The path of each eleutheroembryo was analysed and the total distance travelled (further referred to as swimming movements) was calculated. The sum of all swimming movements (in mm) in 30 min was compared among conditions. Additionally, the proportion of the movements at different swim speeds (3 categories: ≤2 mm/s, 2 - 10 mm/s, ≥10 mm/s) was compared.

2.4.2.2. Morphological scoring

After the behaviour analysis, eleutheroembryos were anaesthetised using 100 mg/L MS-222 (tricaine methanesulfonate, Sigma-Aldrich) adjusted to pH 7.5 using $NaHCO_3$ and morphological abnormalities were scored, including failure to hatch, impaired swim bladder inflation (posterior chamber), tail malformations (curvature, elbow, tissue deviation), oedemas (pericardium, yolk), blood accumulations (heart, yolk (extension), swim bladder), absent blood circulation in the tail, malformations of fins (absence, curved), yolk, mouth, eyes and otoliths. Eleutheroembryos were photographed together with a calibrator, using a camera (Canon EOS 600D, 18 megapixel) mounted on the stereomicroscope, and images were analysed using the Image J software (http://rsbweb.nih.gov/ij/) to determine standard length (± 0.002 mm).

2.5. Analytical measurements

Medium samples were taken at 0 hpf (after equilibration), 36 hpf, 72 hpf (both before and after O-ring and medium renewal) and 120 hpf. At each time point, 3 independent replicate samples were taken from each condition. In each sample, 100 µL from 6 distinct wells was combined (600 µL total). From the spiked medium, samples were taken at 0 hpf (after renewal), 48 hpf (both before and after

renewal), 96 hpf (both before and after renewal) and 120 hpf. Samples were only taken from wells where embryos were alive. Samples were stored in Gas Chromatography (GC) vials (5182-0716, Agilent Technologies, Palo Alto, CA, USA) at -20 °C until GC-MS analysis.

After the sublethal effect assessment at 120 hpf, eleutheroembryos were euthanized using an overdose of 1 g/L MS-222 adjusted to pH 7.5, transferred to a nylon 100 µm filter (BD Biosciences, Durham, NC, USA), rinsed with reconstituted freshwater, transferred to 30 mL of clean reconstituted freshwater twice, and finally transferred to GC vials. For each sample, 4 or 5 eleutheroembryos from the same condition were pooled. The extracts were analysed using an Agilent Technologies 6890 gas chromatograph coupled to an Agilent 5973 mass spectrometer operated in electron ionization mode (EI). For more details, see Appendix.

2.6. Calculations

Effective chemical activities were determined as the ratio of LC_{50} or EC_{50} and the subcooled liquid solubility of phenanthrene (Mayer and Reichenberg, 2006) (Appendix). For calculation of critical (mortality) and effective (sublethal effect) body residue (CBR_{50} and EBR_{50}), final eleutheroembryo weight was calculated based on dimensions measured in photographs, using imageJ (Appendix). Based on the body residues, the bioconcentration factor (BCF) was calculated as the ratio of the body residue at 120 hpf (µg/kg fresh wt) and the average measured medium concentration (µg/L). Total lipid content was determined in 5 samples (of 25, 120 hpf non-exposed eleutheroembryos) according to methods previously described (Vergauwen et al., 2013), and used to calculate the lipid normalized critical body residue (CBR_{LIP50}) as the ratio of the body residue (mmol/kg fresh wt) and the lipid content (w/w, fresh wt).

2.7. Statistical Analysis

Treatments with O-rings that were loaded with methanol (LC) served as controls and reference for the calculation of the various toxicity endpoints. Data were considered significantly different when

p-values were <0.05. Linear regression was used to (1) compare measured to estimated exposure concentrations, (2) relate body residues to exposure concentrations, and (3) relate length to body residue. Proportions of mortality, impaired swim bladder inflation and other sublethal morphological effects were analysed as a function of exposure concentration and body residues using a nonlinear regression estimate (variable hill slope, top and bottom constrained at 100 and 0), and effect values were calculated. Additionally, the frequency data for morphological abnormalities were compared among conditions using a chi-squared contingency table test, with a Bonferroni post-hoc test to adjust for multiple testing. Standard length, total swimming movements and proportions of swimming movements at specific swim speeds were not normally distributed (Shapiro-Wilk normality test), and to determine significant effects, Kruskal-Wallis tests with Dunn's multiple comparison tests were used. All statistical analyses were performed in GraphPad Prism Version 6.00 for Windows (GraphPad Software, La Jolla California, USA).

3. Results and discussion

In this study, we evaluated a passive dosing format, facilitating controlled exposure to hydrophobic chemicals in a FET Test (OECD, 2013). The FET test was extended up to 120 hpf, which is consistent with the definition of non-protected animals (Straehle et al., 2012). We critically examined three requirements such a passive dosing format should meet, corresponding to our study objectives.

3.1. Requirement 1: Linearity and goodness of fit of exposure concentration series

Fig. 1 shows a linear relation between the estimated and measured phenanthrene concentrations in the medium (R^2 = 0.945; Table 1, and Fig. A.3 in appendix, show the medium concentrations in more detail). Measured concentrations were on average 55% of estimated concentrations and ranged from 49 to 60% with standard deviations of 7 to 9% (calculated over all replicates from all time points for each condition) of estimated concentrations during the 5 day exposure. Phenanthrene was not detected in control media (Table 1).

In the conventionally spiked condition without O-rings (Fig. A.3), medium concentrations dropped from 200 µg/L to 1-2 µg/L within 24 h (from 96 to 120 hpf). Riedl and Altenburger (2007) showed that for substances with log K_{ow} > 3, assays in polystyrene multi-well plates underestimate toxicity due to losses. The FET testing guideline mentions that in case adsorption to polystyrene is suspected, inert materials (glass) should be used to reduce losses (OECD, 2013). However, Schreiber et al. (2008) showed that even the use of glass multi-well plates still resulted in a 40% loss of phenanthrene after 48 h. This confirms the need for passive dosing of HOCs, and our results show that polystyrene plates can be used when passive dosing is applied.

Two studies have previously reported the use of passive dosing for zebrafish embryo/larval exposure to PAHs. Butler et al. (2013) used a combination of silicone coated vials and silicone O-rings in a flow-through system to chronically expose zebrafish embryos/larvae to phenanthrene and exposure concentrations were 86 to 104% of estimated concentrations with standard deviations of 10 – 14%.

Recently, Seiler et al. (2014) reported exposure concentrations of 60 to 117% of estimated concentrations and standard deviations ranging from 0 to 23% when exposing zebrafish embryos to 10 PAHs in silicone cast 60 mL jars. Several factors possibly contributed to the deviation from estimated exposure concentrations in our study: Firstly, it is important to be aware that the estimation of exposure concentrations based on partition coefficients is associated with some error. Secondly, the use of polystyrene plates instead of glass, the use of small exposure volumes resulting in a higher surface/volume ratio and therefore more sorption, and not stirring the medium during exposure may have reduced exposure concentrations. However, these are exactly the factors that make the passive dosing format necessary and compatible with the existing FET test guideline (OECD, 2013), and we have shown that even under these circumstances we can produce a linear dilution series with a high goodness of fit. Therefore, we can conclude that the proposed passive dosing format, using silicone O-rings, is a valuable addition to the routinely applied FET test (OECD, 2013) when testing hydrophobic chemicals, provided that analytical measurements of the exposure medium are performed and used as final exposure concentrations to interpret toxicity observations. We suggest the following further improvements to the method: Firstly, the use of glass 24-well plates could reduce sorption and therefore avoid the need to replace the rings after 72 h. Secondly, equilibrating O-rings with medium in glass bottles overnight on a shaker before transferring to the well plates could increase the exposure concentrations.

3.2. Requirement 2: Precise determination of effect concentrations, offering mechanistic toxicity information

3.2.1. Impaired swim bladder inflation was the most pronounced morphological effect

At the four lowest exposure concentrations, the only significant sublethal morphological effect was impaired inflation of the posterior chamber of the swim bladder (Figs. 2-3A). Since the test was ended at 120 hpf, we could not discriminate between a complete lack of swim bladder inflation and delayed swim bladder inflation. Incardona et al. (2004) also reported that different PAHs, including

phenanthrene, caused delayed or failed inflation of the swim bladder. PAHs have multiple modes of action in the aquatic environment, including AhR (aryl hydrocarbon receptor) mediated and AhR-independent (cardio)toxicity and narcosis (Di Toro et al., 2000; Billiard et al., 2006). Phenanthrene in particular has been demonstrated to have low AhR binding affinity, to have a resulting inability to induce CYP1a, to cause cardiotoxicity through AhR independent pathways, and to act as a non-polar narcotic (baseline toxicant), implying that it disrupts membranes due to its lipophilic nature (Hawkins et al., 2002; Barata et al., 2005; Incardona et al., 2005). Villeneuve et al. (2014) used the adverse outcome pathway (AOP) framework to hypothesize possible mechanisms underlying impaired swim bladder inflation. Recent research has shown that compounds from a wide variety of chemical classes and modes of action impair swim bladder inflation (Hagenaars et al., 2014; Lanham et al., 2014; Wang et al., 2014), probably through a variety of underlying mechanisms (e.g., Bagci et al., 2015). However, the current description of AOPs related to swim bladder inflation do not provide information to understand the role of narcosis, and AOPs related to narcosis do not specifically focus on swim bladder inflation effects. The link between these two has therefore yet to be established. Such integrated AOP assessments involving different mechanisms and pathways require an AOP network approach, a concept that is still under development (Knapen et al., 2015).

In the second highest exposure concentration (362 µg/L), mortality was 65%, 43% of survivors were not hatched at 120 hpf (n=3), and all hatched eleutheroembryos (n=4) had uninflated swim bladders and tail malformations (curvature, elbow and/or tissue deviation). Furthermore, surviving embryos (n=7) were severely malformed with 71% oedemas (pericardium and/or yolk), 57% blood accumulations (in the heart, in the yolk (extension) and/or adjacent to the swim bladder), 57% absent blood circulation in the tail, 43% fin malformations (one or both fins absent or curved), 43% yolk malformations and 57% mouth malformations (Fig. 2). These proportions were all significantly different from LC. These observations correspond to what Incardona et al. (2004) termed a characteristic suite of abnormalities in teleost embryos exposed to petroleum-derived PAH mixtures, including cardiac dysfunction, pericardial and yolk sac oedema, reduced size of the jaw and

curvature of the tail. Butler et al. (2013) found similar phenotypes of 120 hpf zebrafish eleutheroembryos exposed to phenanthrene, with oedemas of pericardium and yolk, as well as curvatures of the tail.

3.2.2. Phenanthrene was lethal during the first 24 hours of exposure

All mortalities were already observed at 24 hpf, except for one embryo in the 217 µg/L exposure that had not hatched and died at 120 hpf. Firstly, we can conclude that embryos were already able to take up phenanthrene through the chorion, which was also shown by Petersen and Kristensen (1998). Secondly, LC_{50} values at 96 hpf and 120 hpf can be considered identical (Table 2). Our calculated LC_{50} of 310.0 µg/L was in the range of previously reported 96h LC_{50} values of 234 and 375 µg/L for juvenile *Lepomis macrochirus* and *Salmo gairdneri* respectively (Call et al., 1986), and slightly lower than the 7 day LC_{50} for zebrafish reported by Petersen (1997) of 536 µg/L, and the TLM (target lipid model for toxicity assessment of residual petroleum constituents) predicted 96h LC_{50} for zebrafish of 510 µg/L (McGrath and Di Toro, 2009).

3.2.3. Effect values were within ranges expected for baseline toxicity

Some generalizations have been proposed with regard to baseline toxicity. Firstly, baseline toxicity is assumed to initiate in the chemical activity range of 0.01 to 0.1, which corresponds to 1 to 10% of (subcooled) liquid solubility (Reichenberg and Mayer, 2006; Schmidt and Mayer, 2015). We report an La_{50} of 0.047, which is in this range (for detailed calculations, see Appendix). Ea_{50} values of 0.009 and 0.008 for impaired swim bladder inflation and any sublethal morphological effect respectively, are slightly below the range, as expected.

The second assumption uses critical body residues to compare toxicities among chemicals and test species, because they are a more direct measure of toxicity than indirect metrics such as LC_{50}, which are based on exposure media concentrations and influenced by bioavailability, uptake,

biotransformation, etc. (Mackay et al., 2014). The log BCF was 3.02 ± 0.21 L/kg fresh wt (average ± SD over all samples). Although we did not verify that steady-state was reached in the larvae, Petersen and Kristensen (1998) showed that steady-state conditions for phenanthrene were already reached after 20 h in zebrafish larvae at the end of the yolk sac stage (presumably around 7 dpf), and hypothesized that this was due to the small size of the larvae and thus an increased total surface of the membranes per unit fish weight compared to juveniles/adults. Furthermore, our value was in very good agreement with the experimentally determined log BCF of 3.20 L/kg fresh wt (assuming an 80% water content for conversion from dry weight to fresh weight) reported by Petersen and Kristensen (1998). CBRs are expected to be similar (in the range of 2 to 8 mmol/ kg) among baseline toxicants and small aquatic organisms due to similar mode of action (Van Wezel and Opperhuizen, 1995). Recently, van der Heijden et al. (2015) showed that CBR values for seven narcotics tested in three species ranged from 2.1 to 16.1 mmol/kg fresh wt. McCarty et al. (2013) reviewed a large set of CBR values and reported a mean CBR of 1.80 mmol/kg fresh wt for 29 narcotics in algae, invertebrates and fish. CBR values for phenanthrene in invertebrates ranged from 0.32 to 8.40 mmol/kg (McCarty et al., 2013). Our CBR value of 2.72 mmol/kg fresh wt fits very well into this range (Table 2). Furthermore, our critical and effective body residues (Table 2) are in the same range as the CBR and EBR (E: bent chorda) values of 3.48 and 0.80 mmol/kg fresh wt reported by Petersen (1997) after exposure of zebrafish larvae to phenanthrene.

Thirdly, lipid normalized CBRs are expected to be similar among baseline toxicants and small aquatic organisms due to similarity at target site (cell membranes containing phospholipids). The lipid content of 120 hpf unexposed zebrafish was 1.63% ± 0.29% (SD) (w/w, fresh wt), and our CBR_{LIP50} of 167 mmol/kg lipids was very close to the expected range of 40 to 160 mmol/kg (Van Wezel and Opperhuizen, 1995), but was higher than the mean CBR_{LIP50} of 53.9 mmol/kg lipid reported for 29 narcotics in algae, invertebrates and fish (McCarty et al., 2013). Our observation that the CBR fits nicely into the expected interval while the lipid normalized CBR is rather high relative to what is expected, may be due to the fact that lipid corrected CBR values are subject to variation in both lipid

362 content and composition, which may compromise their accuracy (McCarty et al., 2013). For
363 sublethal effects we report EBR_{LIP50} (swim bladder): 16 mmol/kg lipids and EBR_{LIP50} (sublethal effects):
364 13 mmol/kg lipids, which are below the range, as expected.

365 In this way, our results support that phenanthrene exerted baseline toxicity. They also support the
366 findings of Belanger et al. (2013) who showed that the FET test has good predictability for *in vivo*
367 effects in fish, also for highly hydrophobic compounds (log K_{ow} > 3). Currently, generalized
368 assumptions about narcosis are limited to mortality. Through the application of the same concepts
369 for calculating and comparing sublethal effect values among chemicals and species, our
370 understanding of the narcosis mechanism can be greatly improved.

371 3.2.4. Reduced swimming activity was attributed to baseline toxicity

372 We observed reduced swimming activity in all phenanthrene exposure concentrations compared to
373 LC (Fig. 4A). This was caused by an altered activity pattern as shown in Fig. 4C. Exposed
374 eleutheroembryos made fewer movements at swim speeds higher than 10 mm/s compared to LC.
375 Furthermore, they made significantly more swimming movements at swim speeds lower than 2
376 mm/s, and at 217 µg/L they additionally made fewer movements at swim speeds between 2 and 10
377 mm/s. This shows that exposed eleutheroembryos were able to reach high swim speeds, but were
378 less active. One could expect that this reduced swimming activity is caused by the failure to inflate
379 the swim bladder. However, for the lowest exposure concentration, we were able to compare
380 eleutheroembryos with and without inflated swim bladders and we found no difference (Figs. 4B
381 and 4D). On the one hand, it is possible that the function of the swim bladder was impaired even in
382 those cases where the swim bladder was inflated. On the other hand, narcosis is commonly
383 associated with reduced motility due to decreased respiratory-cardiovascular functions, and with
384 altered energy metabolism which can indirectly influence motility and affect growth (McKim et al.,
385 1987). Barata et al. (2005) also showed decreased motility after exposure of the copepod *Oithona*
386 *davisae* to phenanthrene. When standard length was compared among all exposure conditions, we

found that growth was reduced in eleutheroembryos exposed to 362 µg/L (Fig. 3E), and linear regression analysis showed that length proportionately decreased with increasing body residue (Fig. 3F). This may indicate that energy was diverted from growth to detoxification and homeostasis, in line with the metabolic cost hypothesis (Rowe et al., 2001).

While previous studies were able to investigate the nature of effects of hydrophobic compounds, they were mostly not able to link these effects to stable exposure concentrations in order to establish effect concentrations necessary for ecological risk assessment. We have shown that controlled exposure concentrations facilitate determination of effect concentrations with narrow confidence intervals, and allow for obtaining mechanistic toxicity information.

3.3. Requirement 3: The passive dosing format itself causes no toxicity

There were no mortalities in the internal negative controls. At 120 hpf, mortality in the positive controls was 8, 0, 50 and 100% in 0.5; 1; 2 and 4 mg/L DCA respectively, showing adequate sensitivity of the zebrafish embryos (OECD, 2013). We observed 0% mortality in C, 10% in RC, and 5% in LC, all within the limits set in OECD TG 236 (OECD, 2013), namely a survival of ≥ 90% in the controls. We did not observe any effects of RC or LC on measured endpoints (Figs. 3-4), except for the activity pattern of RC (Fig. 4C), but this does not show a consistent effect of the passive dosing format. Although the embryos are often in physical contact with the O-rings (visual observation), this had no effect on the endpoints that were assessed. Therefore, we conclude that the silicone O-rings provide for a safe format for exposing zebrafish embryos to hydrophobic compounds.

3.4. Conclusion

We showed that the use of silicone O-rings for controlled exposure of zebrafish embryos to phenanthrene met the formulated requirements. Together with the practical advantages, this shows the potential of the format as an extension of the FET test, specifically when testing hydrophobic chemicals. Additional studies should test this passive dosing format with different chemicals, both

411 solids and liquids, in order to assess its applicability in terms of the log K_{ow} range and/or any other
412 chemical characteristics.

413

414 4. Appendix A. Supplementary material

415

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6. Captions

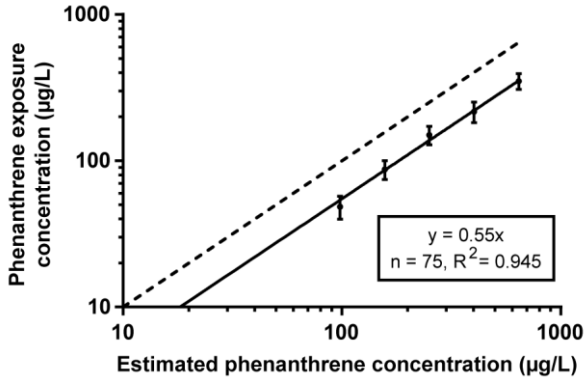
Figure 1: Overview of exposure concentrations in the 120 hpf FET test using passive dosing. The highest concentration (447 µg/L) was not included, since this concentration was only measured at the beginning of the test (all embryos died within 24 h). The full line represents a linear regression fit based on all measurements ($p < 0.001$). A 1:1 relation is included as a visual reference (dotted line). Logarithmic axes are used (average ± SD of all measurements, Table 1).

Figure 2: Representative photographs of 120 hpf eleutheroembryos at the end of the FET test. Measured phenanthrene exposure concentrations are given. The upper left photograph shows a loading control. Clearly visible morphological abnormalities are indicated: thin black arrow: swim bladder not inflated; thick black arrow: oedema of yolk (extension) and pericardium; white asterisk: yolk malformation; white arrow: blood accumulation; †: malformation of eyes and mouth; asterisk in inset: curvature of the tail.

Figure 3: Lethal and sublethal effects of phenanthrene exposure at the end of the 120 hpf FET test. Graphs on the left show effects as a function of exposure concentration, while graphs on the right show effects as a function of body residues. (A) Proportions of eleutheroembryos that died, had impaired swim bladder inflation, or other sublethal morphological effects. (B) Phenanthrene body residues in the surviving eleutheroembryos (average ± SD of 4 biological replicates, Table 1). The line represents a linear regression fit based on all replicate measurements with $y = 0.008x$, $n = 21$, $R^2 = 0.776$, $p < 0.001$. Concentration-response (C) and dose-response (D) curves of mortality, swim bladder inflation and any sublethal morphological effect. Exposure concentrations are indicated in the graph. (E) and (F) Standard length. Boxes show median with 25^{th} and 75^{th} percentiles. Whiskers represent 10^{th} and 90^{th} percentiles. * indicates significant difference from LC. In (F) average ± SD is shown in both directions. The line represents a linear regression fit with $y = -0.15x + 4.07$, $n = 6$, $R^2 = 0.908$, $p = 0.003$.

Figure 4: Effects of phenanthrene exposure on swimming activity at the end of the 120 hpf FET test. (A) Movements in 30 min calculated over all surviving and hatched eleutheroembryos. (B) Movements in 30 min, compared between LC, and exposed eleutheroembryos with inflated and non-inflated swim bladders. (C) Proportions of movements at different swim speeds. (D) Proportions of movements at different swim speeds, compared between LC, and exposed eleutheroembryos with inflated and non-inflated swim bladders. Boxes show median with 25^{th} and 75^{th} percentiles. Whiskers represent 10^{th} and 90^{th} percentiles. * indicates significant difference from LC.

455 Figure 1



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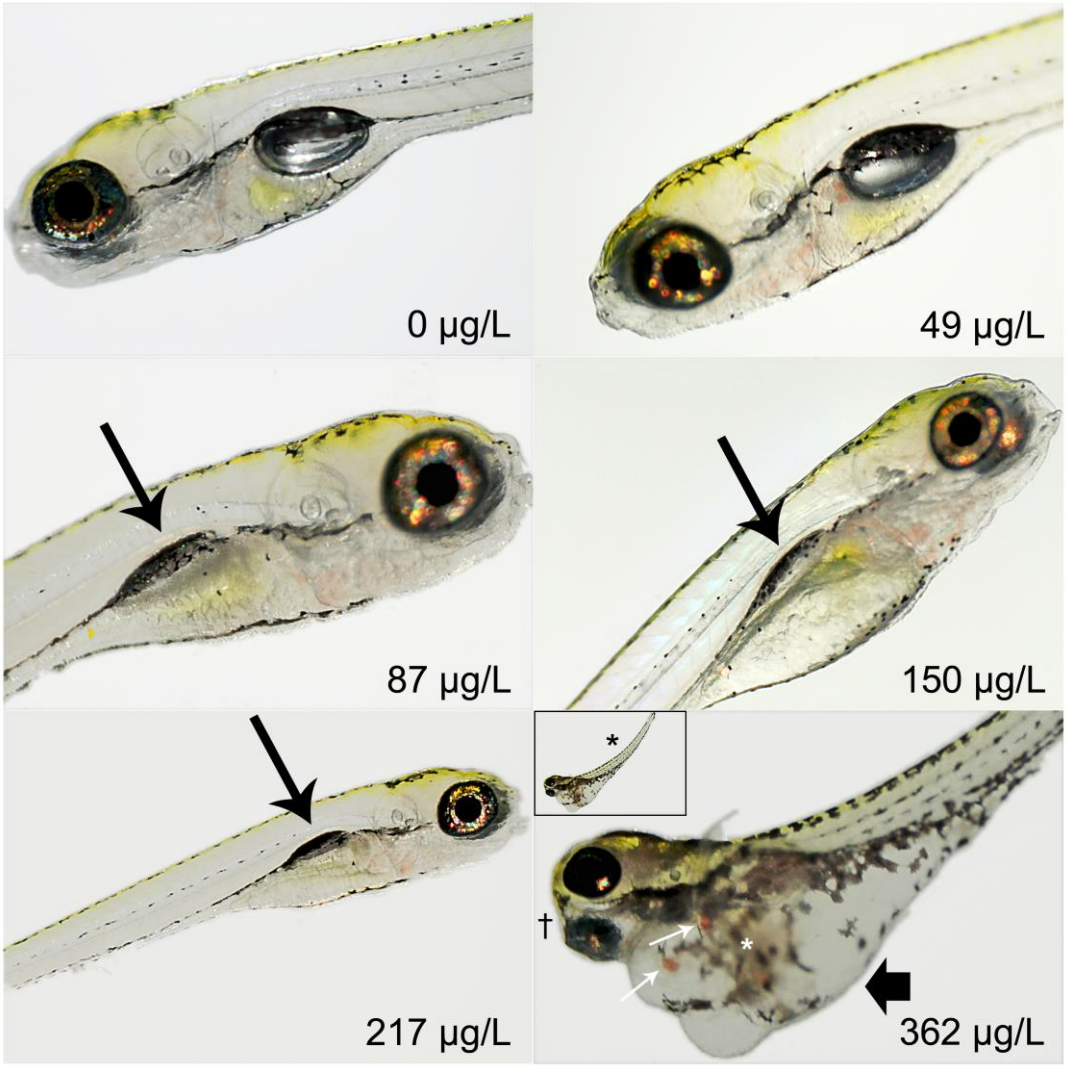
458 Figure 2



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462 Figure 3

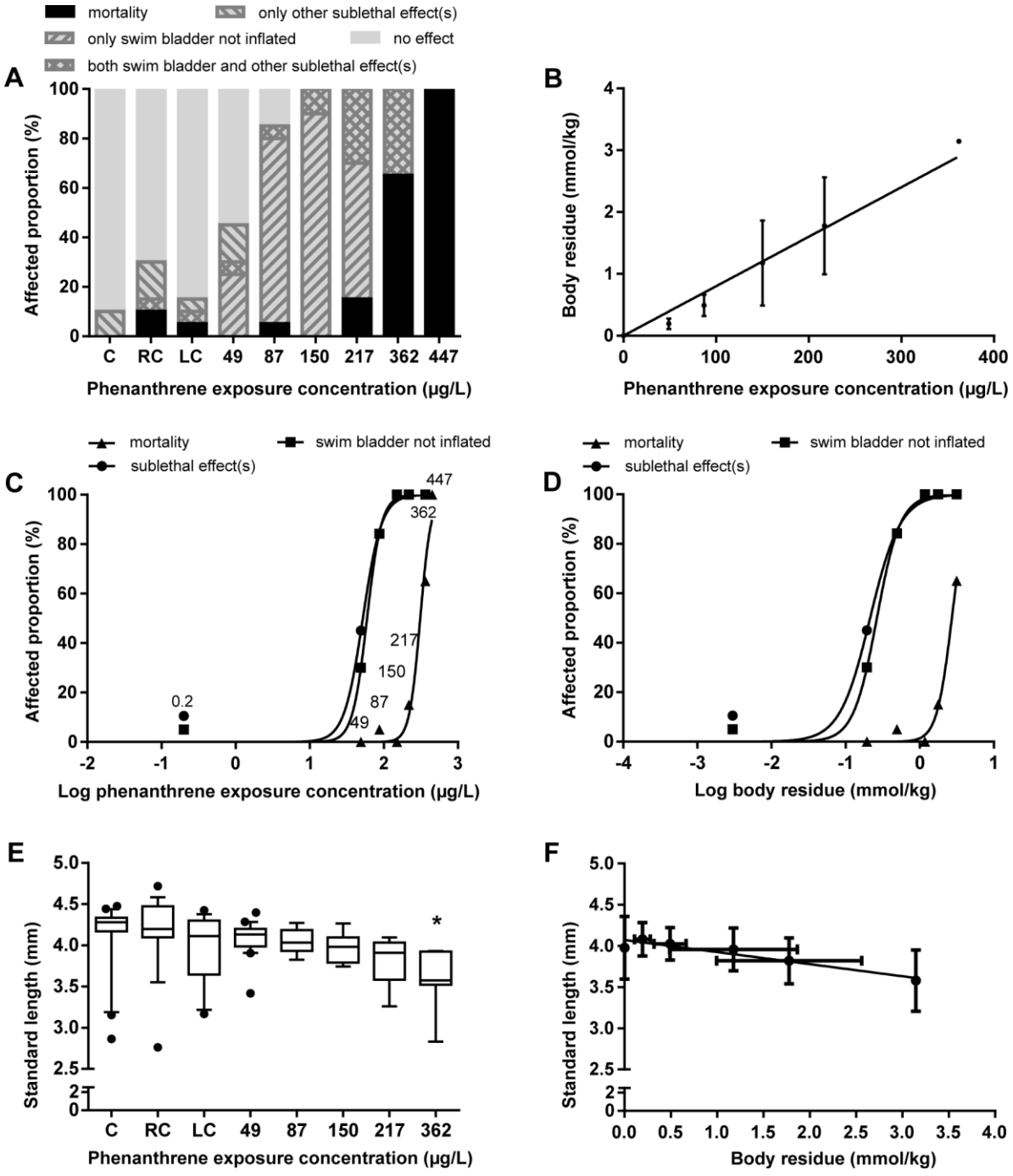

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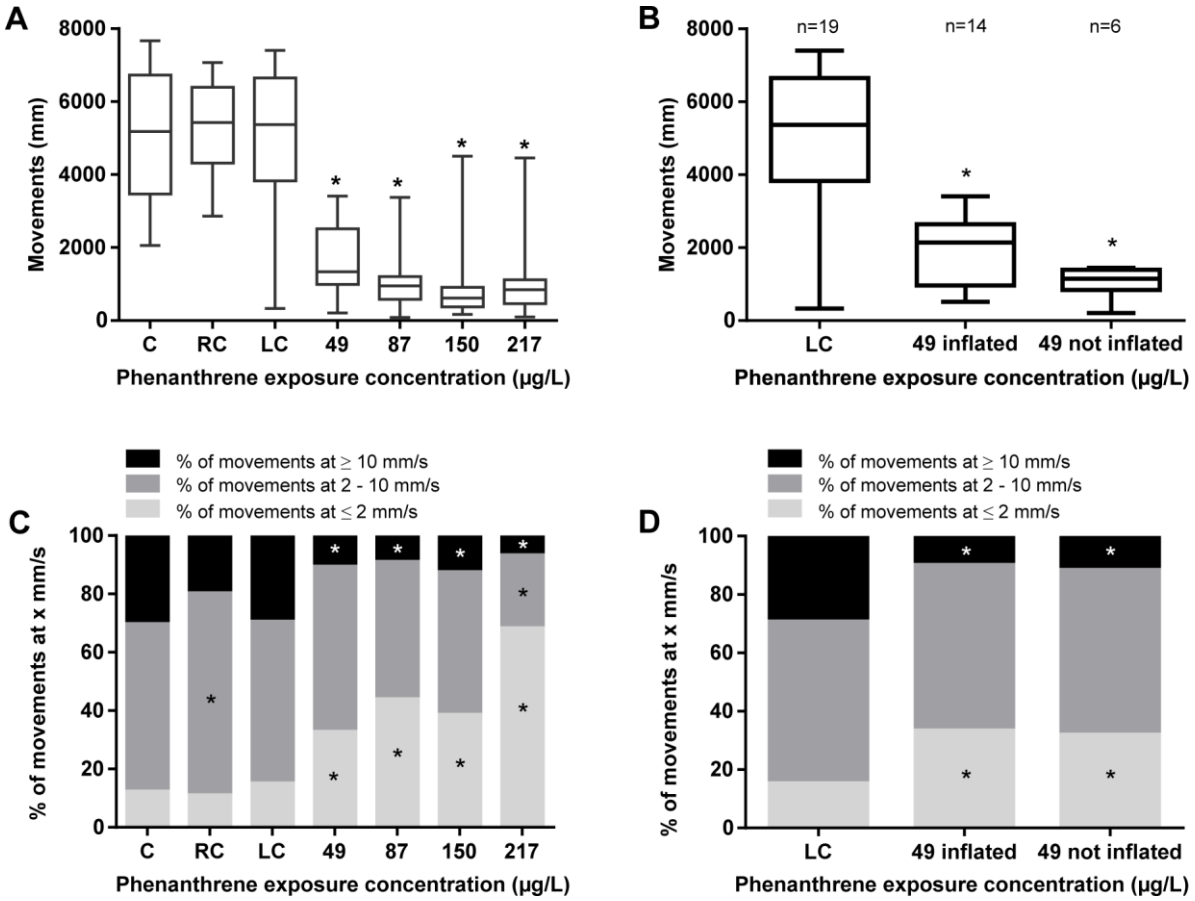
Figure 4


A
Movements (mm)
8000
6000
4000
2000
0
C
RC
LC
49
87
150
217
Phenanthrene exposure concentration (µg/L)
B
n=19
n=14
n=6
LC
49 inflated
49 not inflated
Phenanthrene exposure concentration (µg/L)
C
% of movements at ≥ 10 mm/s
% of movements at 2 - 10 mm/s
% of movements at ≤ 2 mm/s
% of movements at x mm/s
100
80
60
40
20
0
Phenanthrene exposure concentration (µg/L)
D

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