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Dries Knapen ORCID iD: 0000-0003-0472-8114

Lucia Vergauwen ORCID iD: 0000-0002-6534-7473

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Sublethal Effect Concentrations for Non-Polar Narcosis in the Zebrafish Embryo

Riccardo Massei[a,†], Dries Knapen[a], Adrian Covaci[b], Ronny Blust[c], Philipp Mayer[d], and Lucia Vergauwen[a,*]

[a]Zebrafishlab, Veterinary, Department of Veterinary Sciences, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium

[b]Toxicological Centre, Department of Pharmaceutical Sciences, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium

[c]Systemic Physiological and Ecotoxicological Research (SPHERE), Department of Biology, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerpen, Belgium

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[d]Department of Environmental Engineering, Technical University of Denmark, Bygningstorvet B115, 2800 Kgs. Lyngby, Denmark

[†] Present address: Department of Bioanalytical Ecotoxicology, UFZ-Helmholtz Centre for Environmental Research, Permoserstr. 15, 04318 Leipzig, Germany

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Abstract: Non-polar narcosis, also known as baseline toxicity, has been described as the minimal toxicity that an organic chemical may elicit based on its lipophilicity. While lethal effects of narcosis-inducing chemicals (NICs) have been thoroughly investigated, knowledge of sublethal effects is still very limited. We investigated the effects of three well-known NICs (phenanthrene, 1,3,5-trichlorobenzene and pentachlorobenzene) on a variety of organismal endpoints (malformations, swim bladder inflation, respiration, heart rate, swimming activity and turning angles), which can be plausibly linked to narcosis in zebrafish embryos. Baseline toxicity recorded as mortality is typically observed in similar exposure ranges in a wide variety of species including fish, corresponding to a chemical activity range between 0.01 and 0.1. In the present study we found that sublethal effects occurred at concentrations around 5 times below lethal concentrations. Altered swimming activity and impaired swim bladder inflation were the most sensitive endpoints occurring at exposure levels below the generally accepted threshold for baseline toxicity for two out of three compounds. Overall, most effective exposure levels across the sublethal endpoints and compounds did fall within the range typically associated with baseline toxicity, and deviations were generally limited to a factor 10. While there could be benefit in adding sublethal endpoints to toxicity tests, such as the Fish Embryo Acute

Toxicity (FET) test, based on the present sublethal endpoints and available evidence from our and other studies, the underestimation of toxicity due to the sole assessment of mortality as an endpoint in a FET test may be limited for narcosis.

Keywords: aquatic toxicology, non-polar narcotics, narcosis-inducing chemicals, ecotoxicology, baseline toxicity, developmental toxicity

*Address correspondence to lucia.vergauwen@uantwerpen.be

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1. INTRODUCTION

Every day, thousands of anthropogenic pollutants enter into freshwater environments through different pollution sources posing a major risk to the aquatic communities. It has been estimated that nearly 70% of organic pollutants released into the environment may potentially exhibit an unspecific mode of action defined as narcosis (Bradbury et al. 2003). Non-polar narcosis (further referred to as narcosis), also known as baseline toxicity, has been described as the minimal toxicity that an organic chemical may elicit based on its lipophilicity (Wezel and Opperhuizen 1995). Narcosis-inducing chemicals (NICs) exert their toxicity by accumulating in the organism's lipid compartments and disrupting the molecular interactions in cellular membranes, resulting in depressed respiratory-cardiovascular activity, lethargy, loss of equilibrium and, finally, death of the organism (Ankley et al. 2010; Geier et al. 2018; Incardona et al. 2004; McKim et al. 1987; Raftery et al. 2017; Wezel and Opperhuizen 1995). Exposure to NICs can be

harmful to various aquatic species and may lead to a decrease in population fitness (Escher and Hermens 2002; Hedgpeth et al. 2019). Mortality resulting from exposure to NICs has been well described and discussed for different aquatic organisms, and acute toxicity data have been extensively used to develop quantitative structure-activity relationship (QSAR) models that predict mortality for aquatic organisms based on the lipophilicity of chemicals (Adhikari and Mishra 2018; Di Toro et al. 2000; Finizio et al. 2020; Klüver et al. 2016).

While lethal effects of NICs have been thoroughly investigated, studies on identifying and characterizing potential sublethal effects are still very limited. As a result, today narcosis is still generally perceived as lethality, and sublethal effects are rarely taken into account, even though it is plausible to assume that a number of sublethal toxicological events take place after the accumulation of the chemical into the membranes and other lipid compartments, and preceding the death of the organism (Ankley et al. 2010; Bolser et al. 2018; Raftery et al. 2017; Volz et al. 2011). It follows that NICs could cause adverse effects at concentrations lower than those typically associated with baseline toxicity. Consequently, the sole assessment of mortality in the context of narcosis may not allow to capture the full range of more subtle, but possibly important, effects and may lead to an underestimation of the toxicity caused by NICs. This is particularly important since organic pollutants are usually detected at sublethal concentrations in the environment (Brack et al. 2016).

It is currently unclear which sublethal effects can be caused by NICs, at which exposure concentrations they occur, and how relevant and important these effects are in an ecotoxicological perspective. A better understanding of the nature and incidence of such

sublethal effects following exposure to NICs would thus be valuable in a risk assessment context. For example, Hedgpeth et al. (2019) recently drew attention to the importance of sublethal effects caused by petroleum substances (anthropogenic or natural mixtures), exerting toxicity mainly through narcosis. These authors focused on overt developmental abnormalities that are easily observable in zebrafish embryos and showed that morphological malformations occurred at approximately 2.8 times lower exposure concentrations compared to lethal effect concentrations.

Here, we study a broader range of organismal sublethal effects that can be plausibly linked to narcosis and are potentially more sensitive than the ones used in previous studies: general morphological malformations (Hedgpeth et al. 2019; Vignet et al. 2014), oxygen consumption (Raftery et al. 2017), heart rate (Incardona et al. 2004; Raftery et al. 2017) and swimming performance (Vignet et al. 2014). We exposed zebrafish embryos in an adapted Fish Embryo Acute Toxicity (FET) Test, OECD Test Guideline 236 (OECD 2013) to three well-known non-polar NICs (phenanthrene, 1,3,5-trichlorobenzene and pentachlorobenzene) with varying octanol-water partition coefficients (logKow, describing lipophilicity, ranging from 4.0 to 5.2). We defined concentration thresholds for sublethal effects caused by narcosis and compared them to lethal exposure concentrations and concentration ranges typically associated with baseline toxicity. Based on these comparisons, we evaluated the relevance of our findings in an ecotoxicological context.

2. MATERIAL AND METHODS

2.1 Chemicals and standard solutions

1,3,5-trichlorobenzene (TChB, CAS 108-70-3, logKow: 4.02) Phenanthrene (PHN, CAS 85-01-8, logKow: 4.46), and pentachlorobenzene (PChB, CAS 608-93-5, logKow: 5.21) were acquired from Sigma-Aldrich (Merck, purity 98-99%). TChB and PChB are organochlorine compounds widely used in the past to study non-polar narcosis in aquatic organisms (Roex et al. 2001; Schuler et al. 2007; van Wezel et al. 1997). PHN is a polycyclic aromatic hydrocarbon (PAH) and could therefore have additional mechanisms of toxicity other than narcosis, but its toxicity has been shown to be largely aryl hydrocarbon receptor independent (Incardona et al. 2005). Information on the chemical and toxicological proprieties is given in the Supporting Information (SI, Table S1).

2.2 Adult fish breeding and egg collection

Non-exposed adult wildtype zebrafish (strain AB, *Danio rerio*) were maintained in the Zebrafishlab of the University of Antwerp (Antwerp, Belgium) and used for egg production. Zebrafish embryos are not protected under the European legislation on the use of laboratory animals until the age of 120 hours post fertilization (hpf) (EC 2010; 2012), and therefore no ethical approval was required for these experiments. Reconstituted freshwater (45 mg/L $CaCO_3$) was freshly prepared by adding Instant Ocean Sea Salt (Instant Ocean) to reverse osmosis water (RO 40, Werner) 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. Fish were kept in a ZebTEC zebrafish housing system (Tecniplast) at a 14 h/10 h light/dark cycle, at 28 ± 0.2 °C. Ammonium, nitrite

and nitrate levels were measured twice a week with Tetratest kits (Tetra Werke) and remained below 0.25, 0.3 and 12.5 mg/L respectively. The breeding stock was fed four times a day: twice daily with granulated food (Biogran medium, Prodac International) at a rate of 1.5% of their mean wet weight and twice daily with thawed and rinsed *Artemia sp. nauplii, Daphnia sp., Chironomidae* and *Chaoboridae* larvae alternately. Two males and one female were placed in breeding tanks with a perforated bottom the night before egg collection. The next day, the separator between the two sexes was removed when the lights were turned on. Eggs were then collected, washed with reconstituted freshwater and placed in plastic DECA containers until starting the exposure.

2.3 Passive dosing

Passive dosing was used to maintain stable exposure concentrations of lipophilic compounds, since previous studies have shown important losses in conventionally spiked exposure setups in polystyrene multi-well plates for substances with logKow above 3 (Riedl and Altenburger 2007; Vergauwen et al. 2015). Silicone O-rings (outer diameter: 14.4 mm; cross section: 2.4 mm; average weight: 0.22 g; product no. ORS-0096-24, Altec) were loaded from methanol solutions by equilibrium partitioning (Smith et al. 2010). Ring loading and medium equilibration followed the procedure described by Vergauwen et al. (2015). Additional information on loading procedure and passive dosing are given in SI (Annex 1).

2.4 Setup of Fish Embryo Acute Toxicity (FET) test

Zebrafish embryos were exposed from 2 until 72 or 120 hpf (see specific endpoints below) using silicone O-rings loaded with six concentrations of PHN, TChB or PChB or

methanol as negative control. Fertilized eggs were selected according to (Kimmel et al. 1995) and transferred to polystyrene 24-well plates (Cellstar 24 well cell culture plate, sterile, with lid, cat. No. 662 160, Greiner Bio-one) within 2 hpf. Each well contained one ring, one embryo and 2 mL exposure medium. Embryos were carefully placed at the center of the wells to avoid direct physical contact with the O-rings. During the test it was observed that some embryos were in contact with or in close vicinity of the O-rings. Seiler and coworkers (2014) investigated the impact of direct contact with the polymer for a set of PAHs including phenanthrene and reported no difference in mortality between zebrafish embryos with and without direct contact to the silicone cast at the bottom of the test vessel. The authors concluded that the main exposure pathway was via the water phase.

Plates were covered with parafilm and plastic lids, and placed in an incubator at 28.5 °C with a 14 h/10 h light/dark cycle. In addition to specific sublethal endpoints described in the following paragraphs, the four endpoints of acute toxicity described in OECD TG 236 (OECD 2013) (coagulation of fertilized embryo, lack of somite formation, lack of detachment of the tail bud from the yolk sac, and lack of a heartbeat), and hatching were recorded every 24 hours by observing the embryos directly in the wells using a S8 APO stereomicroscope (Leica Microsystems GmbH). Chorions were removed from the test chamber after hatching. In each plate four wells were used as internal negative control (reconstituted freshwater) and the plate was considered valid if no more than one embryo died. In the 120 hours exposures, rings and medium were renewed at 72 hpf to maintain stable exposure concentrations (Vergauwen et al., 2015). One 24-well plate containing 3,4-dichloroaniline (3,4-DCA, 4 mg/L) was used as positive control (OECD 2013). The

medium was renewed every 48 hours and the test was considered valid if 3,4-DCA mortality was ≥ 30% at the end of the exposure. Additional information of the different exposure setups for each endpoint, including numbers of replicates and organisms, is given in SI (Tab. S2).

2.5 Malformations and growth

At 120 hpf eleutheroembryos were anaesthetized using 100 mg/L tricaine methane sulfonate (MS-222, Sigma–Aldrich, adjusted to pH 7.5 using $NaHCO_3$) and morphological abnormalities were scored including failure to hatch, uninflated swim bladder (posterior chamber), tail malformations, oedemas, blood accumulations, absent blood circulation in the tail, malformations of fins, yolk, mouth, eyes and otoliths. It should be noted that observing swim bladder inflation at 120 hpf does not allow for distinguishing between delayed inflation and complete failure to inflate the swim bladder, since the swim bladder could still inflate after the 120 hpf time frame. Eleutheroembryos were photographed together with a calibrator using a camera (Canon EOS 600D, 18 megapixel) mounted on the stereomicroscope, and images were analyzed using the Image J software (http://rsbweb.nih.gov/ij/) to determine standard length (± 0.002 mm). Morphological and growth data for PHN at 120 hpf were obtained from Vergauwen et al. (2015) and reanalyzed. New growth data were obtained at 72 hpf.

2.6 Heart rate and Respiration

Embryonic heart rate was counted manually at 24 hpf during 20 seconds by direct observation of the heart under a stereomicroscope. Well plates were placed on a heating plate at 28.5 ºC during the observations. For respiration analyses, we used a Loligo

OxoDish® Microplate system (Loligo® Systems), a 24 well plate with optical fluorescence oxygen sensors. Zebrafish eleutheroembryos were raised in clean water and exposed in a short exposure window of 14 hours (from 58 until 72 hpf) to avoid effects on growth which would influence respiration. Two 72 hpf eleutheroembryos were placed in each well together with 200 µL of exposure medium. The microplate was then sealed using a silicone pad while making sure that no air bubbles were formed. The treatments were distributed over different runs to avoid confounding by inter-run variation. Oxygen levels were measured for 40 minutes using a water bath at 28.5°C. In each run, four wells containing only exposure medium without larvae were used for blank correction. Linear regression analysis was used to determine the slope of the oxygen decrease and calculate the oxygen consumption per larva as pg O_2 per eleutheroembryo per minute.

2.7 Swimming activity and turning angles

At 120 hpf the swimming activity of all eleutheroembryos was determined using a Zebrabox 3.0 video tracking device (ViewPoint) and ZebraLab software (Version 3.20.5.104, Viewpoint) directly in the 24-well plates, after removal of the O-rings to avoid interference with image detection and after a 10 min acclimation phase in the Zebrabox. The sum of the swimming distance (in mm) in 30 min was used as a metric for swimming activity and the frequency of eight turning angles was determined and normalized using the swimming distance (average turning angles/mm) and the control group as a reference. Eleutheroembryos that had not hatched or showed clear tail malformations were excluded. For PHN, video recordings obtained by Vergauwen et al. (2015) were re-analyzed to obtain these metrics.

2.8 Chemical measurements of exposure medium and body residue

Medium samples were taken at 0 and 72 hpf in 3 replicates. For body residue analyses, at 72 hpf, 3 replicates of 10 eleutheroembryos each were collected. After extraction, the medium and eleutheroembryo extracts were analyzed using a 6890 gas chromatograph (Agilent Technologies) coupled to a 5973 mass spectrometer (GC-MS, Agilent Technologies) operated in electron ionization mode (EI). Detailed information on the GC-MS analysis of the three compounds is given in SI (Annex 2).

2.9 Calculations and statistical analysis

Sigmoidal regressions (4 parameter Hill equation, GraphPad Prism version 8.4.2) were used to calculate the exposure levels at which 50% mortality or effect occurred (EC50 for malformations, uninflated swim bladder and swimming distance and LC50 for mortality). Additionally, effect values based on measured exposure concentrations were converted to effective chemical activities (Ea50: chemical activity at which 50% effect occurred for malformations, uninflated swim bladder, swimming distance and La50: chemical activity at which 50% mortality occurred). Chemical activity quantifies the energetic level of an organic compound relative to the energetic level in its pure liquid form (reference state, chemical activity = 1), and chemical activity is thus defined between 0 and 1. Effective chemical activities were calculated as the effect concentration divided by the subcooled liquid solubility for the specific compound (SI Annex 3). Body residue data expressed per eleutheroembryo were converted to body residues expressed as mmol/kg wet weight using weight estimates based on pictures (Vergauwen et al., 2015). Considering that the

lipid membranes are the primary target of NICs, the measured body residues were lipid normalized and then expressed as mmol/kg lipids (SI Annex 3).

Statistical differences between negative control and treatments for mortality, general malformations, heart rate and respiration were detected using ANOVA with Bonferroni's multiple comparison posthoc. Since data from behavioral analysis are typically not normally distributed, statistical differences between treatments and controls for swimming activity and turning angles were detected by a Kruskal-Wallis test with Bonferroni's multiple comparison posthoc. The lowest exposure level that was statistically different from the control was considered as the lowest observed effect level (LOEL) for the heart rate, respiration and turning angles. For endpoints with dose-response curves, the benchmark dose (BMD) was calculated using the Benchmark Dose Software (BMDS, Version 3.2) from US EPA (Haber et al. 2018). The benchmark response (BMR) was set to 10%, confidence level at 95% and the background was estimated from the dataset using a logistic regression. In order to confirm the absence of excess toxicity after exposure to the NICs, toxicity ratios (TRs) were calculated by dividing the compounds' baseline toxicity estimated with the EPA software ECOSAR by the observed LC50. Compounds with TRs ranging from 1 up to 10 are considered baseline toxicants (Verhaar et al. 1992). All statistical analyses and data processing were performed using Microsoft Excel 2010®, Sigma Plot® (version 12.0.0.182), the software RStudio® (version 1.0.136) and GraphPad Prism (version 8.4.2). Data were considered significantly different when p-values were < 0.05.

3. RESULTS

3.1 Passive dosing performance and body residue

Compound concentrations in the exposure media did not vary more than 16% between 0 and 72 hpf across all exposures (SI, Figs.S1 A1 to C1) and compound accumulation was concentration dependent (SI, Fig. S1 A2 to C2). Average measured medium concentrations and body residues are reported in SI (Tab. S3) and used to construct concentration- and dose-response relationships and calculate effect values (Fig. 1 to 2, Tab. 1, Figs. S2 to 5, Tab. S4a to b).

3.2 Effects on mortality and morphology

Only for PHN and TChB we already observed mortality during the first 24 hours. No differences in mortality were observed between 72 and 120 hpf for any of the compounds (Fig. S5). For PChB, the highest observed mortality was 70% at the highest tested concentration at 120 hpf while 100% mortality was observed at the highest exposure concentrations of the other two compounds (Fig. 1). The LC50 for all three compounds ranged from 41 (PChB) to 1249 µg/L (TChB) and the calculated TRs between 2.2 (PHN) and 5.7 (PChB) (Tab. S4a to c). Moreover, toxicity showed a logKow dependence with PChB being the most toxic followed by PHN and TChB. The effective (50%) concentration in the lipid compartment for all three compounds was in the range of 259 to 970 mmol/kg lipids while the La50 was between 0.02 to 0.06.

For all compounds, the EC50 for malformations was lower than the LC50 ($p < 0.05$), and ranged between 29 and 433µg/L (Ea50: 0.02 to 0.04; 59 to 228 mmol/kg lipids) (Fig. 1

and Table S4a). Impaired swim bladder inflation was the most sensitive endpoint for all three compounds (EC50: 13 to 303 µg/L; Ea50: 0.008 to 0.014; 25 to 69 mmol/kg lipids). Finally, growth was also affected at 72 and 120 hpf, but the decrease in length seemed to be mostly related to general malformations (excluding uninflated swim bladders) for all three compounds. Significant decreases of length occurred at exposure levels causing around 60% incidence of gross malformations (Data not shown).

3.3 Effects on heart rate and respiration

Significant decreases of the heart rate were observed after 24 hours for all three compounds (Fig. 2). In particular, PChB already showed a decrease at 6 µg/L corresponding to a chemical activity of 0.004 (LOEL values in Table 1 and Table S4b). We observed a progressive decrease of respiration at 72 hpf after a 14 hour exposure window, reaching a 37% decrease for PHN and a 20% decrease for TChB (Fig. 2). Interestingly, for PHN a decrease was already observed at a concentration of 44 µg/L corresponding to a chemical activity of 0.007 (Table 1). For PChB, we did not observe a significant decrease in respiration (*p*: 0.117 at 41 µg/L).

3.4 Effects on swimming and turning angles

Swimming activity decreased progressively for all three compounds at concentrations below the EC50 for malformations (Fig. 1). Consequently, we assume that effects are specific and unrelated to defects in the general body morphology**.** In particular, analyses showed an effect on the swimming activity with EC50 values ranging between 19 and 366 µg/L (35 to 106 mmol/kg lipids; Ea50: 0.01 to 0.017) (Tab.1, Tab. S4a). It is important to underline that the decrease in total swimming distance was correlated with

the increasing incidence of uninflated swim bladder (R^2: 0.78, Fig.S6). Consequently, we statistically compared the swimming distance of eleutheroembryos with and without an inflated swim bladder at a concentration below the swimming EC50 (Fig. S7). No statistical differences between the two groups were found for PHN and PChB while in the TChB exposure we did observe an influence of uninflated swim bladders on the total distance. Finally, all three compounds altered the pattern of turning angles (Fig. S8). Both routine behavior (turning angles 0/15° and 15/45°) as well as escaping behavior (turning angles 90/180°) was impacted by the compounds.

3.5 Summary of biological responses to narcosis

BMD and LOEL values were used to summarize the data and compare lethal and sublethal effect levels (Table 1 and Table S4b). Firstly, the logKow dependency of all responses was investigated, to evaluate whether the observed effects had a narcotic component. Overall, the endpoints were logKow dependent, with some exceptions after exposure to phenanthrene (Fig. 3). Secondly, we investigated at which exposure levels sublethal responses occurred compared to mortality. Decreased swimming activity was the most sensitive endpoint, followed by uninflated swim bladder and general malformations. Table 1 (left) shows that sublethal BMD/LOEL values were around 5 times lower than the lethal BMD. Thirdly, we compared sublethal effect values to the typical range defined for baseline toxicity generally interpreted as mortality (chemical activity 0.01 to 0.1) (Mayer and Reichenberg 2006; Wezel and Opperhuizen 1995). Table 1 (right), Figure 4 and S9 show that some of the sublethal effects do occur below the reported range for baseline toxicity.

4. DISCUSSION

Non-polar narcosis is generally perceived as lethality and sublethal effects are rarely considered for the evaluation of the toxicological effects of NICs. In the present study, we characterized sublethal effects in zebrafish embryos, evaluated whether they can be plausibly linked to narcosis, determined the exposure levels at which they occur and compared them to lethal exposure levels and thresholds for baseline toxicity. Finally, we discuss the ecotoxicological relevance of our findings.

4.1 Sublethal effects of narcosis occurred within a factor 10 of the reported chemical activity range for baseline toxicity

Available evidence shows that baseline toxicity recorded as mortality occurs in similar exposure ranges in a wide variety of species including fish. General ranges have been defined in terms of chemical activity (La50: 0.01 to 0.1) and concentration in the lipid compartment (40 to 160 mmol/kg lipids) (Mayer and Reichenberg 2006; Wezel and Opperhuizen 1995). Additionally, van der Heijden et al. (2015) determined a range of lethal (50%) body residue values (1 to 16.1 mmol/kg wet weight) for three aquatic species (*Lumbriculus variegatus, Hyalella azteca, and Poecilia reticulata*). Our mortality data (La50: 0.02 to 0.05, 50% lethal body residue: 4.2 to 16 mmol/kg wet weight) fit well within these ranges (Tab. S4a). The present results are also in good agreement with effective body residue values reported by McCarty et al. (2013) where the mean value for 29 NICs was 1.80 mmol/kg wet weight across algae, invertebrates and fish. When expressing chemical dose as the concentration in the lipid compartment, the data for PHN and PChB (295 mmol/kg lipid and 259 mmol/kg lipid respectively) are above the range

of 40 to 160 mmol/kg lipid but comparable to results from Klüver et al. (2016) reporting a lethal toxicity range of 226 ± 178 mmol/kg lipid based on data retrieved from databases and after exposing zebrafish embryos to a diverse set of chemicals for 96 to 120 h. The higher value of 970 mmol/kg for TChB that was found in the present study could be the result of its deviating accumulation pattern at high exposure concentrations compared to PHN and PChB (Fig. S1). The toxicity ratio for all three compounds was below 10 (Table S4c), confirming that there was no apparent excess toxicity and all three compounds mainly cause adverse effects through a narcotic mode of action (Verhaar et al. 1992). Overall, our mortality data are in line with what has been previously reported for baseline toxicity, and can therefore be used as a reference for comparison of the sublethal effects that were found in our study.

Although only three compounds with a relatively small range of logKow values was investigated and there were some deviations after exposure to phenanthrene, Fig. 3 shows that in general respiration, heart rate, swim bladder inflation, malformations, swimming distance and altered turning angles responded in a logKow dependent manner with a factor 10 difference in effect concentrations across the compounds. This generally confirms that the toxicological mechanism causing these effects is likely to have a narcotic component, although additional mechanisms may be involved (e.g., specific effect of PAHs (Incardona et al. 2004)). Only a limited number of studies have previously compared lethal and sublethal exposure levels across a group of NICs. For the non-polar NICs included in the study of Knöbel et al. (2012), sublethal effect values for general morphological malformations in zebrafish embryos (heart edema, yolk sack edema, deformation of axis, deformation of tail, deformation of the head, slowed heartbeat, and

delay in development) were on average 1.6 times lower than lethal effect values. Hedgpeth et al. (2019) showed that the most sensitive morphological malformations that were observed (pericardial edema, yolk sac edema, tail curvature and non-viability) occurred at approximately 2.8 times lower exposure concentrations (EC25 13.3 mM bioavailable fraction, indicative of the concentration in the lipid compartment as assessed in our study) compared to lethal effect concentrations (LC50 36.6 mM bioavailable fraction) across a range of petroleum substances including mixtures, thought to exert toxicity via narcosis.

In the present study, we show that more subtle sublethal effects on physiology occur at concentrations around 5 times below lethal concentrations (Table 2, left). Altered swimming activity and impaired swim bladder inflation were the most sensitive endpoints occurring at chemical activities below the generally accepted threshold for baseline toxicity for two out of three compounds (Fig. 4, Fig. S9, Table 2, right). Significant effects for these endpoints (BMD and LOEL, Table 1) were observed in a chemical activity range of approximately 0.0007 to 0.013. Although some effects occurred below the generally accepted lower limit for baseline toxicity, in the present study most BMD and LOEL values across the sublethal endpoints and compounds did fall within the reported chemical activity range for baseline toxicity (Ratios ≤ 1 compared to the generally accepted lower limit for baseline toxicity, Table 2, right), and deviations were generally limited to a factor 10. To allow interpretation of our data in a broader toxicological context we performed an exploratory meta-analysis for a total of 21 non-polar NICs using data available in the US EPA ECOTOX Knowledgebase (methods and results can be found in the “Meta-analysis” section of the supporting info). Overall, our

analysis confirmed that for a broader range of non-polar NICs, effective exposure levels for morphological and behavioral effects were either within the range typically associated with baseline toxicity (chemical activity: 0.01 to 0.1) or within a factor 10 lower than this range (chemical activity: 0.001 to 0.1). It should be noted that our analysis was mainly based on toxicity data for chemicals with relatively low logKow values (ranging from 0 to 5.2, logKow < 3 for 50% of the chemicals). The availability of reliable sublethal effect data for non-polar NICs with medium to high logKow values is indeed very limited, which is largely due to the need for dedicated and technically demanding experimental setups (e.g., passive dosing exposure platforms, confirmation of exposure concentrations using analytical chemistry, etc.). The present study contributes to addressing this data gap.

4.2 Ecotoxicological relevance of sublethal effects of narcosis

In ecotoxicology, effects are typically considered ecologically relevant if they occur at environmentally relevant exposure concentrations and if they can be linked to effects on survival, development or reproduction in individual organisms and eventually to effects at the population level. The BMD of the most sensitive endpoint, decreased swimming distance, was 231, 5.6 and 6.5 µg/L for TChB, PHN and PChB respectively (Table 1). For PHN, similar and higher concentrations have been reported in urban runoff of Tehran (Iran, 15.1 µg/L), in the Jiulong River estuary and Western Xiamen Sea (China, 1.37 µg/L) and different surface water bodies in Nigeria (2.83 to 1460 µg/L) (Anyakora et al. 2005; Mahvi and Mardani 2005; Maskaoui et al. 2002; Tongo et al. 2017).

Environmental concentrations of TChB isomers 2 to 3 orders of magnitude below the BMD have been reported, ranging from 0.088 µg/L to 2 µg/L in industrial waste water and surface waters in Greece and China (Fan et al. 2021; Nikolaou et al. 2002; Yuan et al. 2020). For PChB, environmental concentrations 1 to 5 orders of magnitude below the BMD have been reported in industrial waste water and surface waters in China and North America (Bailey et al. 2009; Yuan et al. 2020). Based on these data, it seems that environmental concentrations of PHN are much more likely to cause sublethal effects than those of the chlorobenzenes. It is important to note that environmental water bodies mostly contain mixtures of several compounds that can act as NICs. Therefore, total PAH levels or chlorobenzene levels may be more relevant although difficult to immediately link to effect levels due to the variation in lipophilicity. Total PAH levels ranging from 26 to 8000 µg/L have been reported in China and Nigeria (Anyakora et al. 2005; Maskaoui et al. 2002; Tongo et al. 2017) and total chlorobenzene levels of 6.6 µg/L have been reported in the Tonghui River (Beijing, China) (Zhou et al. 2009). Taken together this shows that especially for PAHs and to a lesser extent for chlorobenzenes, environmental concentrations could cause sublethal effects in fish.

For the most sensitive sublethal endpoints observed in the current study (i.e., swim bladder inflation and swimming performance), biologically plausible links leading to population effects exist. In our study, impaired swim bladder inflation and reduced swimming distance were correlated after exposure to NICs (Fig. S6). Larvae with uninflated swim bladder must move to stay suspended and would be expected to use additional energy to maintain position, plausibly leading to less lateral distance travelled. A link between impaired swim bladder inflation on the one hand and reduced growth

rate, spinal malformations and reduced survival on the other hand has been shown for a range of freshwater and marine fish species with different swim bladder anatomy (Chatain 1994; Czesny et al. 2005; Egloff 1996; Goolish and Okutake 1999; Stinckens et al. 2020; Woolley and Qin 2010). For example, when swim bladder inflation was prevented by holding zebrafish in a closed chamber (preventing air gulping to inflate the swim bladder), larval survival was significantly lower than that of fish held in open chambers whose swim bladders could inflate (Goolish and Okutake 1999). Both in aquaculture systems as well as in the laboratory and in the field, failure to inflate the swim bladder has been shown to reduce growth rates (Czesny et al. 2005; Woolley and Qin 2010). Czesny et al. (2005) showed a link with swimming performance (e.g., prey capturing efficiency, ability to escape predators). Fig.S7 shows that, across compounds, the effect on swimming activity is not solely explained by impaired swim bladder inflation, suggesting that the effect on swimming activity can be caused via one or more additional pathway(s). It should be noted that although impaired swim bladder inflation and swimming performance are clearly linked to decreased larval survival, the extent to which such effects may impact population sizes in a realistic, environmental exposure scenario remains unclear. Under some conditions, reduced larval survival may be compensated by reduced predation and increased food availability, and therefore not result in population decline (Stige et al. 2019).

When considering the ecological relevance of effects, one way to learn more about the links to population level effects, is to use the adverse outcome pathway (AOP) framework. An AOP is a conceptual framework that organizes existing knowledge on toxicological mechanisms starting with a molecular initiating event (i.e.

accumulation/interaction of chemicals in membranes in the case of narcosis) and leading to an adverse outcome at the organism or population level through a series of key events at increasing levels of biological organization. Putative AOPs for narcosis have been outlined (Ankley et al. 2010; Perkins et al. 2015; Volz et al. 2011). In general, these AOPs perceive narcosis as a process that is triggered by the accumulation of NICs in the membranes, leading to membrane disruption. This is thought to negatively affect metabolic rate and respiration, resulting in loss of equilibrium, reduced growth and mortality, which is linked to population decline. Souders et al. (2018) and Dreier et al. (2019) discussed AOPs describing mitochondrial dysfunction, which may be partly involved in narcosis (Bolser et al. 2018). To date, an AOP for narcosis has not been fully described and there is a high level of uncertainty both about the exact nature of the mechanism(s) and about the linkages to population level effects (Perkins et al. 2015). Our data on reduced heart rate and respiration provide empirical evidence for the link between narcosis and reduced metabolic rate and respiration together with earlier reports (Escher and Hermens 2002; Raftery et al. 2017; Souders et al. 2018). Furthermore, impaired swim bladder inflation as well as decreased swimming performance may be added as key events. In fact, these key events have already been included in AOPs describing other (specific) mechanisms in the AOP-Wiki (a public database for AOPs, www.aopwiki.org). This way, a narcosis AOP network will emerge (Knapen et al. 2018), reflecting the idea that many processes are dependent on membranes and thus narcosis can simultaneously affect several pathways. This narcosis AOP network could be further expanded by selecting a range of generic key events from existing AOPs, like the ones we investigated, and determining whether they are logKow dependent across a range of

NICs. The development of such an AOP network for narcosis would facilitate the centralization of knowledge on the mechanism(s) of narcosis and would further aid in evaluating the benefits of including sublethal endpoints in toxicity tests to inform risk assessment. Currently, the FET test only assesses mortality (OECD Test Guideline 236 (OECD 2013)), but various sublethal responses can be observed in the timeframe preceding the limit where zebrafish are protected under the EU legislation on the use of laboratory animals.

In summary, we observed sublethal effects caused by narcosis that can be of ecotoxicological relevance. Although these effects sometimes occur below the thresholds that are generally associated with baseline toxicity typically recorded as mortality, the difference seems to be limited to a factor 10. While there could be benefit in adding sublethal endpoints to toxicity tests such as the FET test, based on available evidence, the underestimation of toxicity due to the sole assessment of mortality as an endpoint in a FET test may be limited for narcosis. It should be noted however that relevant sublethal effects other than those included in the present study may occur at lower exposure levels.

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Data availability statement—Data, associated metadata, and calculation tools are available from the corresponding author (lucia.vergauwen@uantwerpen.be).

Author contributions statement—L. Vergauwen and D. Knapen conceived and designed experiments. L. Vergauwen and R. Massei performed experiments. R. Massei performed statistical analyses. R. Massei, L. Vergauwen and D. Knapen wrote manuscript. A. Covaci performed chemical analyses. P. Mayer provided assistance with passive dosing. P. Mayer, A. Covaci and R. Blust provided technical and editorial assistance.

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FIGURES

Figure 1: Concentration response curves for trichlorobenzene (a), phenanthrene (b) and pentachlorobenzene (c) for mortality (black), malformations (pericardial/yolk edema and tail curvature, red), uninflated swim bladders (blue) and decrease of swimming activity (total distance travelled in 30 minutes, green) at 120 hours post fertilization. Concentration response curves were modelled using a 4-parameter Hill equation. Parameter estimations can be found in Tab.1 and SI (Tables S4a-b).

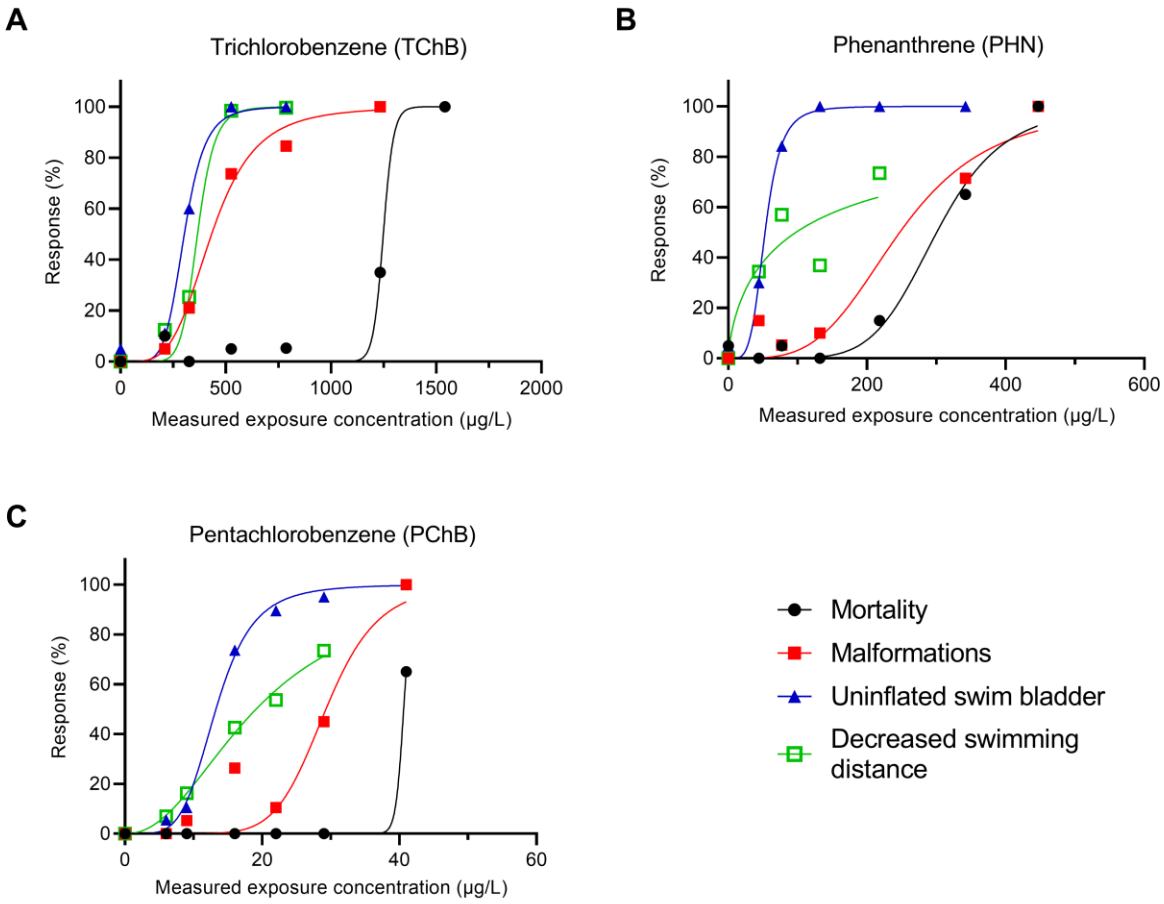


Figure 2: Effect of trichlorobenzene (a), phenanthrene (b) and pentachlorobenzene (c) on the heart rate at 24 hpf and oxygen consumption (respiration) at 72 hpf (after a 14 h exposure window). Symbols represent the average response and error bars show the standard deviation (n = 8 - 9). Data were normalized to the controls. * represents statically significant differences between control and treatment for heart rate (blue) and respiration (red), respectively ($p < 0.05$).

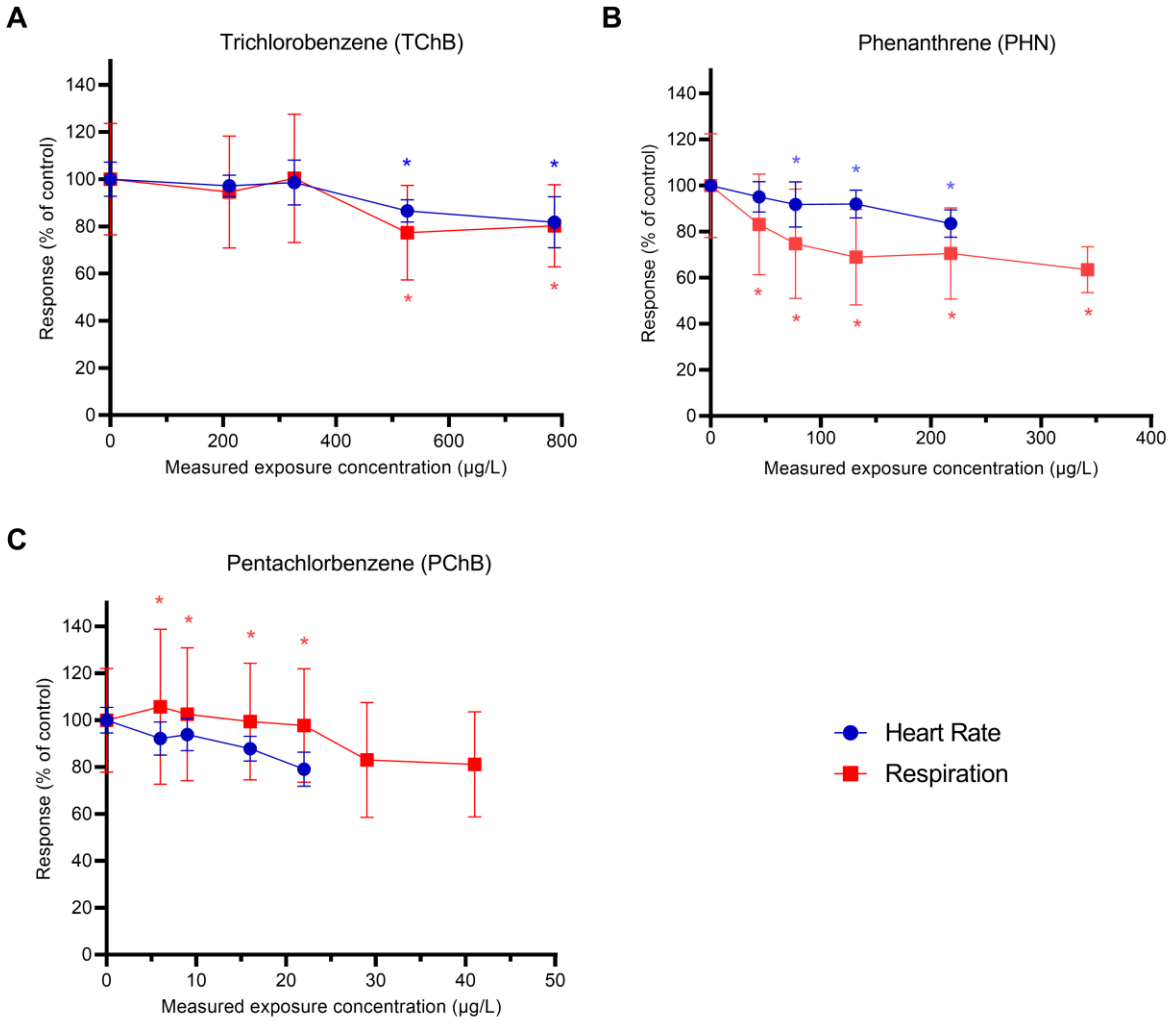


Figure 3: LogKow dependency of all sublethal endpoints in zebrafish embryos after exposure to trichlorobenzene (TChB), phenanthrene (PHN) and pentachlorobenzene (PChB). BDMs and LOEL are expressed as measured exposure concentration on a logarithmic axis.

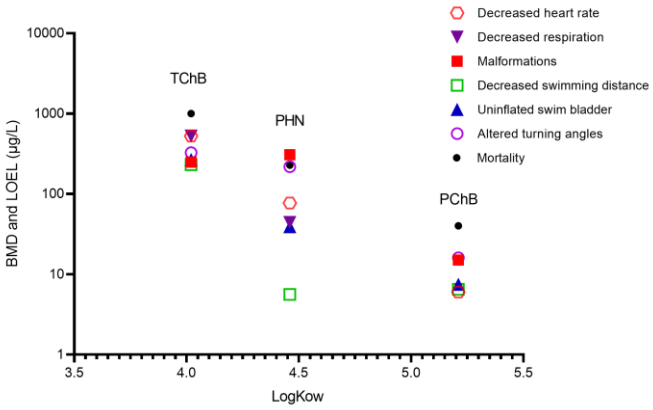
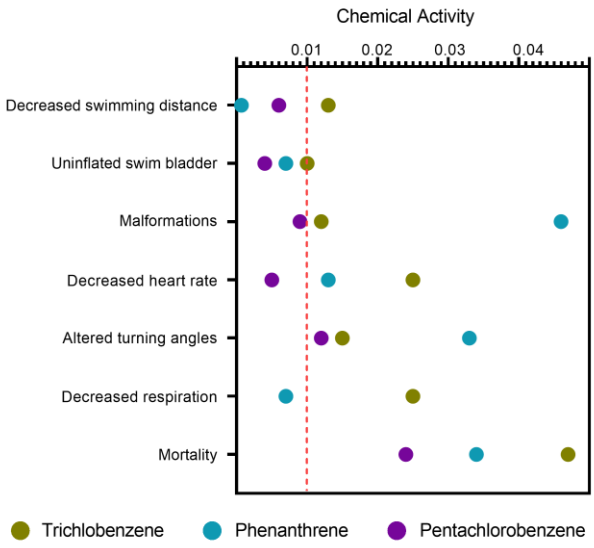


Figure 4: Effect levels (lowest observed effect level and bench mark dose) of all the endpoints expressed as chemical activity. For decreased respiration only two chemicals showed a significant effect. The red dotted line represents the reported lower limit for baseline toxicity (0.01) typically interpreted as mortality (Reichenberg and Mayer, 2006). Endpoints are ordered according to median sensitivities.



TABLES

Table 1: Calculated effect levels for lethal and sublethal endpoints [a]

Endpoint	**Measured exposure concentration (LOEL/BMD, µg / L)** [a]			**Chemical activity** [a]		
	TChB	PHN	PChB	TChB	PHN	PChB
Decreased heart rate	526	77	6	0.025	0.013	0.004
Decreased respiration	526	44	-[b]	0.025	0.007	-[b]

Mortality	999	228	40	0.047	0.034	0.025
Malformations [c]	248	307	15	0.012	0.046	0.009
Uninflated swim bladder	267	39	7.5	0.01	0.005	0.005
Decreased swimming distance	231	5.6	6.5	0.013	0.0007	0.004
Altered turning angles	326	218	16	0.015	0.033	0.010

[a] Lowest observed effect levels (LOEL) and bench mark doses (BMD) for the different endpoints expressed as a function of measured exposure concentration and chemical activity for trichlorobenzene (TChB), phenanthrene (PHN) and pentachlorobenzene (PChB). Effect levels are LOEL for heart rate, respiration, and turning angles and BMD (10% effect) for mortality, malformations, swim bladder inflation and swimming distance.

[b] Respiration was not significantly altered after PChB exposure.

[c] Incidence of pericardial/yolk edema and tail curvature.

Table 2: Comparison of sublethal effect values to lethal effect values (left) and the reported lower limit of baseline toxicity (right)

Endpoint	**Ratio of lethal BMD and sublethal LOEL/BMD [a]**			**Ratio of reported lower limit of baseline toxicity and sublethal LOEL/BMD [b]**		
	TChB	PHN	PChB	TChB	PHN	PChB
Decreased heart rate	2	3	5	0.4	0.8	2.5
Decreased respiration	2	5	- [c]	0.4	1.4	- [c]
Mortality	1	1	1	0.2	0.3	0.4
Malformations [d]	4	1	3	0.8	0.2	1.1

Uninflated swim bladder	4	6	5	1	2	2
Decreased swimming distance	4	41	6	0.8	14	2.5
Altered turning angles	3	1	2	0.7	0.3	1

[a] The lethal BMD was divided by the sublethal LOEL or BMD. Ratios were calculated using effect values based on measured exposure concentrations and are equally applicable to effect values based on chemical activity. Numbers can be interpreted as follows: Decreased swimming distance occurs at a 4 times lower exposure concentration compared to mortality for TChB.

[b] The reported lower limit for baseline toxicity (chemical activity 0.01) was divided by the sublethal LOEL or BMD also expressed as chemical activity. Numbers can be interpreted as follows: For PHN, uninflated swim bladders occur at a chemical activity two times lower than the reported lower limit for baseline toxicity. Values below 1 indicate that effect values were above the reported lower limit for baseline toxicity.

[c] Respiration was not significantly changed after PChB exposure.

[d] incidence of pericardial/yolk edema and tail curvature.