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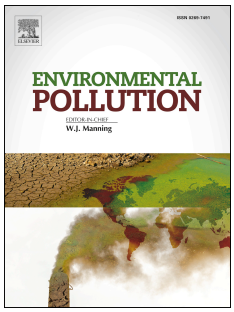

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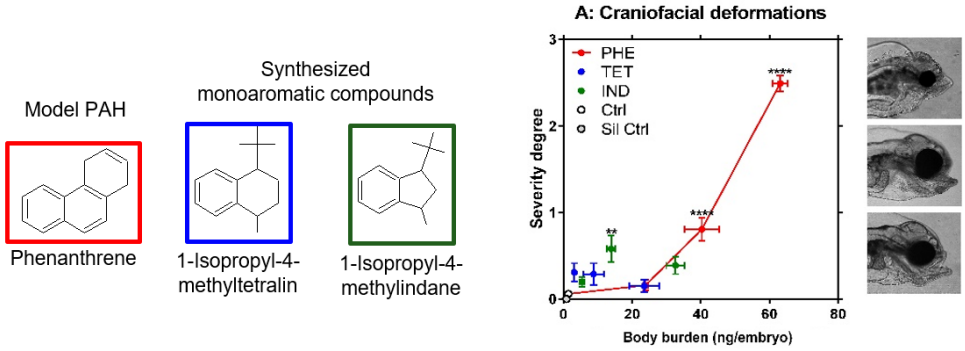

Accumulation and toxicity of monoaromatic petroleum hydrocarbons in early life stages of cod and haddock

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Abstract

A multitude of recent studies have documented the detrimental effects of crude oil exposure on early life stages of fish, including larvae and embryos. While polycyclic aromatic hydrocarbons (PAHs), particularly alkyl PAHs, are often considered the main cause of observed toxic effects, other crude oil derived organic compounds are usually overlooked. In the current study, comprehensive two-dimensional gas chromatography coupled to mass spectrometry was applied to investigate the body burden of a wide range of petrogenic compounds in Atlantic haddock (*Melanogrammus aeglefinus*) and cod (*Gadus morhua*) embryos that had been exposed to sublethal doses of dispersed crude oil. Several groups of alkylated monoaromatic compounds (e.g. alkyl tetralins, indanes and alkyl benzenes), as well as highly alkylated PAHs, were found to accumulate in the fish embryos upon crude oil exposure. To investigate the toxicity of the monoaromatic compounds, two models (1-isopropyl-4-methyltetralin and 1-isopropyl-4-methylindane) were synthesized and shown to bioaccumulate and cause delayed hatching in developing embryos. Minor developmental effects, including craniofacial and jaw deformations and pericardial edemas, were also observed at the highest studied concentrations of the alkylindane.

Capsule: Crude oil derived monoaromatic hydrocarbons accumulate in fish early life stages and may contribute to overall toxicity.

Keywords: Dispersed crude oil, monoaromatic compounds, fish early life stages, Atlantic haddock, Atlantic cod

1 Introduction

As oil exploration is moving further north, and closer to shore, there is a demand to produce accurate and relevant risk assessment models for future oil spills (Misund and Olsen, 2013). The Lofoten-Vesterålen area off the Norwegian coast is an important spawning ground for many economically and ecologically important fish species, such as the Atlantic cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) (Caroll and Smit, 2011; Vikebø et al., 2014). Developing adequate risk assessment tools for evaluating the potential impact of oil exploration in these sensitive areas has become an important focus (Caroll and Smit, 2011; Hjermann et al., 2007; Vikebø et al., 2014). In the aftermath of major spill events, such as the Exxon Valdez spill in the Prince William Sound in 1989 and the Deepwater Horizon event in the Gulf of Mexico in 2010, the detrimental impact of crude oil pollution on early life stages (ELS) of marine fish has received much attention (Beyer et al., 2016). The development of good impact models for the effects of spilled crude oil on ELS of cold water marine fish requires additional empirical data, especially on bioaccumulation and critical body burdens of a wider range of oil compounds (Olsen et al., 2013).

The main toxic responses observed in crude oil exposed fish ELS include mortality, cardiotoxicity and morphogenetic defects (Brette et al., 2014; Incardona and Scholz, 2016; Sørhus et al., 2015a), but the toxicological mechanisms are still not fully understood. Previously, it was believed that only water-soluble oil constituents were responsible for crude oil toxicity toward fish ELS (Barron et al., 2004; Carls et al., 2008; Nordtug et al., 2011b; Wu et al., 2012). However, new observations suggest that the presence of crude oil droplets leads to more severe effects than if only the water-soluble fraction (WSF) is present (González-Doncel et al., 2008; Khursigara et al., 2017). Recently, it was established that the Atlantic haddock is particularly sensitive to dispersed crude oil (Sørhus et al., 2015a; Sørhus et al., 2016). It was hypothesized that this was caused by direct interaction with crude oil droplets adhering to the chorion of the exposed embryos, causing a secondary exposure pathway (Hansen et al., 2018) by allowing direct transfer of crude oil compounds from the droplets to the eggs. This way, water solubility becomes less important for bioavailability and significant accumulation of high log K_{OW}

compounds becomes feasible. This secondary pathway has been demonstrated to cause increased internal body burden of embryotoxic PAHs and alkyl PAHs in haddock eggs (Sørensen et al., 2017), leading to much more severe effects than in similarly exposed cod eggs that are less affected by oil adhesion (Hansen et al., 2018).

The novel indications of potential whole crude oil contribution to embryotoxicity, furthermore raises the question about the contribution to toxic response from other petrogenic compounds, beyond the well-studied PAHs (Hodson, 2017). Alkylated monoaromatic compounds, which are abundant in crude oils (Booth et al., 2007), have comparable molecular weights and water solubilities to 3-4 ring alkylated PAHs (Smith et al., 2001), and therefore might be expected to follow similar uptake pathways in fish embryos. Available literature on the toxicity of crude oil derived monoaromatic compounds is limited. Studies have revealed that alkyl tetralins and indanes are acutely toxic to the mussel *Mytilus edulis* (Booth et al., 2008; Donkin et al., 2003; Smith et al., 2001), but there is no available literature on the toxicity (chronic or acute) of monoaromatic compounds to fish ELS.

The aim of the current study was to investigate the potential for accumulation and toxicity of currently overlooked petrogenic compounds toward fish ELS. Focus was given to monoaromatic compounds in the size range of 3-4 ring PAHs. In a non-targeted approach, two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GCxGC-MS) was applied to resolve and identify the complex mixtures of crude oil constituents accumulating in cod and haddock eggs exposed to dispersed crude oil. Two model monoaromatic compounds (1-isopropyl-4-methyltetralin and 1-isopropyl-4-methylindane) were synthesized and their accumulation and toxicity to haddock ELS was evaluated in comparison with a known embryotoxic PAH (phenanthrene).

2 Materials and methods

2.1 Chemical and materials

Certified standard solutions (100-1000 µg/mL) of *n*-alkanes (C_{14-32}), pristane, phytane, PAHs, alkylated PAHs, heteroaromatics and deuterated PAHs were purchased from Chiron AS (Trondheim, Norway). Phenanthrene (>98 % purity) was purchased from Sigma-Aldrich. Cyclohexylbenzene was purchased from Acros Organics. Linear and branched alkyl benzenes were supplied by Chevron Oronite (Levallois-Perret Cedex, France). C_{10-11} branched alkyl tetralins and indanes were prepared as described by Booth et al. (2008). The deuterated internal standards used as surrogate spike during extractions comprised naphthalene-*d*8, biphenyl-*d*8, acenaphtylene-*d*8 or acenapthene-*d*10, anthracene-*d*10 or phenanthrene-*d*10, pyrene-*d*10 or chrysene-*d*12, perylene-*d*12 and indeno[1,2,3-*cd*]pyrene-*d*12. All solvents were of analytical grade and purity was tested before use.

2.2 Synthesis of C_4 substituted branched alkylindane and tetralins

The syntheses methods were based upon cerium chloride-promoted Grignard additions of isopropyl magnesium bromide to 4-methyltetralone or 3-methylindanone. Cerium III chloride heptahydrate was supplied by Sigma (UK). All solvents were supplied by Rathburn (UK). Briefly, for the reaction with the indanone: cerium chloride (2.5 g) was added to dry magnesium turnings (1.7 g) in dry ether. 2-bromopropane (6.8 g) was added slowly with mild heating. On completion of the reaction, 3-methylindanone (1 g) was added in dry ether after cooling the mixture (ice). After stirring for a further 3h the solution was very cautiously worked up with wet ether and saturated ammonium chloride (1.05 g crude product; 81 %). For the tetralone: cerium chloride (5.3 g) was added to dry magnesium turnings (3.02 g) in dry ether. 2-bromopropane (15.4 g) was added slowly with mild heating. On completion of the reaction, 4-methyltetralone 2.5 g) was added in dry ether after cooling the mixture (ice). After stirring for a further 3h, the solution was worked up with wet ether and saturated ammonium chloride (2.71 g crude product; 85 %). The resultant crude alcohols (also containing alkenes resulting from spontaneous dehydration during work-up) were dehydrated with pyridine/$POCl_3$ and purified

from the other products in the crude mixture by column chromatography. The purified alkenes were hydrogenated to the hydrocarbons with either palladium on carbon or Adam's catalysts (supplied by BDH Chemicals). The hydrocarbons, 1-isopropyl-3-methylindane and 1-isopropyl-4-methyltetralin were assigned by GC-MS (Table S1) and 1-isopropyl-4-methyltetralin also by ^{1}H and ^{13}C NMR spectroscopy (data not shown).

2.3 Animal husbandry and exposure regime

2.3.1 Exposure of cod and haddock eggs to dispersed crude oil

Fertilized Atlantic cod and haddock eggs were collected from brood stocks kept at the Austevoll Research station (Institute of Marine Research, Bergen, Norway), and maintained in incubators at 7±1 °C until transfer to exposure tanks. At 1 day post fertilization (dpf), approximately 12,000 eggs were transferred into circular exposure tanks (50 L) of green PE plastic (giving an initial biomass loading of 0.4-0.5 g/L). The flow through of the tanks was 32 L/hour, and the water temperature was 7±1 °C. The light regime for the exposure tanks was 12 hours light; 12 hours dark with 30 min smooth transitions between light and dark. The light source was broad spectrum 2x36 W Osram Biolux 965 dimmable fluorescent light tubes (Munich, Germany, www.osram.com).

The crude oil used in the exposure was a laboratory weathered crude oil blend from the Heidrun oil field in the Norwegian Sea (Sørensen et al., 2017). The oil exposure system is thoroughly described elsewhere (Nordtug et al., 2011a), and oil exposure was performed as described previously (Sørensen et al., 2017; Sørhus et al., 2015a). In the current study, cod eggs exposed to nominal concentrations of 600 µg/L oil and haddock eggs exposed to 300 µg/L oil (both with droplets present and the water-soluble fraction alone) was examined. Oil droplets were in the size range 10-30 µm. To create the water soluble-fraction (WSF), the 300 µg/L dispersion was filtered through a custom-made filter containing fine glass wool over a Whatman GF/F glassfiber filter (Whatman Ltd., Maidstone, UK) with nominal particle retention of 0.7 µm. To prevent clogging, the filter was replaced every 24 hours. The WSF exposure conditions were otherwise identical to the oil droplet exposures. All exposure experiments were stopped when

50 % hatching of embryos was observed. This happened at 12 dpf (11 days of exposure) for cod and 11 dpf (10 days of exposure) for haddock. Tissue samples (pooled 0.1-1 g eggs) were collected from all exposure groups and controls after nine days of exposure, quickly rinsed in clean seawater to eliminate any free oil droplets from the sample and examined under the microscope to eliminate dead and damaged eggs from the sample. At day 10 (during hatching), 100 individual un-hatched haddock eggs were sampled, and the chorions and embryos manually separated using tweezers to be analyzed separately. The samples were preserved by flash-freezing in liquid nitrogen and stored at -80 °C until further handling.

2.3.2 Exposure of haddock embryos to phenanthrene and monoaromatic compounds

Accumulation and toxicity studies of the two synthesized monoaromatic compounds and phenanthrene were performed using a passive dosing system. AlteSil® translucent Silicone Cords (1 mm diameter, 64 cm length) were loaded with the test compounds (1-isopropyl-4-methyltetralin, 1-isopropyl-4-methylindane or phenanthrene) from methanol using a method adapted from Vergauwen et al. (2015). Briefly, pre-cleaned silicone cords were loaded by partitioning in methanol solution for 72 hours, followed by repeated partitioning in new methanol solution for 24 hours. Methanol solution concentrations are given in Table S2. Loaded silicone cords were rinsed in MilliQ-water three times, followed by equilibration in 80 mL seawater over 48 hours in glass vials. Both loading and equilibration took place at the exposure temperature (8 °C). Viable (n=250, biomass loading 6 g/L) embryos were transferred to the vials at 3 dpf. The exposure temperature was 8 ± 1 °C with a light regime of 12 hours light:12 hours dark. After 72 hours exposure, samples were removed for body burden analysis and remaining live eggs were transferred to filtered (0.22 µm Sterivex®) seawater for development and hatching. Mortality and hatching success were recorded daily and dead eggs or larvae removed. Videos and images of hatched larvae at 3 days post hatching (dph) were taken through a microscope (Eclipse 80i, Nikon Inc., Japan) equipped with a CMOS camera (MC170HD, Leica Microsystems, Germany). All imaged larvae were analyzed for segmented body length, body area, eye diameter, jaw length and eye-to-forehead distance (myotome height) using ImageJ (Schneider et al., 2012). Morphological abnormalities (jaw deformations, craniofacial

deformations, pericardial edema, spine deformations, abnormal pigmentation) were determined according to a severity degree scale (0-3 where 0 is normal, 1 is minor deformation, 2 is moderate deformation and 3 is severe deformation; Sørhus et al. (2015b), examples are given in Figure S10.). Heart rate measurements was performed on videos. All image analysis was performed 'blinded', on randomized samples.

2.4 Chemical analysis

2.4.1 Extraction and purification of fish egg samples

Extraction of tissue samples was performed as described by Sørensen et al. (2016). After addition of surrogate standards (100 ng/g sample), the samples were homogenized in *n*-hexane-dichloromethane (DCM) (1:1 v/v, 3 mL), followed by addition of Na_2SO_4, vortex extraction and centrifugation. The supernatant was collected, and the extraction repeated twice. The combined organic extract was concentrated to approximately 1 mL prior to clean-up by either silica solid phase extraction (SPE) columns as described by Sørensen et al. (2016) (haddock eggs exposed to single compounds) or by gel permeation chromatography (GPC) (haddock and cod eggs exposed to crude oil). The GPC clean-up was optimized to remove the largest lipid molecules, such as triacylglycerols (TAG), phospholipids (PL) and cholesterol, while leaving a larger fraction of crude oil compounds in the extracts. The separation was achieved using an Agilent 1220 Infinity series LC with Waters Envirogel GPC columns (300 x 19 mm) coupled to a diode array detector (DAD) for retention time monitoring. DCM was used as mobile phase at a flow rate of 5 mL/min. Standards of TAG, PL, cholesterol, PAHs and a haddock egg lipid extract (method of Folch et al. (1957)) spiked with PAHs, were used to optimize the GPC. The method was calibrated first with a GPC standard made of soy oil (high content of TAG) spiked with 2-6 ring PAHs (Meier et al., 2005). Standards of cholesterol and phospholipids extracted from herring roe were also analyzed to determine their elution range. Then the method was applied to a lipid extract of haddock eggs prepared as described by Sørensen et al. (2016). Fractions of the eluent were collected and characterized by thin layer chromatography, as described previously (Meier et al., 2006; Olsen and Henderson, 1989; Sørensen et al., 2016). For sample

clean-up, 900 µL samples were injected and the PAH fraction collected from 10-14 minutes. The collected fractions were concentrated by solvent evaporation (Turbovap LV) prior to analysis.

2.4.2 Extraction of water samples

During the cod and haddock exposure studies, water samples (1 L) were taken from each exposure tank at the beginning, during and at the end of each experiment (total three samples). The samples were acidified (HCl, pH<2) and stored dark and cool (4 °C) until further handling. For characterization of the exposure during the haddock egg passive dosing study, water samples (1 mL) were taken on day 0, 1, 2 and 3 of exposure. Deuterated internal standards were added prior to extraction to account for analyte loss during extraction. The samples were extracted three times by partitioning to solvent (30 mL DCM for 1 L samples, 1 mL 1:1 DCM:*n*-hexane for 1 mL samples) and dried with Na_2SO_4. The sample volume was adjusted by gentle evaporation prior to GC-MS or GC-MS/MS analysis.

2.4.1 GC-MS

The GC-MS system for analysis of passive dosing water samples comprised an Agilent 7890A GC and an Agilent 5975 C MS fitted with a DB5 MS UI column (30 m x 0. 25 mm x 0.25 µm). The carrier gas was helium, at a constant flow of 1 mL/min. Samples (1 µL) were injected in pulsed splitless mode at 250 °C. The oven was held at 40 °C (1 min), ramped by 40 °C/min to 120 °C, by 15 °C/min to 300 °C, and finally by 40 °C/min to 320 °C (7 min hold). The transfer line temperature was 300 °C. The MS was operated at 70 eV in selected ion monitoring (SIM) mode with the ion source at 230 °C and the quadrupole at 150 °C. The analytes were identified by their molecular ion. Quantification was based on average response factors relative to internal standard fluorene-*d*10.

2.4.2 GC-MS/MS

An Agilent 7890 gas chromatograph with an Agilent 7010 triple quadrupole mass spectrometer fitted with an EI source and collision cell was used for analysis of body burden samples and oil exposure water samples (Agilent Technologies, Santa Clara, CA, USA). Two Agilent J&W DB-5MS

239 UI GC-columns (15 m × 0.25 mm x 0.25 µm) were coupled in series through a purged ultimate
240 union (PUU). The carrier gas was helium at constant flow of 1.2 mL/min. For analysis of PAHs
241 from crude oil exposures, the oven was held at 60 °C for 1 min, then ramped to 120 °C by 40
242 °C/min and finally ramped to 310 °C at 5 °C/min. For analysis of 1-isopropyl-4-methyltetralin and
243 1-isopropyl-4-methylindane in passive sampling egg tissue samples, the oven was held at 60 °C
244 for 1 min, ramped to 120 °C by 40 °C/min, and then ramped to 310 °C at 5 °C/min. The
245 temperature was held at 310 °C for 5 minutes, while the first column was back-flushed. The ion
246 source temperature was 230 °C and the quadrupole temperature was 150 °C. N_2 was used as
247 collision gas (1.5 mL/min) and helium was used as a quench gas (4 mL/min). Phenanthrene and
248 deuterated PAHs were identified by two unique multiple reaction monitoring (MRM) transitions
249 and quantified by the most intense peak (Sørensen et al., 2016). 1-isopropyl-4-methyltetralin
250 was identified by transitions 145-91 (CE 25 eV) and 188-145 (CE 10 eV) and quantified by the
251 former. 1-isopropyl-4-methylindane was identified by transitions 131-91 (CE 20 eV) and 174-91
252 (CE 40 eV) and quantified by the former.
253
254 *2.4.3 GCxGC-MS*
255 Analysis of tissue samples by GCxGC-MS was performed using an Agilent 7890A GC (Agilent
256 Technologies, Wilmington, DE) interfaced with a Zoex ZX2 GCxGC cryogenic modulator and an
257 Markes/Almsco Bench Tofdx™ Time of Flight MS. The first-dimension column was a 100%
258 dimethyl polysiloxane (60 m x 0.25 mm x 0.25 µm) Rxi®-1ms, and the second-dimension column
259 was a 50% phenyl polysilphenylene siloxane (2.5 m x 0.25 mm x 0.25 µm) BPX50. Helium carrier
260 gas was used and was kept at a constant flow rate of 1.0 mL/min and samples were injected (1
261 µL) into a 250 °C splitless inlet. The temperature of the first oven was held 35 °C for 1 min,
262 ramped by 5 °C/min to 120 °C, then by 2 °C/min to 280 °C, finally by 5 °C/min to 320 °C and held
263 for 10 min. The temperature of the second oven was constantly offset by +50 °C and the hot jet
264 pulse by +70 °C from oven 1. The modulation times were 4 or 6s. MS parameters were as
265 follows: ionization energy 70 eV, scan speed 50 Hz, scan range m/z 50-550. The MS transfer line
266 temperature was 300 °C and the ion source temperature was 250 °C. Data were collected in
267 ProtoTof and processed using GC Image v2.3. Representative standards of different compound

groups were run to verify retention times. Quantification was achieved using ChromSpace software (provided by Markes International Limited, Llantrisant, Wales, UK), by use of linear regression of responses measured as volumes.

2.5 Statistical analyses

Statistical analyses were conducted using R software (R Development Core Team, 2008). Comparisons between treatments were made using the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test for larvae deformation severity data and one-way ANOVA followed by Tukey's multiple comparisons test for heart rate and biometric data. Significance level was set at $p < 0.05$.

2.6 Ethics statement

All methods were performed in accordance with approved guidelines. Embryos and larvae were frozen in liquid nitrogen immediately upon sampling. The Austevoll Aquaculture Research station has permissions for catch and maintenance of Atlantic cod and haddock given by the Norwegian Directorate of Fisheries. Austevoll Research station has a permit to run as a Research Animal facility using all developmental stages of fish, with code 93 from the Norwegian Animal Research Authority; NARA.

3 Results and discussion

The aim of the current study was to investigate the potential for accumulation and toxicity of currently overlooked petrogenic compounds toward fish ELS. There is a need for a better understanding of which oil compounds are responsible for the severe detrimental effects on developing fish. It is crucial that the most toxic oil compounds are included in risk assessment models. Today the main focus is on the PAHs and there is no doubt that the petrogenic PAHs are toxic to fish ELS (Hodson et al., 2007), but PAHs alone far from explain the observed effects after an oil spill (Barron et al., 1999). In the current study, comprehensive two-dimensional gas

chromatography (GCxGC) was used to elucidate potential bioaccumulating oil compounds fish eggs. Focus was given to monoaromatic compounds in the size range of 3-4 ring PAHs.

3.1 GPC clean-up of fish egg extracts

To serve the study aim of non-target screening of a potentially large range of crude oil derived compounds, the clean-up protocol applied needed to be as non-discriminating as possible. Nonetheless, as previously shown, the lipid contents in fish eggs present analytical challenges if not effectively removed (Sørensen et al., 2016). Gel permeation chromatography (GPC) was applied, since this technique has the ability to separate compound groups based on size (as opposed to chemical properties such as polarity and functionalization), and its applicability toward isolating polar and semi-polar compounds relevant to crude oil has been shown previously (Meier et al., 2005). Significant lipid classes present in cod and haddock eggs include triacylglycerols (TAG), several classes of phospholipids (PL), cholesterol and free fatty acids (FFA) (Bachan et al., 2012; Salze et al., 2005; Sørensen et al., 2016). Initial tests of the method showed that TAGs eluted at 7 mins, while 2-6 ring PAHs eluted in the range 11-13 mins (Fig. S1). Cholesterol eluted at approximately 9.5-10 minutes. The method was then applied to an extract of haddock eggs (Fig. S2). Fractions of the eluent were collected and characterized qualitatively by thin layer chromatography (Table S3), which confirmed that TAGs and most PLs eluted in the earliest fractions. Most crude oil compounds eluted in the fraction collected from 10-14 minutes (Fig. S3). Some break-through of cholesterol and free fatty acids (particularly tetradecanoic, hexadecenoic and octadecanoic acids) was observed when the samples were analyzed by GCxGC-MS. However, their presence did not compromise the analysis, because their retention positions were well separated from those of any compounds of interest.

3.2 Accumulation of monoaromatic and polyaromatic hydrocarbons in crude-oil exposed fish eggs

It was previously determined that crude oil droplets adhere to a greater extent on the chorion of haddock eggs than cod eggs (Hansen et al., 2018; Sørensen et al., 2017). It was furthermore

revealed that the adhered droplets on the haddock chorion lead to a significant increase in the portion of PAHs and alkyl PAHs that accumulated in the internal embryo, compared to the non-fouled cod eggs or haddock eggs exposed only to the WSF of oil. The observed increased internal PAH body burden was also correlated to increased toxicological response. However, PAHs only comprise 0.1-1 % of most crude oils (Bence et al., 1996), and there is a plethora of less characterized groups of compounds that might be of toxicological interest. Therefore, in the present study, the aim was to identify other crude oil compounds that also accumulate in fish embryos during oil exposure, with and without oil droplets adhering to the chorion. Confirming the visual observation of crude oil droplets on the haddock eggs, GCxGC chromatograms of oil exposed (and fouled) eggs showed a similar chromatographic profile to those of crude oil samples, although the egg samples were depleted in the most volatile crude oil compounds (Fig. S4). Compounds considered too large to partition through the chorion and thus likely originating from the adhered oil droplets, such as large alkanes, large cycloalkanes and some petroleum biomarker compounds (e.g. hopanes), were identified in these chromatograms (Fig. S5). These compounds were not detected in either control samples, samples of cod eggs, or WSF exposed haddock eggs. To investigate the partitioning of compounds into the embryo, it was necessary to de-chorionate the eggs prior to analysis (Sørensen et al., 2017), in order to analyze the chorion and embryo separately. Through this analysis, it was confirmed that the larger, oil-related, compounds remained on the chorion. Alkanes, large cyclic alkanes and petroleum biomarkers (e.g. hopanes) were observed in samples of the entire haddock egg and separated chorion, but not in the separated embryos.

A range of monoaromatic compounds was tentatively identified in both the WSF and oil droplet exposed haddock and cod embryos. The structures of the observed compounds were partially elucidated by co-injection of authentic compounds, and comparison of the two-dimensional retention positions and mass spectra of these and the unknowns. Among the identified compound groups were C_{5-10} alkylbenzenes, C_{1-2} cyclohexylbenzenes, C_{0-5} alkyltetralins and alkylindanes. Comparison of GCxGC retention times in first and second dimension with those of co-injected authentic compounds is shown for alkylnaphthalenes, alkyltetralins,

cyclohexylbenzenes and alkylbenzenes in Fig. S6. A comparison between mass spectra of the putative alkyltetralins observed in samples and the mass spectra of alkyltetralins available in a NIST library is shown in Table S4. Although the obtained mass spectra gave indications of the alkylation pattern in the petrogenic tetralins, the availability of only a few synthetic compounds meant that identification of specific isomers was not possible in the current study.

Due to their previously demonstrated toxic potential of these two compound groups (Booth et al., 2008), focus was given to the determination of the accumulation and toxicity of alkyltetralins and indanes in the current study. Fig. 1 shows an example of the elution pattern of possible C_4-alkyltetralins (molecular ion *m/z* 188) found both on the the oil-exposed haddock chorion and inside the embryo following oil exposure. For comparison, the same is shown for C_{0-3} phenanthrenes. C_4-alkyltetralins have molecular weights in the same range as some alkylated phenanthrenes, but are more hydrophobic. It is therefore plausible that the bioaccumulation potential of these compounds is high when fish eggs are exposed to crude oil droplets. By co-injection with the synthesized C_4-alkyltetralin and indanes (Table S1), it was possible to obtain semi-quantitative uptake data for identified peaks in the haddock chorion and embryo samples (Fig. 2). Six C_4-alkylindanes were tentatively identified, of which five were quantifiable in both chorion and embryo samples. Seven C_4-alkyltetralins were tentatively identified, of which six were quantifiable in the chorion sample and one was quantifiable in the embryo sample (Fig. 2).

An interesting and unexpected phenomenon was the selective accumulation of certain isomers of each (C_{1-3}) alkyl phenanthrene groups in the embryo (for instance 4/9-methyl-subsituted phenanthrene), whereas the profile of alkyl phenanthrenes on the chorion was similar to that of the crude oil (Fig. 1). Rather than being caused by selective partitioning through the chorion, it is hypothesized that the phenomenon is caused by a reduced potential for biotransformation of certain sterically-hindered isomers. Less pronounced differences were observed for the alkyltetralins (Fig. 1), and this emphasizes the need for further investigations into the effects of accumulated monoaromatic compounds in fish ELS.

3.3 Uptake and toxicity of monoaromatic compounds in haddock embryos

Since several C_4-alkyltetralins and indanes were observed in the cod and haddock embryos, two monoaromatic compounds (1-isopropyl-4-methyltetralin and 1-isopropyl-4-methylindane) were synthesized for the purpose of performing controlled bioconcentration and toxicity studies. Phenanthrene was also included in these studies and used as a 'positive' control for known accumulation and effects (Incardona et al., 2004). The preparation of solutions aimed for maximum solubility in seawater at the experimental temperature (8 °C), and two dilutions. Samples for egg tissue analysis were taken after three days of exposure. The accumulated body burden is shown in Fig. 3. Compared to observed body burden of comparable compounds after nine days crude oil exposure (shown in Fig. 3 of Sørensen et al. (2017)), these levels are much higher (ng/embryo rather than pg/embryo), reflecting the individual compounds exposure levels.

In the oil exposure studies (Sørensen et al. (2017), haddock embryos were exposed to 300 µg oil/L and the body burdens of C4-tetralin (0.02 ng/embryo), C4-indane (0.05 ng/embryo) and phenanthrene (0.02 ng/embryo) were 600-3000 times lower compared with the highest dose of single compound exposures in the current study; 1-isopropyl-4-methyltetralin (24 ng/embryo), 1-isopropyl-4-methylindane (32 ng/embryo), phenanthrene (63 ng/embryo). The oil exposed embryos were severely damaged (corresponding to a malformation degree of 3 or worse, Fig. 5), while in the single compound exposure, similar severe malformation was only observed in the high dose phenanthrene. It should be mentioned that due to the differences in both exposure system and time, the body burden levels cannot be compared directly between the two studies. Nevertheless, the differences in body burden suggest that these three single compounds we have tested cannot be expected to contribute strongly to the very severe toxicity that are observed in the oil exposed embryos. Oil exposures are extremely complex and the high embryotoxicity is expected to be a result of additive effects (and possibly synergistic effects) of many compounds. (Hodson, 2017).

Compared to obtained water concentrations of each test compounds (Fig. S7), the bioconcentration of the three studied compounds are similar (log BCF ~ 2.6-2.8). Due to differences in obtained water concentration (lower for the monoaromatics compounds), the maximum body burden obtained is also lower in the monoaromatic exposed eggs, so the lowest dose phenanthrene body burden (24 ng/embryo) is comparable to the high dose body burden of alkyltetralin (24 ng/embryo) and alkylindane (33 ng/embryo). This should be kept in mind when evaluating the toxicity endpoints.

No clear dose-response relationship of mortality was observed during the single compound exposure study. Heart rate measurements revealed increased heart rate relative to controls (seawater and non-loaded silicone) in exposures with alkylindane and phenanthrene, but not with alkyltetralin (Fig. S8). Hatching was delayed relative to controls in all exposures, and the delay is linked to both compound and concentration (Fig. 4). Biometric measurements in hatched larvae (3 dph) revealed developmental abnormalities (reduced body and jaw length, as well as reduced eye diameter) only in embryos exposed to phenanthrene at the two higher concentrations (Fig. S9), while significant craniofacial deformations, jaw deformations and pericardial edema was observed also for embryos exposed to the two highest doses of 1-isopropyl-4-methylindane (Fig. 5). In the high dose phenanthrene (85±16 µg/L; 33±2 mg/kg body burden) nearly all larvae were severely malformed.

The effects doses in haddock embryo found for phenanthrene in this study are comparable with what has been reported in zebrafish. Vergauwen et al. (2015) found acute mortality at 310 µg/L (LC_{50}; 120 h) (measured body burden of 485 mg/kg) and sublethal effects (malformation) at 52 µg/L (37 mg/kg body burden). Butler et al. (2016) found similar dose thresholds for acute (334 µg/L, LC_{50}; 120 h) and delayed (44 µg/L LC_{10}; 30 days) mortality in zebrafish. The acute toxicity data from the zebrafish studies fits well with the model for base-line toxicity of nonpolar organics (Butler et al., 2016). To compare data from the current study to literature values, we re-calculated the obtained concentrations from ng/embryo to mmol/kg (haddock egg wet weight was determined 1.9 mg/egg). The tissue concentrations in the current study are below

concentrations expected to cause acute (narcosis) effects (<0.2 mmol/kg compared to 2-8 mmol/kg) (McCarty and Mackay, 1993).

Crude oil toxicity in fish ELS at environmental relevant concentration are most often associated with delayed mortality (not acute toxicity). The developing heart has been identified as the primary target of crude oil developmental toxicity (Incardona, 2017; Incardona and Scholz, 2016). Exposure during key periods of embryonic heart development leads to a gradient of oil exposure phenotypes that is concentration-dependent and ranges from outright heart failure with accumulation of edema fluid to more subtle heart malformation. At the high end of this gradient, irreversible heart failure leads to a cascade of secondary effects from loss of circulation and accumulation of edema fluid, resulting in gross spinal and craniofacial abnormalities (Sørhus et al., 2017; Sørhus et al., 2016). At this level of severity, affected fish have jaw deformities and reduced swimming that preclude feeding, and they die as larvae (Hicken et al., 2011; Incardona et al., 2013). In the present study all the larvae with malformation severity degree 2 and 3 (Fig 5, Fig. S10) can be considered to be ecologically dead; they will not have the ability to catch and eat prey either due to destroyed jaws or disrupted swimming behavior. The damaged larvae will be easy prey to natural predators.

Three-ring PAHs, like phenanthrene, are proven to induce cardiotoxicity in fish embryos (Brette et al., 2017; Incardona et al., 2004). However, as shown in the current study, single compound exposure of phenanthrene requires more than thousand times higher exposure dose to generate the same severe malformation in haddock embryo as what is observed in oil exposure studies. While the crude oil exposures are very complex, severe malformations of fish larvae was found in oil exposed embryos at only 3.5 µg total PAH/L exposure concentration (corresponding to 3.3 ng total PAH/embryo body burden) (Sørensen et al. (2017)). In the current study, phenanthrene only gave similar toxic response at a dose of 85 µg/L (63 ng/embryo body burden). This strongly suggests that other compounds than PAHs also contribute to toxicity in the oil exposed embryos.

Based on the current study and the combined research knowledge available, it is proposed that all future crude oil bioaccumulation studies take advantage of the resolution power offered by GCxGC, preferably in combination with high-resolution mass spectrometry for identification of peaks. Knowledge obtained regarding bioaccumulation potential of several petrogenic compound groups should then be combined with targeted effects-directed chemical fractionation of oil, to allow better understanding of which compound groups and what mixture are driving the toxicity in towards fish ELS. Furthermore, it is suggested that more attention is given to the potential toxic effects of metabolites of PAHs and other oil compounds. It is thus a need for developing more sensitive methodologies for analyzing metabolites in small biogenic samples, such as fish ELS.

4 Conclusion

In this study, several groups of petrogenic monoaromatic compounds were identified in cod and haddock embryos after exposure to dispersed crude oil. Although the toxicity of these compounds has been evaluated in only a limited number of studies, they have been proven detrimental to marine species. To investigate the potential toxicity of such compounds to fish embryos, two monoaromatic compounds (1-isopropyl-4-methyltetralin and 1-isopropyl-4-methylindane) were synthesized and subjected to haddock embryo toxicity assay using passive dosing as an exposure pathway. Although the monoaromatic compounds were observed to have comparable bioconcentration factors to phenanthrene, the total uptake was lower, due to the lower concentrations which could be solubilized in seawater by passive dosing. The monoaromatic compounds caused dose-dependent delayed hatching in the exposed embryos. Small, but statistically significant effects, including craniofacial and jaw deformations and pericardial edemas, were also observed at the highest doses of 1-isopropyl-4-methylindane. The results of the current study suggest a need for more research on the sublethal effects of monoaromatic compounds toward fish ELS. This would require additional work on identifying and synthesizing relevant compounds of interest. Of particular interest, would be the study of possible synergistic effects of co-exposure of monoaromatic compounds and PAHs.

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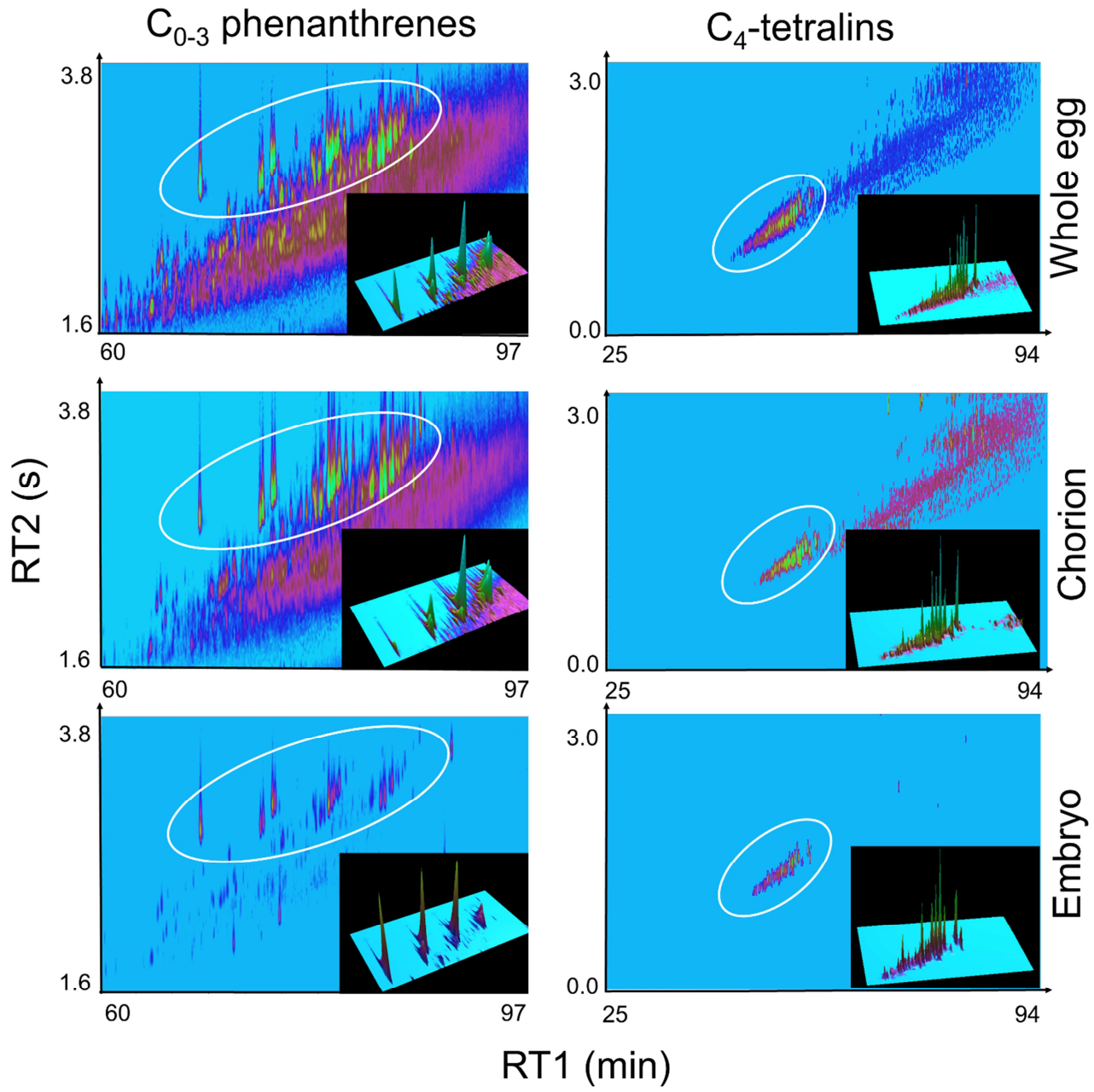
Fig. 1 GCxGC-chromatograms of whole egg, chorion and embryo tissue samples of crude oil exposed haddock. The x-axis (RT1) shows relative retention times in the first dimension (apolar), while the y-axis (RT2) show relative retention times in the second dimension (polar). Circled are extracted peaks for m/z 178, 192, 206 and 220 (molecular masses of C_0-C_3-phenanthrenes) as well as extracted peaks for m/z 188 (molecular mass of C_4-tetralins) in haddock eggs, haddock egg chorion, and haddock embryo separated from the chorion.

Fig. 2 C_4-indanes and C_4-tetralins measured in embryo and chorion samples of crude oil exposed haddock eggs (nine days exposure).

Fig. 3 Body concentrations of phenanthrene (PHE), 1-isopropyl-4-methyltetralin (TET), and 1-isopropyl-4-methylindane (IND) during passive dosing exposure at three different doses. Concentrations in seawater (SW) and silicone controls (Sil Ctrl) samples are shown as reference. Error bars represent standard deviation (n=3).

Fig. 4 Cumulative hatching success (% of embryos surviving to hatch that hatched and at what day of development) of embryos exposed to phenanthrene (PHE), 1-isopropyl-4-methyltetralin (TET) and 1-isopropyl-4-methylindane (IND) at three different doses, viewed relative to seawater (SW) and silicone controls (Sil ctrl).

Fig. 5 Deformation severities in larvae 3 days post hatching after embryonic exposure to phenanthrene (PHE), 1-isopropyl-4-methyltetralin (TET) and 1-isopropyl-4-methylindane (IND) at three different doses plotted as a function of measured body burden (ng/embryo) and viewed relative to pure seawater (SW) and silicone controls (Sil Ctrl). Error bars represent standard error of the mean. Images of a control larvae is provided on top, and examples of larvae with different degrees of deformation severities (1, 2 and 3, bottom to top) is provided on the right side of each graph (1 mm scale bar indicated). Statistical differences between sea water controls and exposed fish (N=31-67 for different groups), using the non-parametric Kruskal-Wallis test, are given as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.



674

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676 **exposed haddock. The x-axis (RT1) shows relative retention times in the first dimension**
677 **(apolar), while the y-axis (RT2) show relative retention times in the second dimension (polar).**
678 **Circled are extracted peaks for m/z 178, 192, 206 and 220 (molecular masses of C_0-C_3-**
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680 **haddock eggs, haddock egg chorion, and haddock embryo separated from the chorion.**
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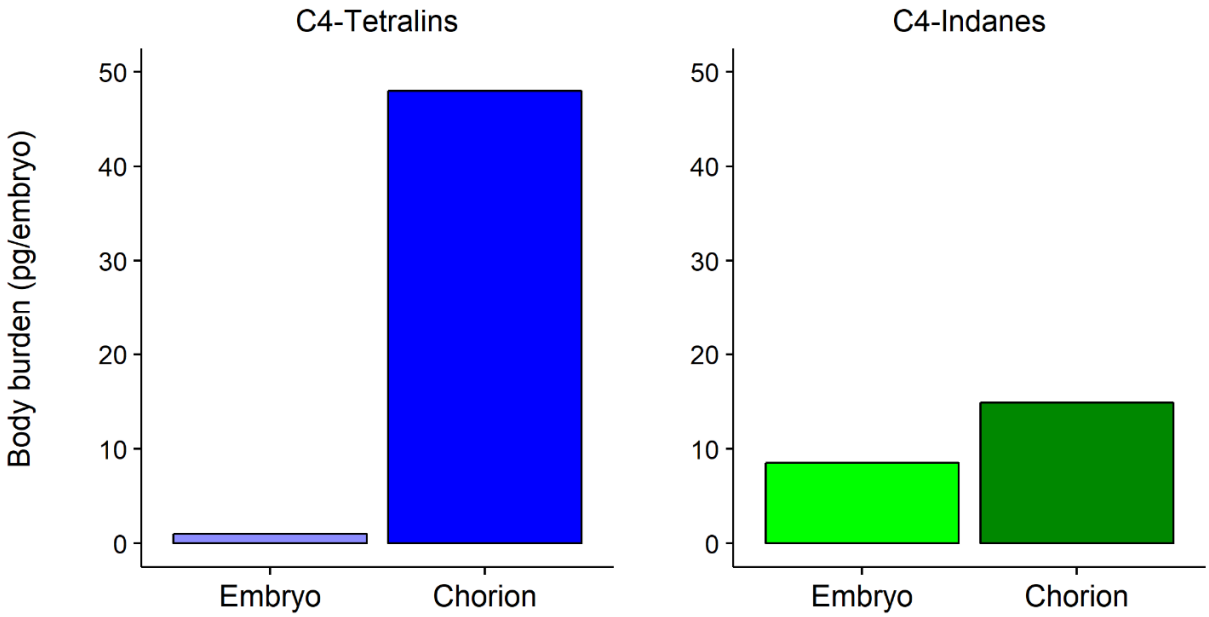

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685 **Fig. 2 C_4-indanes and C_4-tetralins measured in embryo and chorion samples of crude oil**
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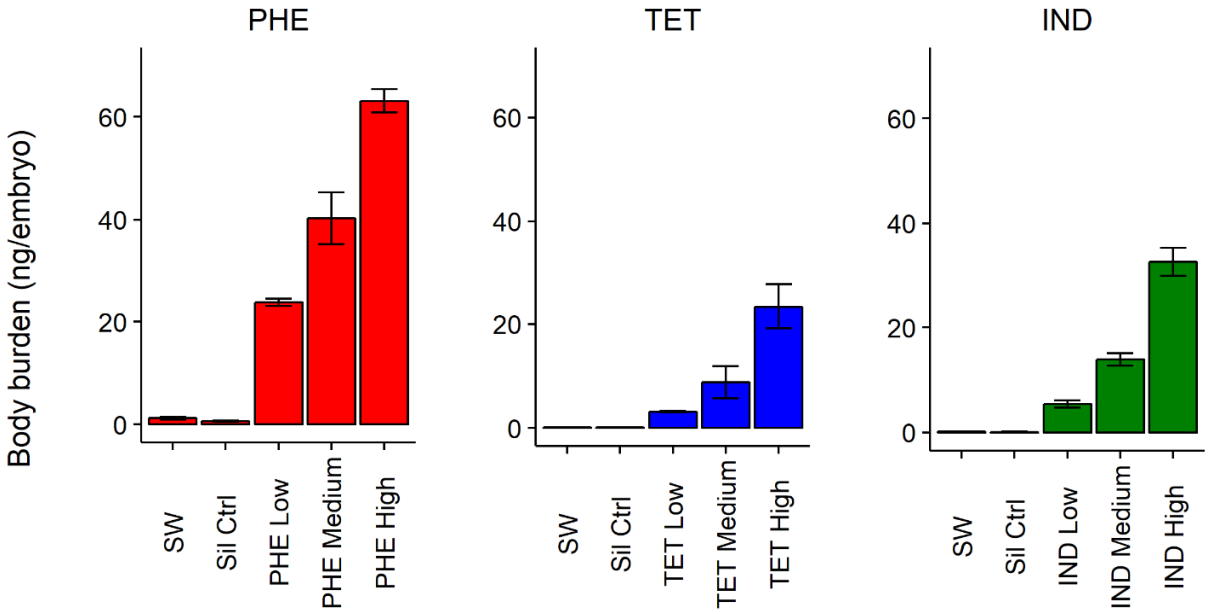

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689 **Fig. 3 Body concentrations of phenanthrene (PHE), 1-isopropyl-4-methyltetralin (TET), and 1-**
690 **isopropyl-4-methylindane (IND) during passive dosing exposure at three different doses.**
691 **Concentrations in seawater (SW) and silicone controls (Sil Ctrl) samples are shown as**
692 **reference. Error bars represent standard deviation (n=3).**

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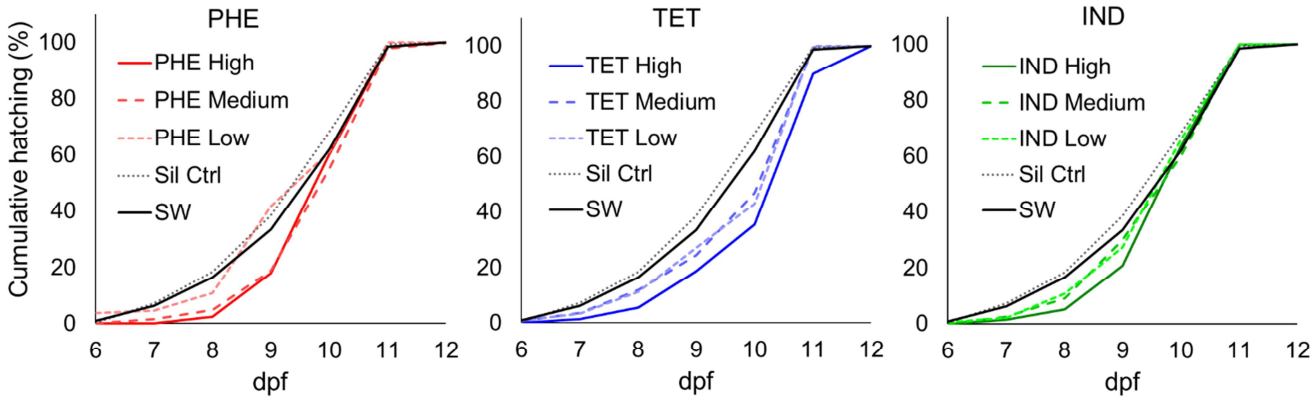

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695 **Fig. 4 Cumulative hatching success (% of embryos surviving to hatch that hatched and at what**
696 **day of development) of embryos exposed to phenanthrene (PHE), 1-isopropyl-4-**
697 **methyltetralin (TET) and 1-isopropyl-4-methylindane (IND) at three different doses, viewed**
698 **relative to seawater (SW) and silicone controls (Sil ctrl).**

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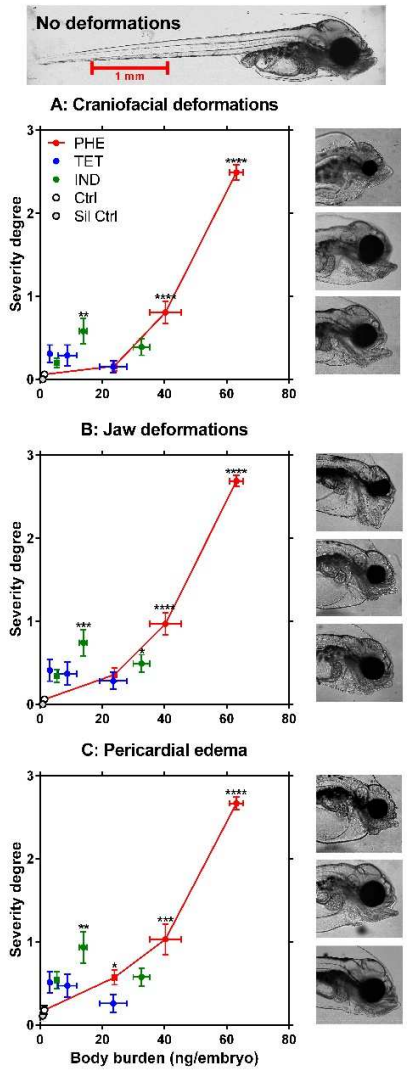

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701 **Fig. 5 Deformation severities in larvae 3 days post hatching after embryonic exposure to**
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703 **at three different doses plotted as a function of measured body burden (ng/embryo) and**
704 **viewed relative to pure seawater (SW) and silicone controls (Sil Ctrl). Error bars represent**
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706 **larvae with different degrees of deformation severities (1, 2 and 3, bottom to top) is provided**
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708 **water controls and exposed fish (N=31-67 for different groups), using the non-parametric**
709 **Kruskal-Wallis test, are given as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.**

Highlights

- Monoaromatic compounds were found to accumulate in crude oil exposed haddock and cod embryos
- Two model compounds were synthesized and bioconcentration and toxicity tested using passive dosing
- Monoaromatic compounds displayed sublethal toxicity towards haddock embryos