Differences in biomarker and behavioral responses to native and chemically dispersed crude and refined fossil oils in zebrafish early life stages

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HIGHLIGHTS
• Oil-specific (sub-)lethal effect concentrations for oil spill risk assessment were established.
• Oil types induced different strength of embryo toxicity not correlated with common PAHs.
• Sublethal exposure concentrations resulted in CYP1A induction but no AChE inhibition.
• Larvae showed strong behavioral impairment already at low exposure concentrations.
• Dispersant contributes to higher toxicity of chemically dispersed oils compared to native oils.

GRAPHICAL ABSTRACT

ABSTRACT
Petroleum products including crude oils and refined distillates are unique environmental pollutants consisting of thousands of compounds with varying physical-chemical properties and resulting toxicity for aquatic biota. Hence, for a reliable risk assessment individual petroleum product toxicity profiles are needed. Furthermore, the influence of oil spill response strategies like the application of chemical dispersants has to be implemented.

The present study addressed the toxicity of water-accommodated fractions (WAFs) of two different oil types on fish early life stages on different biological organization levels in the laboratory model species Danio rerio. Experiments with a 3rd generation dispersant used in loading rated resembling the exposure in experiments with chemically dispersed oils were included, enabling a direct comparability of results. This approach is of high importance as especially the investigation of dispersant toxicity in relevant exposure concentrations is rather scarce. Zebrafish embryos were exposed to different WAFs shortly after and up to 120 hour post fertilization (hpf). Besides phenotypic effects including edema and spine deformations, reduced responses to dark stimuli, increased CYP1A activity and marginal AChE inhibition were observed in sublethal effect concentrations. Both oil types had varying strength of toxicity, which did not correlate with corresponding chemical analysis of target PAHs. Chemically dispersed oils induced stronger acute toxicity in zebrafish embryos compared to native (initial) oil exposure, which was further reflected by very low exposure concentrations for biomarker endpoints. Based on a comparison to the dispersant alone, a higher toxicity of dispersed oils was related to a combination of dispersant

Keywords: AChE, Dispersant toxicity, Embryo toxicity, EROD, Oil spill assessment, Oil toxicity

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1. Introduction

With steadily increasing oil production, consumption, transportation and rapidly moving exploration (Fritt-Rasmussen et al., 2018; Shafiee and Topal, 2008) huge and small diffuse oil spills still pose a high risk for the aquatic environment. In order to accomplish a comprehensive risk assessment of the individual situation, important knowledge about regional ecology, the key oil compounds as well as the ecotoxicological risk of the spilled oil is required (Baker, 1995; Taylor et al., 2018). Petroleum products such as crude oils and refined distillates or blended mixtures of fuels each are unique environmental pollutants consisting of thousands of compounds with varying physical-chemical properties (Singer et al., 2000). As a consequence, the variety of characteristics might lead to a range of potential exposure scenarios and resulting strength of toxicity for aquatic biota. Against the background of a rapid decision making after an oil spill, the unique characteristics highlight the importance of individual petroleum product toxicity profiling.

Especially embryonic and larval stages of developing fish have been found to be highly sensitive towards the exposure to water-accommodated fractions (WAF) (de Soysa et al., 2012; Jung et al., 2013; Xu et al., 2016). It has been concluded that the phenotypical adverse effects caused by oil components in fish embryos are markedly conserved across several freshwater and marine laboratory as well as endemic species of oil-impacted areas (Hodson, 2017; Incardona, 2017; Incardona et al., 2013). Frequently reported common adverse effects in fish embryos include craniofacial deformations (de Soysa et al., 2012; Hansen et al., 2019), cardiotoxicity (Brette et al., 2014; Incardona et al., 2013; Jung et al., 2013; Khursigara et al., 2017; Paula et al., 2011; Philibert et al., 2016) and impaired swimming behavior patterns (Hicken et al., 2011; Mager et al., 2017; Philibert et al., 2016). Especially the measurement of biomarker activities in fish early life stages can be sensitively indicative for active defense mechanisms or severe damage caused by oil components. With respect to the toxicity of PAHs and co-planar structures especially the phase I oxidation during biotransformation, catalyzed by CYP enzymes is a reliable and highly sensitive biomarker and measured as the EROD activity (Sanni et al., 2017; Van der Oost et al., 2003). Also neurotoxicity is emerging as an important endpoint in ecotoxicological risk assessment with biomarker measurements (in this study AChE inhibition) and swimming behavioral assays as suitable screening tools already at early life stages (Legradi et al., 2018). The combination of different biomarker measurements can improve the knowledge on toxicity mechanisms and indicate adverse effects even before visible at phenotypical level.

In the context of oil spill toxicity assessment, alterations in the exposure scenarios caused by different oil spill response strategies have to be considered. The deployment of chemical dispersants is one important measure for wide-area response. Hence, especially in the case of a larger oil spill, it is quite likely that the exposure situation gets altered with the introduction of another chemical mixture. Through reducing the interfacial tensions and breaking down the oil slick, dispersants minimize the risk of smothering seabirds and coastal shorelines. However, besides being a chemical burden by itself, this response action results in the enrichment of the water column with dissolved and particulate crude oil compounds, thus potentially increasing the exposure of the pelagic community towards potentially toxic oil compounds (Dupuis and Ucán-Marin, 2015; Ramachandran et al., 2004). Even though chemically dispersed petroleum products had been addressed intensively, the knowledge of toxic effects attributed to the dispersant itself being a complex mixture of surfactants and hydrocarbon solvents in relevant exposure concentrations is rather scarce and conclusions about the benefit of dispersant use were contradictory (Prince, 2015).

Against this background, the present study addressed the toxic impact of two different oil types on early life stages of zebrafish (Danio rerio). The zebrafish is a well-established laboratory teleost model species in ecotoxicology and has already been proven a valid test organism in the context of oil spill research (de Soysa et al., 2012; Incardona et al., 2013; Paula et al., 2011). By investigating morphological effects and mechanism-specific endpoints (biomarker activity, behavior changes) at low and simultaneously more environmentally relevant exposure concentrations, the study in detail focused on a) establishing individual petroleum toxicity profiles due to individual chemical composition and b) the dispersant role in chemically dispersed petroleum products by including experiments with a 3rd generation dispersant alone in loading rates resembling the exposure in the experiments with chemically dispersed crude oil, thus enabling a direct comparability of results. Through comparing effects of chemically dispersed oil and dispersant alone it was intended to understand whether and to what extent the dispersant contributes to acute fish embryo toxicity.

2. Material and methods

2.1. Petroleum product and dispersant samples

The present study focused on two different petroleum products of varying processing degree. As a crude oil a naphthenic North Sea crude oil (NNS) characterized by low viscosity and high proportion of low molecular weight saturates and aromatics was selected. Additionally, a distillate marine gas oil (MGO), representing a commonly used fossil fuel, was used. The MGO is supplemented with the green dye Dyeguard Green MC25 produced by John Hogg Technical Solutions. Both oil types were tested as native (initial) samples without additional artificial weathering or any other treatment. Representative for a typical oil spill response technique, the 3rd generation dispersants Finasol OSR 51® and 52® (Total Special Fluids, France) were included. Finasol OSR 51® was combined with the NNS, while chemically dispersion of MGO was realized using Finasol OSR 52®. In order to investigate the influence of dispersants on petroleum product toxicity, Finasol OSR 51® was additionally investigated as representative for an exposure to dispersant alone. Dispersants and specific oil combinations were selected based on recommendations with respect to oil type treatment. An overview of the different approaches can be found in Table 1.

2.2. Preparation of water-accommodated fractions (WAF) for fish exposure

In general, low-energy water-accommodated fractions (LEWAF) for native (initial) petroleum product alone, chemically enhanced water-accommodated fractions (CEWAF) for dispersed petroleum product and high energy water-accommodated fractions (HEWAF) for dispersant alone were prepared according to Singer et al. (2000). Briefly, WAFs were prepared in aspirator glass flasks (500 mL) by application of oil (LEWAF), a dispersant oil mixture (CEWAF; dispersant-to-oil ratio (DOR) 1:10) or dispersant alone (HEWAF) on the surface of fish medium at an oil/dispersant-to-water ratio of 1:50 (LEWAF), 1:200 (CEWAF) or 1:2000 (HEWAF). The LEWAF setup was carefully stirred avoiding a vortex while for the CEWAF and HEWAF setup stirring speed was adjusted to create a 25% vortex of the water column. WAFs were incubated stirring at 10 °C for 40 h followed by 1 h settling time.
Dilution series were prepared in fish medium from the 100% stock solutions that had been drained off carefully, and were warmed up to 26 °C before embryos were exposed to the samples. Artificial fish medium was prepared, aerated, warmed up and adjusted to a pH of 7.5 ± 0.5 one day before using.

2.3. Zebrafish culture and egg production

This study utilized wildtype zebrafish of the WestAquarium strain (Bad Lauterburg, Germany) from the facilities of the Institute for Environmental Research, RWTH Aachen University. The breeding groups consisted of 100–120 adult zebrafish from 1 to 3 years of age. Zebrafish were kept in 170 L tanks in a flow-through system with a water exchange rate of 40% per week and a biological filter and UV light as cleaning steps. Fishes were fed twice a day with dry flakes (TetraMin®, Tetra GmbH, Melle, Germany) and larvae of Artemia spec. (JBL GmbH & Co. KG, Neuhofen, Germany). A constant day-night rhythm (14:10) and temperature (26 ± 1 °C) was maintained. Spawning took place from 30 min after the onset of light. Fertilized eggs were collected 2 h after the onset of spawning.

2.4. Zebrafish embryo exposure

Shortly after fertilization embryos were divided in groups of 5 individual and transferred into 10 mL air sealed glass vials with sparsely head space containing the exposure solutions. The glass vials have been proven to minimize the evaporation of volatile WAF compounds in pretests. For the fish embryo acute toxicity test (FET) 20 embryos per treatment dilution were exposed to WAF samples according to the OECD guideline 236 (OECD, 2013). To investigate the swimming behavior 16 (control) and 20 (treatment) individual embryos were used per treatment dilution, while for 7-Ethoxyresoru (control) and 20 (treatment) individual embryos were transferred to the samples. Artifi cial fish medium from the 100% stock solution was prepared fresh daily. All zebrafish experiments of the present study included negative (fish medium, all endpoints) and positive (3,4-dichloranilin (4 mg L⁻¹), FET) controls to verify test validity and basal biomarker responses.

2.5. Fish embryo acute toxicity test (FET)

The prolonged fish acute embryo toxicity test up to a maximum of 120 hour post fertilization (hpf) was performed according to OECD guideline 236 (2013) with minor modifications with respect to sample type specifications (details described below). All experiments were terminated with the final measurement shortly before 120 hpf. Zebrafish embryos and larvae below 120 hpf are not protected animal stages according to EU Directive 2010/63/EU (European Parliament and Council of the European Union, 2010) (see also: (Strähle et al., 2012), TierSchG (Tierschutzgesetz) and the respective regulation TierSchVerV (Tierschutz-Versuchsierverordnung). After termination, larvae were euthanized by prolonged immersion in a benzoic acid solution.

Observation of embryonic development was carried out throughout the exposure period every 24 h and lethal and sublethal effects according to criteria defined in the OECD guideline 236 were documented. Additionally, medium pH was measured every 24 h. An experiment was classified valid if no >10% of negative control and at least 30% of positive control eggs showed lethal effects according to the OECD 236 guideline. All data presented in the present study (3 independent experiments) were valid according to these criteria.

In order to identify sublethal effect concentrations, a 4-parameter non-linear regression model (GraphPad Prism v 6, San Diego, USA) was used to fit a concentration-response curve to the data. Top and bottom were fixed to 0 and 100, respectively. Equation: \( Y = 100 / (1 + 10^{(\text{logEC50-X}) \times \text{HillSlope}}) \).

2.6. Zebrafish swimming behavior in a light/dark transition test

Larval swimming behavior alterations were investigated using 96 hpf zebrafish larvae and a light/dark transition test. 16 (control) to 20 (WAFs) zebrafish larvae per treatment concentration were transferred individually alongside 200 μL exposure solution into wells of a 96-well plate. They were exposed to 2 cycles of alternating light (10 min) and dark (4 min) periods after an initial acclimatization period of 10 min. The swimming performance of control and WAF-treated larvae in sublethal effect concentrations was tracked during the experiment. Experiments were conducted using a DarioVision observation chamber and EthoVision tracking software (Noldus, The Netherlands). Data processing was conducted in spreadsheets (Microsoft Excel 2016). In detail, the mean distance moved of 16–20 larvae per treatment and 1 min time bin was used for the evaluation of swimming patterns. In total, 3 independent experiments were performed and hence data were plotted and analyzed as mean (3 experiments) of mean (20 individuals per experiment) distance moved per 4 min interval in order to ensure a comparability of light and dark phase. Graphs were plotted using the software GraphPad Prism (version 6, San Diego, USA).

2.7. Biomarkers in whole-specimen homogenates

Biomarker activity in unexposed and WAF-treated hatched zebrafish larvae was investigated at 96 and 120 hpf. In total, 40 embryos per treatment concentration were pooled after the exposure, anesthetized using a saturated benzoic acid solution and washed twice with cold phosphate buffer saline (PBS, Sigma-Aldrich). Anesthetized larvae were quickly transferred to 1.5 mL tubes and excess solution was replaced with 700 μL of phosphate buffer (1.8 L 0.1 M Na₂HPO₄ adjusted with 0.5 L 0.1 M NaH₂PO₄ to pH 7.8). Larvae were immediately shock frozen in liquid nitrogen and stored at ~80 °C until further use.
2.7.1. Preparation of homogenates

For measurement of enzyme activity larvae in buffer were carefully thawed on ice and homogenized for 10 s using an electric dispersing device (VDI 12, VWR International GmbH, Germany). Afterwards, homogenates were centrifuged for 15 min at 4 °C and 10,000g. Subsequently, the supernatant was instantly transferred to new tubes and placed on ice. Both EROD and AChE were measured successively in order to avoid re-freezing and thawing of the samples.

2.7.2. Measurement of 7-ethoxyresorufin-O-deethylase (EROD) activity

The investigation of phase I metabolism induction as a biomarker of exposure to PAHs and similar co-planar structures was conducted by means of the fish embryo 7-ethoxyresorufin-O-deethylase (FE-EROD) assay. The test was performed according to the protocol described by Schiwy et al. (2015) with modifications regarding egg number and quantification method (kinetic measurement) as described in Meyer-Alert et al. (2019). Sample supernatants of treated and control larvae were tested in triplicates on a 96-well plate. The substrate 7-ethoxyresorufin (2.4 μM) was added to each well and subsequently incubated for 10 min at 26 °C in darkness. Shortly before the kinetic measurement of fluorescence for 25 min (step 1: kinetic cycles: 15, interval time: 20 s, step 2: kinetic cycle: 30, interval time: 40s) in a microplate reader (Infinite® M 200, Tecan Group, Switzerland), NADPH (3.35 mM) was added to initiate the enzymatic reaction. Substrate deethylation was determined by measuring the formed resorufin at 540 nm excitation and 590 nm emission wavelength. Quanti

2.7.3. Measurement of acetylcholinesterase (AChE) activity

As a surrogate endpoint for neurotoxicity the acetylcholinesterase (AChE) activity was measured according to the initial protocol established by Ellman et al. (1961) with modifications described by Velki et al. (2017) regarding adoptions to a 96-well plate format. 7.5 μL sample supernatant and assay reagents (180 μL sodium phosphate buffer (0.1 M, pH 7.8), 10 μL 5.5'-dithiobis-2-nitrobenzoic acid (DTNB 1.6 mM), 10 μL acetylcholine iodide (156 mM)) were added in triplicates to a 96-well plate. The increase in absorbance was immediately measured in triplicates at 412 nm for 10 min in 10 s intervals using a microplate reader (Infinite® M 200, Tecan Group, Switzerland).

Resulting data were controlled for linearity in absorbance increase (R² ≥ 0.98) and minimum increase of absorbance over time (ΔA3min ≤ 0.1). Only data fulfilling the control criteria were used for further evaluation. Enzymatic activity was calculated as nmol AChE min⁻¹ mg⁻¹. For the calculations the molar extinction coefficient of 13,600 M⁻¹ cm⁻¹ was used. Data processing including normalization was performed as described for the EROD activity.

2.7.4. Measurement of protein

In parallel to enzyme activity measurements, protein in whole-specimen homogenates was determined. A Bicinchoninic Acid kit (Sigma Aldrich GmbH, Germany) was used according to the manufacturer’s instructions. Protein quantification was carried out with a dilution series of bovine serum albumin (BSA) as an external standard (0.31–1 mg mL⁻¹). For protein measurement the sample supernatants were diluted 1:2.

2.8. PAH analysis in water-accommodated fractions

Solid phase micro extraction (SPME) as described in detail in Poter and Pawliszyn (1994) was used to quantify a set of 18 target PAHs in native LEWAFS of both oil types. Details can be found in the SI (Section 1).

3. Results

3.1. Acute toxicity on zebrafish embryonic development

Native and chemically dispersed WAFs from both oil types induced a concentration-related increase in sublethal and lethal effects (Fig. 1) in the prolonged fish embryo acute toxicity test (FET). While zebrafish embryo exposure to undiluted LEWAF stocks (1:50) of both oil types led to 100% mortality in 120 hpf larvae, the CEWAF stocks (1:200) were markedly more toxic to zebrafish embryos. Dilutions of 12.5% (NNS) and 5% (MGO) still induced 100% mortality at the test end. In a direct comparison of both oil types, a higher embryotoxicity was observed for the distillate in both LEWAF and CEWAF approaches (Fig. 1). Calculated EC50 values of MGO with 25.2% (LEWAF) and 1.1% (CEWAF) of stock were below the EC50 values resulting from NNS exposure (33% for LEWAF and 2.0% for CEWAF, Table 2).

In order to ensure a direct comparability of dispersant HEWAF to the (NNS) CEWAF approach, the resulting EC50 value was expressed as % of 1:200 CEWAF stock containing corresponding dispersant amounts (DOE: 1:10). Similar to LEWAF and CEWAF the exposure to the dispersant alone did induce concentration-related embryotoxicity. With 50% of zebrafish larvae at 120 hpf showing malformations at exposure dilutions of 4.4% of stock (EC50), the HEWAF toxicity was in a comparable range but still below the acute toxicity induced by the corresponding CEWAF (2.0%). Importantly, the embryotoxicity of the dispersant HEWAF, in contrast to oil LEWAF and CEWAF, was strongly dependent on the embryonic developmental stages. Embryos that were protected by the chorion did not show any harmful effect even for the highest exposure concentrations. After hatching set in around 72 hpf, these normal developed embryos died until the final inspection at 120 hpf (coagulation).

In general, most prominent sublethal morphological effects on LEWAF- and CEWAF-treated embryos were heart deformations, pericardial and yolk sack edema associated with bradycardia as well as spine deformations independent of the oil type (SI, Fig. 1A–C). In contrast, no cardiovascular effects were observed for the HEWAF treatment, but hatched larvae showed deformations of caudal fins, mainly manifested in nodes occurring in fin ray structures (SI, Section 2, Fig. 1D). Fin deformations of HEWAF-exposed embryos were observed for up to 60% of embryos at test end. Interestingly, while hatching success was not affected for MGO-exposed zebrafish embryos, NNS caused significantly reduced hatching rates in sublethal exposure dilutions at 120 hpf (Fig. 2). Also HEWAF-exposed larvae showed a tendency towards hatching delay for 72 and 96 hpf embryos compared to the untreated control.

3.2. EROD activity

In general, the chemically dispersed oils (CEWAF) showed the trend to stronger EROD induction compared to the native oils (LEWAF, Fig. 3). Zebrafish embryos exposed to LEWAFs of both oil types did show marginally increased EROD activities up to 2-fold normalized to the untreated control with the exception of larvae at 120 hpf exposed to MGO LEWAF, reaching a maximum induction of 3.1-fold. In contrast, while for the crude oil CEWAF no changes in EROD activity were detected in 96 hpf larvae, a concentration-related increase in CYP450IA induction up to a maximum of 4.7-fold at the highest exposure
ANOVA with Dunnett’s post hoc test, p<0.05. AChE activity

No clear concentration-related AChE inhibition was observed (Fig. 4), and also no significant difference could be detected (One-Way ANOVA with Dunnett’s post hoc test, p<0.05). However, sublethal exposure dilutions of crude oil (12.5 and 25% of stock) resulted in decreased AChE activities at 96 hpf (50% of NC). All remaining exposure concentrations, independent of oil type or developmental stage, led to AChE activities corresponding to their unexposed control. Additionally, also the dispersant Finasol OSR 51® did not show AChE inhibition.

3.4. Swimming behavior

Unexposed control larvae did respond to the photo-alternating cycles in a normal manner, with high activity during dark and low activity during light phases (Fig. 5). In contrast, native and chemically dispersed MGO treatments led to a constantly low baseline swimming activity independent of the light/dark transitions in all exposure concentrations. Larvae exposed to NNS crude oil showed similar behavior of no response to dark stimulus. Detailed locomotion patterns of NNS treatments will be published and described within a separate study. No differences in response to the dark stimulus were observed between LEWAF and CEWAF exposure groups.

3.5. PAH analysis in water-accommodated fractions

The most prominent target PAHs in undiluted WAFs from both oil types were naphthalene, phenanthrene and fluorene. Naphthalene was found in concentrations >290 μg L⁻¹ with higher concentrations in the NNS, while fluorene and phenanthrene were detected in the low μg L⁻¹ range. All remaining target PAHs were in the low ng L⁻¹ range. Details can be found in the SI (Table 1).

4. Discussion

4.1. Acute zebrafish embryo toxicity

Morphological effects including cardiotoxicity and spine deformations were in compliance with previous studies focusing on WAF-induced acute toxicity in early life stages of zebrafish (de Soysa et al., 2012; Incardona et al., 2005; Incardona et al., 2013; Jung et al., 2013; Pauka et al., 2011; Perrichon et al., 2016) as well as other species (Jung et al., 2015; Khursigara et al., 2017; Martin et al., 2014; Rodrigues et al., 2010; Xu et al., 2017) for a variety of other crude and refined petroleum products compared to the present study. Overall, a higher toxicity of the distillate compared to the crude oil was observed. This, however, did not correlate with the chemical profile of target PAHs. With the exception of higher naphthalene concentration in NNS crude oil, the concentrations of other target PAHs were only marginally increased in MGO LEWAF (see SI, Table 1). The lack of effect explanation from the present chemical analysis, mainly based on the US-EPA priority PAHs, indicates that either PAH-derivates or other uncharacterized WAF compounds were responsible for the differences in embryotoxicity, which was concluded also in previous studies (Anderson and Achten, 2015; Meador and Nahrgang, 2019). Especially alkylated PAHs and crude oil fractions containing alkylated congeners have been found to induce much stronger embryotoxicity than the mother compounds (Adams et al., 2014; Incardona, 2017). Furthermore, it has to be considered that the MGO is supplemented with a green dye. The toxicity of this additive could not be addressed in the present study and hence a contribution of the dye towards increased toxicity cannot be excluded.

Phenotypical adverse effects have been attributed mainly to tricyclic PAHs and their alkylated derivates, which act via AhR-dependent and independent mechanisms - in the latter case often through interruptions of calcium signaling (Brette et al., 2014; Brette et al., 2017; Hodson, 2017; Incardona, 2017; Sarhus et al., 2016). In contrast, it was recently suggested that crude oil toxicity might be linked to narcosis instead of receptor-mediated pathways (Meador and Nahrgang, 2019).

With focus on molecular processes in oil exposed fish larvae based on

Table 2

Effect concentrations (EC) of the prolonged fish embryo acute toxicity test (120 hpf) with D. rerio exposed to WAF dilutions. EC values were calculated based on a 4-parameter non-linear regression model with variable slope (see Fig. 1).

|             | LEWAF | CEWAF (HEWAF) |
|-------------|-------|---------------|
|             | EC₁₀ [%] | EC₉₀ [%] | EC₁₀ [%] | EC₉₀ [%] |
| Naphthenic North Sea crude oil (NNS) | 23.2 | 33.0 | 1.2 | 2.0 |
| Marine gas oil (MGO) | 15.6 | 25.2 | 0.7 | 1.1 |
| Dispersant Finasol OSR 51® | 2.7 | 4.4 |  |  |

* Finasol OSR 51® alone was tested as high energy water-accommodated fraction (HEWAF) with DOR rates corresponding to CEWAF preparation (n = 2 (HEWAF) + 3 (LEWAF, CEWAF)).
whole transcriptional or microarray analyses, it becomes obvious that WAFs, being complex pollutant mixtures, initiate multiple and simultaneously interacting molecular mechanisms (Hook et al., 2010; Xu et al., 2016), and that the pathways of crude oil toxicity remain partially unknown.

Overall, despite identical phenotypical effects the different strength in toxicity caused by the two oil types emphasizes again the importance of individual ecotoxicological profiles in the context of a reliable risk assessment. In general, it has to be mentioned that the present experimental setup with respect to WAF stock solutions and chronic embryo exposure displayed a worst case scenario. The present concentrations of dissolved oil compounds exceeded concentrations found in the environment after an oil spill. Mean total PAH concentrations in the water column during the Deepwater Horizon Blowout were, for example, mainly <10 μg L⁻¹ (Echols et al., 2015), which is 10 times below the effective concentrations found for the WAF investigated in the present study. Additionally, as already discussed previously, chronic exposure without a recovery phase is not representative for open water conditions (Bejarano et al., 2014). However, also short-time exposure towards crude oil WAF resulted in adverse effects, indicating the time window around hatching to be the most sensitive (Mager et al., 2017). The fact that biomarker responses were altered already at more realistic sublethal exposure concentrations (around EC₁₀) further supports the relevance of the present results.

4.2. Toxicity alterations induced by chemical dispersant application

Results from FET indicate a contribution of the dispersant to the higher toxicity of crude oil CEWAF compared to LEWAF. In compliance to some WAF treatments the dispersant alone did induce a trend towards a hatching delay in zebrafish embryos. This effect seems to be a general response to dispersant exposure, as recently similar patterns in hatching delay have been observed for zebrafish embryos exposed to a different chemical dispersant (Li et al., 2018). However, the increased adverse effects in embryos cannot exclusively be explained by the dispersant itself as the HEWAF exposure resulted in higher effect concentrations than the CEWAF exposure. Hence, a combination of dispersant toxicity and an elevated crude oil compound bioavailability, due to increased partitioning kinetics, is most likely responsible for the high CEWAF toxicity. Dispersant application leads to the formation of small droplets with high surface to volume ratio (Prince, 2015), resulting in increased dissolved fractions in the water column. Increased relative PAH concentrations in aqueous solution of WAFs after the application of a dispersant have been observed in several studies (Cohen et al., 2015; Lozada et al., 2016).
Importantly, the increase of dissolved oil compounds indicates that the initial WAF system was not in a stable state of equilibrium, in which droplets should not change PAH partitioning. However, also the influence of droplets in micro- or even nano-scale should be considered as a route of exposure (Hansen et al., 2019). Interestingly, HEWAF-exposed embryos of the present study did not show typical adverse morphological effects (blue sac disease) during embryonic development as oil exposed embryos and lethal effects became apparent only after larvae were hatched and no longer protected by the chorion. Additionally, in contrast to CEWAF and LEWAF treatments no mechanism-specific bioactivity via CYP1A induction was observed. These results might indicate narcosis (baseline toxicity) via membrane disruption as the predominant cause of effect of the dispersant. In contrast, even though narcosis was suggested as an important toxicity mechanism identical mode of actions have been concluded in a previous study since highly similar gene expression profiles were identified for oil and dispersant treatments (Hook and Osborn, 2012).

### 4.3. Xenobiotic metabolism in WAF exposed zebrafish early life stages

Biphasic biotransformation predominantly occurs in vertebrate livers and efficiently transforms lipophilic xenobiotics into polar hydrophilic metabolites that can be eliminated via excretion (Schlenk et al., 2008). The EROD activity has been found to be indicative for petroleum product fish exposure in several studies across a variety of developmental stages (Oliveira et al., 2007; Perrichon et al., 2016; Sanni et al., 2017; Van der Oost et al., 2003; Whyte et al., 2000). The biotransformation
capacity of fish to efficiently metabolize and eliminate PAHs, depending on physical-chemical properties or the mixture composition, is particularly high (Logan, 2007; Van der Oost et al., 2003) relativizing the risk of pollution indicated by CYP activity biomarker. Nonetheless, especially during early organogenesis a quite limited capacity for metabolism is described, and hence a higher bioconcentration of dissolved PAHs in embryos expected (Incardona, 2017; Jung et al., 2015). Furthermore, biotransformation can generate bio-activated metabolites (e.g. DNA-adducts) that can elicit a variety of effects if fish. EROD activities observed in the present study were in a comparable range found in zebrafish larvae (Pauka et al., 2011; Perrichon et al., 2016) and adults (Arukke et al., 2008) exposed to a set of fresh and weathered crude oils. The EROD activity in zebrafish larvae was measured in the present study during in the most sensitive time windows of post-hatching development (96 and 120 hpf) within the limits of animal free testing, as has already been shown before (Bräunig et al., 2015; Kais et al., 2017). Even though cyp450 gene expression has been determined in early embryonic stages prior to hatching, post-translational
modifications were suggested to impact the enzyme activity leading to low responses on protein level (Meyer-Alert et al., 2018) within the first 2–3 days of development.

Interestingly, the distillate induced stronger EROD activity compared to the crude oil even in comparable WAF exposure dilutions. The potential to induce CYP1A in fish for the most prominent PAHs namely naphthalene, phenanthrene and fluorene varies enormously throughout literature. While those PAHs were described as weak CYP1A inducer when tested as single compounds (Barron et al., 2004; Billiard et al., 2004; Hawkins et al., 2002), other studies reported high CYP1A activity in zebrafish embryos exposed to complex sediment extracts with phenanthrene being one of the dominant PAHs (Kais et al., 2017). Results from several studies focusing on modes of action in fish embryo toxicity further expanded the complexity of potential molecular mechanisms by demonstrating AhR-dependent and -independent CYP1A-dependent and -independent mechanisms (Incardona, 2017; Incardona et al., 2005; Incardona et al., 2006; Scott et al., 2011). Finally, from previous and present results it can be concluded that oil WAFs represent a complex mixture of weak and strong CYP1A inducer acting additively, synergistically or even antagonistically via different pathways.

4.4. Neurotoxicity in WAF exposed zebrafish early life stages

Neurotoxicity in early life stages of fish is emerging as an important endpoint in ecotoxicological risk assessment with biomarker measurements (AChE inhibition) and behavioral assays as suitable screening tools (Legradi et al., 2018). In zebrafish, the brain and the nervous system are nearly fully developed around 96 hpf (e.g. reviewed in (Legradi et al., 2015)). The AChE expression and activity in zebrafish increases within the first days of embryonic and larval development (Bertrand et al., 2001; Yen et al., 2011) and is largely inhibited by, for example, neuroactive pesticides in 96–120 hpf larvae (Velki et al., 2017). In some studies crude oil compounds such as PAHs have been found to inhibit AChE in fish based on in vitro studies with purified AChE (Jett et al., 1999; Kang and Fang, 1997) or AChE biomarker measurements in adult fish tissue (Akaishi et al., 2004), while other studies failed to determine PAH (Tang et al., 2003) or oil contamination-related inhibitory (Jung et al., 2011) response. In concordance with the present study whole specimen-homogenates of zebrafish embryos showed reduced AChE activity after the exposure towards sediment extracts, highly contaminated with PAHs (Kais et al., 2015). Stronger AChE inhibitory effects in the crude oil compared to the distillate might be related to higher bio-transformation and detoxification in MGO-treated embryos as indicated by the CYP1A activity. Also more bioavailable compounds in NNS that interact with AChE cannot be excluded based on the limited chemical analysis of the present study.

Changes in swimming behavior patterns can further indicate a neurotoxic potential of complex mixtures. Petroleum product WAF-exposed larvae had shown reduced sensitivity towards touch stimulus (de Soysa et al., 2012). Furthermore, in compliance with the present study, reduced swimming activities have been observed for oil-exposed larvae and juveniles in locomotor and light dark/transition tests (Mager et al., 2014; Perrichon et al., 2016). However, at least one previous study has found no effects on swimming behavior for Arabian light crude oil (Perrichon et al., 2016). Importantly, previous studies have further shown that impaired swimming activity can manifest as delayed effects in fish that were originally exposed to crude oil WAFs during an early time window of embryonic development, even after a prolonged time window without exposure (Hicken et al., 2011; Mager et al., 2014). However, the mode of actions behind behavioral changes can be attributed to several pathways. They might not exclusively be related to neuroactive effects, but rather a secondary effect due to several other physiological events. Deformations in the muscular system (de Soysa et al., 2012) as well as cardiotoxicity-based reduction in swimming activity (Hicken et al., 2011) or changes in brain monoamine contents (Vignet et al., 2017) have been suggested to explain behavioral disruptions. Furthermore, recent results indicate impairments of normal eye development, which might explain lacking response to light/dark alterations simply by interrupted visual perception. In this context, significant down-regulation of genes involved in functional eye development (Xu et al., 2017; Xu et al., 2016) and histological changes including reduced eye size or disruption and apoptosis in retinal cell layers (Huang et al., 2013; Kawaguchi et al., 2012) have been reported for fish larvae exposed to crude oil WAFs or PAHs.

5. Conclusion

In summary, the present findings were in compliance with findings for other fish species and oil types indicating that at least most petroleum products are able to induce a common set of adverse effects in fish early life stages. In terms of oil spill risk assessment, the present results further complement the knowledge about possible environmental impacts of oil contaminations and help evaluating their severity based on the determined effect concentrations. However, crude oil toxicity in fish seems to be diverse. Further studies should focus on the modes of action of those complex mixtures on different biological organization levels. Additionally, future work should include more insight into the toxicity as well as the mode of actions of dispersants in relevant exposure concentrations.

For the extrapolation of the present results to endemic species of oil-spill affected regions in the context of oil spill risk assessment it has to be considered that response sensitivities can significantly vary among different species (Incardona, 2017; Jung et al., 2015; Perrichon et al., 2016). Also the extrapolation to later developmental stages including juveniles and adults is limited due to potential changes in metabolism or tissue-specific CYP1A activity. Another aspect that urgently needs to be addressed in more detail is the role of oil weathering due to accompanying changes in physical-chemical properties as well as...
exposure scenarios and toxicity, since the weathering starts immediately after the spill. Overall, each oil spill displays a unique situation for response strategies and data including the results obtained from the present study can contribute to a better understanding of oil toxicity.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.136174.

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