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Adverse outcome links for zebrafish - Report on adverse outcome links and transcriptomic fingerprinting for zebrafish regarding oil and dispersant

D3.12

WP3: Oil impacts on biota using biomarkers and ecological risks assessment

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Executive summary

The overreaching goal in ecotoxicology is to be able to predict adverse effects of chemical groups on the environment (Ankley et al. 2010, Schirmer et al. 2010) supporting a comprehensive risk assessment and regulatory decision making. However, bridging the causal link of events on molecular level over changes on organismic - up to population level is difficult. The present report addresses petroleum toxicity induced links of molecular/cellular events with adverse outcomes in zebrafish using crude and refined oil product WAF exposure. Deviating from the adverse outcome pathway (AOP) concept, exposure and species specific adverse outcome links (AOL) in respect to the identification of possible modes of action are established.

Based on the results on different biological organization levels including transcriptional response, biochemical enzyme activities, swimming behavior and morphological alterations in zebrafish embryos (up to 120 hpf) and adults, modes of actions like xenobiotic metabolism, potential neurotoxicity, antioxidant defense and cardiotoxicity are discussed. Within this, links between the different biological organization levels have successfully been established. The results and recent literature indicate well-described AhR-mediated but, most importantly, also AhR-independent regulatory pathways.

The results of the present report will contribute to the big picture of petroleum product toxicity towards fish using the well-established model zebrafish. First, as every crude oil is a unique sample, the results provide a detailed toxicity profiling of the selected naphthenic North Sea crude oil tested as crude and dispersed water-accommodated fractions. Second, the findings of the present report in the context of actual knowledge from literature provide perspectives for future work in the field of crude oil risk assessment. A possible crude oil induced oculotoxicity will be addressed in more detail with experiments on additional histological and behavioral changes in crude oil exposed zebrafish in order to bridge the gap from gene expression to behavioral alterations that has been observed in the present study. Additionally, results of the comprehensive adult zebrafish study as well as results of the fuel oil types in embryo experiments will contribute to the understanding of petroleum product toxicity. Common or unique modes of actions regarding the chemical composition will be compared. Furthermore, especially the role of dispersant needs to be addressed in more detail in future work, as the droplet induced changes in exposure scenarios are often ignored in several studies but absolutely required to be included in a complete risk assessment.

I Introduction

The overreaching goal in ecotoxicology is to be able to predict adverse effects of chemical groups on the environment (Ankley et al. 2010, Schirmer et al. 2010) supporting a comprehensive risk assessment and regulatory decision making. In this context time and cost efficient assessment of tons of chemicals in respect to the 3 R principle (reduction, refinement and replacement of animal testing) is possible by focusing on specific selected endpoints on low biological organization level. However, bridging the causal link of events on molecular level over changes on tissue - or organismic - up to population level is difficult. Not only practical aspects of exposure conditions like scales of varying exposure concentrations affecting different biological levels, but also the availability of analytical tools triggers the establishment of so called mechanisms of action. By definition, *mechanism of action* is clearly differentiated from the term *mode of action* including a complete and detailed knowledge of causal and temporal relationships (mechanism of action) instead of linking a direct initiating event with the adverse outcome allowing gaps in cascade of events (mode of action) (Ankley et al. 2010). In this context the adverse outcome pathway (AOP) concept was established to portray existing knowledge of links between a direct molecular initiating event (MiE) with adverse outcome (AO) on organism or population level (Ankley et al. 2010). Modular key events, including MiE and AO, range from receptor interaction over biochemical processes or morphological malformations covering different biological organization levels. AOPs are defined to be chemically and species independent living documents that evolve over time with the inclusion of new knowledge (Villeneuve et al. 2014, Vinken 2013).

The present report addresses petroleum toxicity induced connections of molecular/cellular events with adverse outcomes in zebrafish. Hence, deviating from the AOP concept, chemically specific adverse outcome links (AOL) in respect of investigating the *mode of action*, are established as not every key event in the cascade was addressed with different bioassays. The AOL concept in GRACE aims not at a full understanding of the toxicity mechanism, but focuses on establishing molecular surrogate endpoints for apical effects to increase versatility of the zebrafish as a widespread model organism in ecotoxicology for the bioanalytical detection and investigation of oil contaminations. Figure 1 illustrates the examined endpoints using the zebrafish model on different biological organization levels to define linkages between crude oil toxicity on gene transcriptional level and phenotypical outcome on the organismic level. The approach includes next generation sequencing analysis as well as biochemical processes on protein level up to morphological and behavioral changes in individual zebrafish. In the GRACE project, three different oil types from crude over intermediate (IFO) to distilled (MGO) have been used to identify common or unique toxic modes of action in zebrafish early life stages. Furthermore, a comprehensive study on naphthenic North Sea crude oil exposed adult zebrafish was conducted focusing on

responses on several biological organization levels including molecular, biochemical and histopathological endpoints in short- and long term exposure regimes.

Since for the embryo investigations of MGO and IFO and for adult experiments some biological endpoints are currently under evaluation, the AOL discussion will focus mainly on the selected naphthenic North Sea crude oil toxicity. Available results from other oil types and from adult experiments will be included in corresponding sections of the present report.

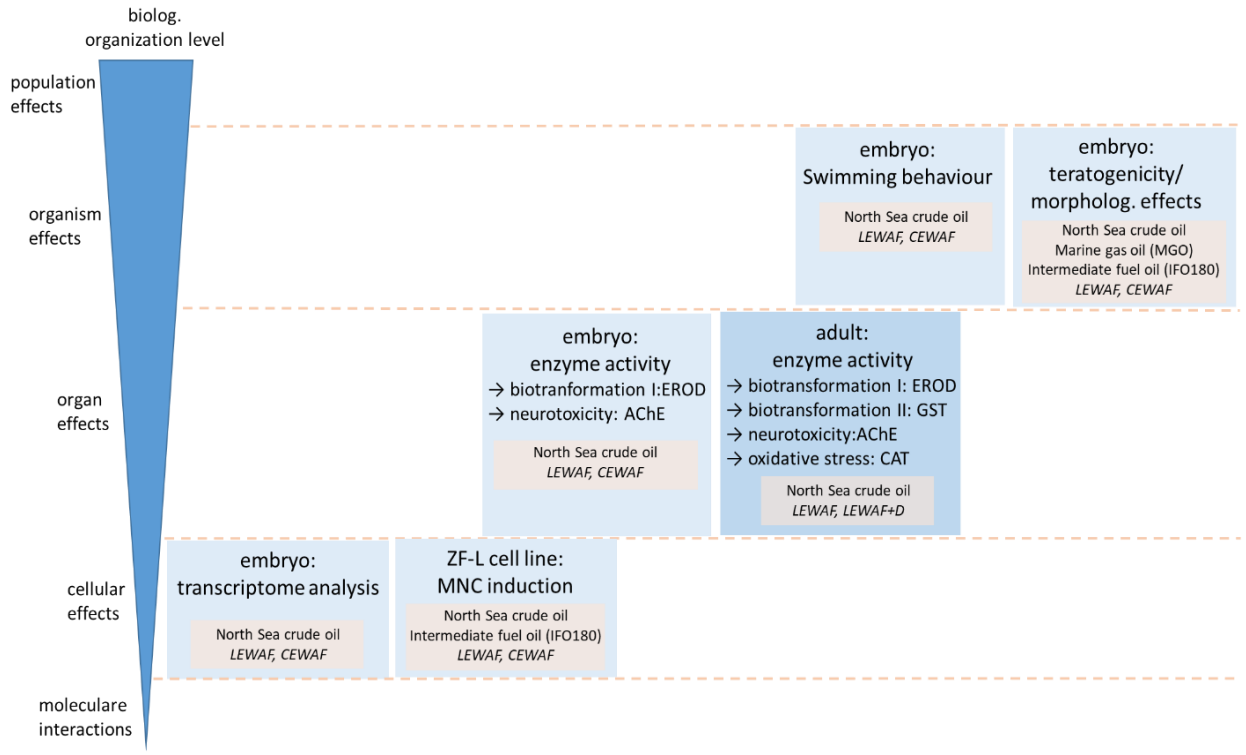

Figure 1 Overview of investigated endpoints on different biological organization levels to identify the modes of action of crude and refined oil toxicity in the zebrafish model. Organization levels were based on previous suggestions from established AOP concept (Ankley 2010, AOP Wiki).

Even though marine species, which are more relevant to the study region of the GRACE project, were described to be more sensitive towards crude oil or polycyclic aromatic hydrocarbons (PAH) exposure, the zebrafish is a useful model in this field of research. Especially in respect to molecular events and pathways a well-established model is crucially needed to guarantee the availability of specific information on, for example, gene expression patterns or species-specific receptor activities.

As crude oil is one of the most complex sample types in ecotoxicology it is likely that multiple targets involve multiple and simultaneously interacting molecular mechanisms. The morphological impacts of crude oil exposure on early life stages of fish have been investigated intensively in the last years. Common adverse effects like craniofacial deformations, cardiotoxicity or impaired swimming activity are described across several freshwater and marine laboratory and regional relevant species of oil spill impacted areas.

Also several studies focusing on the adverse effects of crude oil toxicity in juvenile or adult life stages of fish are available. However, independent of the huge amount of studies available in this field of research, the underlying molecular pathways of crude oil toxicity are not fully understood. Besides AhR-mediated pathways also other molecular aspects like calcium homeostasis receive increasingly attention as important drivers of crude oil toxicity and will be discussed in more detail in this report.

The results of the present report will contribute to the big picture of petroleum product toxicity towards fish using the well-established model zebrafish. First, as every crude oil is a unique sample, the results provide a detailed toxicity profiling of the selected naphthenic North Sea crude oil. Second, the findings of the present report in the context of actual knowledge from literature provide perspectives for future work in the field of crude oil risk assessment. Additionally, the results of the transcriptomic study exposing zebrafish to crude and dispersed naphthenic North oil in sublethal effect concentrations (EC_{10}) will be freely available in order to allow comparison to data on other species or new pathways that will be established in future research.

II Sample background

A naphthenic North Sea crude oil was selected as the crude and untreated petroleum sample. It is a light crude oil with low viscosity characterized by a high proportion of low molecular weight saturates and aromatics. A detailed chemical profile of the naphthenic North Sea crude oil was generated in the GRACE project.

In addition to the crude oil a marine gas oil (MGO), a distillate formed during the fractional distillation of a crude oil, was used in this study. This MGO is supplemented with the green dye Dyeguard Green MC25 produced by John Hogg Technical Solutions.

Furthermore, an intermediate fuel oil (IFO 180) which is a blend of heavy fuel oil and gas oil was selected as an intermediate stage of petroleum products purity between crude and marine gas oil. The intermediate fuel oil is characterized by a high viscosity (maximum viscosity = 180 centistokes) and a sulphur content of less than 3.5 %.

To investigate the influence of dispersants on petroleum product toxicity Finasol OSR 51 and Finasol OSR 52 produced by Total were included. The selected chemical dispersants are complex mixtures of anionic and neutral surfactants and hydrocarbon solvents. Both dispersants are relevant in the study region of the GRACE project and the treatment of the selected oil types. The selected third-generation dispersants have slightly different chemical composition (see Table 1). Finasol OSR 51 contains 15-30 % non-ionic and 0.2-0.5 % anionic surfactants, while Finasol OSR 52 contains >30 % non-ionic surfactants and 15-30 %

anionic surfactants. Finasol OSR 52 is compliant with all the three regulations on the market (EPA, MMO, CEDRE), while Finasol OSR 51 is compliant with two of them (MMO, CEDRE) (Total Special Fluids 2019).

In general, while MGO and IFO 180 samples were exclusively combined with the dispersant Finasol OSR 52, the naphthenic North Sea crude oil was combined with Finasol OSR 51 based on the recommendation from previous studies of the project partners.

Table 1. Information on ingredients and composition of the dispersants Finasol OSR 51 and Finasol OSR 52 (Total®). All information is based on the safety data sheets (SDS 30033 (2012); SDS 30034 (2015)).

Finasol OSR 51		**Finasol OSR 52**	
chemical name	**weight %**	**chemical name**	**weight %**
hydrocarbons, C11-C14, n-alkanes, isoalkanes, cyclics, <2% aromatics	60 - 70	hydrocarbons, C11-C14, n-alkanes, isoalkanes, cyclics, <2% aromatics	15 - 20
docusate sodium	0.2 - 5	docusate sodium	20 - 25
		(2-methoxymethylethoxy)propanol	15 - 20
		carboxylic acids, di, C6-12 cmpds, with ethanolamine, boric acid cmpd with ethanolamine	0 - 2
		ethanolamine	0 - 1
non-ionic surfactants	15 - 30 %	non-ionic surfactants	> 30 %
anionic surfactants	0.2 5 %	anionic surfactants	15 - 30 %

III Material and Methods

1. Preparation of water-accommodated fractions

In general, all different water-accommodated fractions were prepared according to Singer et al. (Singer et al. 2001). The different types of WAFs used for embryonic testing are low energy water-accommodated fractions (LEWAF) for oil exposure only, chemically enhanced water-accommodated fractions (CEWAF) for the combination of oil and dispersant exposure. Furthermore, for adult zebrafish experiments a LEWAF+D instead of a CEWAF was used.

1.1 WAF preparation in zebrafish embryo experiments

Briefly, LEWAFs and CEWAFs were prepared in aspirator glass flasks (500 mL) by application of oil or a dispersant-oil mixture (1:10) on the surface of artificial fish water at an oil-to-water ratio of 1:50 (LEWAF) or 1:200 (CEWAF), respectively. The LEWAF setup was carefully stirred with low energy avoiding a vortex in the water phase while CEWAF was stirred at higher stirring speeds to create a 25 % vortex of the water phase. LEWAFs and CEWAFs were incubated stirring at 10°C for 40 h and followed by 1 h settling time. Afterwards, water fractions were carefully drained off. Different dilutions prepared from the 100 % stock solutions (1:50 WAF, 1:200 CEWAF) were warmed up to 26 °C before embryos were exposed to the samples.

1.2 WAF preparation in adult zebrafish experiments

LEWAFs and LEWAF+Ds were prepared in glass flasks (5-20 L) by application of oil (LEWAF) or a dispersant-oil mixture (1:10, LEWAF+D) on the surface of artificial fish water (remineralized osmosis water at 600 µS/cm and pH around 7.2) at an oil-to-water ratio of 1:200. Both setups were stirred with low energy avoiding a vortex in the water phase. Avoiding a vortex is the main difference between CEWAF and LEWAF+D. WAFs preparation lasted 40 h at 20 °C, with a higher temperature due to practical operation possibilities being the second difference to WAF preparation in zebrafish embryo experiments. Again, water fractions were then collected from a stop cock at the bottom part of the flask and immediately used for exposure. Exposure tanks with a capacity of 45 L were filled with artificial fish water (control) or different exposure dilutions of 25 % LEWAF, 5 % LEWAF, 5 % LEWAF+D.

2. Chemical analysis of LEWAF using SPME

In general, a comprehensive chemical analysis profile for the naphthenic North Sea crude oil is available. Additionally, in combination with the next-generation sequencing study a basal chemical analysis of the LEWAF was performed. A set of target PAHs that are expected to be relevant in water-accommodated fractions was selected based on the comprehensive chemical profile of the crude oil. The aim of the chemical analysis during the exposure experiment was to determine the stability of the LEWAF in the air-tight exposure vessels.

Exposure concentrations for selected PAHs were measured daily in samples of the freshly prepared LEWAFs and in samples from the LEWAFs that were removed in the daily medium exchange regime. Target PAHs were extracted directly from the medium using silicone coated solid phase micro extraction fibers. External (S-4008-100-T) and perdeuterated internal standards (S-4124-200-T) were purchased from Chiron (Chiron AS, Trondheim, Norway). Extraction was conducted for 2 h to enable quantification of low concentrations of target PAHs in the test medium. Loaded SPME fibers were then analyzed using an Agilent Technologies GC system (7890 A GC System and 5975 C inert XL MSD with Triple-Axis-Detector, Agilent Technologies Deutschland GmbH, Böblingen, Germany).

Also for the adult zebrafish experiment the distribution and behavior of selected target PAHs was monitored during the time of one exposure cycle of 3 days. For each treatment 2 complete exposure cycles were investigated using SPME with twisters instead of fibers. As the results are still under evaluation, no detailed method description is included in the present report.

3. Zebrafish culture

Wildtype zebrafish of the WestAquarium strain (Bad Lauterburg, Germany) from the facilities of the RWTH Aachen University were used in the present study for embryonic endpoints. Breeding groups of 100 to 150 adult zebrafish from 1 to 2 years of age were kept in 170 L tanks of a flow-through system with an automatically water exchange rate of 40 % per week. Tank water was cleaned through a biological filter and UV light. Fishes were fed twice a day with dry flakes and larvae of *Artemia* spec. 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.

As adult zebrafish experiments were performed at UPV/EHU, the locally available zebrafish strain was used. Wildtype fish of AB Salk strain were grown until 5-6 months of age in the facility of the University of the Basque Country. Fish were maintained in a temperature-controlled room at 28 ± 1 °C with a constant light-dark rhythm (12:12) in 100 L tanks provided with mechanical and biological filters. Water was in continuous movement triggered by the action of an aeration siphon. Water was previously conditioned by passage through a deionization system and then buffered to pH 7.2 with Sera pH plus (Sera, Heinsberg, Germany) and to 600 µs Ω with commercial marine salt (Sera). Fish were fed twice a day with live brine shrimp larvae of 24 h post hatch and Vipagran baby (Sera).

4. Methods in embryo toxicity testing

4.1 Fish embryo acute toxicity test

The prolonged fish acute embryo toxicity test up to a maximum of 120 hours post fertilization (hpf) using the model species *Danio rerio* was performed. All experiments were terminated with the final measurement shortly before 120 hpf, so that no animal test authorization was required. Zebrafish embryos and larvae below 120 hpf are not protected animal stages according to EU Directive 2010/63/EU (European Uniion 2010) (see also:(Strähle et al. 2012)), TierSchG (Tierschutzgesetz) and the respective regulation TierSchVerV (Tierschutz-Versuchstierverordnung). After termination, larvae were euthanized by prolonged immersion in a benzocaine ethanol solution.

The embryo toxicity assay was performed according to the OECD guideline 236 (2013) with minor modifications in respect to the sample type. Briefly, 20 embryos per sample concentration were transferred to sample dilutions shortly after fertilization. Embryos were incubated at 26°C using a semi-static approach with periodic medium exchange (every 24 h). Artificial fish medium was prepared, aerated and warmed up one day before using. The pH of all media was adjusted between 7.0 and 8.0.

Embryos were exposed in air-sealed 10 mL glass vials with sparsely head space to minimize the evaporation of volatile, water-soluble compounds (5 embryos per vial). In each experiment negative controls (artificial water) and positive controls (3,4-dichloranilin 4 mg/L) were included. Embryos were investigated for lethal and sublethal effects every 24 h. Additionally, medium pH was controlled every 24 h. An experiment was classified valid if no more than 10 % of negative control eggs and at least 30 % of positive control eggs showed lethal effects according the OECD 236 guideline. Afterwards concentration-response curves were established using the software GraphPad Prism version 6 (GraphPad, San Diego, USA) and ECx (effect concentrations inducing x % of effects) and LCx (lethal concentrations inducing x % of effects) values were calculated. Based on the concentration-response curves sublethal effect concentrations (EC_{10} to EC_{20}) for all following experiments focusing on molecular mechanisms behind observed phenotypic embryotoxicity were determined.

4.2. Next Generation Sequencing study

In general, detailed information about the experimental design and the evaluated data of the zebrafish transcriptome can be found in (Nüßer et al. in prep). Methods are described briefly in the following sections.

4.2.1 Exposure regime

The EC_{10} based exposure concentrations for the next generation sequencing study, prepared from 100 % stock solutions, were 12.5 % of stock (1:400 dilution, LEWAF) and 0.78 % of

stock (1:25000, CEWAF), respectively. The exposure of zebrafish embryos was performed according to the exposure scenario described for the fish embryo acute toxicity test with minor modifications. Most importantly, 40 embryos per treatment and control were transferred to exposure solutions shortly after fertilization (5 embryos per vial). Exposure was terminated at 119 hpf. All 40 larvae were pooled, anesthetized using saturated Benzocaine solution and washed twice with cold phosphate buffer saline (PBS, Sigma-Aldrich). Anesthesized larvae were transferred to 1.5 mL tubes and excess solution was replaced by 200 µl of RNA later. Subsequently embryos were shock frozen in liquid nitrogen.

4.2.2 Statistical analysis and functional annotation of transcriptome data

Functional annotation analysis was conducted with all significantly regulated genes found for CEWAF and LEWAF treated groups. In a second analysis the list of genes regulated 1.5 fold greater or lesser compared to the control group were analyzed in order to display only the highly regulated pathways. The software Cytoscape with GlueGo plugins calculates enrichment and depletion tests for terms and groups based on the statistical method of hypergeometric distribution. The genes were functionally annotated by assigning Gene Ontology (GO) terms for the domains biological process, molecular function and cellular component, and by assigning genes to KEGG Pathways. More details can be found in Nüßer, Johann et al. (in prep).

4.3 Light/dark transition test

30 embryos for each treatment were used to assess differences in behavioral responses of the embryos in the morning of the termination of the test. For the behavioral assays embryos were individually transferred to a 96 well plate and subsequently kept in the incubator for recovery from the stress for 30 min prior to the tests. The behavioral test was conducted using a DanioVision observation chamber and EthoVision tracking software (Noldus, Netherlands). Embryo swimming activity was challenged by a light dark transition test. After an initial acclimatization time of 10 min zebrafish larvae were exposed to alternating short light (10 min) and dark (10 min) periods. Resulting data were analyzed according to the method described in Nüßer et al. (2016).

4.4 7-Ethoxyresorufin-O-deethylase (EROD) activity in zebrafish embryos

The fish embryo 7-ethoxyresorufin-O-deethylase (FE-EROD) assay was performed according to Schiwy et al. (2015) with modifications regarding egg number and a kinetic measuring method. Briefly, 40 embryos per treatment were exposed to 3 concentrations of different WAF dilutions ranging from below EC_{10}, around EC_{10} and around EC_{20} concentrations. The exposure scenario is already described in detail in chapter III.3 (fish

embryo acute toxicity test) with the exception of 10 instead of 5 embryos per glass vial. At 3 different time points (48hpf, 96hpf and 120hpf) exposure was terminated and embryos were pooled, anesthetized using saturated Benzocaine solution and washed twice with cold phosphate buffer saline (PBS, Sigma-Aldrich). Anesthetized larvae were transferred to 1.5 mL tubes and excess solution was replaced by 700 µl of buffer (1.8 L 0.1 M Na_2HPO_4 adjusted with 0.5 L 0.1 M NaH_2PO_4 to pH 7.8). The embryos were immediately shock frozen in liquid nitrogen and stored at -80 °C until further use.

To measure EROD activity, embryos 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 at 10000 g and 4 °C for 15 min. After centrifugation, the supernatant was immediately transferred in new tubes and placed on ice. A 1:2 dilution series of the reference resorufin (0.5 µM in buffer) was prepared in duplicates in a 96 well-plate (100 µL). 100 µL of each samples were added in triplicates on the plate. Afterwards, 100 µL of 7-ethoxyresorufin (2.4 µM) were added to each well followed by 10 min incubation at 26 °C in darkness. Shortly before 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), 50 µL NADPH (3.35 mM) were added. Substrate deethylation was determined by measuring the formed resorufin at 540 nm excitation and 590 nm emission wavelength. Whole protein of each sample was measured in parallel using a Bicinchoninic Acid kit (Sigma Aldrich GmbH, Germany) according to the manufacturer's instructions and quantified with a dilution series of bovine serum albumin (BSA) as an external standard (0.31 - 1 mg/mL). For protein measurement the sample supernatants were diluted 1:2.

4.5 Acetylcholinesterase (AChE) activity in zebrafish embryos

To investigate a petroleum based potential to induce neurotoxicity in zebrafish larvae, the measurement of acetylcholinesterase (AChE) activity was conducted according to the initial protocol established by Ellman et al (1961) with modifications according to Velki et al. (2017) regarding adaptions to 96-well plate format. Exposure conditions as well as treatment concentrations were described in previous chapters. A pool of 40 embryos per treatment was sampled after 96 hpf and 120 hpf to conduct AChE activity measurement.

For AChE measurement 7.5 µL sample supernatant as well as 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 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). Whole protein amount was measured with the Bicinchonincic Acid kit (Sigma Aldrich GmbH, Germany) described in

detail in the previous chapter on FE-EROD. Resulting data were controlled for linearity in absorbance increase ($R^2 \geq 0.98$), and minimum increase of absorbance over time ($\Delta t_{3min} \geq 0.1$). Only data fulfilling these criteria were used for further calculations. Enzymatic activity was calculated as nm acetylcholine hydrolyzed per min and mg protein. For the calculations the molar extinction coefficient of 13600 M^{-1} cm^{-1} was used.

5. Micronucleus induction in ZF-L cells

Based on previous studies at RWTH (DiPaolo, in prep) it was concluded that the micronucleus assay with primary zebrafish cells isolated from larvae up to a maximum developmental stage of 120 hpf is not sensitive enough to determine potential genotoxic chemicals or environmental samples. Even highly potential chemicals such as 4-Nitroquinoline 1-oxide (NQO) or Cyclophosphamide (CPP), which are commonly positive controls in standardized micronucleus assay protocols, did not show significant increase in micronuclei formation compared to an untreated control. Hence, it was decided to use the zebrafish liver cell line to perform the micronucleus assay.

5.1 Zebrafish liver cells (ZF-L)

The permanent ZF-L cell line was cultured in L15 medium (Leibovitz, with L-glutamine, Sigma Aldrich, L4386), supplemented with 10 % fetal calf serum (Biowest, France) in 75 cm^2 flasks at 28 °C. Cells were passaged regularly when reaching 90 % of confluence. A 3x-concentrated assay medium was prepared from L15-powder, which was finally supplemented with FCS (charcoal stripped, Biowest, France), and penicillin-streptomycin. 1x-concentrated assay medium was prepared by diluting the 3x-concentrated assay medium with sterile ultrapure water.

5.2 Micronucleus assay with ZF-L cells

The assay was performed according to the ISO guideline 21427-2 (2004). However, major modifications in respect to assay optimization based on the selected cell line and the sample types mainly influencing the incubation periods and exposure materials were established in pretests. In accordance with other bioassays of this report, the viability of ZF-L cells exposed to dilution series of all WAF samples was evaluated using the MTT assay (data not shown). The two highest non-cytotoxic sample concentrations of each treatment resulting in a viability of at least 80 % were selected as exposure dilutions.

Briefly, cell suspension (in 1x concentrated assay medium) at a density of $5*10^4$ was seeded in sterile small glass petri dishes (40 mm, VWR, Germany) containing sterile cover slips (20 x 20 mm, VWR, Germany), on which cells attach after settling. 24 h after seeding, the attached cells were exposed to dilution series of LEWAF, CEWAF and HEWAF samples in duplicates.

To evaluate the test validity a negative control (assay medium only), a positive control (4-Nitroquinoline 1-oxide, exposure concentration 6.22 * 10^{-8} M, stock in DMSO) and a solvent control (0.1 % DMSO) were included. After 48 h of exposure the cover slips with attached cell layers were fixed with MeOH: acetic acid (4:1, each for 5 min), air dried and finally sticked onto glass slides using Aquatex adhesive (Merck KGaA, Darmstadt, Germany). The cells on microscopy slides were stained using the Acridine Orange dye. An Eclipse 50i epifluorescence microscope (Nikon Instruments, Düsseldorf, Germany) with 400 x magnification was used to generate pictures in which micronucleated cells were identified according to the following criteria (ISO 21427-2):

a) the maximum size of a micronucleus was one-third of the main nucleus, b) micronuclei had the same staining intensity as normal nuclei and c) micronuclei were clearly separated from the nucleus, and d) only cells with clear plasmatic outlines were included. A total number of 2000 cells per treatment were evaluated for micronuclei formation. Validity criteria were met when in negative and solvent controls not more than 3 % of counted cells were micronucleated and positive control induced a significant increase in micronucleated cells. Statistical analysis was done by Chi² test with Yates correction using the program SigmaStat 12.5 (Systat Software, 2007) for each replicate.

6. Methods in adult zebrafish toxicity testing

6.1 Exposure setup and sampling regime

The adult zebrafish study was conducted in October 2018 with the approval of animal care committee under project authorization number NoRefCEID: M20/2017/173.

In general, control and exposure solutions were already filled into the tanks 3 days before fish were added in order to saturate the system. Tank internal water circulation using a circulatory pump, a light:dark rhythm of 14:10 h and 28 °C were maintained in 2 tanks per treatment. 4 treatment groups (control, 5 % LEWAF, 25 % LEWAF, 5 % LEWAF+D) were established in total. Biological filters were not used during the experiment to avoid interference of with the exposure. 50 adult zebrafish (AB Salk) at the age of 5 to 6 months were placed in each tank with sex ratio of 50:50. Exposure medium was exchanged periodically (every 3 days) using a 75 % exchange rate in order to avoid additional stress for the fish. Fish were fed twice a day with larvae of *Artemia* spec. (INVE Aquaculture, Salt Lake City, Utah, USA).

After 3 days of exposure, the first sampling of short term exposure with 40 fish per treatment was conducted. For this, fish were individually anesthetized in a benzocaine solution (200 mg/L) prepared in an ethanol-water (1:10 (v/v)) solution and immediately dissected

according to a set of individual endpoints planned for the experiment. As several biomarker activities are reported to be influenced by sex differences, males and females were clearly separated for individual biomarkers. Briefly, besides immediate preparation of blood smears on microscopy slides, livers, gills and brains of male individuals were dissected for gene expression analysis. Female livers, gills and brains were dissected for biomarker analysis. Furthermore, visceral mass and gills were sampled for histological investigations. All carcasses were collected and preserved at -20 °C for chemical analysis. Liver tissue for gene expression analysis was embedded in RNA later (Sigma-Aldrich, Germany) and immediately frozen in liquid nitrogen and stored at -80°C until further usage. Liver, gills and brain dissected for biochemical biomarker analysis was also immediately frozen in liquid nitrogen and stored at -80°C. For histopathological evaluation, visceral mass and gills were fixed in 10 % formalin solution for 24 h prior dehydration and embedded in paraffin wax.

Remaining fish (10 individuals) were transferred to the second tank of exposure. After 21 days all fish of the experiment were anesthetized and dissected. The distribution behavior of selected target PAHs during an exposure cycle of 3 days was also investigated for the different treatments using a solid phase membrane extraction.

So far, data on the biomarker levels in the different tissues are complete, while remaining data on gene expression, micronucleus induction, histological investigations or chemical analysis are still under evaluation. Hence, the following sections focuses on the biomarker methods which's results are presented in the present report.

6.2 Preparation of homogenates for biomarker analysis

A pool of 3 liver, gill or brain tissues that were stored individually at -80°C were homogenized in 300 µL cold homogenization buffer (50 mM potassium phosphate buffer, pH 7.5, with 1mM EDTA ,0.5 mM DTT and 0.4 mM PMSF) for 15 sec on ice using an electric disperser (PELLET PESTLE® Cordless Motor, Kimble Kontes, U.S.A) resulting in 4 samples per treatment (12 individuals). Subsequently, homogenates were centrifuged at 10,000 *g* and 4 °C for 15 min (Eppendorf 5415R refrigerated centrifuge, Sigma-Aldrich, Germany). Afterwards, supernatant was carefully transferred to new micro test tubes and aliquoted on ice for different enzymatic and protein measurement in order to avoid repeated thawing and freezing. Supernatants were stored at -80 °C until further usage.

6.3 7-ethoxyresorufin-O-deethylase (EROD) activity in adult zebrafish liver and gills

Measurement of EROD activity in liver and gill supernatants was performed according to Brinkmann et al. (2010) with minor modifications. Briefly, 20 µL of samples triplicates and a resorufin calibration series (1:2 dilution series from 0.004 to 1 µM) in duplicates were transferred to a 96-well plate. Resorufin standard and stock solutions were prepared in Tris-

KCl buffer (pH 7,4; Trizma base 0.1 M, KCl 0.15 M). 200 µL of 7-ethoxyresorufin (0.5 µM) were added to each well followed by 10 min incubation at room temperature in darkness. Shortly before kinetic measurement of fluorescence for 25 min (kinetic intervals: 30 s) in a microplate reader (FLx800, Bio-Tek Instruments, INC, Winooski, Vermont, U.S.A), 20 µL NADPH (1 mM) were added. Substrate deethylation was determined by measuring the formed resorufin at 540 nm excitation and 590 nm emission wavelength. Quantification of EROD activity was performed based on the resorufin calibration and expressed in pmol resorufin/mg/min.

6.4 Glutathione-S-transferase (GST) activity in adult zebrafish liver and gills

Measurement of Glutathione-S-transferase in zebrafish liver and gills was performed according to the protocol developed by Habig and Jakoby (1981) with modifications regarding the adaption to 96-well plate described in Velki et al. (2017). 12 µL of supernatant as well as 180 µL of CDNB (1 mM, dissolved in phosphate buffer pH 7.2) and 50 µL of reduced glutathione (25 mM, dissolved in MilliQ) were added to a 96-well plate. Immediately thereafter the increase in absorbance as a result of S-(2,4-dinitrophenyl) glutathione formation was measured in triplicates at 340 nm for 15 min in 10 s intervals using a microplate reader (Thermo Scientific Multiskan Spectrum, Thermo Fisher Scientific Oy, Vantaa, Finland). Resulting data were controlled for linearity in absorbance increase ($R^2 \geq 0.98$) and minimum increase of absorbance over time ($\Delta t_{3min} \geq 0.1$). Only data fulfilling these criteria were used for further calculations. Enzymatic activity was calculated as nmol conjugated GSH per min and mg of protein. The molar extinction coefficient of 9600 M^{-1} cm^{-1} was used.

6.5 Acetylcholinesterase (AChE) activity in adult zebrafish liver and gills

Measurement of acetylcholinesterase (AChE) in brain tissues was conducted according to the initial protocol established by Ellman et al. (1961) with modifications according to Velki et al. (2017) regarding adaptions to 96-well plate format. Briefly, 7.5 µL sample supernatant as well as 180 µL potassium phosphate buffer (0.1 M, pH 7.2) 10 µL 5,5′-dithiobis(2-nitrobenzoic acid (DTNB 1.6 mM), 10 µL acetylcholine iodide (156 mM) were added to a 96-well plate. The increase in absorbance was immediately measured in triplicates at 412 nm for 25 min in 10 s intervals using a microplate reader (Thermo Scientific Multiskan Spectrum, Thermo Fisher Scientific Oy, Vantaa, Finland). Resulting data were controlled for linearity in absorbance increase ($R^2 \geq 0.98$) and minimum increase of absorbance over time ($\Delta t_{3min} \geq 0.1$). Only data fulfilling these criteria were used for further calculations. Enzymatic activity was calculated as nm acetylcholine hydrolyzed per min and mg protein. For the calculations the molar extinction coefficient of 13600 M^{-1} cm^{-1} was used.

6.6 Catalase (CAT) activity in adult zebrafish liver and gills

Measurement of Catalase (CAT) activity in zebrafish liver and gill supernatants was performed according to the initial protocol developed by Claiborne (1985) adapted to UV 96-well plates (Thermo Fisher Scientific Oy, Vantaa, Finland). 5 µL supernatant were added to 295 µL of H_2O_2 solution (20.28 mM). Immediately thereafter the decrease in absorbance was measured kinetically for 5 min in 10 s intervals (Thermo Scientific Multiskan Spectrum, Thermo Fisher Scientific Oy, Vantaa, Finland). Additionally, the absorption of a H_2O_2 dilution series (0.4 - 20.28 mM) was measured for quantification of H_2O_2 consumption. Calibration series as well as samples were measured in quadruplicates. Based on the increase of measurement-disturbing O_2 bubbles, the linear part of the reaction (until 1 min) was used for calculations. Enzyme activity was expressed as nmol H_2O_2 consumption per min and mg protein using the calibration series.

6.7 Protein measurement

Whole protein of supernatants was measured in triplicates using a DC protein assay kit (BioRad) according to the manufacturer's instructions and quantified with a dilution series of bovine serum albumin (BSA) as an external standard (1.5 - 0.15 mg/mL) measured in quadruplicates. For protein measurement supernatants were diluted 1:2 and 1:4.

IV Relevance of the zebrafish model for the study regions Baltic sea and Northern Atlantic - temperature and salinity regime

The zebrafish is known to be a quite eurythermal teleost fresh water species, with the optimal thermal niche between 26 ° C and 28.5 ° C (Avdesh et al. 2012, Sfakianakis et al. 2011). However, the study region of the GRACE project, characterized by cold temperatures and elevated salinity conditions from brackish to sea water, is far from the optimal environmental conditions for the zebrafish. The suitable range of the environmental parameters temperature and salinity was evaluated in detail in deliverable 3.2. In report 3.2 the zebrafish embryo tolerance to a range of temperatures and salinity conditions was analyzed using literature data and experimentally investigated using crude oil samples and model chemical substances with known effects on the embryonic development.

It was concluded that temperature conditions in zebrafish experiments cannot be changed towards the temperatures that are relevant for the study regions of the GRACE project. Even temperatures marginal below the optimum would delay developmental processes like the time point of hatching. Especially in embryotoxicity testing up to a maximum of 120 hpf the hatching is a very critical point that can increase the toxicity as the embryos are no longer protected by the chorion. Salinity was found to be a far better adjustable environmental parameter. Zebrafish embryos tolerated salinities up to 6 ‰ without any adverse effect and also no influence on sublethal, sensitive measurements such as behavior. However, as water-accommodated fractions of crude and dispersed naphthenic North Sea crude oil exposure prepared in artificial fresh - and brackish water led to comparable concentration- and time dependent adverse effects in the embryos, it was decided to use the optimal conditions for further investigations using the zebrafish. Especially in the context of focusing on regulatory pathways behind physiological adverse outcomes in the embryos, additional stressors in respect to possible osmotic stress at higher salinities should be avoided.

The zebrafish has been proven to be a suitable model even in the field of oil toxicity research. Several studies on oil toxicity demonstrated that specific crude oil constituents such as PAHs induce a range of typical physiological effects like edema, cardiovascular dysfunctions or craniofacial deformities (blue sac disease) in this freshwater species (see discussion AOL chapter VII, e.g., (De Soysa et al. 2012, Incardona et al. 2013, Pauka et al. 2011). Even though the scientific literature suggests a broad variation of effect thresholds across different teleost species (Stieglitz et al. 2016), these findings from the zebrafish investigations were found to be consistent for the regionally relevant species truly exposed to oil (e.g.,(Brette et al. 2014, Khursigara et al. 2017). Comparison to indigenous species can be found in the discussion of different AOL below (chapter VII). Furthermore, the zebrafish is

one of the best described and understood fish models in ecotoxicology, which is crucially required to define the molecular pathways for affected biological functions.

Nonetheless, for the interpretation of effect data it always has to be kept in mind that deviating exposure medium conditions exert a great impact on the test system. Specifically, the composition and the amount of soluble compounds partitioning from crude oil into the water phase is altered under varying salinities and temperatures (Whitehouse 1984). Hence, a basic chemical analysis on priority PAHs of the water-accommodated fractions was included to support the interpretation of induced toxicity.

V Multilevel responses of zebrafish embryos exposed to crude and refined petroleum products

1. Fish embryo acute toxicity

The first step in developing the adverse outcome links is to characterize the adverse outcome on a phenotypic level. Thus, the embryonic development of zebrafish exposed to different petroleum products with varying processing degree from crude to refined was investigated. All data met validity criteria for FET according to OECD guideline 236.

For all oil types a concentration-related increase in sublethal and lethal effects was observed (**Figure 2**). LEWAFs of crude and refined oils resulted in 100 % mortality after 120 hpf for the undiluted stock concentration (1:50). CEWAF stocks (1:200) were extremely toxic to the early life stage of zebrafish and still concentrations of 12.5 % of stock for crude oil and 5 % of stock for marine gas oil led to 100 % mortality. Even though the dispersant Finasol OSR 51 induced concentration-dependent sublehtal and lethal effects in developing zebrafish embryos (data not shown), the higher toxicity of the CEWAF is rather likely based on a higher availability of crude oil compounds due to a larger surface area and thus earlier partitioning equilibrium. The embryos were not affected at dispersant concentrations corresponding to the CEWAF proportion. The marine gas oil, a fuel distillate, was the most toxic out of the three investigated petroleum products. The role of the supplemented green dye for MGO induced embryotoxicity is planned to be investigated in future.

Zebrafish embryos chronically exposed to LEWAF and CEWAF showed typical effects referred as blue-sac disease in the literature. Especially during the embryonic development, the cardiovascular system or the development of the craniofacial structures are affected at low aqueous concentration of PAHs originating from the crude oil (De Soysa et al. 2012, Perrichon et al. 2016). Most prominent, heart deformation and yolk sac or pericardial edema have been observed in all oil type exposure dilutions down to 16.7 % of stock (LEWAF MGO) or 0.78 % of stock (CEWAF of MGO), respectively. Several spine deformations occurred especially in MGO and IFO 180 treated embryos. A delayed hatching in sublethal effect concentrations was observed for exposure to naphthenic North Sea crude oil (see **Figure 3**). While more than 90 % of unexposed embryos (NC) were hatched after 96 hpf, a 40 - 60 % hatching rate was detected for LEWAF and CEWAF treatments. No hatching delay was observed for MGO and IFO180.

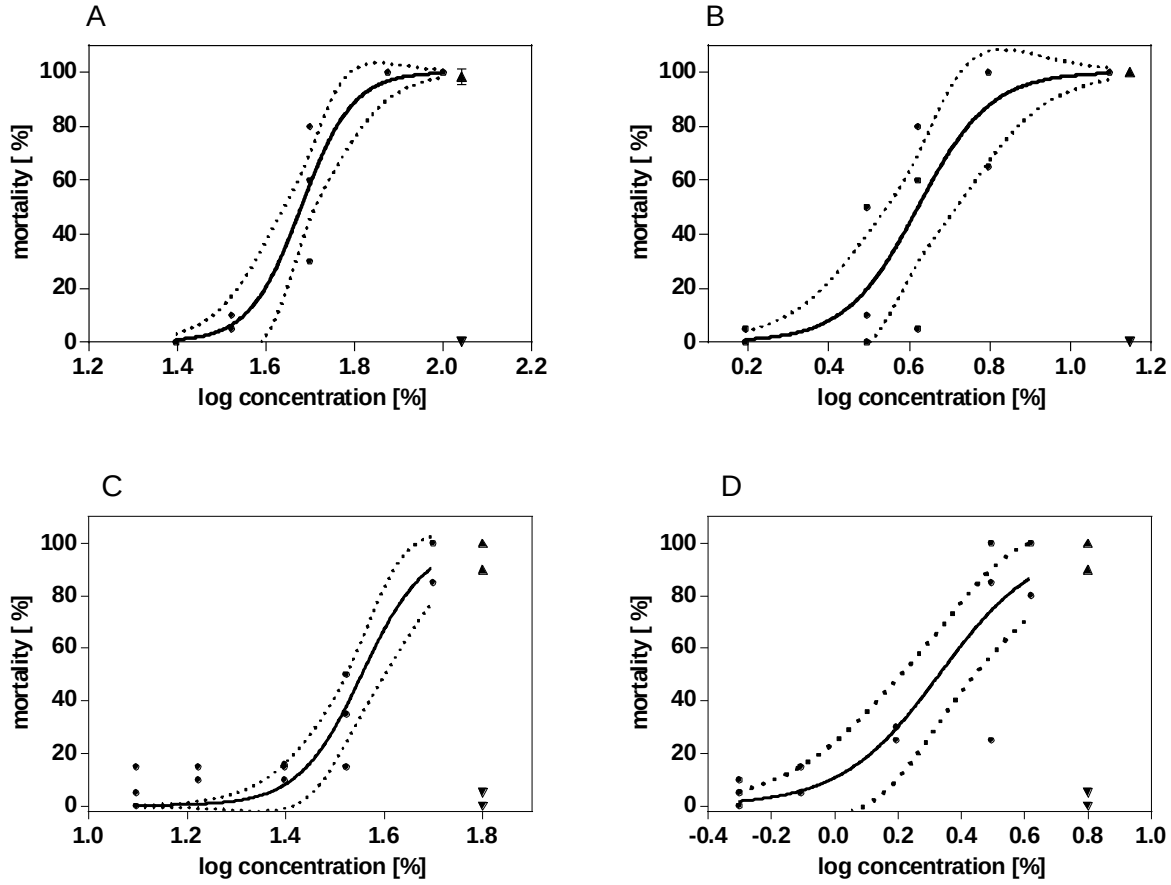

Figure 2 Mortality of zebrafish larvae (120 hpf) exposed to LEWAF and CEWAF dilutions of crude and refined petroleum products. A) LEWAF exposure with naphthenic North Sea crude oil (stock: 1:50 (w/v), B) CEWAF exposure with crude oil and Finasol OSR 51 (stock: 1:200 (w/v). C,D) corresponding WAFs with MGO and Finasol OSR 52. E) corresponding LEWAF of IFO180. Points denote the mortality of chronically exposed embryos (each point represents 1 out of 3 replicates). Triangles show the negative (artificial medium, pointing downwards) and positive controls (3,4-dichloraniline). Semi-static exposure conditions were used (medium exchange every 24 h). Sigmoidal concentration-response curves were added in GraphPad Prism 6 using the 4-parameter non-linear regression model, top and bottom variables were set to 100 and 0, respectively. Dotted lines indicate 95 % confidence band (Equation: Y= 1/(1+10^((LogEC50-X)*HillSlope).

Based on the established concentration-response curves EC_{10} and EC_{20} values, concentrations leading to 10 or a maximum of 20 % of the determined effects, were calculated by interpolation (EC_{10} values see **Table 2**). With this low-effect exposure concentrations different key events leading to the adverse outcome are aimed to be characterized as described in the following chapters focusing on mechanism-specific responses in zebrafish embryos and adults.

Table 2 Calculated 10 % effect concentrations (EC10) of different oil types in the acute fish embryo toxicity test with *D. rerio*. at 120 hpf. Data are based on 3 valid and independent experiments. EC values are based on a sigmoidal concentration-response curve fitted to the data. Top and bottom were set to 100 and 0, respectively (Equation: Y= 1/(1+10^((LogEC50-X)*HillSlope).

Oil type	WAF	EC_{10} [% of stock]
naphthenic North Sea crude oil	LEWAF	23.17
	CEWAF	1.23
marine Gas oil (MGO)	LEWAF	15.60
	CEWAF	0.65
intermediate fuel oil (IFO 180)	LEWAF	16.18

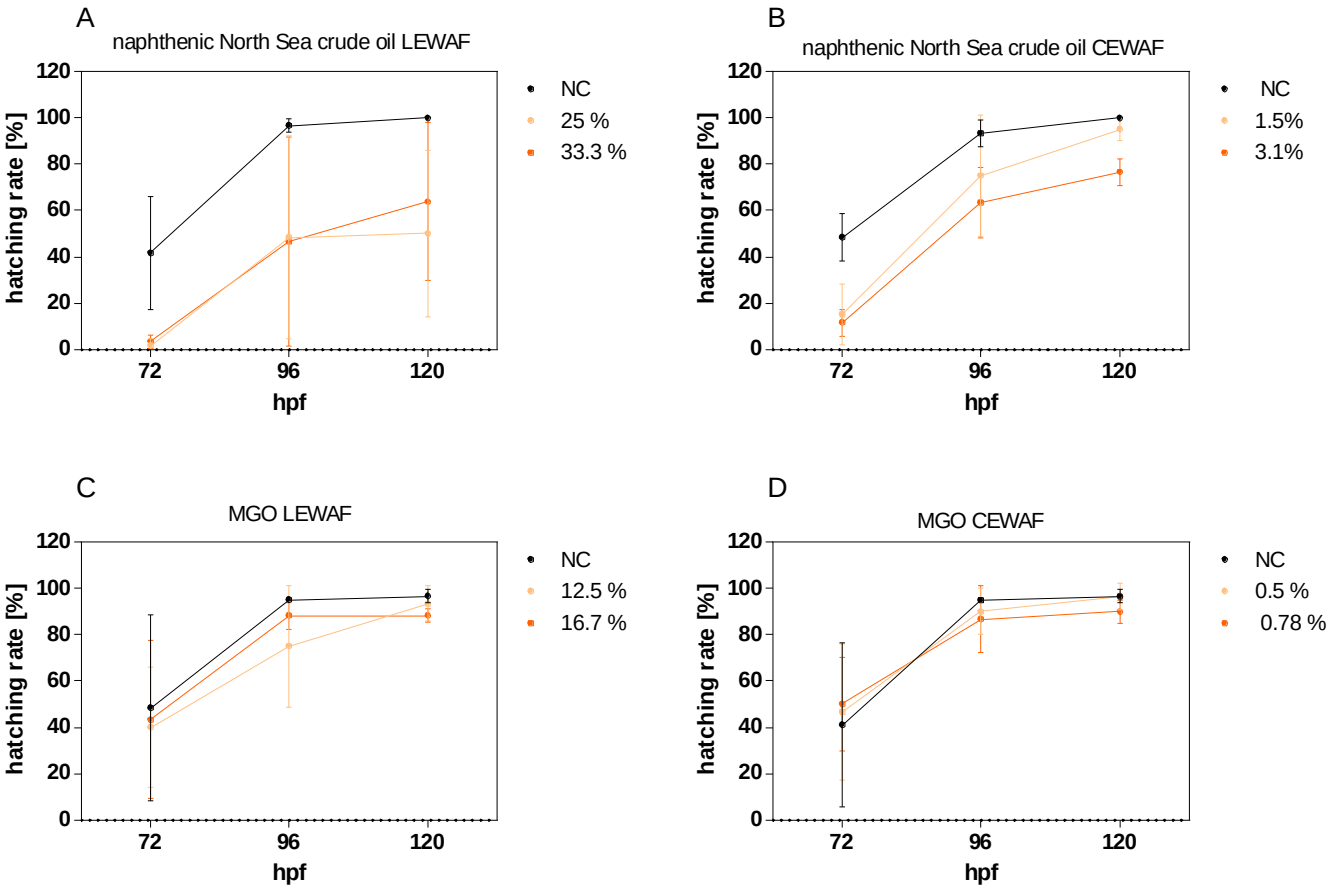

Figure 3 Hatching success of zebrafish embryos exposed to sublethal effect dilutions of crude and refined oil. Hatching rate was recorded every 24 hours post fertilization (hpf) in an acute fish embryo toxicity test. For each treatment of naphthenic North Sea crude oil exposure (A: LEWAF, B: CEWAF) and marine gas oil (MGO; C,D) the 2 lowest exposure concentrations expressed in % of stock as well as a negative control (NC) are shown. Points and error bars denote mean and standard deviation of 3 independent replicates.

2. Next Generation Sequencing

Understanding the underlying molecular pathways for the affected biological functions is of major interest. The results of transcriptome analysis provide an initial understanding of the underlying processes leading to the disruption of biological functions and can be used to focus subsequent studies using mechanism-specific bioassays or biomarkers. Hence, the RNA-sequencing results helped to characterize biomarkers for inclusion into the evaluation of oil toxicity to developing fish. For next generation sequencing as well as swimming behavior assay, zebrafish embryos were exposed to sublethal effect concentrations around EC_{10} of LEWAF and CEWAF.

2.1 Chemical analysis

During the exposure of the next generation sequencing study mainly 3 low-molecular weight PAHs were detected in fresh exposure medium before application to the vessels and old exposure medium that was removed every 24 h. The 2-ring PAH naphthalene was detected in the highest concentrations followed by the 3-ring PAHs phenanthrene and fluorene. Other PAHs of higher molecular weight were either below the detection limit of the applied method or not present in the LEWAF samples. From the comparison of fresh and old exposure LEWAFs composition it was concluded that the exposure conditions in the exposure vessels were relatively constant and that the exposure exchange regime prevented complete loss of freely available compounds over the course of 24 h.

2.2 Regulated pathways

The statistical analysis of mRNA levels between control and LEWAF/CEWAF groups found a significantly different (p value = 0.01) expression for 2223 genes (LEWAF) and 4048 genes (CEWAF), respectively. For the pathway enrichment analysis all significantly regulated genes with a fold change ≥ 1.5 were selected in order to identify most relevant regulated pathways in zebrafish embryos that were exposed to naphthenic North Sea crude oil LEWAFs and CEWAFs. For the analysis of the gene list with all genes regulated more than 1.5-fold 66 terms of significant enrichment were found for CEWAF treated embryos. For LEWAF treated embryos 57 significantly enriched pathways were found.

A detailed description of regulated pathways in LEWAF and CEWAF treated embryos can be found in Nüßer, Johann et al. (in prep). Briefly, especially biological processes summarized in group leading terms like *response to xenobiotic stimulus* were identified in both treatment groups. Furthermore, the pathway enrichment analysis showed that multiple genes associated with the development of the eye and lens and genes associated with *phototransduction*, *visual perception* and *sensory perception of light stimulus* were significantly regulated. Also terms like *response to oxidative stress and gluthathione metabolism* were identified to be significantly regulated pathways.

3. Alterations in swimming behavior

In the light-dark transition test both sublethal LEWAF and CEWAF exposure concentrations led to altered swimming behavior of zebrafish embryos at the age of 119 hpf during the dark phases compared to untreated control embryos. A significant decrease in swimming activity was found for swimming distances pooled for 1 min (min 24) for both treatments in all replicates except the LEWAF exposure in replicate 3. For the directed swimming trajectories, all treatments were found to be significantly different to the control group. Detailed results of zebrafish swimming activity can be found in Nüßer, Johann et al. (in prep).

4. Biomarker of exposure in zebrafish embryos

4.1 EROD induction – biotransformation I enzyme activity

The EROD assay is a well-established bioassay that monitors the activity of the xenobiotic metabolizing enzyme CYP1A. Monooxygenase CYP1A enzymes catalyze the hydroxylation of xenobiotics to more hydrophilic compounds, which can be later conjugated in biotransformation phase II before metabolite excretion will occur. The expression and activation of CYP enzymes is mediated via the Aryl-hydrocarbon receptor (AhR). Dioxines and dioxine-like compounds including PCDDs, PCDFs and PCBs but also a set of PAHs bind the cytosolic AhR. The activated AhR-ligand complex is translocated in the nucleus where it binds to specific DNA sequences, so called dioxine-responsive elements, and functions as a transcription factor to induce the expression of a set of genes including CYP enzymes.

Zebrafish embryos exposed to three dilutions of naphthenic North Sea crude oil LEWAFs in the range of below, around and above established EC_{10} did not show significantly increased EROD activity at any of the tested time points (**Figure 4** Panel A). In contrast, the CEWAF exposure resulted in a concentration-related increase in EROD activity compared to the untreated control (**Figure 4** Panel B). While after 48 hpf only a slight increase in EROD activity was observed, a clear EROD induction was observed after 96 hpf and 120 hpf. However, significant increase compared to untreated control was observed in zebrafish embryos exposed to the 2 highest test concentrations (1.56 and 3.13 % of stock) only at 120 hpf.

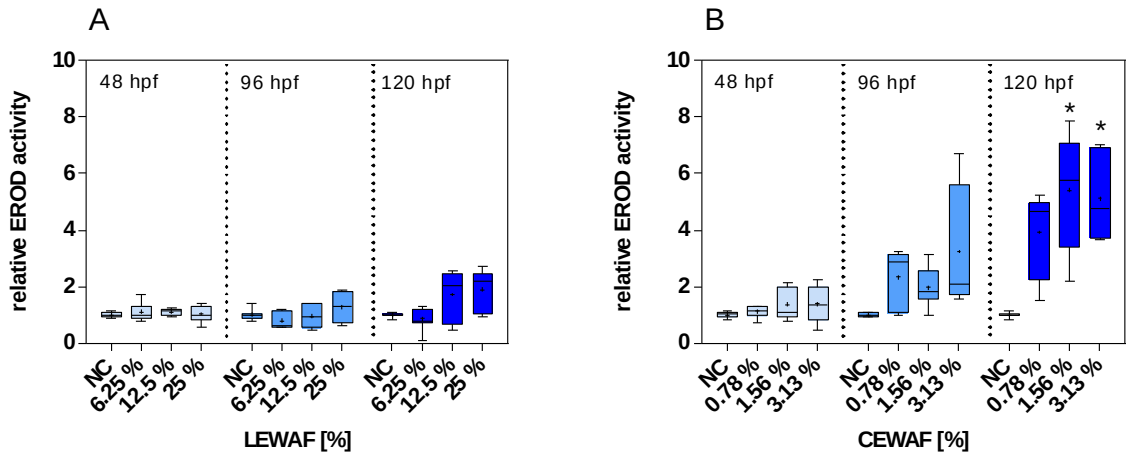

Figure 4 Relative 7-ethoxyresorufin-O-deethylase (EROD) activity in zebrafish embryos exposed to LEWAF and CEWAF dilutions of a naphthenic North Sea crude oil and Finasol OSR 51. Embryos were exposed to WAF dilutions of crude oil only (LEWAF, A) and dispersed crude oil (CEWAF, B). Specific EROD activity of 3 individual experiments each with 3 technical replicates were normalized to the untreated control (NC). Boxes show the 25 - 75 percentile, while median and mean are indicated as vertical line and +, respectively. Whiskers represent the min and max values. One Way ANOVA with Dunnett's post hoc test was used for statistical analysis. Asterisk indicate statistically significance of exposure groups compared to control group ($p<0.05$).

4.2 Acetylcholinesterase inhibition – neurotoxicity

As a biomarker of neurotoxicity, the acetylcholinesterase activity in zebrafish embryos was investigated. In general, no clear exposure concentration-related inhibition of the enzyme AChE was observed (see **Figure 5**). However, both LEWAF and CEWAF exposure resulted in significantly reduced AChE activity compared to the untreated control. Sublethal LEWAF dilutions of crude oil only resulted in significantly decreased AChE activity at 96 hpf. No AChE inhibition was observed in embryos exposed for 120 h. In contrast, CEWAF exposure led to AChE activity corresponding to the untreated control at 96 hpf, while inducing a singnificant inhibition in zebrafish embryos at 120 hpf.

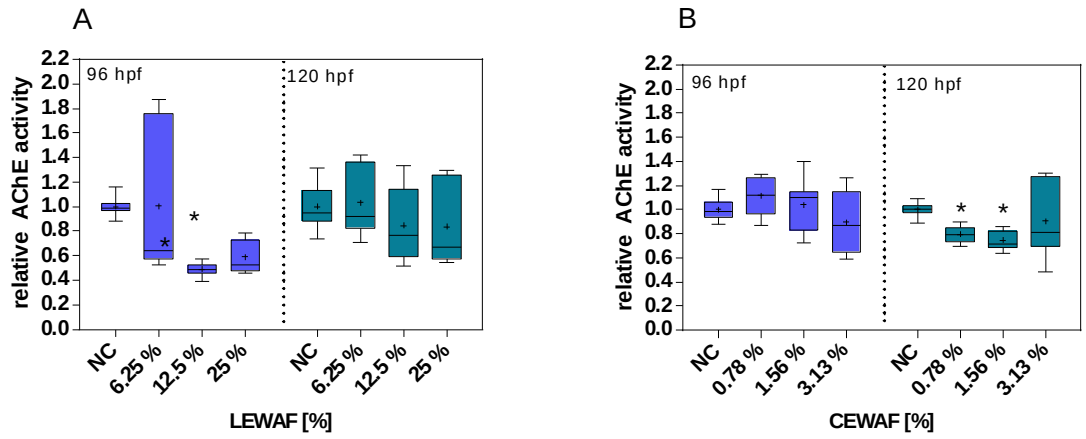

Figure 5 Relative acetylcholinesterase activity in zebrafish larvae (96 hpf, 120 hpf) exposed to WAF dilutions of a naphthenic North Sea crude oil and Finasol OSR 51. Zebrafish were exposed to WAF dilutions of crude oil only (LEWAF) and dispersed crude oil (CEWAF) at sublethal effect concentration. Data are represented as median (line), and mean (+) with boxes showing the 25 - 75 percentile and whiskers representing min to max values of 9 replicates based on 3 independent experiments (3 technical replicates per experiment). One Way ANOVA with Dunnett's post hoc test was used for statistical analysis with specific AChE activity. In case normal distribution or equal variance tests failed, non-parametric Kruskal-Wallis One Way ANOVA on ranks with Dunn's post-hoc test was used. Asterisk indicate statistically significance of exposure groups compared to control group ($p<0.05$).

5. Micronucleus induction ZF-L cells – genotoxicity

Even though the isolation of primary cells from zebrafish larvae has been shown to be not sensitive enough to detect micronuclei formation, the micronucleus assay using the permanent zebrafish liver cell line (ZF-L) was performed to implement the endpoint genotoxicity in this study.

Chromosomal fragments, so called micronuclei, that are located in the cytoplasm after cell division occur as a result of spontaneously or, to a higher extent, chemically induced chromosome breakage or inhibition of the spindle apparatus (Countryman and Heddle 1976, Reifferscheid et al. 2007).

The exposure concentrations were selected based on cell viability examinations (MTT bioassay) to guarantee normal cell growth and avoid false negative results based on cytotoxicity (data not shown). The validity criteria of a maximum of 3 % micronucleated cells in negative and solvent control and a significant increase in micronuclei formation in positive control were met for all the data presented. In general, both LEWAF and CEWAF of the naphthenic North Sea crude oil induced significantly increased micronuclei formation compared to the unexposed negative control (see Panels A, B of **Figure 6**). While for LEWAF exposure only the highest concentration was significantly different to the control, the chemically dispersed crude oil led to higher micronuclei induction occurring in both

treatments already at much lower exposure concentrations. Cells exposed to the intermediate fuel oil (IFO 180, see **Figure 6** Panels C,D) did not show significantly increased micronuclei formation compared to untreated control.

The observed higher potential of chromosomal aberration in cells exposed to dispersed crude oil WAFs was rather likely based on a higher bioavailability of the crude oil compounds and not on the dispersant toxicity. The dispersant Finasol OSR 51, which is used in combination with the crude oil to produce a CEWAF, was also tested for micronucleus induction (data not shown) in the CEWAF-corresponding concentration range. Observed micronuclei formation was in the range of the untreated control. Hence, the genotoxic potential of CEWAF was presumably induced by the dispersion effect of micelle formation leading to an increased amount of dissolved hydrocarbons reaching the equilibrium of water-oil partitioning much faster during WAF preparation.

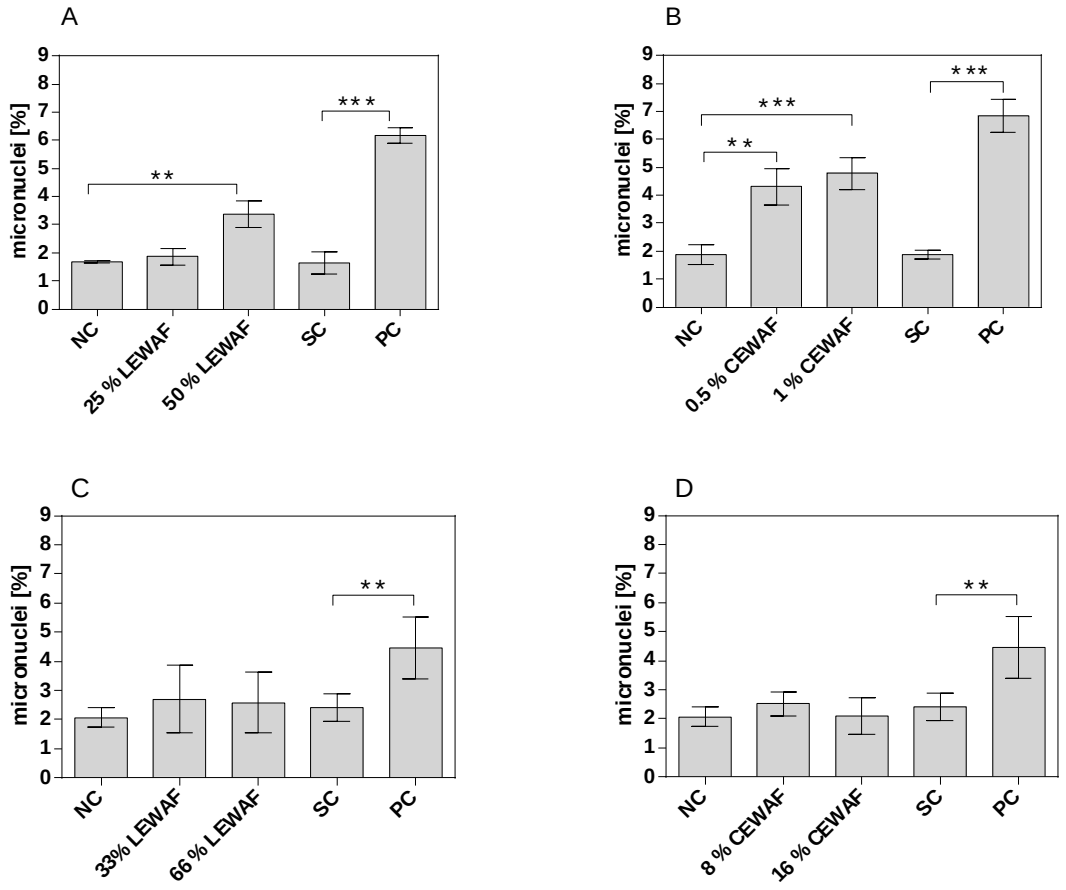

Figure 6 Micronucleus induction in ZF-L cells exposed to LEWAF and CEWAF dilutions of a crude and intermediate fuel oil. Bars represent the mean percentage of micronucleated cells (out of 2000 counted cells) for A) naphthenic North Sea crude oil LEWAF (100% stock= 1:50), B) dispersed crude oil CEWAF (100 % stock= 1:200 (oil/dispersant(1:10):water (w/v)) and C) and D) corresponding intermediate fuel oil (IFO 180) LEWAF and CEWAF dilutions. Error bars indicate the standard deviation (n=3). Negative- (NC), solvent- (SC, 0.1 % DMSO) and positive (PC, 4-Nitrochinolin-1-oxide in DMSO) controls were added. Chi² test with Yates correction was used

for statistical analysis. Asterisks indicate significantly higher micronuclei induction compared to controls. *p<0.05, **<0.01, ***<0.001.

VI. Biomarker responses of adult zebrafish exposed to crude oil

As part of a comprehensive experiment with adult zebrafish, several effect biomarkers were investigated after short term (3 d) and long term (21 d) exposure to naphthenic North Sea crude oil WAFs. Deviating from embryo experiments, the dispersant Finasol OSR 52 was used in the experiment as it is planned to compare those results to previous experiments using the same dispersant. During the experiment only the dispersed crude oil (LEWAF+D) treatment led to a mortality rate of 23 %. Control and LEWAF treatments resulted in 100 % survival.

Overall, the dispersed crude oil LEWAF (5 % LEWAF+D) showed the highest effects on changes in biomarker levels. Furthermore, trends of increased (EROD, GST, CAT) or decreased (AChE) enzyme activity were consistent for 3 d and 21 d of exposure with the exception of catalase activity in gills. Catalase activity levels were elevated after long-term exposure compared to short term exposure.

As a biomarker of neurotoxicity, the acetylcholinesterase activity in zebrafish brain was investigated. The exposure to a highly diluted naphthenic North Sea crude oil WAF (5% LEWAF) led to a slight increase in AChE activity compared to the untreated control (**Figure 7**).

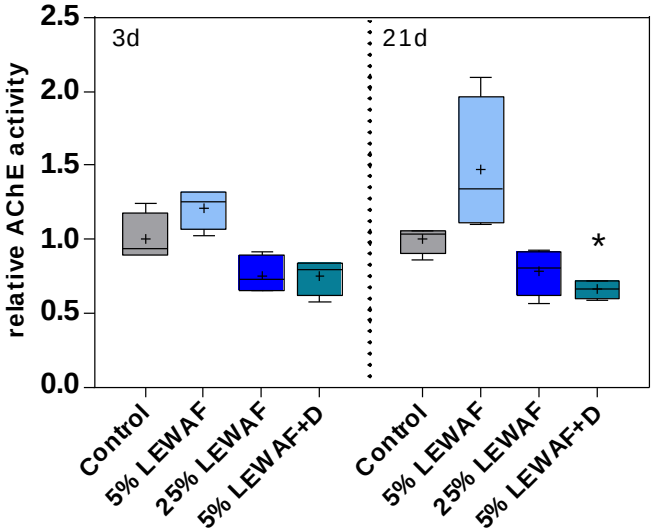

Figure 7 Relative acetylcholinesterase (AChE) activity in adult zebrafish brain tissue exposed to water-accommodated fractions (WAF) of a naphthenic North Sea crude oil and the dispersant Finasol OSR 52. Adult zebrafish were exposed to WAF dilutions of crude oil only (LEWAF) and dispersed crude oil (LEWAF+D). Brain homogenates of 4 replicate groups per treatment (3 individual brains pooled) were sampled after short term (3d) and long term (21 d) exposure. Data are represented as median (line), and mean (+) with boxes showing the 25 - 75 percentile and whiskers representing min to max values. One Way ANOVA with Dunnett's post hoc test

was used for statistical analysis with specific AChE activity. In case normal distribution or equal variance tests failed, non-parametric Kruskal-Wallis One Way ANOVA on ranks was used. Asterisk indicate statistically significance of exposure groups compared to control group ($p<0.05$).

In contrast, zebrafish that were exposed to a 25 % dilution of the LEWAF and the dispersed LEWAF did show an inhibition of the enzyme compared to the untreated control. However, a statistically significant difference to control was only observed for dispersed crude oil after 21 d of exposure.

In liver and gill homogenates biomarkers for biotransformation enzymes of xenobiotic metabolism phase I (EROD) and II (GST) as well as the biomarker of the antioxidant defense mechanism (CAT) were investigated. All biomarkers showed a higher activity in livers than in gills.

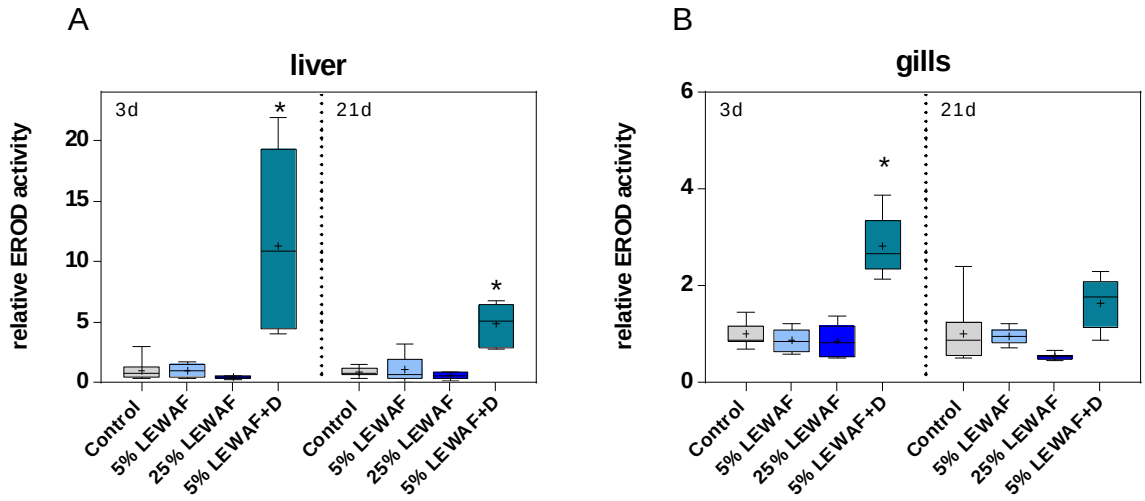

Figure 8 Relative 7-ethoxyresorufin-O-deethylase (EROD) activity in zebrafish tissues after short and long term (3 d, 21 d) exposure to WAF dilutions of a naphthenic North Sea crude oil and the dispersant Finasol OSR 52. Adult zebrafish were exposed to WAF dilutions of crude oil only (LEWAF) and dispersed crude oil (LEWAF+D). Data represent the EROD induction in liver (A) and gill (B) homogenates of 4 replicate groups per treatment (1 group= pool of 3 individual tissues). Boxes show the 25 - 75 percentile, while median and mean are indicated as vertical line and +, respectively. Whiskers represent the min and max values. One Way ANOVA with Dunnett's post hoc test was used for statistical analysis of specific EROD activity. In case normal distribution or equal variance tests failed, non-parametric Kruskal-Wallis One Way ANOVA on ranks was used. Asterisk indicate statistically significance of exposure groups compared to control group ($p<0.05$).

No changes in EROD activity after the exposure to an elevated and low concentration of crude oil LEWAF were observed in both tissues sampled after 3 d and 21 d of exposure (**Figure 8**). The dispersed crude oil approach (LEWAF+D) resulted in significantly increased EROD activity up to 10-fold of control group. Interestingly, in contrast to initial expectations a reduced but still significant EROD induction over exposure time was observed (3d: 10-fold

induction, 21d: 5-fold induction). As this treatment was the only exposure scenario with occurring mortality, the observed reduced EROD activity might be related to consequences of generalized toxicity. In case of general acute toxicity, mechanism-specific responses might be masked by non-specific secondary effects. However, this effect of reduced specific response was not observed in the other biomarkers measured in the present study.

The activity of the biotransformation phase II enzyme GST was determined to be increased especially for the high test concentration of crude oil LEWAF (25 % LEWAF) and the dispersed crude oil WAF (**Figure 9**). Highly diluted LEWAF (5% LEWAF) led to no significant changes in GST response with the exception of zebrafish liver after long term exposure.

As already indicated above, catalase was the only biomarker with consequently higher activity after the long term exposure compared to short term exposure in both tissues (**Figure 10**). Interestingly, an increased CAT activity in liver tissue was observed only for the highly diluted crude oil WAF, which is in general the least affecting treatment of this experiment. However, in gills all treatments led to increased CAT activity after 21 d of exposure with dispersed crude oil being significantly different from the control group.

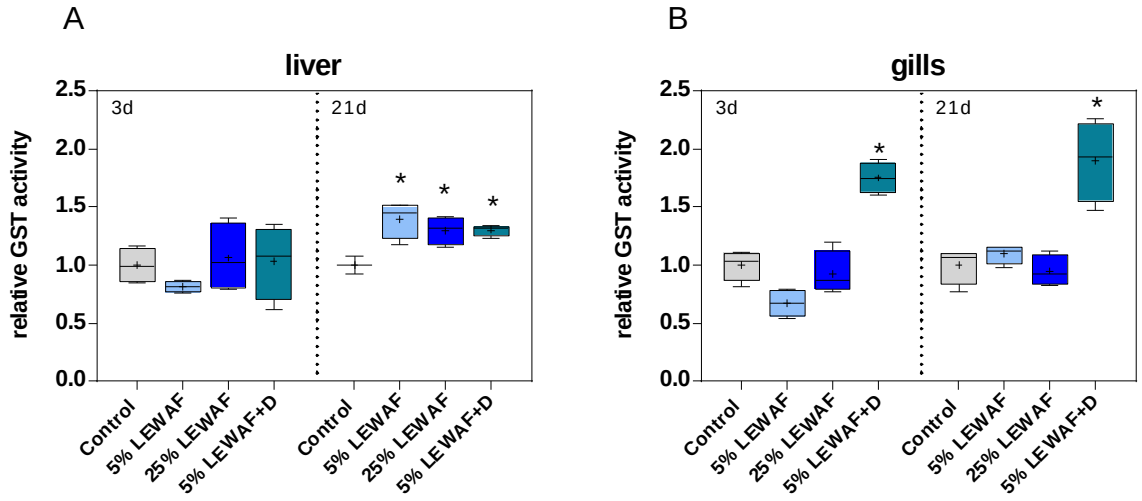

Figure 9 Relative glutathione-S-transferase (GST) activity in zebrafish tissues after short and long term (3 d, 21 d) exposure to WAF dilutions of a naphthenic North Sea crude oil and the dispersant Finasol OSR 52. Adult zebrafish were exposed to WAF dilutions of crude oil only (LEWAF) and dispersed crude oil (LEWAF+D). Data represent the CAT activity in liver (A) and gill (B) homogenates of 4 replicate groups per treatment (1 group= pool of 3 individual tissues). For control gropu of liver, only 2 valid replicates are available. Boxes show the 25 - 75 percentile, while median and mean are indicated as vertical line and +, respectively. Whiskers represent the min and max values. One Way ANOVA with Dunnett's post hoc test was used for statistical analysis. In case normal distribution or equal variance tests failed, non-parametric Kruskal-Wallis One Way ANOVA on ranks was used. Asterisk indicate statistically significance of exposure groups compared to control group ($p<0.05$).

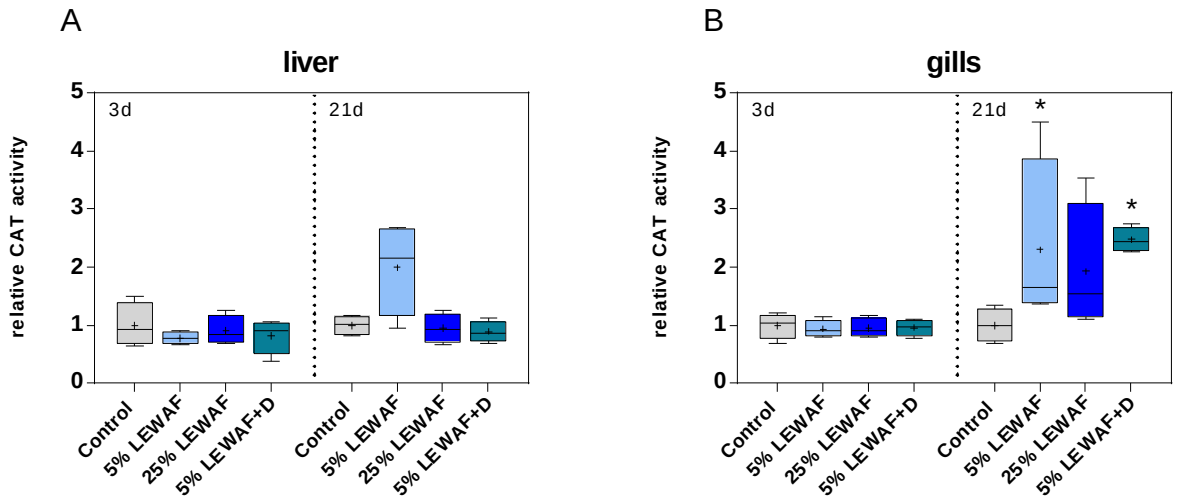

Figure 10 Relative catalase (CAT) activity in zebrafish tissues after short and long term (3 d, 21 d) exposure to WAF dilutions of a naphthenic North Sea crude oil and the dispersant Finasol OSR 52. Adult zebrafish were exposed to WAF dilutions of crude oil only (LEWAF) and dispersed crude oil (LEWAF+D). Data represent the CAT activity in liver (A) and gill (B) homogenates of 4 replicate groups per treatment (1 group= pool of 3 individual tissues). Boxes show the 25 - 75 percentile, while median and mean are indicated as vertical line and +, respectively. Whiskers represent the min and max values. One Way ANOVA with Dunnett's post hoc test was used for statistical analysis of specific CAT activity. In case normal distribution or equal variance tests failed, non-parametric Kruskal-Wallis One Way ANOVA on ranks was used. Asterisk indicate statistically significance of exposure groups compared to control group ($p<0.05$).

VII. Adverse outcome links in oil exposed zebrafish

In the following section, different modes of actions behind crude oil toxicity towards zebrafish embryos and adults will be discussed. **Table 3** in the appendix summarizes the WAF induced effects at different biological organization levels presented in the present report, which are brought together in the context of a zebrafish adverse outcome link.

1. Neurotoxic potential

The zebrafish is an emerging alternative model in neurotoxicity studies as the nervous system structures and functions are highly conserved across vertebrates (Kopp et al. 2018). Neurotoxicity is defined as the capacity of agents (chemical, physical, biological) to cause adverse functional or structural changes in the nervous system (Legradi et al. 2018). Acute neurotoxic effects including for example the inhibition of receptors, which interferes the intra- or intercellular network, are distinguished from neurotoxic effects related to neurodevelopmental processes like the formation of axons (Legradi et al. 2018). In the field of neurotoxicity research several approaches are continuously developing to identify neurotoxic mode of actions in respect to detect neurotoxic pollutants. Besides biomarkers (e.g. AChE inhibition) or *in-vitro* assays focusing on molecular mechanisms also behavioral assays are promising tools for neurotoxicity assessment. In respect to the zebrafish model even early life stages are suitable for behavioral assays. The brain and the nervous system are nearly fully developed at early embryonic stage of 96 hpf and the visual system is completely functional from around 120 hpf (Legradi et al. 2015). In the following sections the neurotoxic potential of crude oil water-accommodate fractions in the zebrafish is discussed based on enzymatic and behavioral investigations.

1.1 Acetylcholinesterase inhibition

The primary function of the enzyme acetylcholinesterase is to regulate the activity of the neurotransmitter acetylcholine (ACh). In its function it cleaves the acetylcholine, which was released into the synaptic cleft as a result of an action potential in the presynaptic neuron, into choline and acetate. The cleavage leads to the release of the neurotransmitter from the postsynaptic muscarinic receptor of neuromuscular junctions or chemical synapses of the central and vegetative nervous system (Behra et al. 2002, Soreq and Seidman 2001) regenerating the induced impulse of the action potential.

Neurotoxic substances like organophosphate - and carbamate-type pesticides or heavy metals lead to an inhibition of the enzyme AChE (Soreq and Seidman 2001, Velki et al. 2017, Yen et al. 2011), resulting in several described adverse effects in the threated organisms like spasms, paralysis or even death. In general, these downstream effects of AChE inhibition

(molecular initiating event) are described in a set of key events of a corresponding suggested adverse outcome pathway, which is under development of the OECD work plan (AOP wiki).

The AChE can be found in almost all taxonomic groups with highly conserved primary amino acid sequence across vertebrate and invertebrate species (Russom et al. 2014). Insects and crustaceans are the most sensitive species for AChE inhibitors due to targeted effects of the neurotoxic pesticides (Russom et al. 2014). Fish and amphibians were described to be moderate sensitive followed by mollusks and annelids in decreasing sensitivity. The investigation of AChE inhibition as a biomarker of neurotoxicity is well described in fish species and more specifically and simultaneously more relevant for the present study, in zebrafish. Even early life stages of zebrafish have been determined to be sensitive for the exposure towards organophosphate pesticides like chlorpyrifos, diazinon or diuron (Velki et al. 2017, Yen et al. 2011).

In the present study a significant reduction in AChE activity in zebrafish embryos and adults exposed to crude oil water-accommodated fractions has been observed. Even though the AChE expression appears to be present in all life stages (Russom et al. 2014), determined already from 5 to 7 somite stage in zebrafish (Behra et al. 2002, Bertrand et al. 2001), our results indicate that a stable activity in embryos and larvae can be measured from 96 hpf (data not shown in the report). In addition to our findings previous studies observed an increase in AChE expression and activity within the first days of embryonic and larval development (Bertrand et al. 2001, Kienlea et al. , Yen et al. 2011). Hence it can be concluded that the AChE inhibition is a useful biomarker for neurotoxic compounds in zebrafish embryos, when used in late embryonic developmental stages (from around 96 hpf).

In respect to crude oil relevant compounds it is indicated in literature that also PAHs might be able to inhibit the AChE in fish. The purified AChE isolated from the electric eel was found to be inhibited by a set of PAHs (Jett et al. 1999, Kang and Fang 1997) in *in-vitro* studies. The authors found higher molecular weight PAHs to be more effective in the inhibition of the enzyme then the PAHs containing 2 - 3 aromatic rings PAHs like naphthalene or fluorene. However, another *in-vitro* study investigating the role of PAHs as AChE inhibitors failed to detect a decrease in enzyme activity after the exposure to a set of PAHs including anthracene, chrysene and benzo[a]pyrene (Tang et al. 2003). Based on chemical analysis data of the present study, especially low molecular weight PAHs were found in the WAFs, indicating that also these PAHs could contribute to an inhibition of the AChE.

Also studies directly investigating the AChE activity in fish tissues of crude oil WAF exposed animals led to controversial conclusions about PAHs as AChE inhibitors. Acute exposure of a tropic freshwater species to water soluble fractions of a crude oil for 96 h resulted in a decrease in AChE activity (Akaishi et al. 2004). However, the AChE activity was measured in

muscle tissues. In contrast, Jung et al (2011) monitored the AChE activity in brain tissues of 2 fish species for 1 year after the Hebei Spirit oil spill and could not correlate a reduced AChE activity to the chemically measured PAH levels in fish.
Another study conducted by Kais et al. (2015) investigating the neurotoxic potential of complex sediment extracts was able to detect a reduced AChE activity in zebrafish larvae. Based on chemical analysis data of the extracts PAHs were identified as the main driver of toxicity in the sediments.

In respect to AChE inhibition, like for all toxicity mechanisms, the metabolization and detoxification has to be considered. For some organophosphate pesticides it has been shown that oxidized products are the AChE inhibiting compounds which are produced during xenobiotic biotransformation. Hence, as xenobiotic metabolization occurred in embryos and adults (see chapter below) not only mother compounds of the WAFs but also metabolites might have acted as AChE inhibiting compounds. Furthermore, also a detoxification of AChE inhibitors has to be considered as the AChE inhibition is a reversible process.

In conclusion, the present study emphasizes again the conclusion by Kais et al (2015) that PAHs in complex mixtures at least might contribute to AChE inhibition. However, based on the present results and based on discussions in literature, it is not understood how PAHs might interact with the AChE. A direct interaction with the enzyme via competitive-binding is not exclusively the reason for a decreased enzyme activity. Also changes in the expression and synthesis of the enzyme or a general neuronal cell degeneration cannot be ruled out.

In general, a direct comparison of the inhibitory potential of different crude oils has to be treated carefully, as every crude oil is a unique complex sample with widely varying contents of low and high molecular weight PAHs and additionally other possible neuroactive compounds such as heavy metals might have an influence on observed toxicity.

1.2 Alterations in swimming behavior and potential influence on eye development

Zebrafish larvae at the age of 119 hpf that were exposed to the naphthenic North Sea crude oil WAFs of the present study showed significantly reduced swimming activity compared to untreated control during the dark phase of a light/dark transition test.

Altered swimming behavior in adult and embryonic or larval zebrafish exposed to crude oil samples or typical crude oil compounds such as PAHs have been reported in several studies. Within this, altered behavior has been demonstrated throughout developmental stages from early embryo (around 48 hpf) over larvae (typically around 120 hpf or later) and juveniles up to adults. De Soysa showed that the Macando crude oil exposure impaired the escape behavior in 48 hpf old fish (De Soysa et al. 2012) using a touch response behavior

assay. Embryos were less sensitive to touch stimulus, showed significantly reduced frequency of body bend and swam less then untreated embryos. Also other studies investigating the behavioral response of later developmental stages of fish larvae or juveniles in locomotor or light/dark transition tests determined reduced swimming activity in PAH or crude oil WAF exposed individuals (Mager et al. 2014, Perrichon et al. 2014). However, opposed to these observations increased swimming activity of PAH exposed Japanese medaka larvae during the dark phase of a light/dark transition test have been reported (Le Bihanic et al. 2014). Furthermore, anxiety-related alterations in shelter-seeking behavior in zebrafish larvae (7dpf) exposed to WAFs of crude oils and dilbit was documented (Philibert et al. 2016).

In another study using weathered water soluble fractions (WSF) of the naphthenic North Sea crude oil adult zebrafish exposed in a flow-through system showed reduced swimming activity in 100 % WSF stock during the first 24 h of exposure, while a recovery was observed until the end of exposure (72 h) (Arukwe et al. 2008). Higher dilutions of WSF did not alter the fish behavior. The observations of the present study exposing adult zebrafish up to a maximum of 25 % LEWAF were in compliance with the results described by Arukwe et al. However, fish showed clear behavioral changes like reduced swimming activity, increased breathing and reduced feeding after the exposure to dispersed crude oil (LEWAF+D). Even in this treatment a recovery was observed at the second day of one exposure cycle (3 d) in the experiment. Another study investigating the behavioral disruption of adult zebrafish after dietary PAH exposure using a comprehensive set of behavioral tests found that PAHs led to greater mobility, lower levels of exploratory activity and higher levels of anxiety (Vignet et al. 2014).

Interestingly, it has been shown that the impaired swimming activity manifested as a latent effect even after a prolonged time without exposure in fish that were originally exposed to crude oil WAFs during an early time window of embryonic development (Hicken et al. 2011, Mager et al. 2014).

However, also no effects on swimming behavior of zebrafish exposed to crude oil WAFs have been reported. In a study conducted by Perrichon and coworkers zebrafish exposed to a light crude oil and a heavy fuel oil showed different results in a light/dark challenging assays (Perrichon et al. 2016). While the heavy fuel oil exposure impacted the swimming behavior of zebrafish larvae, the light crude oil exposure, in contrast to present findings of light crude oil exposure, did not induce altered swimming behavior of 120 hpf larvae.

In summary, it can be concluded that several crude oils and more specifically some PAHs are able to induce adverse consequences like disorganized swimming patterns. However, the identification of modes of actions behind the behavioral changes is quite difficult.

Especially the application of sublehtal effect concentrations close to mortality in some studies makes the interpretation of observed altered swimming behavior beyond obviously impacted phenotypes with edema or spine deformations difficult. Observed reduced swimming activity might not be exclusively related to neurotoxic effects but rather be a secondary effect due to several other physiological issues. De Soysa et al. (2012) focused on more details of observed altered locomotion in zebrafish embryos by assessing anatomical changes of neural circuity and skeleton muscle. The authors were able to detect deformations especially in the muscular system. Even though no anatomical effects on neurons of the central nervous system have been observed, the authors did not exclude an effect in central nervous system function which might additionally explain the observed altered swimming behavior. In another study a cardiotoxicity based reduction in swimming activity of adult zebrafish after a short term exposure in early life stages was suggested (Hicken et al. 2011). Adult zebrafish showed significant corresponding changes in ventricular shape, indicative of reduced cardiac output. In a follow-up experiment on dietary PAH exposure, Vignet et al. (Vignet et al. 2017) focused on the changes in brain monoamines and found that PAH exposure changed the monoamine contest which could underlie observed behavioral disruption.

One major finding of the transcriptomic study performed in GRACE was that the expression of several genes involved in structural and functional development of the zebrafish eye were significantly regulated in LEWAF and CEWAF treatments. Hence, the results indicate that normal development or function of the eyes might be impacted, which might further have contributed to observed altered swimming behavior of reduced reaction to dark onset stimulus. It has to be emphasized that sublethal exposure concentrations used for the present experiment did not result in anatomic changes like spine curvature or heart deformations in the embryos. Hence, behavioral changes as secondary effect of embryonic malformation can be excluded. However, a reduced fitness or secondary effect based on molecular events cannot be ruled out at the current state of experimental investigations.

The significant down-regulation of genes involved in functional eye development has also been determined for other fish species' transcriptomes after the exposure to Macando crude oil WAFs (Xu et al. 2017, Xu et al. 2016). Furthermore, different studies that observed impacted swimming behavior of PAH or crude oil WAF-exposed fish simultaneously found morphological alterations in the eye like reduced size or disruptions and apoptosis in retinal cell layers (Huang et al. 2013, Kawaguchi et al. 2012). The key events of molecular mechanisms and especially the molecular initiating event underlying these effects are not fully understood but might be a AhR-dependent regulatory pathway as suggested in different studies. Huang et al. (2013) focused on possible mechanisms involved in the interrupted ocular development of zebrafish exposed to phenanthren using behavioral, morphological,

histological and molecular methods of investigation. The authors suggested an phenanthrene-induced activation of the AhR, which results in a cascade involving the regulation of genes like *Zeb1* or *Mitf* followed by the reduction of the *Pax6*, the ultimate regulatory gene in eye development (Gehring 2005). The reduction of *Pax6* might then be associated with reduced cell proliferation and increased apoptosis in the zebrafish retinal cells.

Another study conducted by Aluru et al. emphasizes again the important role of the AhR pathway in zebrafish eye development. The authors showed that the knockdown of the AHRRa in zebrafish, which has been demonstrated to be needed for normal embryonic development in previous knock-down experiments, resulted in the down-regulation of several genes related to photoreceptor function (Aluru et al. 2014).

However, the development of the vertebrate eye is a complex process and hence also other pathways might be involved in disrupting these processes by chemical stressors. More recent findings for example indicated that thyroid disruption in zebrafish lead to pathologic alterations in the zebrafish eye (Baumann et al. 2016). It is known that the vertebrate eye development is partially regulated via thyroid hormones and different modes of actions of thyroid disruptors leading to the same apical endpoint have been suggested.

In conclusion, the results of the present study might indicate a role of impaired eye development in observed altered swimming behavior. However, further experiments on eye morphology will be conducted in the near future to bridge the gap between cellular response on mRNA level and adverse outcome on organismic behavioral level.

2. Detoxification mechanism

The two-phase process of xenobiotic biotransformation predominantly occurring in the liver of vertebrates is catalyzed by enzymatic reactions of oxidation, reduction and hydrolysis (biotransformation phase I) followed by conjugation (biotransformation phase II) in order to transform lipophilic chemicals to polar hydrophilic metabolites that can be eliminated via excretion (Schlenk et al. 2008). The xenobiotic biotransformation of PAHs in fish is described in several studies and reviews. PAHs are, depending on physical-chemical properties or the mixture composition, efficiently metabolized and eliminated in fish (Logan 2007, Van der Oost et al. 2003). However, especially for early stages of organogenesis a quite limited capacity for metabolism is described, and hence a higher bioconcentration of dissolved PAHs in embryos is the consequence (Incardona 2017, Jung et al. 2015). The exposure to PAHs elicits a variety of effects in fish through bio-activated metabolites, the extent of which is dependent on several factors like species or life stage. Although the liver is the most important organ for xenobiotic detoxification, the gills are directly and much earlier exposed

to a chemical stressor of the aqueous phase. As reviewed by Schlenk et al. several studies have demonstrated the expression of biotransformation enzymes also in this tissue (Schlenk et al. 2008).

In the present study it was found that compounds of the crude oil WAFs (PAHs) were bioavailable for exposed embryonic and adult zebrafish as a clear biotransformation response was determined on different biological organization levels. Based on the analysis of significantly regulated genes, group leading terms associated with biotransformation (e.g. *drug metabolic process)* were highly regulated pathways with typical upregulated genes like *cyp1a*, *cyp1c1*, *cyp1b1*. In respect to gene expression analysis the chemically dispersed crude oil led to more and higher regulated genes in terms of xenobiotic metabolism. Also on protein level a high biotransformation potential has been shown with CYP1A activity (EROD) significantly increased in embryos and CYP1A and GST activity significantly increased in adult gills and livers. However, while on mRNA level also the LEWAF treatment indicated high xenobiotic metabolism via *cyp,* a significant change in EROD activity on protein level was observed in dispersed crude oil exposed zebrafish (CEWAF, LEWAF+D) only. In contrast to this, LEWAF treatment led to significantly increased GST activity in livers of adult zebrafish.

The measurement of EROD activity is one of the most robust effect biomarkers in terms of sensitivity and dose-responsiveness and has been used in numerous studies focusing on the toxicity of crude oil WAFs in many freshwater and marine species across a variety of developmental stages (e.g (Oliveira et al. 2007, Perrichon et al. 2016, Sanni et al. 2017, Van der Oost et al. 2003, Whyte et al. 2000)).

Laboratory studies using the zebrafish model found mainly increased EROD activities after the exposure to petroleum product WAFs. In a study conducted by Perrichon et al.(2016) the EROD activity in 96 hpf zebrafish embryos exposed to light crude oil WAFs was increased up to 3-fold compared to the untreated control. In parallel, the exposure to a heavy fuel oil resulted in even higher elevated values of EROD activity. The light crude oil LEWAF used in the present study resulted in a maximum induction of 2 fold at comparable developmental stages of 96 and 120 hpf. However, the dispersed crude oil exceeded the detected CYP1A activity in the range of 4- to 6-fold. Also in 2-week old zebrafish larvae exposed to crude oil WSFs for 96 h a significant increase in EROD activity compared to the untreated control was detected (Pauka et al. 2011). Furthermore, a study using adult zebrafish and WAFs of the weathered naphthenic North Sea crude oil showed a significantly increased EROD activity in whole-body microsomal fractions (Arukwe et al. 2008).

In general, the most sensitive life stage of PAH-induced toxicity has been reported to be early embryo or larval development (e.g. (Whyte et al. 2000)). In the present study, 3

sampling time points from 48 to 120 hpf were selected to investigate the CYP1A induction via the EROD activity. Even in the highly xenobiotic metabolism-activating CEWAF treatment, no significant EROD induction was observed in embryos at 48 hpf. A weaker response in earlier pre-hatching stages has been described before (Bräunig et al. 2015). Even though the *cyp* genes expression have been determined in those early embryonic stages, a recent study suggested that a direct temporal relation between increased *cyp* expression and their biotransformation activity in those early life stages is not given and hence, post-translational modifications impact enzyme activity (Meyer-Alert et al. 2018). Nonetheless, the EROD activity in zebrafish embryos is a sensitive biomarker when investigated in different time windows including post-hatching developmental stages in order to exclude of false negative effects.

Besides in zebrafish, significantly increased EROD activity was observed in different developmental stages from early larvae (Couillard et al. 2005) to juveniles in other fish species exposed to water-accommodated fractions of crude oils (Adams et al. 2014, Kennedy and Farrell 2006, Oliveira et al. 2007, Ramachandran et al. 2004). Elevated hepatic CYP1A levels were also observed in fish species monitored after the exposure to crude oil compounds in real environmental conditions as a consequence of an oil spill (Dubansky et al. 2013).

In addition to EROD activity, also GST activity was measured in adult zebrafish liver and gills. The interpretation of GST activity in biotransformation is difficult as GST plays an important role for a set of biotransformation phase II independent regulatory pathways. Especially the role in oxidative stress response via reduction of cellular peroxides is of particular importance (Schlenk et al. 2008).

Results from the present study indicate no clear concentration-response related increase in GST activity. While in liver homogenates an increased GST activity compared to the untreated control was observed in every treatment only after long-term exposure, a significantly increased GST activity in gill homogenates, the relative induction of which was even higher compared to inductions in liver, was observed after short and long term exposure but only in dispersed crude oil samples.

Some studies discussed above in the context of biotransformation phase I also investigated the GST activity in fish exposed to crude oil WAFs. A significant GST increase in 2-weeks old zebrafish larvae acutely exposed to crude oil WSFs have been reported (Pauka et al. 2011), which was comparable to the induction observed in EROD activity. Simonato (2008) investigated the biomarker response in juvenile streaked prochilode (*Prochilodus lineatus*) exposed in different short and long term exposure scenarios to diesel oil WAFs and found a time dependent increase in hepatic GST activity with significantly higher inductions in fish that were acutely (96h) and semi-chronic exposed (15 d). However, another study exposing

goldfish (*Carassius auratus*) larvae to diesel oil WAFs detected only a slight increase in GST activity over an exposure period of 25 days (Zhang et al. 2004). Additionally, larvae of *Astyanax sp*. exposed to water samples from an oil spill affected area showed slightly increased GST activities after short term exposure of 96 h (Silva et al. 2009). While several studies indicated changes in GST levels, other studies failed to detect a significantly increased GST activity after the exposure to petroleum product WAFs.
As indicated in the present discussion with a high variability in hepatic GST levels also some reviews have discussed the GST to be no stable biomarker in respect to clear dose-response relationships across several fish species (Sanni et al. 2017, Van der Oost et al. 2003). Other biotransformation phase II enzymes like UDPGTs were suggested as better indicators (Van der Oost et al. 2003). However, internal studies in GRACE failed to establish a functional protocol for UDPGT measurement.

In general, CYP and GST activities are described to be regulated via the AhR pathway (e.g. (Van der Oost et al. 2003)). Interestingly, the dominant PAH of the naphthenic North Sea crude oil WAFS (naphthalene, fluorene and phenanthrene) all have been described as weak AhR ligands (Barron et al. 2004, Huang et al. 2013, Incardona 2017) but at the same time good CYP1A substrates. In general, especially higher molecular-weight PAHs are known to be strong AhR agonists. Hence, AhR independent mechanisms of observed toxicity might play a significant role. However, crude oil is one of the most complex sample types, potentially containing hundreds of different compounds in the water-accommodated fractions that act as a mixture of weak and strong CYP substrates and/or possible AhR ligands. Furthermore, in the context of AhR ligands, the role of weathering has to be taken into account. While for example the parent compound phenanthrene is a poor AhR agonist, alkylated forms have been described to show AhR-mediated toxicity (e.g. retene) (Scott et al. 2011). Even though a weathering was avoided as much as possible in respect to crude oil stock storage and WAF preparation, the process starts immediately with loading oil on the water surface and hence, changes in WAF composition cannot be excluded. Furthermore, from the detailed chemical profile of the naphthenic North Sea crude oil it is known that several alkylated PAHs are present in relatively high concentrations.

The activation of the phase I and II metabolism and specifically the AhR mediated pathway is often the primary reaction of organisms to chemical exposure and has several implications for the developing fish embryos. As a consequence of the upregulation of the detoxification mechanisms not only reactive PAH-metabolites could be formed but also the production of reactive oxygen species (ROS) is possible (Van der Oost et al. 2003), which might additionally impact the organism via other toxicity mechanisms like oxidative stress response as discussed in the following chapter.

3. Antioxidant defense assessment

A basal oxyradical production due to oxygen consumption in different biochemical processes like CYP activity is a normal process in aerob organisms. A complex antioxidant system counteracts the produced reactive oxygen species (ROS) (Regoli et al. 2002). In case the ROS production exceeds the limit of antioxidant defense mechanisms, oxidative stress of varying extent is the consequence.

The role of oxidative stress and corresponding defense mechanisms in naphthenic North Sea crude oil toxicity was investigated on gene transcriptional (embryos) and protein levels (adults). On mRNA level the pathway enrichment analysis of genes regulated more than 1.5-fold in the LEWAF treatment group found the GO term *cellular response to oxidative stress* to be enriched. The genes were also regulated in the CEWAF treatment groups. However, no terms or pathways connected to oxidative stress were significantly enriched for the CEWAF treatment group basically because of statistical calculations indicating several other higher regulated pathways in this treatment. Interestingly, genes of antioxidant enzymes like catalase, superoxide dismutase or glutathione peroxidase were not up-regulated in zebrafish embryos. However, peroxiredoxin 1 and Nrf2a and Nrf2b, all clearly associated with oxidative stress response, were significantly regulated. On protein level, the activity of the antioxidant enzyme catalase was investigated in the livers and gills of exposed adult zebrafish. While in livers no significantly increased CAT activity compared to untreated control was observed, both crude and dispersed crude oil treatments resulted in significantly increased CAT activities after long-term exposure.

The function of catalase is limited to the removal of hydrogen peroxide, which is metabolized to molecular oxygen and water. As the enzyme is also located in the peroxisomes of most cells and involved in fatty acid metabolism (Van der Oost et al. 2003) an interpretation of observed changes should always keep in mind the limited informative value for oxidative stress response.

An increase in the oxidative stress biomarker catalase in different fish species exposed to petroleum product WAFs has been reported in previous studies. Achuba et al (2003) investigated the CAT activity in different tissues of crude oil dispersion exposed catfish and found significantly increased CAT activity in livers and gills after long term exposure of 21 and 28 d. Also Zhang (2004) and Silva (2009) found the catalase to be activated in long-term (25 d) and short term (96 h) exposure experiments of juvenile goldfish or *Astyanax sp.* treated with diesel oil WAFs or water samples of an oil spill affected area, respectively. However, while for the short term exposure experiment in *Astyanax sp*. a significantly increased CAT activity was observed, the long term exposure in goldfish led to a trend of

increased antioxidant enzyme activity that did not meet statistical significance. Another study working with diesel oil WAF did not detect changes in CAT activity in streaked prochilod even using an elevated concentration range compared to the above mentioned study (Simonato et al. 2008). However, as different fish species have been used the comparability of the results is even more limited than already based on WAF preparation and unique diesel characteristics. Milinkovitch et al. (2011), focusing on biomarker responses in gills of juvenile golden grey mullets, showed that WAFs of a light crude oil (Brut Arabian), independent of chemically or mechanically dispersion, had no effects on antioxidant enzymatic activity in an acute exposure experiment (48h).

Several studies also investigated the oxidative stress potential of single PAHs via antioxidant enzymes. In this context, different fish species have been exposed to sublethal effect concentrations of phenanthrene with resulting significantly increased CAT activities in livers and gills (Jee and Kang 2005, Sun et al. 2006). However, no clear trend of antioxidant defense enzyme activity was obvious in terms of dose- and exposure time-response and significant differences to control groups were quite limited.

As indicated in the present discussion about widely varying results of petroleum compounds-induced catalase activities, the relevance of oxidative stress biomarkers in ecotoxicology is quite controversially discussed in the literature. On the one hand those biomarkers have been described as sensitive and useful biomarkers in respect to PAH toxicity (Milinkovitch et al. 2011). On the other hand, the antioxidant defense system was discussed as less sensitive with little evidence to be an important mode of toxicity of petroleum products (Perrichon et al. 2016). Besides measuring the catalytic activity of antioxidant defense enzymes, also non enzymatic antioxidants like glutathione (GSH) or some vitamins have to be taken into account. Relations of reduced to oxidized glutathione levels (GSH:GSSG) can be indicators for the oxidative stress status as reduced GSH is not only the substrate for GST in phase II metabolism but reacts with oxyradicals and hence act as antioxidant itself. A shifted GSH:GSSG status (normal > 10:1, (Van der Oost et al. 2003)) indicates the cells health status. Interestingly, for LEWAF treated embryos the pathway enrichment analysis of significantly enriched genes found the KEGG pathway *Glutathione metabolism* to be significantly regulated, which might indicate an oxidative stress potential. In respect to GSH, also the activity of GST as antioxidant defense mechanism has to be taken into account as discussed in previous chapter on xenobiotic biotransformation.

Furthermore, biochemical indices of oxidative stress like lipid peroxidation (LPOX) are often investigated in respect to PAH toxicity. However, even the LPO measurement provided strong or weak correlations with oxidative stress induced toxicity of petroleum compounds in different studies (Achuba and Osakwe 2003, Milinkovitch et al. 2011). As already discussed

by Regoli et al (Regoli et al. 2002) measuring a single biomarker cannot be a general marker for oxidative stress. The response of individual antioxidants might be specific and sensitive but it is critical for understanding the big picture of organismic health and defense status. Hence, more levels of organization should be taken into account.

In limited available studies using electron paramagnetic resonance (EPR) detection methods for radical formation it has been shown that naphthalene and phenanthrene, which are the prominent PAHs in water-accommodated fractions of the present studies, did induce the formation of radicals in goldfish liver tissue in a concentration range comparable or slightly increased to the present LEWAF (Shi et al. 2005, Sun et al. 2006). Interestingly, the enzymatic activity of CAT, SOD and GST, additionally tested in those studies, was only significantly increased in some sampling time points or the highest test concentrations emphasizing again that oxidative stress induces a complex response taking place via several biochemical processes.

In general, oxidative stress response can be a first indicator for genotoxicity as reactive radicals can interact with cellular macromolecules possibly leading to DNA damage (Regoli et al. 2002, Van der Oost et al. 2003). In the present study, the genotoxic potential towards crude oil exposed zebrafish was indirectly investigated by micronucleus induction in a zebrafish liver cell line (Z-FL). Results from the micronucleus assay showed that both, crude and dispersed, WAFs did induce significantly increased micronuclei rates with a stronger induction in dispersed oil treatment. Hence, a possible genotoxic potential of naphthenic North Sea crude oil WAFs is indicated. However, a direct extrapolation from the results of the cell culture to the whole organism is limited due to several factors like divergent bioavailable exposure fraction in cells and organs or whole organisms. Furthermore, genotoxicity is not exclusively correlated to oxidative stress.

In conclusion, based on biochemical and transcriptional results of the present study and based on the relatively high potential of the WAF-dominating PAHs to induce ROS, activating the antioxidant defense mechanisms, our findings indicate that crude and dispersed oil treatment induces oxidative stress in zebrafish embryos and adults. This oxidative stress seems to be related to the induction of detoxification mechanisms as indicated with elevated biotransformation enzyme activity. However, for a clear picture of oxidative defense mechanism additional biomarkers should be investigated. In respect to the adult zebrafish experiments, oxidative stress markers on gene expression levels as well as genotoxic potential via micronucleus induction in erythrocytes are currently under evaluation.

4. Cardiotoxicity

Even though cardiotoxicity was investigated only on morphological level in the acute fish embryo toxicity test, a short discussion of this endpoint should not be missing in the present report, as cardiotoxicity is one of the main driver in petroleum compounds toxicity on fish early life stages.

Impairments of cardiac phenotypes in zebrafish embryos have been observed across all tested petroleum products from crude oil over heavy fuel oil (IFO 180) up to purified oil distillates (marine gas oil). Interestingly, the crude and marine gas oil exposure resulted in more cardiotoxic effects than the intermediate fuel oil which might be correlated to relative contents of low or high molecular weight PAHs, as discussed in this section. Pericardial and yolk sack edema as well as heart deformation, bradycardia and circulatory interruptions were found in LEWAF and CEWAF treated embryos. Several studies investigating the embryotoxicity of crude oils found corresponding effects on the cardiovascular system. Bradycardia as well as edema have been reported in laboratory model species like the zebrafish and in indigenous species like mahi mahi or pacific herring that were exposed to crude oils after oil spills during the last decades (Deepwater Horizon, Exxon Valdez, Hebei Spirit) (De Soysa et al. 2012, Incardona et al. 2008, Incardona et al. 2013, Incardona et al. 2012, Jung et al. 2013, Perrichon et al. 2018). Interruptions of cardiac performance during embryogenesis can lead to death or severe consequences for adult fish like reduced fitness or altered swimming behavior as discussed in the previous chapter (Hicken et al. 2011, Vignet et al. 2017).

The typical morphological malformations connected to cardiotoxicity in crude oil exposed fish like edama or craniofacial skeleton interruption seem to be secondary effects originating from reduced blood circulation with the heart to be the primary target of crude oil toxicity on developing fish (Incardona 2017, Incardona et al. 2004). Studies focusing on details of cardiotoxicity in fish early life stages usually detect changes in heart beat rate and calculate factors like atrial contractility or stroke volume based on imaging and video tracking of heart dimensions. As a consequence of exposure, abnormal heart chamber looping, reduced heart beat rate and decreased stroke volume or atrial contractility have been observed (Edmunds et al. 2015, Esbaugh et al. 2016, Incardona et al. 2008, Khursigara et al. 2017). But also alterations in the cardiac jelly layer, which is a matrix important for the elasticity of the heart, and disruption of erythropoiesis have been reported in retene exposed zebrafish embryos (Scott et al. 2011). As stated by Perrichon et al. (2018), especially measuring the heart rate is not the ultimate parameter for cardiac performance as contrary results are available. Some studies found significantly reduced heart beat rates in 48 h or 96 h crude oil WSF exposed zebrafish embryos (Pauka et al. 2011, Philibert et al. 2016). However, no real concentration-

response relationship has been established (Philibert et al. 2016). In contrast to these significant alterations, *in situ* measurements of cardiac parameters did not reveal altered heart rate in mahi mahi (*Coryphaena hippurus*) exposed towards Deepwater Horizon crude oil HEWAFs in a short term experiment (24 h exposure) compared to untreated control while other cardiac parameters were significantly affected (Nelson et al. 2016). Furthermore, Edmunds et al (2015) did not detect effects of oil exposure on the heart rate while they observed clear cardiotoxicity based on poor looping and atrial contractility.

In the context of crude oil toxicity towards fish early life stages PAHs are defined to be the driver of toxicity. The water-accommodated fractions of the present crude oil are characterized by relatively high concentrations of naphthalene, fluorene and phenanthrene. While naphthalene was not described to induce malformations in zebrafish embryos, phenanthrene and fluorene were identified as embryotoxic compounds (Incardona et al. 2004). Also for embryonic and larval stages of rainbow trout and bass the toxicity decreased from phenanthrene to naphthalene (Black et al. 1983). These observations were confirmed by another study in which a crude oil sample was fractionated for toxicity testing. The authors concluded that fractions containing 2 ring PAHs like naphthalene had lower toxicity to rainbow trout embryos compared to fractions containing 3 ring PAHs (Adams et al. 2014).

Even though the phenotypes of specific morphological malformations resulting from cardiotoxicity seem to be consistent for different oil types and different freshwater and marine fish species, the molecular mechanisms behind cardiotoxicity seem to be diverse and are not fully understood in detail (De Soysa et al. 2012, Incardona 2017, Incardona et al. 2008, Perrichon et al. 2018). In a recent comprehensive review on cardiotoxicity in fish, Incardona (2017) explicitly differentiates between AhR-mediated and AhR-independent cardiac malformations: Especially higher molecular weight PAHs (4 to 6 benzene rings) are well known for their AhR-mediated cardiotoxicity, which can be prevented by AhR gene knockdown, while lower molecular weight PAHs may interfere directly with cardiomyocyte physiology. Hence, cardiotoxic effects observed in zebrafish embryos of the present study are probably more likely to be independent of an AhR-mediated pathway as it has been discussed that especially the dominant low molecular weight PAHs of LEWAF (naphthalene, fluorene, phenanthrene) are not typical AHR ligands but for example in the case of cardiotoxicity rather directly influence the excitation-contracting coupling mechanism in cardiomyocytes. The Ca^{2+} homeostasis plays a fundamental role in this context. In normal function a contraction in cardiomyocytes is induced via an action potential accompanying the influx of extracellular Ca^{2+} ions into cardiomycytes which also subsequently triggers the sarcoplasmatic reticulum to release even more Ca^{2+} in the cytosole (Brette et al. 2017, Incardona 2017). An increased intracellular Ca^{2+} concentration activates the actin/myosin

filaments resulting in the muscle contraction. Action potential repolarization and relaxation is driven by Ca^{2+} reflux via calcium natrium exchangers. Brette et al (2014) have demonstrated that complex crude oil mixture disrupted the excitation coupling mechanism in cardiomyocytes via prolonged action potential and decreased calcium cycling. As a consequence, the myofilament activation and contractility was reduced. In a follow-up study the authors tested several single PAHs, including those relevant for present study (naphthalene, fluorene and pehnanthrene) and found naphthalene to have no effects on Ca^{2+} dynamics, which supports again the described low embryotoxicity of naphthalene exposure described above. In contrast, the three ring PAH phenanthrene impaired the EC coupling (Brette et al. 2017). On transcriptional level genes involved in cardiac muscle and calcium homeostasis have been identified to be regulated after oil exposure in Atlantic haddock and mahi mahi (Edmunds et al. 2015, Sørhus et al. 2016, Xu et al. 2016). However, first analysis of the zebrafish embryo transcriptome found no significant alteration in genes suggested for early detection of cardiotoxicity. A detailed interpretation of molecular cardiotoxic markers is currently under evaluation.

VIII. Conclusion and outlook

In the present report, the effects of different petroleum products varying from crude to distillate on the developing zebrafish have been investigated. Furthermore, first results of a recent comprehensive experiments with adult zebrafish have been included. To obtain insight into the mode of actions of petroleum product toxicity, different biological organization levels have been discussed for the crude oil. In the near future, results for all endpoints in embryonic testing of MGO and IFO180, except for transcriptomic analysis, will be available in order to compare potential individual toxicity profiles in respect to oil composition.

Based on the four discussed modes of actions it can be concluded that crude oil toxicity is diverse and several pathways are closely connected (e.g. xenobiotic metabolism and oxidative stress response) and not fully understood. The cardiovascular system of fishes at the onset of organogenesis is described as the major target for petroleum compounds like PAHs. Besides cardiotoxicity, also other biological functions that seem to be independent of cardiac function like the development of the visual system are suggested to be affected during embryogenesis, which is indicated also by the present report. In previous years, several apical endpoints in PAH toxicity have been linked to AhR-mediated initiating mechanisms. However, results from recent research bring AhR independent trigger mechanisms like calcium homeostasis in the context of PAH toxicity. Furthermore, crosstalks between the AhR-mediated mechanisms to other pathways like for example the endocrine system are becoming more the focus of attention.

In general, we were able to detect a set of morphological phenotypes and linked those to expected biomarker responses and gene expression changes. Furthermore, also additional regulated pathways that are not detailed discussed in the context of crude oil toxicity literature have been found (see discussion Nüßer, Johann et al. (in prep). Nonetheless, the limitations of next generation sequencing being a snapshot of all genes at a given time as well as the limitations of biomarker non-pollution related variability should be considered for interpretation.

Concerning the detailed analysis of existing data contributing to the ecotoxicological risk assessment of oil samples in respect to results of the present study, the limitation of comparability posed a major challenge as already discussed previously (Bera et al. 2018, Redman and Parkerton 2015). First, several WAF preparation methods have been used in oil toxicity studies, directly affecting the exposure conditions. Open versus closed test systems, variations in temperatures and salinity of the exposure water or divergent headspace in WAF preparation vessels are described to name just a few. The dissolved hydrocarbons, generally determining the aquatic toxicity, necessarily depend on those factors of WAF preparation method, headspace and substance composition (Redman and Parkerton 2015).

Furthermore, the application of a dispersant will change this situation completely by adding more available droplets into the water column and changing the amount of bioavailable dissolved hydrocarbons.

Second, it has to be kept in mind that each crude oil is a unique sample with unique composition and varying physical-chemical properties. As indicated in the AOL discussion of the present report, a mixture can contain compounds acting or not acting via an established pathway of toxicity (e.g. AhR-mediated regulation). Complicating the interpretation of the data even more, the weathering, beginning immediately after the oil application on a water phase, can change the crude oil composition. The weathering process initiates the complete loss of aromatics like the volatile BTEX group or an increase in alkylated forms of 2 ring PAHs (Dupuis and Ucán-Marín 2015, Wang et al. 2003). It has been shown that alkylated PAHs induce different toxicity compared to their mother compounds (Adams et al. 2014, Incardona 2017, Lee et al. 2017, Scott et al. 2011).

The focus of the present study was to define possible molecular mechanisms leading to adverse outcome in crude oil exposed zebrafish. Hence, several interesting and important aspects of all the data represented in the present reports have not been discussed in detail. Issues like the role of dispersants and additives in oil toxicity, a correlation to chemical composition as well as a discussion of zebrafish responses in respect to other bioassays of a detailed *in-vitro* based toxicity profiling will be addressed in another context of the GRACE project.

For future perspective, a set of experiments has been established based on the present results and the actual literature review process to focus on more details of toxicity mechanisms. The oculotoxicity for example will be addressed in near future with experiments on additional histological and behavioral changes in crude oil exposed zebrafish in order to bridge the gap from gene expression to behavioral alterations that has been observed in the present study. Furthermore, results of the comprehensive adult zebrafish study as well as results of the fuel oil types will contribute to the understanding of petroleum product toxicity.

Especially the role of dispersant needs to be addressed in more detail in future work. Our results in GRACE so far indicate no dispersant toxicity in the concentration range used for CEWAF preparation and hence no influence on higher toxicity of CEWAF compared to LEWAF samples is suggested.

Passive dosing methods have recently been suggested as practical strategies to maintain dissolved oil concentrations (Bera et al. 2018) but they exclude the scenario that droplets can have additional effects in the system. The main focus of crude oil toxicity testing is most often on water-soluble fractions but also dispersed droplets in nm or µm scale might get into direct physical contact with biological membranes or float on the water surface. Limited knowledge

about the quantitative role of the droplets exist or is ignored in several studies (Nordtug et al. 2011b). WAF deviating exposure setups generating uniform oil droplets for the exposure in a flow through system (Nordtug et al. 2011a, Nordtug et al. 2011b) seem promising tools in the field of oil toxicity research to guarantee a controlled generation of dispersion.

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X. Appendix

Table 3 Summary of endpoints in embryo and adult zebrafish exposed to WAF dilutions of a naphthenic North Sea crude oil. Low energy water-accommodated fractions (LEWAF), chemically enhanced water-accommodated fractions (CEWAF) of dispersed crude oil (embryo experiments) or low energy dispersed crude oil (LEWAF+D, adult experiments) have been used to investigate the toxicity of the naphthenic North Sea crude oil and the dispersants Finasol OSR 51 and 52. Color scheme indicated no (grey), weak (light green) or strong effects (dark green). * Detailed report on regulated pathways can be found in Nüßer, Johann et al (in prep).

	LEWAF		CEWAF/ LEWAF+D	
	exposure [% of stock]	**effects**	**exposure [% of stock]**	**effects**
endpoints in embryos				
morphological changes	6.25-100	edema (yolk sack / pericard), heart deformations, circulatory interruptions, spine curvature, hatching delay	1.56 – 12.5	edema (yolk sack / pericard), heart deformations, circulatory interruptions, spine curvature, hatching delay
deep sequencing KEGG and regulated pathways	12.5	Drug metabolic process, visual perception, phototransduction, GSH metabolism, response to oxidative stress,…*	0.78	Phototransduction, drug metabolic process, visual perception, tissue development, regeneration, translation,…*
biotransformation I enzyme activity EROD induction	6.25, 12.5, 25	-	0.78, 1.56, 3.13	+
neurotoxicity enzyme activity AChE inhibition	6.25, 12.5, 25	+ (96 hpf)	0.78, 1.56, 3.13	+ (120 hpf)
endpoints in adults				
biotransformation I enzyme activity EROD induction	5, 25	- (liver) - (gills)	5	+ (liver) + (gills)
biotransformation II enzyme activity GST induction	5, 25	+ (liver) - (gills)	5	+ (liver) + (gills)
oxidative stress enzyme activity CAT induction	5, 25	- (liver) + (gills)	5	- (liver) + (gills)
neurotoxicity enzyme activity AChE inhibition	5, 25	+ (brain)	5	+ (brain)