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Multilevel responses of adult zebrafish to crude and chemically dispersed oil exposure

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Abstract

Background: The application of chemical dispersants is a common remediation strategy when accidental oil spills occur in aquatic environments. Breaking down the oil slick into small droplets, dispersants facilitate the increase of particulate and dissolved oil compounds, enhancing the bioavailability of toxic oil constituents. The aim of the present work was to explore the effects of water accommodated fractions (WAF) of a naphthenic North Sea crude oil produced with and without the addition of the chemical dispersant FINASOL OSR 52 to adult zebrafish exposed for 3 and 21 d. Fish were exposed to environmentally relevant concentrations of 5% and 25% WAF_{OIL} (1:200) and to 5% WAF_{OIL+D} (dispersant–oil ratio 1:10) in a semi-static exposure setup.

Results: The chemically dispersed WAF presented a 20-fold increase of target polycyclic aromatic hydrocarbons (PAHs) in the water phase compared to the corresponding treatment without dispersant and was the only treatment resulting in markedly bioaccumulation of PAHs in carcass after 21 d compared to the control. Furthermore, only 5% WAF_{OIL+D} caused fish mortality. In general, the undispersed oil treatments did not lead to significant effects compared to control, while the dispersed oil induced significant alterations at gene transcription and enzyme activity levels. Significant up-regulation of biotransformation and oxidative stress response genes (*cyp1a*, *gstp1*, *sod1* and *gpx1a*) was recorded in the livers. For the same group, a significant increment in EROD activity was detected in liver along with significant increased GST and CAT activities in gills. The addition of the chemical dispersant also reduced brain AChE activity and showed a potential genotoxic effect as indicated by the increased frequency of micronuclei in erythrocytes after 21 d of exposure.

Conclusions: The results demonstrate that the addition of chemical dispersants accentuates the effect of toxic compounds present in oil as it increases PAH bioavailability resulting in diverse alterations on different levels of biological organization in zebrafish. Furthermore, the study emphasizes the importance to combine multilevel endpoints for a reliable risk assessment due to high variable biomarker responses. The present results of dispersant impact on oil toxicity can support decision making for oil spill response strategies.

Keywords: Bioaccumulation, Biomarkers, Biotransformation, Chemical dispersants, Crude oil, Genotoxicity, Oil spills, Oxidative stress, Water accommodated fractions, Zebrafish

Background

Oil spills represent one of the most important potential sources of pollution in aquatic environments [140]. Anthropogenic activities such as offshore oil exploitation and transportation, or other contaminations caused by shipwrecks and industrial discharges, pose a high risk of an oil spill that could result in devastating consequences to aquatic organisms, such as fish [19, 52, 55]. Crude

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oils are complex mixtures of different toxic compounds that volatilize, dissolve, emulsify or disseminate through the water column [19, 115, 128]. Oil composition and environmental factors such as temperature and salinity are key elements determining the magnitude of the biological impact of an oil spill which—along with natural weathering and the selected remediation strategies—may influence the adverse aftermath of these accidents [18, 69, 106]. Polycyclic aromatic hydrocarbons (PAHs), for example, represent up to 60% of crude oil constituents present in water accommodated fractions (WAF) [40]. Their capacity to provoke diverse toxic effects in aquatic organisms is well known. These effects range from cardiotoxicity and malformations at early developmental stages up to behavioural alterations and carcinogenesis in adults [27, 54, 78, 129].

The use of chemical dispersants is a common practice applied to limit the spreading of the oil slick, thus preventing it from reaching coastal areas and mitigating its possible toxicological impacts on mainly birds and mammals populations [19, 44, 70, 90]. Chemical dispersants are composed of hydrocarbon solvents with anionic and non-ionic surfactants, displaying both hydrophilic and lipophilic properties that enable a rupture in the interfacial tension between oil and water [32, 44, 70, 90]. Thus, they are capable of breaking down the oil slick into smaller droplets which have been suggested to be more easily degraded by bacteria or photo-oxidation [137]. However, through a massive surface increase and direct ingestion of the droplets, chemical dispersion simultaneously enhances the bioavailability of toxic oil constituents, such as PAHs, shifting the ecotoxicological damage towards pelagic species [19]. The potential adverse outcomes of the application of chemical dispersant remains a subject of major concern [17, 44, 46], which is why their effectiveness, benefits and impacts resulting from their use need to be further evaluated.

The EU Horizon 2020 funded project GRACE focused on a holistic approach to investigate the environmental effects of oil spills and response measures [65]. As a part of this, the present study focused on adult zebrafish due to multiple advantages as an (eco)toxicological model including small size, fast development, low maintenance cost, ease of reproduction and large spawn. Furthermore, the zebrafish is a well-established animal model for the assessment of oil spill toxicity [34, 66, 126] and response strategies, such as chemical dispersion [75, 101]. Biomarker changes in gene transcription, development, metabolism, or even behaviour can be efficient tools to provide evidence of an exposure to pollutants [10, 31]. Measuring biomarkers on molecular and enzymatic level can indicate the exposure to toxic substances before an evident tissue-level damage occurs or even when pollutant concentrations in the media are below detection limits, supplying valuable information for environmental health evaluations [106, 121].

One of the sensitive biomarkers selected for the current study, which is linked to crude oil exposure, is the induction of phase I metabolism [10, 29, 121]. Alterations of ethoxyresorufin-O-deethylase (EROD) activity manifest changes on the expression of cytochrome P450 (e.g., the CYP1A subfamily), an essential mechanism along with the likewise investigated glutathione S-transferase (GST) activity in the phase II biotransformation reactions of xenobiotic detoxification [48, 78]. Furthermore, when these two phases of metabolism are not well coupled, the reactive metabolites produced in phase I may generate DNA adducts, which might lead to genotoxic effects [54, 94, 138]. The potential of oil constituents such as PAHs to induce DNA damage has been demonstrated previously, making genotoxicity also a relevant endpoint for crude oil toxicity assessment [12, 79, 112]. Since DNA adducts might intercalate into the DNA inducing strand breaks, genotoxicity assessment in the current study includes the investigation of micronuclei formation in peripheral blood samples in addition to selected molecular marker genes related to cell cycle control and apoptosis (*tp53*, *casp3a*). An imbalance between the appearance of reactive oxygen species (ROS) from phase I biotransformation and enzymatic antioxidant defence mechanisms, such as glutathione peroxidase (GPX) or catalase (CAT) activities [116, 121], can lead to an oxidative damage of cells and tissues, which, ultimately, could be translated into necrosis, apoptosis or carcinogenesis [53, 78, 100, 116, 121]. The assessment of the redox status or health status in general through biochemical or molecular techniques is frequently addressed in gills as the first barrier and contact with crude oil compounds present in the WAF, or in liver as a main detoxifying organ [42, 121] and hence was selected in the current study as well. Finally, the neurotoxic potential of crude oil constituents was investigated by measuring acetylcholinesterase (AChE) inhibition in brain. Though not commonly investigated in oil toxicity studies, recent studies indicate that petroleum hydrocarbons can impact neuronal development and induce neurotoxicity via transcriptional alterations, neurotransmitter regulations or behaviour analyses [45, 125, 133].

Comprehensive studies addressing different levels of biological organization in parallel and offering conclusions about the mechanisms associated with crude oil and the response strategy's toxicity are rather scarce. Therefore, the aim of the present study was to explore the transcriptomic, biochemical and genotoxic effects in adult zebrafish (*Danio rerio*) exposed to different WAF dilutions of a naphthenic North Sea (NNS) crude oil,

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produced with and without the addition of the chemical dispersant FINASOL OSR 52. Furthermore, by means of a comprehensive chemical analysis of target PAHs from the exposure water phase and fish tissue, the biological responses were aimed to be linked to a potential bioaccumulation of oil constituents. Sensitive effect-based methods and biomarkers could further support the need for the implementation of biological monitoring for water quality assessment in the Marine Strategy Framework Directive (MSFD) [130].

Materials and methods
Zebrafish maintenance
Wild-type zebrafish of AB Salk strain from the facilities of the University of Basque Country (UPV/EHU) were used in the present study. Hatched larvae were grown until 5–6 months of age prior to the experiment. Fish were maintained in a temperature-controlled room at 28 ± 1 °C with a constant light–dark rhythm (12:12) in 100 L tanks equipped 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 an osmosis membrane and then buffered to a pH of 7.2 with Sera pH plus (Sera, Heinsberg, Germany) and remineralized to a conductivity of 600 µs cm^{-1} with commercial marine salt (Sera). Fish were fed twice a day with live brine shrimp larvae (*Artemia sp*, INVE Aquaculture, Salt Lake City, USA) and Vipagran baby (Sera).

Preparation of water accommodated fractions
A naphthenic North Sea (NNS) crude oil (Equinor, Stavanger, Norway) and the dispersant FINASOL OSR 52 (TOTAL Special Fluids, Paris, France) were used in the present study. Water accommodated fractions (WAF) of oil (WAF_{OIL}) and chemically dispersed oil (WAF_{OIL+D}) were prepared in 5 L (WAF_{OIL+D}) or 20 L (WAF_{OIL}) glass flasks according to Singer et al. [114] with modifications. Briefly, oil or a dispersant–oil mixture (1:10, w/w) was gently applied on the surface of formulated fish water (remineralized osmosis water) at an oil-to-water ratio of 1:200 (w/w). Regardless glass flask volumes the ratio of water, oil and dispersant and headspace were kept constant. Both setups were stirred for 40 h at 20 °C in dark with low energy avoiding a vortex in the water phase. Water fractions were then carefully drained off from a stopcock at the bottom part of the flask and immediately used for exposure.

Exposure regime
Exposure concentrations were selected based on pretests. A pilot experiment was carried out to test the general toxicity of 25% WAF_{OIL} and 5% WAF_{OIL+D} on adult zebrafish exposed for 6 d with a solution renewal at day 3 resulting in a 100% survival. For the main experiment, eight glass tanks with 45 L capacity covering 1 control and 3 treatment groups (5% WAF_{OIL}, 25% WAF_{OIL} and 5% WAF_{OIL+D}) in duplicate were established in a temperature-controlled room (28 °C) with 12:12 light:dark rhythm. Formulated fish water for the control group, and exposure solutions for the treatments were already filled into the tanks 3 d before fish were added in order to saturate the system. Biological and mechanical filters were not used during the experiment to avoid interference with the exposure. Tank internal water circulation was maintained using a circulatory pump. Fifty adult zebrafish (AB Salk) at the age of 5–6 months were placed in each tank at a sex ratio of approximately 1:1. Exposure medium was renewed every 3 d at a 75% exchange rate. During the experiment, fish were fed twice a day with live brine shrimp larvae. After 3 and 21 d of exposure, 50 adult zebrafish per treatment were individually anesthetized in a benzocaine solution (200 mg L^{-1}) prepared in a 1:9 (v/v) ethanol–water stock, and immediately dissected. For both sampling timepoints one of the two replicate tanks was used. As several biomarker responses are reported to be influenced by fish gender [36, 121, 132], males and females were separated for individual biomarkers as described in detail below.

Chemical analysis of exposure media and fish tissue
Chemical analysis of target PAHs in exposure media
Chemical analysis of exposure media was performed using stir-bar (twister, Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany) sorptive extraction (SBSE) technique according to Prieto et al. [104]. Twisters (20 mm length and 0.5 mm film thick) were placed on magnetic stirrers in the control and treatment tanks and exchanged at 6, 12, 24, 36, 48, 60 and 72 h during two complete exposure cycles of 3 d in order to evaluate the concentrations of PAHs in exposure media along the experiment.

Detection of PAHs was performed using gas chromatography–mass spectrometry (GC (Agilent 6890)–MS (Agilent 5975), Agilent Technologies, Santa Clara, USA) analysis according to the protocol described in Prieto el al., [104] with modifications. A mix standard solution of 16 PAHs (NS 9815: S-4008-100-T, Norwegian Standard supplied by Chiron, Trondheim, Norway) was used for calibration (Additional file 1: Table S1).

PAHs were desorbed from the twisters using a commercial thermal desorption TDS-2 unit connected to a CIS-4 injector (Gerstel GmbH & Co. KG, 10 min, 300 °C, flow 23 mL min^{-1}, cryo-focusing -50°, 7 psi). An Agilent DB-5MS+DG column (Agilent Technologies) was used to separate analytes in a helium stream (1.3 mL min^{-1}).

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Transfer line, ion source and quadrupole analyser temperatures were maintained at 300, 230 and 150 °C, respectively. The following temperature program was used for target PAHs and lineal hydrocarbons: 170 °C for 5 min; ramp at 30 °C min^{-1} to 260 °C; ramp at 8 °C min^{-1} to 300 °C and hold 15 min. The mass selective detector was operated in selected ion monitoring for quantification of target compounds.

Chemical analysis of target PAHs in fish tissue

After 21 d of exposure, fish carcasses from the biological endpoints (described below) were preserved at − 20 °C for the analysis of the bioaccumulation of PAHs. The analysis of PAHs in the fish samples was carried out according to Navarro et al. [93]. Briefly, the fish tissue samples were pooled for each group before analysis. Tissue sample (0.5 g) was accurately weighed in the extraction vessel and 5 mL of acetone were added. The PAHs extraction was performed by focused ultrasound solid–liquid extraction using a SONOPULS HD 2070 (Bandelin electronic GMBH & Co. KG, Berlin, Germany) provided with a 3 mm titanium microtip at 45% of ultrasound amplitude and 0 °C during 120 s. The extracts were filtered by Millex® HV PVDF 0.45 µm (Millipore, Carrigtwohill, Ireland) and concentrated to ~ 0.5 mL under a nitrogen stream (TurboVap LV, Zymark, Barcelona, Spain) after the addition of ∼ 1 mL of iso-octane. The concentrated extracts were cleaned with 5 g Florisil cartridges previously conditioned with n-hexane, and eluted with 25 mL of n-hexane:toluene 75:25 mixture. Subsequently, they were concentrated to dryness, redissolved in iso-octane and kept at − 20 °C in the dark until the GC–MS analysis (previously described).

Gene transcription analysis

Livers of 15 male zebrafish per experimental group and sampling time were dissected, transferred individually to cryovials prefilled with RNA later (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and immediately frozen in liquid nitrogen. Samples were preserved at − 80 °C until analysis. We prioritised the use of males for the gene transcription levels based on the results obtained in previous studies [71, 72, 83].

Total RNA was extracted from a pool of 3 male fish livers with 5 replicates for each of the 4 experimental groups and for each sampling timepoint, except in the case of 5% WAF$_{oil+D}$ group sampled at 21 d with 4 replicates due to the mortality recorded in this treatment. Tissue was homogenized in 300 µL of Trizol using an electric disperser (Pellet Pestle® Cordless Motor, Kimble Kontes, Merck KGaA). RNA was extracted using the Trizol reagent method (Invitrogen, Thermo Fisher Scientific, Waltham, USA) following the manufacturer's

instructions with minor modifications. Samples were transferred to Phase Lock Gel Heavy pre-filled tubes (Quantabio, Beverly, USA) prior to the first centrifugation at 12,000 g and 4 °C for 15 min. RNA was diluted in 50 µL RNAse- and DNAse-free distilled water (Invitrogen). Sample concentration and quality control were evaluated through an Agilent 2100 Bioanalyzer System (Agilent Technologies) using the Agilent RNA 6000 Nano Kit and the corresponding 2100 Expert Software.

First-strand cDNA was synthesized from 2 µg of RNA using the AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies) following the manufacturer's protocol. The conditions for cDNA synthesis were: primer annealing at 25 °C (5 min), cDNA synthesis at 42 °C (15 min) and reaction termination at 95 °C (5 min). cDNA was stored at − 20 °C. The Quant-iT OliGreen ssDNA Assay Kit (Thermo Fisher Scientific) was used for quantification of cDNA samples according to manufacturer's instructions using a Synergy HT Multi-Mode Microplate Reader with Gen5 Microplate Reader and Imager Software (BioTek Instruments, Agilent Technologies, Santa Clara, USA).

Quantitative real-time PCR was performed using TaqMan Gene Expression Assays (Applied Biosystems, Thermo Fisher Scientific, reaction efficiency reported from manufacturer $100 \pm 10\%$) inventoried for *cyp1a* (Dr03112441_m1), *gstp1* (Dr03118992_g1), *cat* (Dr03099094_m1), *sod1* (Dr03074068_g1), *gpx1a* (Dr03071768_m1), *tp53* (Dr03112086_m1), and *casp3a* (Dr03131690_m1). The reaction mixture had a total volume of 20 µL containing 10 µL TaqMan Gene Expression Master Mix (2X), 1 µL TaqMan gene expression assay (20X), 7 µL RNAse-free distilled water (Invitrogen), and 2 µL cDNA sample dilution. Samples and process controls (cDNA synthesis control and no-template control) were performed using a 7500 Real-Time PCR system (Applied Biosystems) at the manufacturer's standard thermal cycling conditions: initial incubation at 50 °C (2 min), activation at 95 °C (10 min), 40 cycles of denaturation at 95 °C (15 s) and annealing and extension at 60 °C (1 min). Transcript levels were normalized by the cDNA concentrations previously quantified according to Valencia et al. [120]:

$$RQ = \left[\frac{(1 + \text{Efficiency})^{-\Delta CT}}{cDNA(ng)} \right]$$

where $\Delta CT = CT$ sample − CT plate internal control.

Biochemical marker analysis

Livers, gills and brains of 12 female zebrafish per treatment were dissected, individually transferred to cryovials and shock frozen in liquid nitrogen. Samples were stored

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at − 80 °C until further usage. A pool of 3 livers, gills or brains were homogenized in 300 µL cold homogenization buffer (50 mM potassium phosphate buffer, pH 7.5, 1 mM ethylenediaminetetraacetic acid, 0.5 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride) for 15 s on ice using an electric disperser (Kimble Kontes) resulting in 4 samples per treatment. Subsequently, homogenates were centrifuged at 10,000 g and 4 °C for 15 min (Eppendorf 5415R refrigerated centrifuge, Sigma-Aldrich). Afterwards, the supernatant was carefully transferred to new 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.

7-Ethoxyresorufin-O-deethylase (EROD) activity in livers and gills

Measurement of EROD activity in liver and gill supernatants was performed according to Brinkmann et al. [25] with minor modifications. Briefly, 20 µL of sample (in triplicate) and a resorufin (Sigma-Aldrich) 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, Sigma-Aldrich) 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, BioTek Instruments), 20 µL NADPH (1 mM, Sigma-Aldrich) were added. Substrate deethylation was determined by measuring the formed resorufin at 540 nm excitation and 590 nm emission wavelengths. Quantification of EROD activity was performed based on the resorufin calibration and expressed in pmol resorufin mg^{-1} min^{-1}.

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

Measurement of GST activity in zebrafish liver and gills was performed according to Habig et al. [49] with modifications regarding the adaption to 96-well plates described in Velki et al. [123]. 12 µL of supernatant as well as 180 µL of 1-chloro-2,4-dinitrobenzene (1 mM, dissolved in phosphate buffer pH 7.2, Sigma-Aldrich) and 50 µL of reduced glutathione (25 mM, Sigma-Aldrich, dissolved in MilliQ water) 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 (Multiskan Spectrum, Thermo Fisher Scientific). Resulting data were controlled for linearity in absorbance increase ($R^2 \geq 0.98$) and minimum increase of absorbance over time ($\Delta t3min \geq 0.1$).

Only data fulfilling these criteria were used for further calculations. Enzymatic activity was calculated as nmol conjugated glutathione min^{-1} mg^{-1}. The molar extinction coefficient of 9600 M^{-1} cm^{-1} was used.

Catalase (CAT) activity in liver and gills

Measurement of catalase (CAT) activity in zebrafish liver and gill supernatants was performed according to the initial protocol developed by Claiborne [28] adapted to UV 96-well plates (Thermo Fisher Scientific). 5 µL supernatant were added to 295 µL of H_2O_2 solution (20.28 mM, Sigma-Aldrich). Immediately thereafter the decrease in absorbance was measured kinetically at 240 nm for 5 min in 10 s intervals using a microplate reader (Multiskan Spectrum). In addition, the absorption of an 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 µmol H_2O_2 consumption min^{-1} mg^{-1} using the calibration series.

Acetylcholinesterase (AChE) activity in brain

Measurement of AChE in brain tissues was conducted according to the initial protocol established by Ellman et al. [43] with modifications according to Velki et al. [123] for 96-well plates. 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 (1.6 mM, Sigma-Aldrich), and 10 µL acetylcholine iodide (156 mM, Sigma-Aldrich) 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 (Multiskan Spectrum). Resulting data were controlled for linearity in absorbance increase ($R^2 \geq 0.98$) and minimum increase of absorbance over time ($\Delta t3min \geq 0.1$). Only data fulfilling these criteria were used for further calculations. Enzymatic activity was calculated as nm acetylcholine hydrolyzed min^{-1} mg^{-1}. For the calculations, the molar extinction coefficient of 13,600 M^{-1} cm^{-1} was used.

Protein measurement

Whole protein of supernatants (brain, liver, gill) was measured in triplicates using a DC protein assay kit (BioRad, Hercules, USA) according to the manufacturer's instructions and quantified with a dilution series of bovine serum albumin as an external standard (1.5– 0.15 mg mL^{-1}) measured in quadruplicates.

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Micronucleus frequency in peripheral erythrocytes

Peripheral blood samples of 7–11 individual males per treatment were taken from the caudal blood vessel of euthanized individuals after cutting the fish tail. The assessment of micronuclei in males and not in females was related to the sampling logistics and not to sex. One smear per individual was immediately prepared on microscopy slides. After drying, samples were fixed using a Hemacolor rapid staining kit according to manufacturer instructions (Merck KGaA) and mounted using Kaiser's glycerine gelatine (Merck KGaA).

A microscope (Eclipse 50i Nikon Instruments, Düsseldorf, Germany) with $100 \times$ magnification and the associated software NIS element (v.5.11, Nikon, GmbH) was used to generate images of erythrocytes. The proportion of micronuclei in peripheral erythrocytes was determined for 1000 cells per individual sample with the following scoring criteria according to Carrasco et al. [26] and Bolognesi and Hayashi [21] for the identification of micronucleated cells: (a) cells with oval appearance and intact cytoplasm, (b) micronuclei less than or equal to one third of the nucleus, (c) micronuclei clearly separated from the main nuclei and d) the micronuclei had the same staining intensity as normal nuclei.

Data analysis

Statistical analyses were performed using SPSS Statistics 26th version (IBM, New York, USA; $p < 0.05$). Metric data including gene transcription level, enzyme activity and micronucleus frequency analysis were tested for normal distribution (Shapiro–Wilk) and equal variance (Levene). For data fulfilling those criteria, a one-way ANOVA with HSD Tukey's post-hoc test was performed to identify significant differences between treatment groups. Data that were not normally distributed and/or of unequal variances were logarithmically transformed. If the transformation failed, data were analyzed using non-parametric Kruskal–Wallis on ranks with Dunn–Bonferroni test. For statistical comparison of exposure regimes (3, 21 d) Student's *T* test was performed for normally distributed and homoscedastic data. For data not fulfilling the criteria, the Welch test or non-parametric Mann–Whitney's *U* test were applied depending on the normality of data.

Results

Chemical analysis of target PAHs

Target PAHs in exposure media

PAH levels in the tanks were measured over two consecutive exposure cycles. In control tanks, concentrations of all target PAHs were below the limits of detection (LOD). The concentration of total PAHs was reduced for all treatments along the exposure cycle in the open test systems (Fig. 1). After exposure solutions were exchanged, a

new peak in dissolved PAH concentration was measured. The concentration of total PAHs at the beginning of the cycle increased from 224.4 ± 17.7 ng L^{-1} (5% WAF$_{OIL}$, 6 h) over $1,724.3 \pm 497.5$ ng L^{-1} (25% WAF$_{OIL}$, 12 h) up to $5,744.1 \pm 119.8$ ng L^{-1} (5% WAF$_{OIL+D}$, 6 h). With medium exchange, the chemically dispersed WAF (WAF$_{OIL+D}$) contained target PAH in concentrations > 20-fold higher than the corresponding dilution without the dispersant application (5% WAF$_{OIL}$). Details of the concentration of individual target PAHs in corresponding exposure media can be found in Additional file 1: Tables S2–S4.
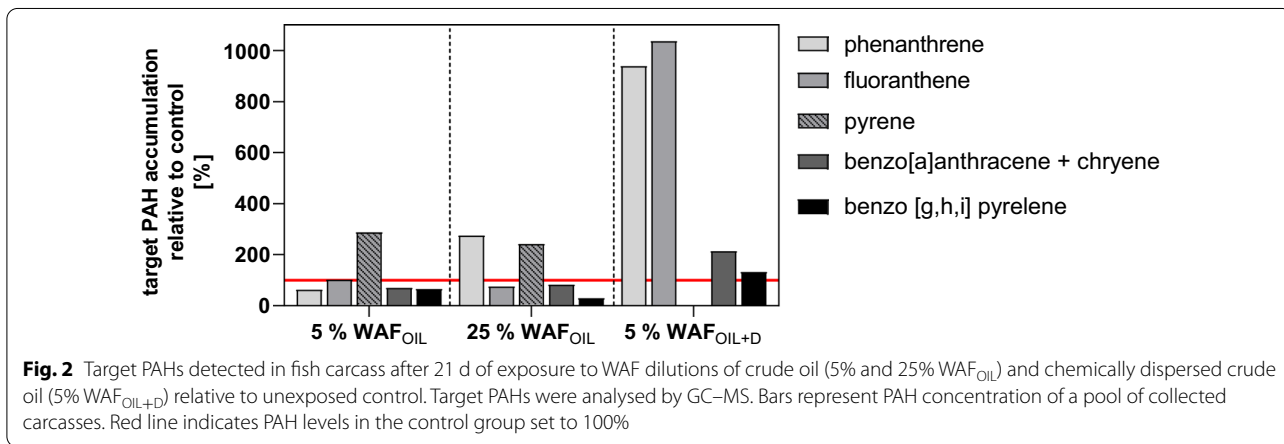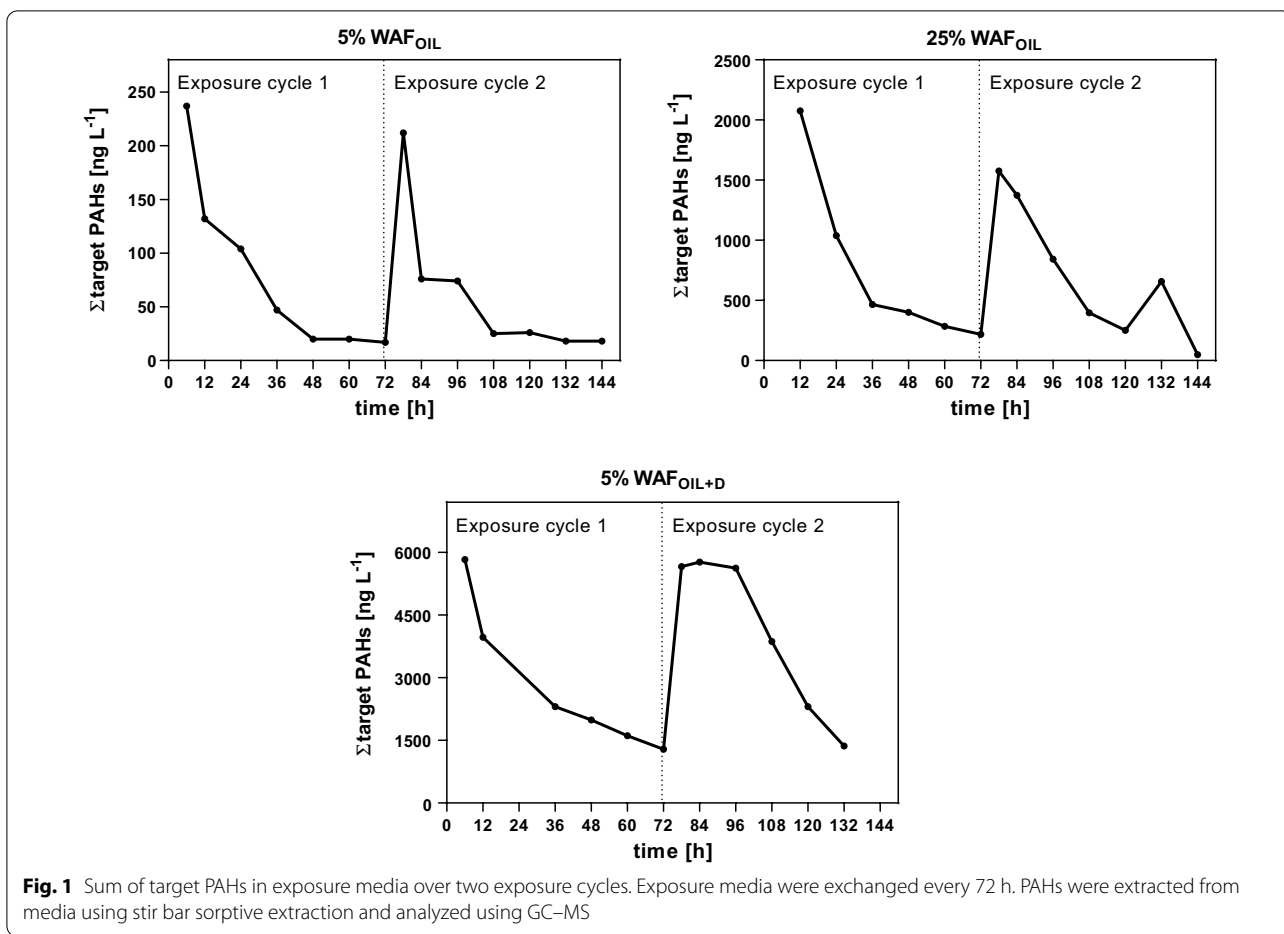
The most dominant PAH across all WAF exposure media was phenanthrene followed by pyrene and fluoranthene (Additional file 1: Tables S2–S4). Phenanthrene was detected in concentrations up to 2674.4 ± 229.8 ng L^{-1} in chemically dispersed WAF within the first 6 h sampling interval after medium exchange. The highest concentrations of phenanthrene shortly after medium exchange (start of exposure cycles) in the case of the WAF$_{OIL}$ dilutions were 147.8 ± 18.0 ng L^{-1} (6 h) and 1251.7 ± 324.1 ng L^{-1} (12 h) in 5% and 25% WAF$_{OIL}$, respectively. Overall, the concentration of dissolved target PAHs increased with decreasing log Kow of individual compounds. High molecular weight PAHs were not quantifiable or even below the detection limits in both WAF$_{OIL}$ groups, while the application of the chemical dispersant resulted in the detection of benzo[a]anthracene, chrysene and benzo[b] fluoranthene in ng L^{-1} ranges.

Bioaccumulation of target PAHs in fish tissue

At the end of the experiment (21 d) target PAHs were detected in fish carcass across all treatments including the unexposed control (Fig. 2). Overall, with the exception of phenanthrene and pyrene, PAH levels in fish exposed to both undispersed oil treatments were in a comparable range of the unexposed control. A markedly increased bioaccumulation of target PAHs was found only for the dispersed oil treatment (5% WAF$_{OIL+D}$). Low molecular weight PAHs such as phenanthrene and fluoranthene were detected up to 782.1 ng g^{-1} and 238.4 ng g^{-1}, respectively. Furthermore, higher molecular weight PAHs including benzo[a]anthracene, chrysene, indeno[1,2,3-cd]pyrene or benzo[ghi]pyrelene, which were scarcely detected or even below detection limits in the water phases of all treatments (Additional file 1 Tables S2–S4), were detected in the fish tissues, with the highest concentrations up to 604.0 ng g^{-1} (benzo[a]anthracene + chrysene) (Additional file 1: Table S5).

Survival and peculiar behaviour

The chemically dispersed oil treatment (5% WAF$_{OIL+D}$) led to a mortality rate of 23% at 21 d, whereas 100% survival was observed for the 5% and 25% WAF$_{OIL}$ as

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Fig. 1 Sum of target PAHs in exposure media over two exposure cycles. Exposure media were exchanged every 72 h. PAHs were extracted from media using stir bar sorptive extraction and analyzed using GC–MS

Fig. 2 Target PAHs detected in fish carcass after 21 d of exposure to WAF dilutions of crude oil (5% and 25% WAF$_{OIL}$) and chemically dispersed crude oil (5% WAF$_{OIL+D}$) relative to unexposed control. Target PAHs were analysed by GC–MS. Bars represent PAH concentration of a pool of collected carcasses. Red line indicates PAH levels in the control group set to 100%

well as the control. Up to 6 deceased fish were found from day 3 of exposure until the end of the experiment in 5% WAF$_{OIL+D}$ group. Overall, chemically dispersed oil exposed fish presented a cyclic stunned behaviour, with fish swimming abnormally close to the surface in a tilted position and apparently ignoring the provided food shortly after each medium exchange. This demeanour was progressively attenuated within the first 24 h of the exposure cycle. Fish exposed to 5% and 25% WAF$_{OIL}$ did not show abnormal swimming behaviour and were comparable to the control individuals.

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Fig. 3 Transcription levels of biotransformation (*cyp1a, gstp1*), oxidative stress response (*cat, sod1, gpx1a*), cell cycle (*tp53*) and apoptosis (*casp3a*) genes in male adult zebrafish livers after 3 and 21 d of exposure to WAF dilutions of crude oil (5% and 25% WAF_{OIL}) and chemically dispersed crude oil (5% WAF_{OIL+D}). Data are represented as median (line) with boxes showing the 25–75 percentile and whiskers representing min to max values of 4–5 replicate groups per treatment. Letters indicate statistically significance between treatments within the same sampling point and asterisks indicate statistically significance between sampling timepoints ($p < 0.05$)

Gene transcription levels

In an overview, only the exposure to 5% WAF_{OIL+D} caused statistically significant alterations on mRNA levels after short- and long-term exposure (Fig. 3).

Both investigated biotransformation metabolism-related genes (*cyp1a, gstp1*) were significantly up-regulated for the 5% WAF_{OIL+D} treatment after 3 d of exposure (Fig. 3A, B). After 21 d the *cyp1a* and *gstp1* mRNA levels of fish exposed to chemically dispersed WAF were significantly decreased compared to the first sampling timepoint but still remained significantly higher compared to the control (Fig. 3A, B).

The oxidative stress response genes *sod1* and *gpx1a* were statistically significant up-regulated by the 5% WAF_{OIL+D} treatment after 3 d of exposure (Fig. 3D, E). Transcription levels of *cat* did not show statistically significant differences between treatment groups. The highest induction of *cat* was recorded in fish exposed to 25% WAF_{OIL} at 3 d (Fig. 3C). Overall, the up-regulation of oxidative stress genes (*sod1, gpx1a* and *cat*) significantly declined from short- (3 d) until the end of long-term (21 d) exposure, albeit not differing from the control. Fish exposed to 5% WAF_{OIL+D} still showed the highest values (Fig. 3C–E).

In contrast, no differences on the transcription levels of cell cycle- and apoptosis-related genes (*tp53, casp3a)* were found across treatments or compared to unexposed fish at any sampling timepoint (Fig. 3F, G).

Biochemical marker responses

Overall, the chemically dispersed crude oil (5% WAF_{OIL+D}) showed the strongest effects on enzymatic biomarker levels. Enzyme activities corresponding to the xenobiotic metabolism phase I (EROD) and II (GST) as well as the antioxidant defence mechanism (CAT) were investigated in zebrafish liver and gill.

No notable changes in EROD activity were recorded for both undispersed crude oil treatments (5 and 25% WAF_{OIL}) independent of the sampled tissues or the exposure period (Fig. 4A, B). In contrast, the chemically dispersed crude oil exposure (5% WAF_{OIL+D}) resulted in significantly increased EROD activity in liver after both exposure periods up to tenfold compared to the unexposed control. Gill GST was found to be significantly increased after the exposure to chemically dispersed crude oil at both exposure times (Fig. 4D). For liver, no

alterations were recorded after 3 d of exposure in any treatment. However, after 21 d all treated groups showed higher activity, which was significantly higher compared to the previous sampling in the case of 5% WAF_{OIL} and 5% WAF_{OIL+D}.

While for liver tissue the 5% WAF_{OIL} treatment did not lead to significant differences in CAT activity after 21 d when comparing treatments to the control group, in gills the chemically dispersed WAF presented significant higher CAT activity than the control group after 21 d (Fig. 4E, F). However, long-term exposure induced an overall trend of markedly increased CAT activity in fish gills exposed to the remaining treatments compared to control.

As a biomarker of neurotoxicity, the acetylcholinesterase (AChE) activity in brain was significantly decreased in fish exposed to the chemically dispersed oil (5% WAF_{OIL+D}) compared to the corresponding non-dispersed WAF dilution (5% WAF_{OIL}) at both short and long-term exposure (Fig. 5). Nevertheless, AChE activity was not significantly different for any treatment compared to the control group.

Relative induction of enzyme activity individually normalized to control can be found in the Additional file 1: Table S6.

Micronuclei frequency in peripheral erythrocytes

Both WAF_{OIL} exposure dilutions resulted in micronuclei formation in a comparable range to the unexposed control (Fig. 6). The long-term exposure to the chemically dispersed oil WAF (21 d) was the only treatment inducing significantly more micronuclei in erythrocytes than the 5% WAF_{OIL} exposure with a resulting induction factor of 2.2 (all induction factors can be found in Additional file 1: Table S7).

Discussion

PAH exposure and bioaccumulation

With the time-resolved chemical analysis of the water phase across 2 exposure cycles we were able to show the dynamic partitioning of target PAHs in 2 ways. First, a fast concentration decrease of analyzed PAHs in the water phase of up to 92% was observed within 3 d, which is most likely associated with physical (evaporation, absorption) and biological (uptake by fish) responses of the system [115]. These findings
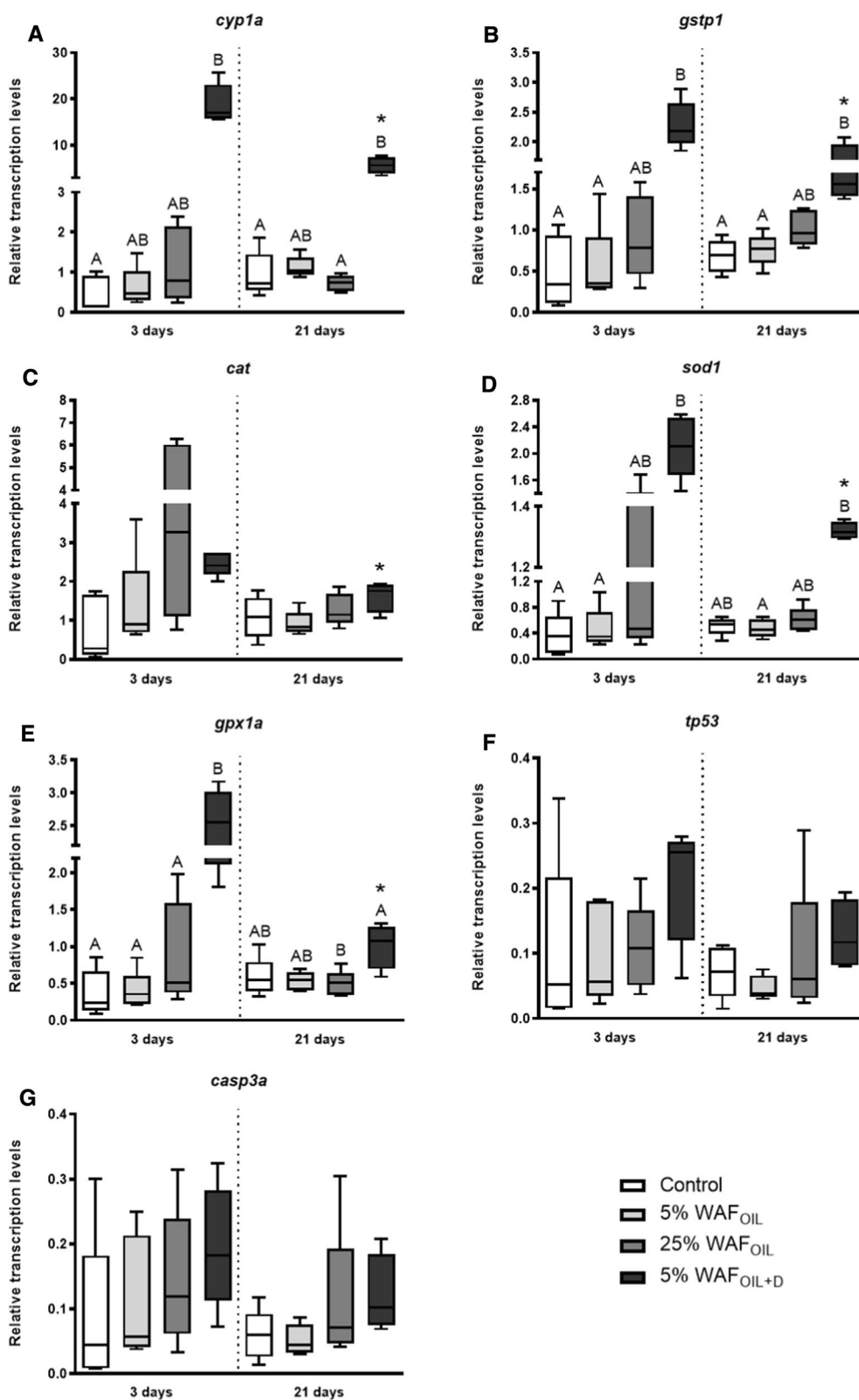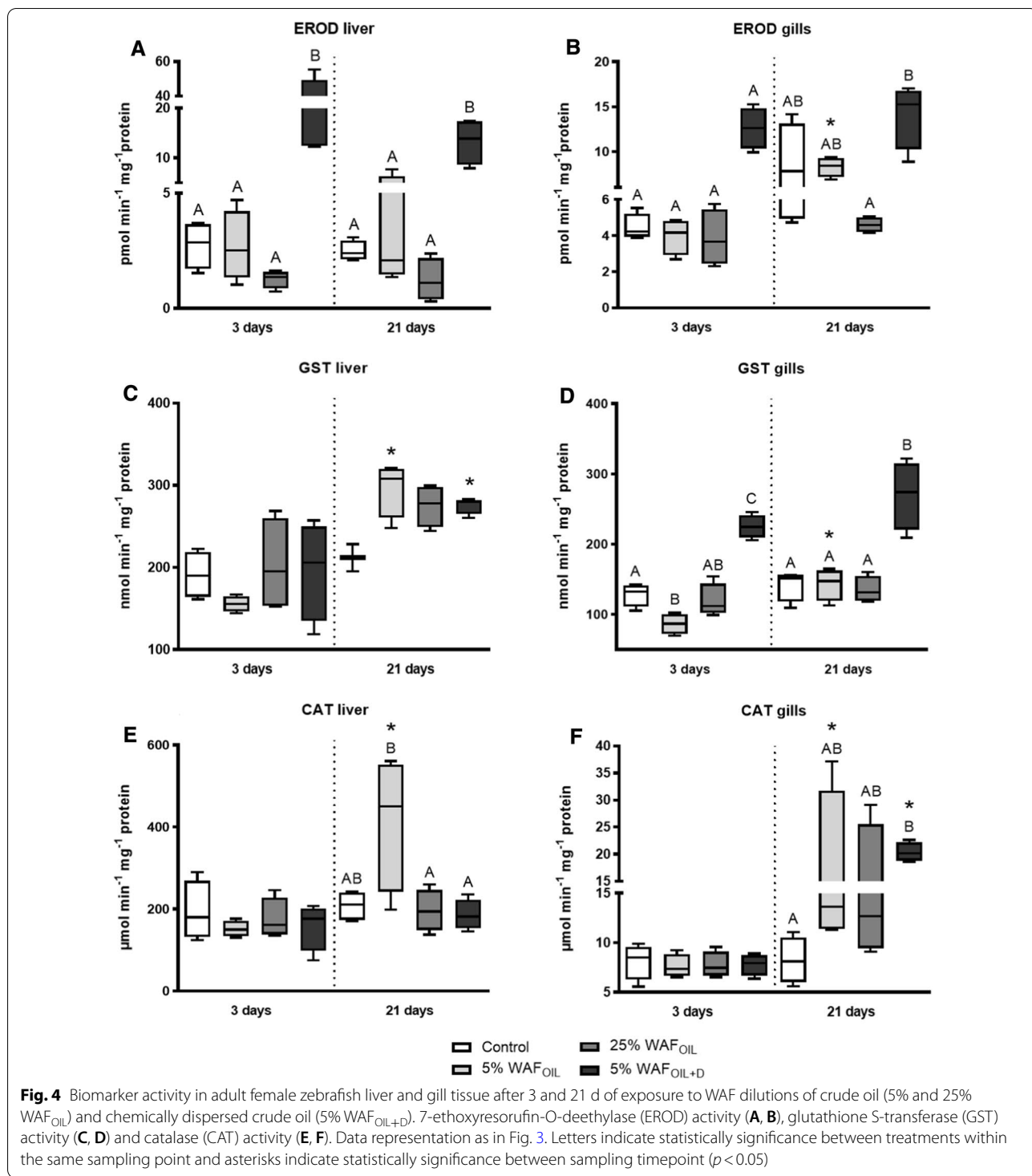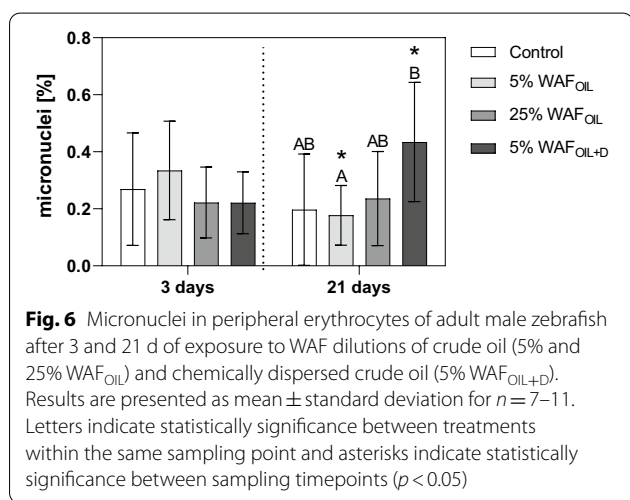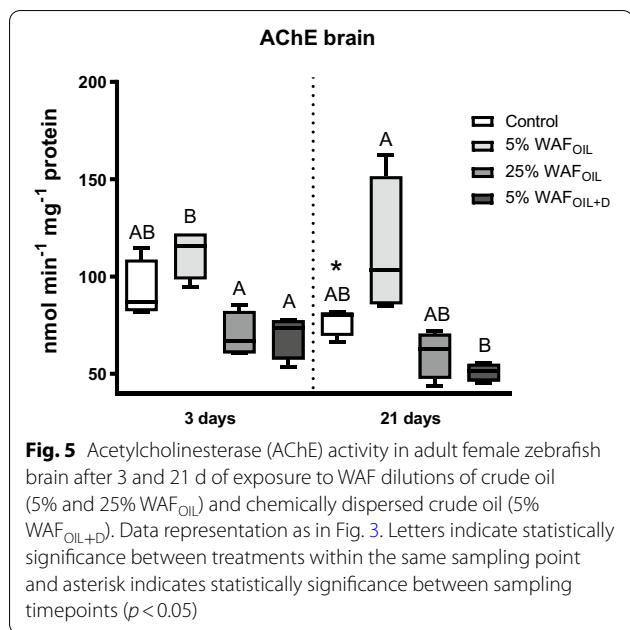
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Fig. 3 (See legend on previous page.)

Fig. 4 Biomarker activity in adult female zebrafish liver and gill tissue after 3 and 21 d of exposure to WAF dilutions of crude oil (5% and 25% WAF$_{OIL}$) and chemically dispersed crude oil (5% WAF$_{OIL+D}$). 7-ethoxyresorufin-O-deethylase (EROD) activity (**A**, **B**), glutathione S-transferase (GST) activity (**C**, **D**) and catalase (CAT) activity (**E**, **F**). Data representation as in Fig. 3. Letters indicate statistically significance between treatments within the same sampling point and asterisks indicate statistically significance between sampling timepoint ($p < 0.05$)

demonstrate the dynamic peak exposure in semi-static open systems and hence the overall importance of continuous exposure solution analysis as well as careful selection of the exposure setup according to the research question.

Second, the present results showed the strong impact of the dispersant on dissolved PAHs in the water column. The observed oil compound mobilization caused by the addition of the chemical dispersant has already been reported in several studies [5, 33, 75, 89]. This

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Fig. 5 Acetylcholinesterase (AChE) activity in adult female zebrafish brain after 3 and 21 d of exposure to WAF dilutions of crude oil (5% and 25% WAF$_{OIL}$) and chemically dispersed crude oil (5% WAF$_{OIL+D}$). Data representation as in Fig. 3. Letters indicate statistically significance between treatments within the same sampling point and asterisk indicates statistically significance between sampling timepoints ($p < 0.05$)

Fig. 6 Micronuclei in peripheral erythrocytes of adult male zebrafish after 3 and 21 d of exposure to WAF dilutions of crude oil (5% and 25% WAF$_{OIL}$) and chemically dispersed crude oil (5% WAF$_{OIL+D}$). Results are presented as mean ± standard deviation for $n = 7$–11. Letters indicate statistically significance between treatments within the same sampling point and asterisks indicate statistically significance between sampling timepoints ($p < 0.05$)

phenomenon seems to be the result of the ability of dispersants to break the oil into a high number of small droplets, incrementing the contact surface of oil and water, while at the same time, the reduced size and the high surface-to-volume ratio of these oil droplets contribute to the partitioning of PAHs from the oil into the water column [88, 105]. Furthermore, the chemical dispersant altered the composition of the PAH mixture present in WAF, which might further affect the complex toxicity. Within this, high molecular weight PAHs such as benzo[a]pyrene and benzo[g,h,i]perylene were only quantifiable in the chemically dispersed oil WAF, showing that the dispersants do not interact equally with all

hydrocarbons and are prone to ease the dissolution of the less hydrophilic compounds [86].

Lower molecular weight PAHs such as fluorene and phenanthrene are known to be more easily dissolved in water and hence more bioavailable for fish than PAHs of higher molecular weight [16] leading to the assumption that those cause the observed adverse effects. In fact, tricyclic PAHs detected in the WAFs, such as phenanthrene as the most dominant target PAH, are well known toxicity drivers in fish resulting in cardiotoxicity, malformations and behavioral changes (reviewed, e.g., in [52, 54]). However, though detected in relatively low concentrations in the present study, high molecular weight PAHs are potent agonists of the aryl hydrocarbon receptor, the molecular initiating event of several mechanisms related to toxic effects in fish, such as cardiotoxicity [54]. Hence, the observed adverse effects in zebrafish are likely a response to the complex mixture toxicity of high and low molecular weight PAHs and even other crude oil constituents, such as alkylated and heterocyclic derivatives, which have been shown to cause even stronger effects in different fish models compared to parent PAHs [4, 24, 82, 110]. Each crude oil is a unique complex sample that can induce various degrees of toxicity via diverse pathways depending on the mutual interference of constituents [76]. It has to be considered that only a limited fraction of oil constituents was investigated. However, even a very comprehensive chemical profile would not guarantee the explanation of the observed mixture toxicity by individual compounds, since some previous studies successfully established such a correlation between biological effects and chemical profiles [73], while others did not establish a very clear correlation [56].

Due to a high PAH biotransformation capacity of fish [76, 121] an efficient metabolization and elimination was expected [116]. This was experimentally indicated in the present study by the activity of corresponding molecular and enzymatic biomarkers (e.g., CYP1A activity, detailed discussion in Sect. 3 below). However, bioaccumulation of low and high molecular weight PAHs was notable in fish exposed to the chemically dispersed WAF. The accumulation of individual PAHs has been observed in several previous studies addressing dispersed oil toxicity [16] or toxicokinetic of PAH accumulation in fish [126, 127]. The accumulation of PAHs might be related to waterborne uptake via the gills and the skin as demonstrated for exposure of adult zebrafish to phenanthrene (100 ng L^{-1}) reaching an equilibrium concentration in all tissues measured within 4 d of exposure [127]. Though detected in low concentrations in the water phase, the relatively strong enrichment of high molecular weight compounds (benzo[a]anthracene + chrysene, benzo[ghi] pyrelene, indene[1,2,3-cd]pyrene in carcass tissues after 21 d of

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exposure was potentially related to lower detoxification rates compared to lighter PAHs with lower logKow. Furthermore, it has to be kept in mind that bioaccumulation of PAHs could have been caused also by oil droplet ingestion followed by desorption through the gut [15, 139], since stable micro-droplets were expected in the water column of the present set up due to constant water circulation. This contribution of oil droplets for bioaccumulation could have been more relevant for PAHs of higher than for those of lower molecular weight. This has been indicated in previous studies [51] and shown in the present study in particular for benzo[g,h,i]pyrelene, which was scarcely detected in the water phase of WAF_{OIL+D} (54.2 ng L^{-1} at 60 h, see Table S4) but in concentrations up to 215 ng g^{-1} in the WAF_{OIL+D} fish tissues.

Mortality and peculiar behaviour

The mortality observed in the chemically dispersed oil WAF exposure tanks might have been caused by the increased concentration of PAHs compared to the undispersed WAFs. Despite the diversity of oils or chemical dispersants and the variety of tested dilutions, which limits a direct comparison between studies, similar results of elevated toxicity after the application of a chemical dispersant have been reported for zebrafish [75, 101]. In addition, the results of no mortality in 5% and 25% WAF approaches were in compliance to a previous study with zebrafish exposed to the same oil type even in higher exposure concentrations compared to the present WAFs [10].

The administration of the exposure media containing dispersant triggered a certain cyclical stunned behaviour in exposed fish of occupying the upper part of the tank for 1 d every time the solution was renewed. This behaviour correlated with the measured PAH concentration in the tanks, which sharply decreased after 24 h. Acute and chronic exposure to PAHs found in high concentrations in our study (fluorene, phenanthrene and pyrene) has been shown to reduce locomotion, induce lethargy in fish and increase boldness behaviour in exploration tests [47, 50]. In addition, the exposure to water-soluble fractions of native and chemically dispersed crude oil significantly reduced the critical swimming speed of fish in a concentration-dependent manner [135]. Moreover, the mixture of oil and the chemical dispersant FINASOL OSR 52 induced several alterations on fish exploration behaviour [7]. The fish posture description indicated by Aimon et al. [7] of still and anaesthetic-like sedative state agrees with our observations.

Different studies link altered swimming behaviour caused by oil constituents to cardiac alterations as an initiating adverse effect leading to altered metabolic rates and the observed behaviour [77, 124]. Another explanation might be an avoidance behaviour of fish due to odor and medium turbidity in combination with hypoxia. Though oxygen was not limited in the water phase (data not shown), chemical dispersants have been found to affect gill ion regulation and overall increase gill lesions in oil WAF exposed fish indicating the disturbance of oxygen transport [6, 39]. Gill membrane functionality could also simply be affected by the physical accumulation or disruption of oil droplets and hydrophobic oil constituents such as the high molecular weight PAHs. However, this previously suggested baseline toxicity of membranes (narcosis) [37, 84] is controversially discussed [54]. It is important to consider behavioural disruptions, since they may imply severe consequences to fish survival and reproductive fitness.

Biotransformation response

Biotransformation biomarkers have been applied in several studies focusing on the toxicity pathways behind PAH exposure in zebrafish [53, 64]. Representative for biotransformation phase I oxygenase activity, mRNA levels of *cyp1a1* and protein levels of EROD activity have been found to be up-regulated in fish after the exposure to individual PAHs, such as phenanthrene [80, 97]. Besides, Arukwe et al. [10] observed an increment in *cyp1a1* mRNA levels and EROD activity in a comparable experiment to the present study applying the identical oil type and zebrafish. In our study, the biotransformation activities were not affected by exposure to the two dilutions of undispersed WAF, emphasizing again the role of the dispersant for the toxicity of crude oil by enhancing the bioavailability of PAHs. A concurrent induction of both mRNA and enzymatic levels of CYP1A suggests a de novo synthesis of the protein most likely initiated by AhR activation by typical receptor agonists [54, 78]. In addition, in accordance with the present findings, up-regulation of phase I biotransformation strongly initiated by the application of a chemical dispersant has been observed in several previous studies [5, 29, 101, 105] including in particular experiments with the presently used FINASOL OSR 52 [35, 41], which has also mainly been related to the increment of PAHs in the exposure solution [105].

After the transformation of PAHs to more hydrophilic compounds by phase I enzymes, the resultant molecules experience a conjugation by phase II enzymes, such as GST, in order to promote its excretion [57]. In this context, levels of *gstp1* were also significantly increased at both sampling timepoints in fish exposed to the chemically dispersed crude oil. However, the enzymatic activity of GST in liver was not correspondent with the increased transcription, but its lack of significance could be related to instable measurement on protein level with not all replicates meeting the established quality criteria.

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The discrepancy could also be related to the endpoints itself, since previous studies concluded that the induction responses of phase II systems, such as GST activity, are less uniform and, therefore, less reliable compared to phase I enzymes [68, 121]. In this context, while different PAHs have been shown to increase GST activity in fish [33, 53, 58, 92], other studies found that PAHs decreased GST activity [80, 98, 129], some showed contrary results of both inhibition and induction [116], or even no alterations of GST activity by PAH exposure [68] in liver, gills or kidney of fish.

As the most immediate organ in proximity with the exposure media and, therefore, the compounds present in it, gills can rapidly metabolize PAHs before reaching the liver and thus playing an important role in the biotransformation process [64]. Hence, an elevated biotransformation activity might be expected in gills. However, when comparing both liver and gill tissues, no consistent trend of biotransformation activity was observed. Even though its induction has been described as a biomarker for crude oil contaminants in fish [2, 3, 64], in our study, the EROD activity in gills of fish exposed to dispersed oil was nearly three times lower than in livers and not significantly different from control. In contrast, for GST activity we observed a significantly higher induction only in gills. These findings of variable biotransformation activities in different tissues highlight the complexity of biological responses and in particular the sensitivity of liver samples in chronic exposure studies with petroleum constituents.

Oxidative stress response

Transcriptional and biochemical results of the present study indicate that the chemically dispersed oil induced oxidative stress in fish, which is in compliance with the overall knowledge on WAF-dominating PAHs inducing ROS [78, 92, 111, 116, 118]. For catalase, no direct correlation between transcriptomic and enzymatic responses could be observed. The significant increase in CAT activity in gills but not in liver in our study indicates that gills represent an important organ for detoxification acting as a primary defence line for fish. The lack of CAT induction in liver might be explained by its suppression or impairment of the system caused by a severe oxidative damage, as indicated in a previous study [116]. Overall, several studies indicate that CAT could play a secondary role on enzymatic defence against the oxidative stress [33, 98]. The varying results after oil WAFs or PAH exposure have led to the assumption that CAT is a less stable biomarker and rather enzymes such as GPX are principal actors responding to ROS, such as H_2O_2 [33, 91, 98]. In this context, zebrafish embryos exposed to a WAF of Arabian Light crude oil for 96 h showed the same pattern as our

results, with an upregulation of *gpx* and *sod* genes but no regulation of *cat* [100]. However, while other studies on different fish species observed comparable trends [11, 97], Milinkovitch [87, 89] did not report significant differences of CAT, SOD and GPx activities in fish exposed to chemically dispersed crude oil. All these different results reflect how the antioxidant response is conditioned by the organ or species studied and, certainly, by the conditions of the exposure to crude oil compounds. Our findings support the higher relevance of SOD and GPx as oxidative stress biomarker over CAT due to the concomitant and strong transcriptomic induction of *sod1a* and *gpx1a*. In addition, the high EROD activity observed in the present study is in line with oxidative stress, as superoxide anion radicals ($O_2^{\bullet-}$) are a type of ROS that can be produced by a non-well-functioning process of the phase I mixed function oxygenase system [121]. Moreover, Luch [78] indicates that some PAHs can generate superoxide or hydroxyl radicals through autoxidation or PAH-mediated inflammatory processes. Thus, the increase in cellular superoxide ($O_2^{\bullet-}$) production would be appeased by the SOD activity generating in return an excess of H_2O_2 that GPX enzymes would transform into water using the reductive power of a glutathione (GSH) molecule [116, 121]. Further studies on GSH content, SOD and GPX activity or the assessment of lipid peroxidation could aid to prove these assumptions.

Cellular stress including oxidative stress and DNA damage can lead to the activation of cell cycle arrest, programmed cell death (apoptosis), and repair pathways [38]. Associated marker genes were not significantly regulated in the present study independent of the exposure solution or sampling timepoint. As previously reviewed, PAHs are well known for their potential to induce apoptosis in fish [9]. In this context, p53 acting as a key transcription factor to initiate the different cascades including extrinsic apoptosis pathways involving caspases [9] has been found to be strongly upregulated by PAH [136] or petroleum oil exposure [30, 109]. In contrast, also a decrease of p53 activity compared to control has been reported for adult fish after dietary exposure to BaP [131]. It has to be considered that only two marker genes were investigated in the present study, which do obviously not cover the whole complexity of p53-regulated pathways including apoptosis.

AChE activity

While previously the brain AChE activity has not been found to be altered in wild fish populations of heavily petroleum contaminated areas [67], the present study observed a significant AChE inhibition after both short- and elongated exposures to chemically dispersed crude oil compared to the same dilution of undispersed

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WAF$_{OIL}$ but not when compared to the control. In addition to complex petroleum mixtures, also individual oil constituents had led to ambiguous results regarding AChE inhibitory potential in fish [96]. Selected PAHs (e.g., phenanthrene) were found to inhibit either isolated AChE enzymes from fish [60] or brain homogenates [59] in exposure concentrations comparable to the present study, whereas another study reported no influence of PAHs on AChE activity [117]. In addition, our previous experiments with early life stages of zebrafish exposed to WAFs prepared from the identical NNS crude oil batch did not indicate AChE inhibition [62], even though embryonic stages have been described to be reliably responsive to neurotoxic exposure [123, 134]. However, since a high variability of AChE activity across the different treatments was observed in the current study, the observed AChE inhibition might be related to a generally reduced fitness of fish. Nonetheless, neurotoxicity of crude oil cannot be excluded simply by the measurement of one enzyme involved in neurotransmission. Previous studies focusing on whole transcriptome analysis, physiological changes in brain tissue, monoamine neurotransmitter regulation, and locomotor behaviour observed that petroleum exposure can affect the neuronal system in fish early life stages [45, 125, 133], indicating a neurotoxic potential of petroleum constituents.

Genotoxicity
Genotoxicity is a commonly addressed endpoint in crude oil toxicity assessment and can be investigated on several levels of biological organization [1, 20]. The micronucleus assay showing clastogenic or aneugenic chromosomal aberrations is a very sensitive indicator for DNA damage [21, 95]. This endpoint has proven the ability to reveal long-term genotoxic effects in fish several years after large oil spills during past decades in various laboratory and monitoring studies [23, 102, 103]. In the present study, only the chemically dispersed crude oil exposure resulted in significantly elevated micronuclei formation. This might be related to the increased bioavailability of higher molecular weight PAHs caused by the dispersion. Low molecular weight PAHs such as the most dominant phenanthrene have inconsistently been reported as inducers for chromosomal aberration. While Peng et al. [99] reported no significant micronuclei formation even for higher exposure concentrations compared to the present study [99], other studies detected significantly increased micronuclei rates for concentration ranges comparable to our work [97, 113]. In contrast, high molecular weight PAHs such as benzo[a]pyrene are well known to initiate a genotoxicity cascade via biotransformation activity (e.g., CYP1A, resulting in the formation of DNA adducts and oxidative radicals that can lead to DNA strand breaks and thus micronuclei formation [79, 108, 119, 138]. Molecular and biochemical results of the present study indicate a high biotransformation activity in oil exposed fish supporting the genotoxicity pathway. Overall, the micronucleus rate observed for 21 d exposed zebrafish exposed to dispersed oil (IF = 2.2) was in the lower range of reported micronucleus rates in erythrocytes found in petroleum product WAF-exposed fish in comparable laboratory studies (IF = 2–5) [14, 22, 85, 122]. Though a comparison using induction factors accounts for inter-species variability [21], a direct comparison of different oil exposure studies is limited due to a variety of selected exposure conditions and oil types. Nonetheless, in a previous study using the identical NNS crude oil batch we also found stronger micronucleus formation in acutely exposed (48 h) permanent zebrafish liver cells [61]. The higher sensitivity of the cell line compared to erythrocytes demonstrates the lack of toxicokinetics in in vitro bioassays and highlights the in situ micronucleus assay as a relevant endpoint for a more reliable ecotoxicological risk assessment of oil WAFs.

Critical considerations for dispersant toxicity interpretation in chronic exposure studies
Overall, our results highlight the importance of considering the effect of chemical dispersants in the toxicity assessment of oil spill response measures, very noticeable when comparing the biological endpoints of identical oil loadings with and without the addition of FINASOL OSR 52. As concluded in several previous studies (e.g., [107]), further supported by the current time-resolved chemical analysis, the dispersant-induced increased toxicity has been attributed to an enhanced bioavailability of toxic compounds present in oil. However, the overall toxicity interpretation should be considered cautiously as oil and dispersant toxicity seems to be species-specific and is further influenced by the exposure methods and oil types. In a worst-case scenario, this might lead to an underestimation of the risk towards more sensitive species compared to zebrafish.

While the present chemical analysis supported the overall theory of a dispersant-induced increase in dissolved PAH fractions, a toxicity of the dispersant itself cannot be excluded with the experimental setup. According to previous studies FINASOL OSR 52 has been considered as moderately toxic [17]. In particular, compared to Corexit EC9500A, another chemical dispersant frequently used as oil spill response measure, a higher toxicity has been found for FINASOL OSR 52 in different fish species (*Menidia beryllina*, *Cyprinodon variegatus*) after short and long-term exposure [13, 35]. It is important to mention that an additional control group with the dispersant alone was not included

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within the current study to experimentally address the role of the dispersant. From our previous studies with the dispersant Finasol OSR 51, which shows high comparability to Finasol OSR 52 used in the present study, we have to emphasize that the toxicity of the dispersant alone or in combination with an inert oil cannot be excluded per se [61–63]). Nonetheless, the dispersant-induced general toxicity (oxidative stress induction) or acute toxicity to zebrafish embryos and larvae were found in exposure concentrations highly elevated compared to the exposure concentrations used in the present study. Hence, it could be assumed that in the present scenario no effects from the dispersant itself might be expected.

Another aspect that needs to be considered for toxicity interpretation is the applied dispersant to oil ratio (DOR). While the DOR used in the current study (1:10) is rather recommended for heavy oils due to relatively high viscosity and hence a higher dosage need for dispersion success [74], also other studies working with much lower DORs (e.g., 1:800) observed adverse effects in fish [39]. Hence, the current DOR can be interpreted as a worst-case scenario with environmental relevance. Besides species sensitivity variations and DOR the exposure concentrations need to be critically addressed. The exposure concentrations of the present study can be considered as environmentally relevant. PAH concentrations detected in the water phase (200–5700 ng L^{-1}) were in the lower range of concentrations detected in the water column after oil spills (up to 600,000 ng L^{-1}) as summarized by Perrichon et al. [100]. After the Deep Water Horizon blowout, concentrations up to 189,000 ng L^{-1} were reported [19]. However, it has to be considered that even though environmentally relevant exposure concentrations were used, the chronic exposure remains a worst-case scenario. A previous study acutely exposing fish to chemically dispersed crude oil in a concentration range of tenfold increase compared to the current setup did conclude no ecological impact after a long-term recovery phase of fish population in semi-natural mesocosms [81]. This might indicate that the observed sublethal effects in the current study are maximum temporary without severe consequences. However, long-term studies are still scarce, and moreover, recovery phases need to be addressed in future research. Especially additional stressors under real field conditions such as ecological or anthropogenic pollution might impact the overall stress tolerance and survival and alter the overall biomarker responses complicating reliable forecasts. In this context, it has been shown that, e.g., global warming-induced temperature increase has a huge impact on acute and chronic stress physiology in fish [8].

Conclusion—combining multilevel endpoints for risk assessment

Due to the high complexity of oil exposure and the high variability of biological responses, it can be concluded that for a reliable ecotoxicological risk assessment, it is important to combine different biomarkers across different levels of biological organization. With significant molecular and enzymatic alterations of biotransformation and antioxidant defences, the present study supports previously reported endpoints for oil toxicity testing as sensitive biomarkers in adult zebrafish. In addition, while the micronucleus induction in peripheral blood erythrocytes is only one apical endpoint in genotoxicity and several other mechanisms can lead to DNA damage, the observed oxidative stress and biotransformation system activation supports the hypothesis of genotoxicity indicated by this biomarker. Comparing biomarker analyses between different studies can be a difficult task due the complexity discussed above. However, it is undeniable that a comprehensive investigation possesses an inherent value. Especially the low exposure concentrations as well as the elongated exposure period up to 21 d are valuable information for the scientific community in order to characterize oil toxicity and can assist decision making for oil spill response strategies. Nonetheless, future experiments should include an elongated recovery phase to take into account an environmentally relevant short-term peak exposure scenario. The present study further emphasizes the importance of a temporally resolved chemical characterization of the exposure water phase for effect interpretation. In addition, further research should different toxicity drivers among oil constituents and study results must be cautiously interpreted leading always to protective actions based on the essential precautionary principle that ensures the environment preservation.

Additional file 1: Table S1. List of Abbreviations and limits of detection of target PAHs analyzed in exposure solutions and carcasses. **Table S2.** Concentrations of target PAHs (ng/L) in 5% WAF$_{OIL}$ exposure media over two cycles (exposure media was changed every 72 h) and analyzed using GC–MS. Values below detection limits are expressed as BDL. **Table S3.**

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