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Report on suitable environmental parameters for investigation of oil spills and oil spill responses using zebrafish

D3.2

WP3: Determination of oil and dispersant impacts on biota using effect-based tools and ecological risk assessment

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Executive Summary

The aim of this study was to report suitable environmental parameters for the investigation of oil spill and oil spill response actions using zebrafish. Firstly, the tolerance of zebrafish embryos to the two environmental factors temperature and salinity mainly influencing the exposure conditions was investigated. Secondly, it had to be investigated whether zebrafish embryos adapted to specific environmental parameters outside their optimum conditions still react similar to the stressors of interest like chemicals, oil samples and dispersants.

Temperature conditions in zebrafish experiments cannot be changed towards the temperatures that are relevant for the study regions of the GRACE project. While zebrafish have a relatively broad thermal niche, temperatures 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 dramatically increase the toxicity (see chapter 3.3.2) 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. Thus, zebrafish embryo experiments can be conducted at salinities relevant for brackish water conditions of the study area in the Baltic Sea (5 ‰). This was verified with comparable results for all embryo toxicity experiments conducted simultaneously in artificial freshwater and brackish water conditions.

Besides the evaluation of suitable environmental parameters, this report presents the first results of WAF (prepared of naphtenic North Sea crude oil), dispersant (Finasol OSR 51) and CEWAF (combined naphtenic oil and Fiansol) toxicity on the early life stages of *D. rerio*. With the comparison to previous studies on crude oil induced fish toxicity we demonstrate that these data fit well in the available literature data on oil research.

Finally, from this study as well as from literature data that were discussed across the present report it can be concluded that the zebrafish is a suitable model organism in oil spill research. Nonetheless, some limitations have to be considered and integrated in the evaluation of study results.

1. Introduction

Work package 3 focuses on the oil impacts on biota from the Northern Atlantic and the Baltic Sea. Therefore, monitoring campaigns and laboratory experiments with ecologically relevant target species at a regional scale are conducted. Besides regional relevant species, the zebrafish is integrated as a laboratory model species in the work package. The zebrafish (*Danio rerio*) has been established as a popular model in a variety of scientific fields including biomedical research and (eco)toxicology (Strähle et al. 2012). Even in the context of oil spill research *Danio rerio* has been proven to be a suitable teleost model. Several studies on oil spills demonstrated that specific crude oil constituents induce a range of typical physiological effects like edema, cardiovascular dysfunctions or craniofacial deformities (blue sac disease) in this freshwater species, resulting from the interruption of specific molecular mechanisms (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 (Brette et al. 2014, Khursigara et al. 2017).

However, many studies do not consider the relevance of different environmental conditions as important additional stressors to crude oil exposure. Several abiotic factors can influence not only animal behavior but also physiology of individuals and population dynamics and size. In the context of oil spill research, it is well documented that besides physical and chemical properties of crude oil also environmental conditions like temperature, salinity and radiation influence the fate and behavior of spilled oil in the environment (Dupuis and Ucán-Marín 2015). Even in laboratory experiments with crude oil samples exposure medium conditions exert a great impact on the test systems. Specifically, the composition and the amount of soluble compounds partitioning from crude oil into the water phase is altered under varying salinities and temperatures. Consequently, the toxicity of these so-called water-accommodated crude oil fractions can depend on the exposure medium conditions.

Hence, this study firstly aimed to evaluate the tolerance of zebrafish embryos to the two environmental factors temperature and salinity that mainly influence experiments with crude oils and oil spill response chemicals like dispersants. Secondly, this study investigated whether zebrafish embryos adapted to specific environmental parameters out of their optimum

conditions react to model chemicals and real oil samples similar to when exposed in freshwater conditions, and if not, how the responses differ.

3,4-dichloranilin was selected as the model chemical, because its teratogenicity and embryotoxicity is well documented and it is used as a positive control in standardized embryotoxicity assays (DIN ISO 2007, OECD 2013). To evaluate adapted zebrafish sensitivity to oil samples, experiments with water-accommodated fractions (WAF) and chemically enhanced water-accommodated fractions (CEWAF) prepared at different salinities were conducted.

2. Material and Methods

2.1 Fish embryo acute toxicity tests

To investigate the salinity tolerance of zebrafish embryos (*Danio rerio*) and also the sensitivity of salinity-adapted zebrafish embryos to different sample types prolonged fish acute embryo toxicity tests up to a maximum of 120 hours post fertilization (hpf) were performed. All experiments were terminated at 120 hpf, since zebrafish embryos and larvae below 120 hpf are not considered animals (Strähle et al. 2012), and hence no animal test authorization is required according German legislation. The final measurements were conducted shortly before 120 hpf. After termination, fishlarvae were euthanized by prolonged immersion in a benzocaine ethanol solution. A wildtype zebrafish strain from WestAqaurium (Bad Lauterburg, Germany) was used in this study. Breeding groups of 100 to 150 adult zebrafish were kept in 170 L tanks of a flow-through system with a water exchange rate of 40 % per week. Tank water was cleaned through biological filter and a UV filter. 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.

The embryotoxicity assay was performed according to the OECD guideline 236 (OECD 2013) with minor modifications with 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. Higher salinity artificial fish medium was adjusted with sea salt (Tropic Marine®). The pH of all media was adjusted between 7.0 and 8.0. For all experiments with WAF, CEWAF and dispersants 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). Experiments with the model compound 3,4-DCA were performed in 24-well plates (1 embryo per well). 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.

2.2 Preparation of WAF and CEWAF

For all experiments a naphtenic North Sea crude oil and the commercially available third generation chemical dispersant Finasol OSR 51 (Total France) were used. Finasol OSR 51 was favored over OSR 52 for these experiments, as preliminary test results from project partner SYKE indicated better suitability for the low levels of salinity in this investigation. The preparation of WAFs and CEWAFs was performed according to Singer et al. (Singer et al. 2000). Briefly, WAFs 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 (WAF) or 1:200 (CEWAF), respectively. The WAF setup was carefully stirred with low energy avoiding a vortex in the water phase while the CEWAF was stirred at higher stirring speeds to create a 25 % vortex of the water phase. WAFs 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.

3. Results and Discussion

3.1. Thermal niche of zebrafish

Temperature is known as one of the most important environmental factors affecting behavior and developmental processes in fish (Polo et al. 1991, Schaefer and Ryan 2006, Sfakianakis et al. 2011). In the literature several studies focus on the temperature tolerance of adult fish while data on early life stages is scarce. Furthermore, most studies investigated fish tolerance to temperatures above their optimum temperature regime. Indeed, for most fish species the zone of tolerance shifts rather towards higher temperatures than lower temperatures

(Rombough 1997). The natural increase in the water temperature regime occurring during the spring and summer season could be a biological reason for the observed trend.

However, especially embryos and larvae are assumed to be more sensitive to temperatures beyond the optimum temperature niche than juvenile or adult individuals (Brett 1970, Schirone and Gross 1968). The limited abilities to regulate membrane fluidity, acclimation of metabolic rates or behavioral thermoregulation was suggested as a potential explanation for this phenomenon (Brett 1970). Reviewing compiled temperature tolerance data from multiple sources for a large number of fish species, Rombough (1997) calculated the width of the temperature zone tolerance for juveniles to be around 20-25 °C, while this zone is only about 12°C wide for embryos. Hence, the theory of a higher embryonal temperature sensitivity was verified with data on different fish species in that review.

In literature also focused studies are available investigating the influence of temperature regimes on early life stages of the zebrafish. In general, the zebrafish is considered to be a quite eurythermal teleost species in nature (Sfakianakis et al. 2011). The optimal thermal niche for laboratory cultures is described to be between 26 and 28.5 °C (Avdesh et al. 2012). The development of fertilized zebrafish eggs incubated at a variety of temperatures ranging from 13 to 35 °C was observed by Schirone and Gross (Schirone and Gross 1968). The authors found a typical development of zebrafish eggs raised at temperatures between 23 and 34 °C. Incubation at temperatures from 13 to 22 °C as well as above 34 °C resulted in 100 % mortality of the embryos. Furthermore, they detected a temperature dependence in reaching specific stages of development. Embryos raised at comparatively lower tolerable temperatures (e.g. 23 °C) required a longer period of time to complete specific morphological developmental stages.

These results were verified in a study conducted by Hallare and colleagues which focused on the combined effects of temperature changes and cadmium exposure on developmental parameters for zebrafish embryos (2005). The authors transferred fertilized zebrafish eggs to 3 temperature conditions (21, 26, 33 °C) and described an increased mortality rate for embryos exposed to 21 °C. Additionally, a faster development up to hatched larvae was described for embryos incubated at 33 °C (hatching started at 48 hpf).

Comparing the zebrafish embryo development at two temperature regimes (25 °C and 33 °C) Kimmel et al. (1995) concluded that the developmental rate is a linear function of the

incubation temperature. Hence, the speed of the embryonal development can be calculated in a certain range of tolerable incubation temperature.

Interestingly, some studies go even further by investigating the temperature-affected development and behavior of zebrafish that were reared in varying thermal regimes from fertilization to juvenile or adult. In addition to higher mortality rates and delayed development in early life stages incubated at extreme thermal conditions (e.g. 22 or 31 °C) also physiological alterations in juveniles and adults were observed. Body mass and body length as well as morphological characteristics were significantly affected by the incubation temperature. The growth rate increased with increasing incubation temperature (Barrionuevo and Burggren 1999, Schaefer and Ryan 2006, Sfakianakis et al. 2011).

Within this current study no further testing of the temperature tolerance of zebrafish was carried out, because literature data is consistent, which is moreover in terms with the 3R-principle of reducing animal testing. Based on all the data described above it can be concluded that the zebrafish can live and reproduce in a thermal regime roughly ranging from 23 to 33 °C with an optimum niche of ca. 26 to 28.5 °C. Zebrafish experiments in GRACE will be conducted animal free, which is statutorily determined to a maximum of 120 hpf. Hence, the temperature tolerance of early developmental stages from zygote to larvae is the relevant criterion. Study regions of the GRACE project are characterized by cold climate conditions with maximum temperatures of 10 - 15 °C. Especially based on the results by Schirone and Gross as well as those by Hallare et al. it is deemed possible to expose zebrafish embryos at temperature conditions relevant for the study region. Rearing embryos at these temperatures would lead to 100 % mortality. Hence, to use zebrafish embryos and larvae up to 120 hpf as models for investigating the effects of oil and dispersants on fish species in cold marine environments, temperature conditions have to be adapted to the thermal niche of zebrafish. As a consequence, knowledge has to be produced on how the deviating temperature can affect experimental outcomes in terms of processes such as uptake, transport and metabolisation, and in terms of partitioning and availability of oil and dispersant components. This can be achieved by comparison to experiments using endemic fish, e.g., a stickleback species, as is planned in the project for future investigations.

3.2 Salinity tolerance of zebrafish embryos

Salinity tolerance experiments with zebrafish embryos were conducted in the range of normal freshwater (0.5 ‰) up to brackish water conditions of 15 ‰. A concentration-dependent increase of mortality with increasing salinity was observed. However, zebrafish embryos tolerated a broad range of brackish water salinities (see Figure 1). Artificial medium with 15 ‰ salinity led to 100 % mortality while 4 ‰ induced negligible mortality comparable to the freshwater negative control (0.5 ‰). The mortality did not increase with increasing incubation time from 24 hpf up to 120 hpf as implied by quite similar concentration-response curves for each time point in Figure 1. Embryos that tolerated higher salinities protected by the chorion during early developmental stages did not show effects after hatching (around 72 - 96 hpf).

In general, no trend of an increasing occurrence of specific sublethal effects like edema or spine deformities was observed. Most embryos were either coagulated or completely normally developed. Based on concentration-response curves that were fitted to the mortality data an LC_{50} of 9.4 ‰ was calculated (see Table 1). Below a salinity of 6.6 ‰, less than 10 % of the embryos are expected to show any effect (EC_{10}).

The salinity tolerance data from this study are comparable to previous studies found in literature investigating the salinity tolerance of zebrafish embryos. In a study by Loerks on the salinity effect on 48 hpf embryos, zebrafish eggs were transferred to salinities ranging from 4 to 9 ‰ shortly after fertilization (2010). No embryotoxic effects were recorded up to a salinity of 5.8 ‰. At a salinity of 7.9 ‰ 50 % of the zebrafish embryos showed adverse effects (EC_{50}) while at a salinity of 6.6 ‰ only 10 % of the embryos were affected (EC_{10}). The resulting salinity tolerance of zebrafish embryos was identical to the tolerance of the present study (see Table 1; EC_{10} values for both studies: 6.6 ‰).

In another study, Sawant et. al transferred zebrafish embryos at 2 - 4 cell stages to artificial fish medium containing 2, 4, 6, 8 and 10 ‰ salinity followed by the incubation until hatching (Sawant et al. 2001). The authors found zebrafish embryos to be tolerating salinities less than 4 ‰. Embryos that were incubated at salinities higher than 4 ‰ were not able to undergo gastrulation and hence died during further incubation. Compared to the present study zebrafish eggs were more sensitive to increased salinity conditions in the study by Sawant et al. However, the range of salinity tolerance clearly below 10 ‰ was still comparable. Our study shows that salinity effects are not altered by (a) the chorion as a barrier, (b) the developmental

stage of the unhatched zebrafish embryo, and (c) the physiological changes occurring during and after hatching.

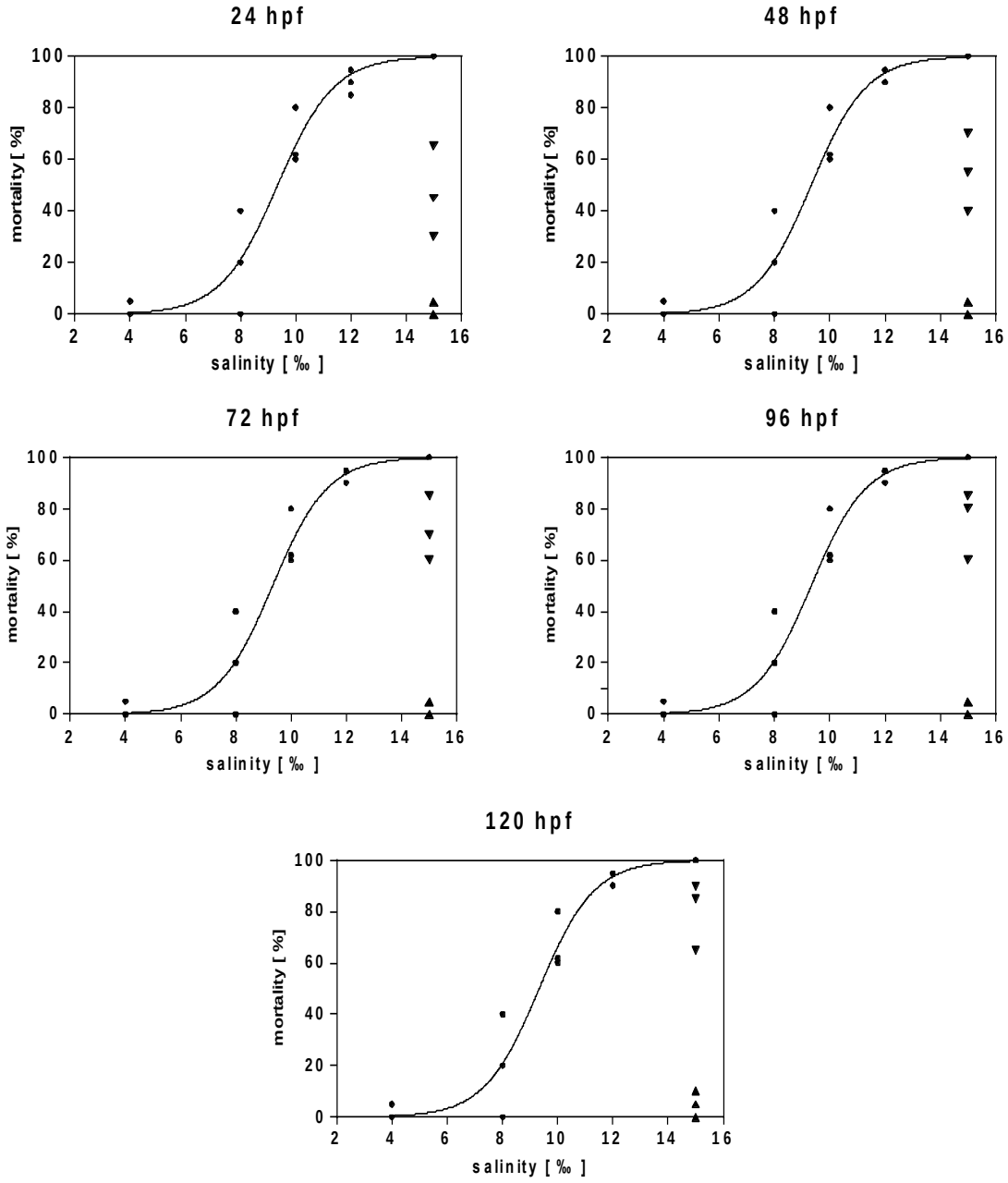

Figure 1. Mortality of zebrafish embryos at different developmental stages exposed to different brackish water salinities. Points denote the mortality of exposed embryos (one point represents 1 out of 3 independent experiments), triangles represent the negative control (freshwater: 0.5 ‰ salinity, pointing upwards) and positive control (3,4-dichloranilin, 4 mg/L). Sigmoidal concentration-response curves (variable slope) were added with top and bottom variables set to 100 and 0, respectively. Equation: $y = \frac{100}{1+10^{(\log EC50 - x)*hillslope}}$.

Table 1 Calculated effect - (EC) and lethal concentrations (LC) of brackish water salinities for zebrafish embryos. The values are based on the data of 3 independent replicates (see Figure 1). LC_{50} indicates the concentration inducing 50 % mortality. Sigmoidal concentration-response curves with variable slope were fitted to the data. Top and bottom variables were set to 100 and 0, respectively. Equation: $y = \frac{100}{1+10^{(\log EC50 - x)*hillslope}}$.

	LC_{50}	EC_{50}	EC_{20}	EC_{10}
salinity at 120 hpf [‰]	9.3	8.8	7.4	6.6

For all following experiments investigating the sensitivity of brackish water adapted zebrafish embryos towards chemicals, artificial brackish water at 5 ‰ was used. This salinity is clearly below any adverse effect level but still relevant for brackish water conditions of the study area in the Baltic Sea. The harbor of Helsinki, e.g., shows seasonal fluctuating salinities around 5.6 ‰.

3.3. Comparison of chemical and oil sample induced embryotoxicity at different salinities

Based on the results described in the previous chapters we further investigated whether zebrafish embryos that were adapted to the selected brackish water conditions still react to model chemicals and real oil samples similar to when exposed in freshwater conditions, and if not, how the responses differ.

3.3.1 Embryotoxicity induced by 3,4-dichloraniline

Zebrafish embryo exposure to the model chemical 3,4-DCA led to increasing mortality with increasing 3,4-DCA concentrations ranging from 0.5 to 4 mg/L. The mortality and effect rate increased with the incubation time from 24 hpf to 120 hpf. The different approaches using artificial freshwater or artificial brackish water resulted in comparable concentration-response relationships, as can be seen in Figure 2. The concentration-response curve shape and slope for freshwater conditions (Panels A and B) were similar to those of the brackish water conditions (Panel C and D) (see also hillslope values in Table 2). The highest test concentration (4 mg/L) induced 70 - 95 % mortality in both approaches while embryos exposed to the lowest test concentration (0.5 mg/L) developed completely normal and comparable to negative control embryos during the whole test duration.

Moreover, the high conformity of embryo sensitivity incubated in both salinities becomes apparent by comparing the calculated LC values (see Table 2). The calculated concentration resulting in 50 % embryo mortality at 120 hpf (= LC_{50}) was 3.0 or 2.9 mg DCA/L. It can be

concluded that zebrafish embryos reared in 5 ‰ salinity react similar to 3,4-DCA exposure as zebrafish embryos exposed to this chemical under normal freshwater conditions.

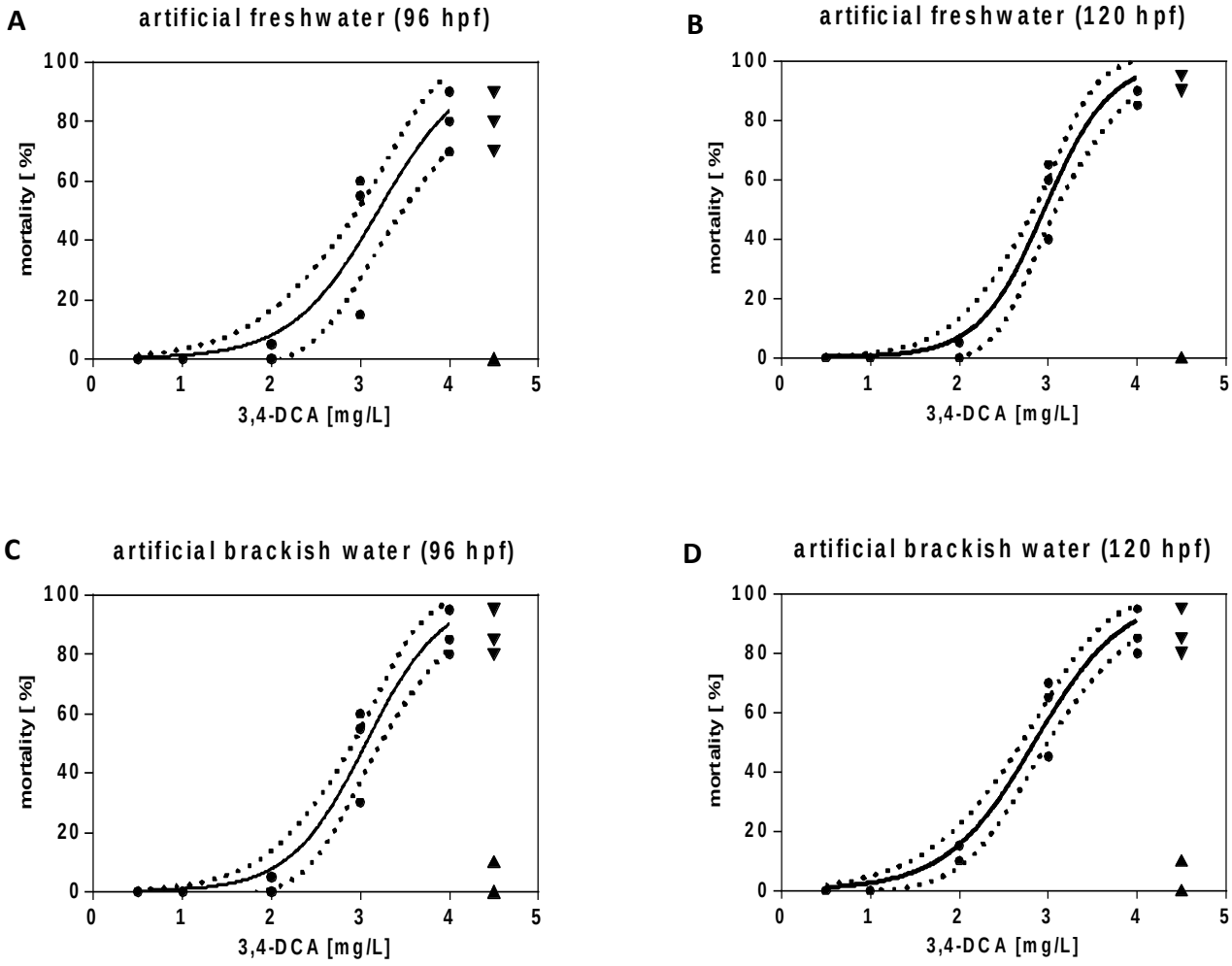

Figure 2. Mortality of zebrafish larvae (96 and 120 hpf) exposed to 3,4-dichloranilin at two different medium salinities. Panels A and B show the larval mortality induced by 3,4-DCA prepared in artificial freshwater medium (0.5 ‰), while panels C and D show larval mortality induced by 3,4-DCA prepared in artificial brackish water medium (5 ‰). Points denote the mortality of chronically exposed embryos (each point represents 1 out of 3 independent experiments). Triangles show the negative (artificial medium, pointing upwards) and positive controls (3,4-dichloraniline, 4 mg/L). Semi-static exposure conditions were used. Sigmoidal concentration-response curves were added (equation see Figure 1), top and bottom variables were set to 100 and 0, respectively. Dotted lines indicate 95 % confidence band.

Table 2 Calculated lethal concentrations (LC) of 3,4-DCA on zebrafish embryos at 120 hpf. Data are based on 3 valid and independent experiments (see Figure 2). Sigmoidal concentrations-response curves were fitted to the data. Top and bottom were set to 100 and 0, respectively. Equation: $y = \frac{100}{1+10^{(\log EC50 - x)*hillslope}}$.

	Artificial freshwater	Artificial brackish water (5 ‰)
LC_{10} [mg/L]	2.2	1.8
LC_{20} [mg/L]	2.5	2.2
LC_{50} [mg/L]	3.0 (hillslope: 1.2)	2.9 (hillslope: 0.9)

3.3.2 Embryotoxicity induced by the dispersant Finasol OSR 51

Finasol was selected as one dispersant of interest in the GRACE project, because its usage is relevant in the study region and the treatment of the selected oil types. Before investigating the combined effects of dispersant and crude oil, the toxicity of Finasol OSR 51 itself has to be evaluated. As a third generation dispersant it is a complex mixture of surfactants and solvents with reported less toxicity and higher efficiency compared to earlier generations developed during the 1960s to late 1980s (BfR 2016). The amphiphilic character with both hydrophobic and hydrophilic properties triggers their purpose acting at oil-water interfaces with the result of reduced interfacial tension. Dispersant/oil micelles get into the water column and break down the oil slick.

The fish acute embryo toxicity assay with Finasol OSR 51 was performed with dispersant contents ranging from 0.0016 to 0.25 % of the respective media. These concentrations were selected based on range finding experiments with Finasol OSR 51 (data not shown). Additionally, this concentration range complies roughly with the amount of dispersant used for CEWAF testing, which is described in chapter 3.3.4.

The embryo toxicity of this dispersant was strongly dependent on the developmental stages of zebrafish embryos. Embryos that were protected by the chorion did not show any harmful effect even for the highest test concentrations. After hatching, these inconspicuous embryos died within the following 24 h of exposure (see Figure 3). Using artificial freshwater medium, the two highest concentrations (0.025 and 0.0125 %) led to 100 % mortality after hatching, whereas using the brackish water medium 100 % mortality was detected only in the highest dispersant concentration (0.025 %). No or low embryo toxic effects up to a maximum of 20 %

mortality were recorded for the two lowest Finasol concentrations (0.0016 and 0.0032 %) in both approaches.

During the experiments with Finasol OSR 51 a recurrent sublethal morphological effect was that hatched larvae had strongly deformed caudal fins as indicated in Figure 4 by comparing a normally developed caudal fin to the fin of exposed larvae. This effect was observed for up to 60 % of the embryos and for nearly all test concentrations (≥ 0.003 %). Only the lowest Finasol OSR 51 concentration had no or negligible effects on caudal fin morphology.

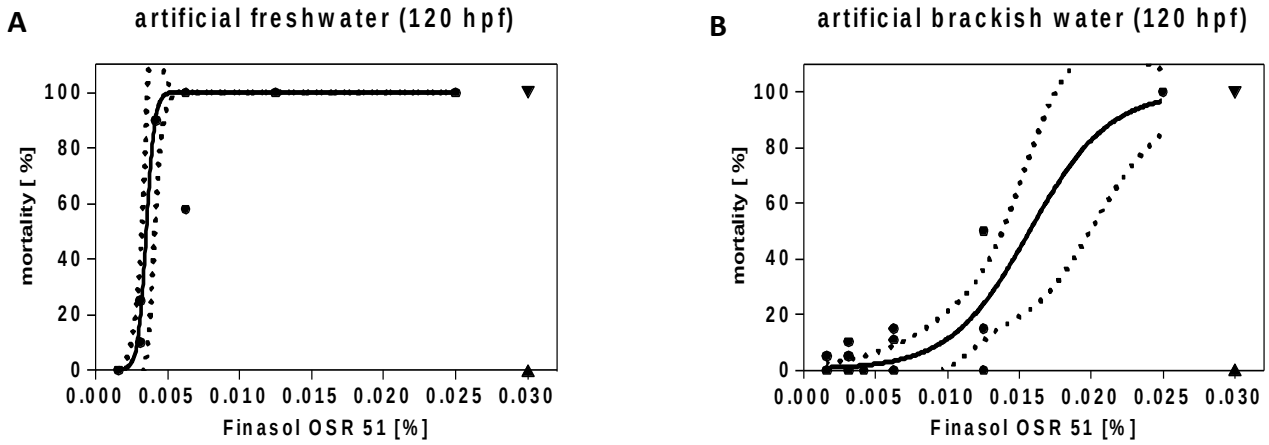

Figure 3. Mortality of zebrafish larvae (120 hpf) exposed to Finasol OSR 51 at two different medium salinities. Panel A shows the larval mortality induced by Finasol prepared in artificial freshwater medium (0.5 ‰), while panel B shows larval mortality induced by Finasol prepared in artificial brackish water medium (5 ‰). Points denote the mortality of chronically exposed embryos (each point represents 1 out of 2 (panel A) or 3 (panel B) independent experiments). Triangles show the negative (artificial medium, pointing upwards) and positive controls (3,4-dichloraniline, 4 mg/L). Semi-static exposure conditions were used. Sigmoidal concentration-response curves were added, top and bottom variables were set to 100 and 0, respectively. Dotted lines indicate 95 % confidence band.

As indicated in the graphs of Figure 3 both media approaches varied in resulting effect and mortality data. Finasol solution prepared in artificial freshwater medium seems to be more toxic to zebrafish embryos than Finasol solution prepared in brackish water medium. This can also be emphasized with resulting LC values (see

Table **3**). The freshwater approach resulted in an LC50 of 0.0035 %, while the brackish water approach resulted in an LC50 of 0.016 %. However, even though resulting concentration-response curves differ in the shape and hill slope, embryos that were incubated in higher

salinity still react sensitive to dispersant exposure. It has to be noted that for the freshwater approach two instead of three valid replicates are available and that one additional experiments is necessary to validate the data. However, we can already conclude that the salinity has an influence on the toxicity of this model dispersant. When assuming similar findings for other dispersants it is advised that dispersant testing using the zebrafish model should be conducted under brackish water conditions, not to overestimate potential hazard of the dispersant.

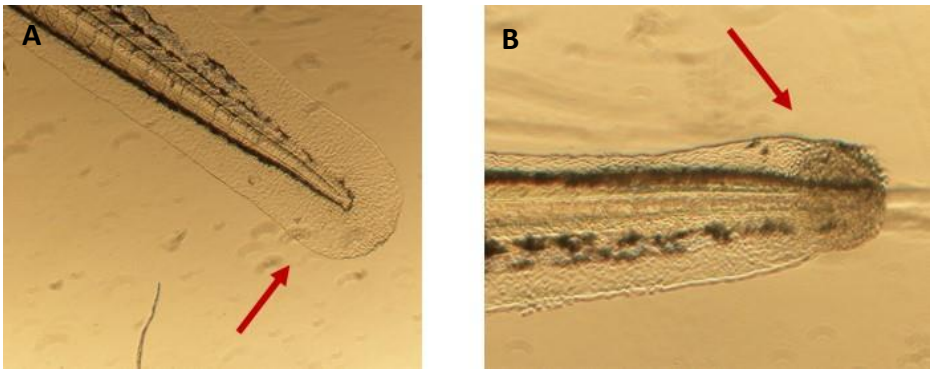

Figure 4 Effects of the dispersant Finasol OSR 51 on caudal fins of hatched zebrafish larvae. In panel A normal caudal fin of a non-exposed zebrafish embryo is shown while panel B represents the caudal fin malformation of more than 60 % exposed zebrafish embryos at dispersant concentrations ≥ 0.003 % at the test end (120 hpf).

Table 3 Calculated lethal concentrations (LC) of Finasol OSR 51 on zebrafish embryos at 120 hpf. Data are based on 2 (freshwater) and 3 (brackish water) valid and independent experiments (see Figure 3). Sigmoidal concentration-response curves were fitted to the data. Top and bottom were set to 100 and 0, respectively. Equation: $y = \frac{100}{1+10^{(\log EC50 - x)*hillslope}}$.

	Artificial freshwater	Artificial brackish water (5 ‰)
LC_{10} [%]	0.0029	0.0097
LC_{20} [%]	0.0031	0.0120
LC_{50} [%]	0.0035	0.0158

3.3.3 Embryotoxicity induced by water-accommodated fractions of a naphtenic North Sea crude oil

Crude oil is one of the most complex sample types for ecotoxicological characterization, as it consists of thousands of compounds with widely varying physico-chemical behavior. One approach to evaluate crude oil impacts on biota is to investigate water-accommodated fractions (WAF) simulating oil fractions that partition into the water column after an oil spill accident, and being available as dissolved contaminants causing adverse effects on aquatic biota. In the context of the GRACE project a naphtenic North Sea crude oil, which is relevant for the study region, was selected. In the present study WAF stocks of 1:50 ratio (oil:water) were prepared. Zebrafish embryos were chronically exposed to a dilution series ranging from 25 to 100 % of this stock. In both salinity approaches a concentration-dependent increase of mortality with increasing WAF concentration was observed (see Figure 5). Even though crude oil is a complex and challenging sample type, induced lethal and sublethal effects were highly reproducible in independent replicates. The WAF stock (1:50) led to 100 % mortality at 120 hpf, while the two lowest concentrations (25 and 33.3 % of stock) led to mortality rates up to a maximum of 10 %, which is comparable to the maximum mortality rate of non-exposed embryos.

A set of morphological effects including tail, yolk sack or pericardial edema, heart deformation, blood circulatory interruptions and spine deformations were observed in both approaches across the whole concentration rage. In the three highest concentrations (100, 75, 50 % of stock) these effects were present in nearly each embryo, while in the lowest concentration (25 % of stock) these deformations were exceptions (up to 20 % of the embryos). Figure 6 illustrates exemplarily these effects in different embryonal and larval developmental stages. Furthermore, interrupted locomotor behavior of embryos that were exposed to concentrations in the range of LC20 or even below LC10 was observed. These data are described and discussed in deliverable D1.4 of the GRACE project (WP1).

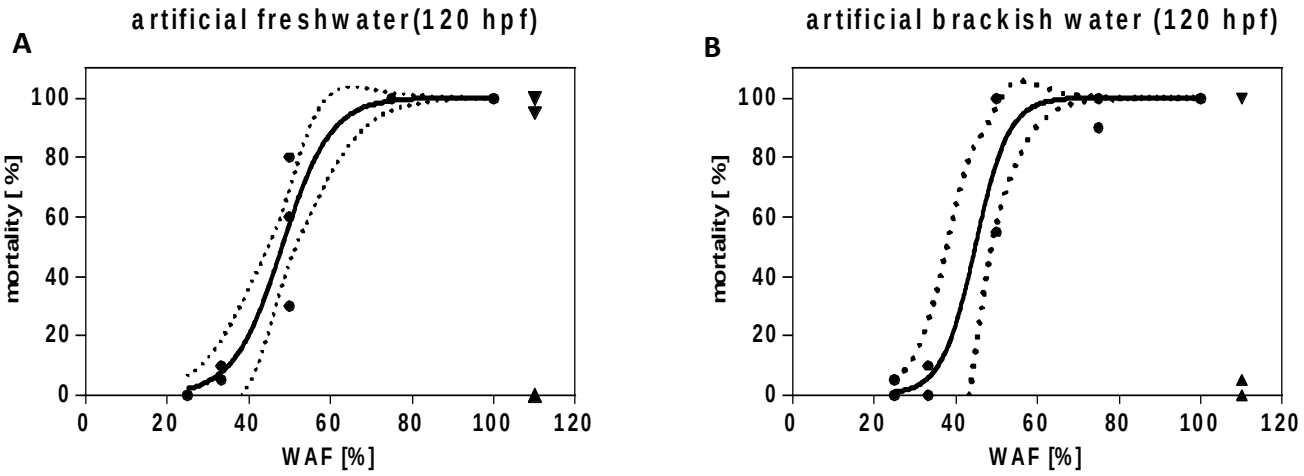

Figure 5. Mortality of zebrafish larvae (120 hpf) exposed to naphtenic North See crude oil WAFs at two different medium salinities. Panels A shows the larval mortality induced by WAFs prepared in artificial freshwater medium (0.5 ‰), while panel B shows larval mortality induced by WAFs prepared in artificial brackish water medium (5 ‰). Points denote the mortality of chronically exposed embryos (each point represents 1 out of 3 (A) or 2 independent replicates (B)). Triangles show the negative (artificial medium, pointing upwards) and positive controls (3,4-dichloraniline). Semi-static exposure conditions were used. 100 % WAF = 1:50 oil:water (w/w). Sigmoidal concentration-response curves were added, top and bottom variables were set to 100 and 0, respectively. Dotted lines indicate 95 % confidence band.

WAF-induced lethal and sublethal effects were quite similar for the two different media approaches as also indicated by the comparable shape and hillslope of resulting concentration-response curves in Figure 5. The calculated LC values indicate that 50 % of exposed embryos died at a concentration of 48 % (artificial freshwater) or 45 % (artificial brackish water) of the WAF stock, respectively (see Table 4).

Table 4 Calculated lethal concentrations (LC) of crude oil WAFs on zebrafish embryos at 120 hpf. Data are based on 2 (freshwater) and 3 (brackish water) valid and independent experiments (see Figure 5**Figure 3**). Sigmoidal concentration-response curves were fitted to the data. Top and bottom were set to 100 and 0, respectively. Equation: $y = \frac{100}{1+10^{(\log EC50-x)*\text{hillslope}}}$.

	Artificial freshwater	**Artificial brackish water (5 ‰)**
LC_{10} [%]	35.4	36.0
LC_{20} [%]	40.2	39.2
LC_{50} [%]	48.4	45.0

A large amount of studies has generated detailed information on the aquatic toxicity of oil using laboratory experiments or monitoring data after huge oil spills from the past decades like the Deepwater Horizon accident in 2010. Across literature, especially data on different fish species are available. The morphological effects like edema or heart deformations observed in the present study seem to be a common characteristic of oil affecting early life stages of fish, as they were also described for many other oil types like Alaska North Slope, Mesa Light Iranian Heavy across different fish species (de Soysa et al. 2012, Hicken et al. 2011, Incardona et al. 2013, Jung et al. 2013, Pauka et al. 2011, Perrichon et al. 2016, Philibert et al. 2016).

Interestingly, even though the morphological effects are consistent, the lethal and sublethal effect concentrations vary widely across different crude oil samples tested in laboratory experiments using zebrafish early life stages.

In a study by de Soysa et al. (2012) zebrafish embryos were exposed to WAFs of Macando crude oil from the Deepwater Horizon oil spill (1:10 oil:water). In compliance with the present study the authors observed the same morphological effects, but no mortality was observed at a 1:10 WAF. The present study resulted in 100 % mortality at even lower exposure concentrations. Hence, the naphtenic crude oil used in the present study seems to be more toxic to zebrafish embryos.

Another study conducted by Pauka and colleagues (2011) found a higher zebrafish embryo toxicity for a Brazilian crude oil WAF compared to the Macando crude oil. In accordance with the study by de Soysa et. al a 1:10 oil:water stock was used, while already the 50 % fraction (diluted with artificial water) induced 88 to 93 % mortality. Even the 15 % WAF induced embryo mortalities up to 67 %, which is more comparable to the highest concentration used in the present experiments.

A study by Philibert et al. (2016) investigated a 1:10 oil:water WAF stocks of Canadian sweet blend and medium sour composite crude oils. The authors exposed zebrafish embryos up to 7 dpf (=168 hpf). At the highest WAF concentrations 80 - 100 % of zebrafish died within the 7 dpf. A 20 % WAF induced a mean mortality of 20 %. Compared to the naphtenic crude oil of the present study the Canadian crude oils seem to be less toxic.

Compared to the present study even lower WAF concentrations still induced larval mortality. Environmentally realistic concentrations of WAFs prepared from Arabian Light and an Erika heavy fuel oil (103 mg oil/ L and 55 mg oil/L) were investigated for zebrafish embryo toxicity

(Perrichon et al. 2016). Significant larval mortality was observed in the recovery phase after embryos were exposed to heavy fuel oil WAF for 96 h.

It has to be considered that the varying acute zebrafish embryo toxicity can not only be caused by the individual composition and chemical properties of a crude oils leading to more or less toxic WAFs. Also deviating exposure conditions and WAF preparation procedures could result in varying toxicity data. Even though a common WAF preparation protocol is suggested by Singer et al. (2000), the discussed studies varied in oil load, mixing duration and settling time. Furthermore, different exposure scenarios were represented within the data. Experiments were conducted with static or semi-static approaches, air-sealed vials or open petri dishes. Furthermore, different developmental stages for the initial exposure were used, which can have a huge influence on embryonal reactions. Hence, ranking the different oil types regarding their toxicity has to be done carefully.

Finally, an important question is which initial molecular events cause the typical set of cardiac defects or edema observed in all crude oil exposure studies. It was suggested that the most embryotoxic compounds in crude oils are PAHs that furthermore induce the same morphological abnormalities when tested as single compounds (Incardona et al. 2004). A strong effect on cardiomyocytes action potential duration caused by toxicity-related blockages of potassium and calcium ion channels was described (Brette et al. 2014). This could lead to impaired cardiac functions. Furthermore, effects on the amount of atrium cardiac jelly, which is important for the elasticity of the early tubular heart, were found after exposure to crude oil compounds leading to reduced diastolic filling (Scott et al. 2011). The authors suggest an AhR-mediated and CYP1A-independent mechanism for the observed effects. However, the underlying mechanisms are still under controversial discussion.

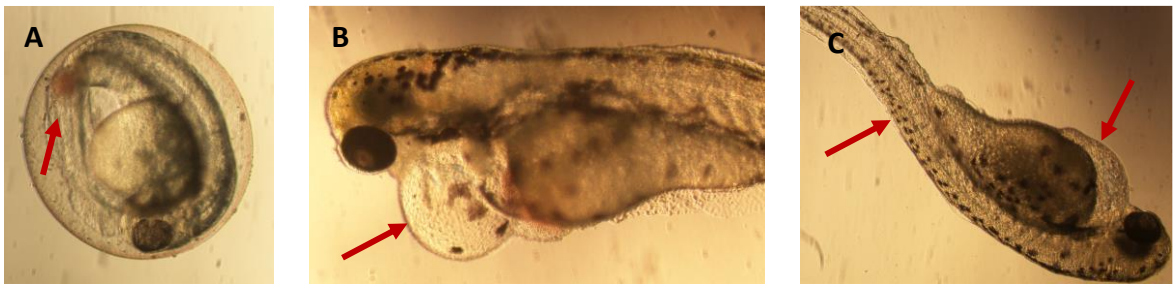

Figure 6 Typical morphological effects induced by crude oil WAFs. Panel A shows an WAF-exposed embryo at 48 hpf (exposure with 75 % of 1:50 WAF stock) with a tail edema. Panels B and C show WAF-exposed embryos at 96 hpf (exposure with 50% of WAF stock) having pericardia and yolk sac edemas, heart and spine deformations.

3.3.4 Embryotoxicity induced by chemically enhanced water-accommodated fractions of a naphtenic North Sea crude oil and Finasol OSR 51

The application of dispersants on an oil slick is discussed controversially. After an accident crude and fuel oils will spread rapidly on open water. The complete removal of oil by mechanical recovery systems is difficult if not impossible due to physical limitations of the mechanical skimmer equipment (Lee et al. 2011, Prince 2015). Hence, based on net environmental benefit analysis spill response coordinators have to decide whether the benefit of dispersant usage overweighs an additional damage that could be caused by the application. It is a fact that the application introduces even more chemicals into an already impacted environment. It was discussed that the water column under the recently dispersed oil slick is significantly transiently more toxic to organism due to the higher concentration of oil droplets in the water column in combination with a higher bioavailability of oil and dissolved oil constituents for organisms (Dussauze et al. 2015, Prince 2015).

From the present study it can be concluded that dispersed naphtenic crude oil was far more toxic than untreated crude oil. A 1:10 dispersant:oil ratio (DOR) was used, which is in accordance with other experiments conducted in the GRACE project. To obtain a typical concentration-response relationship with acute toxicity between 0 and 100 % a 1:200 oil/dispersant:water ratio stock was used, which was even further diluted to 12.5 % of stock to generate the highest test concentration inducing 100 % mortality in all replicates (see Figure 7). As shown in Panel A of Figure 7 embryo exposure to the lowest test concentration of 1.6 % induced low mortality rates up to 5 %, which is again comparable to the non-exposed controls. Unfortunately, the mortality data for the brackish water approach were not consistent across the three available replicates (Figure 7, Panel B). The model fit on the experimental data was therefore unsuitable as indicated by the wide 95 % confidence band in Panel B of Figure 7. Experiments have to be repeated for a solid evaluation of acute embryo toxicity. Hence,

Table 5 represents the calculated LC values for the artificial freshwater approach only. The calculated LC50 of 4.3 % of the stock (1:200) underlines again the higher toxicity compared to crude oil WAF exposure only (48.4 % of a 1:50 stock).

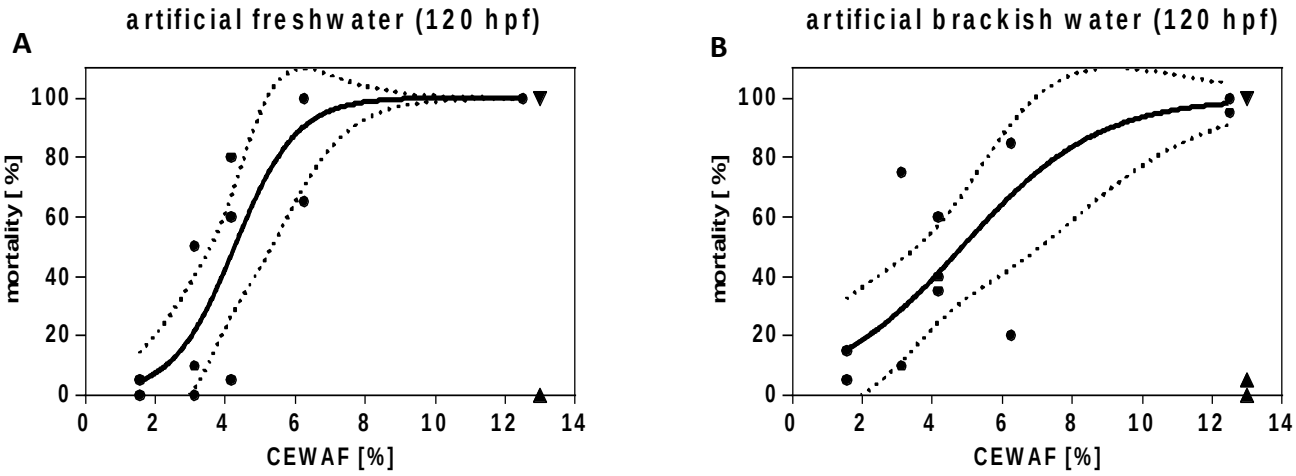

Figure 7. Mortality of zebrafish larvae (120 hpf) exposed to CEWAF of a naphtenic North See crude oil and Finasol OSR 51 at two different medium salinities. Panel A shows the larval mortality induced by CEWAFs prepared in artificial freshwater medium (0.5 ‰), while panel B shows larval mortality induced by CEWAFs prepared in artificial brackish water medium (5 ‰). Points denote the mortality of chronically exposed embryos (each point represents 1 out of 3 independent replicates). Triangles show the negative (artificial medium, pointing upwards) and positive controls (3,4-dichloraniline). Semi static exposure conditions were used. Dispersant:oil = 1:10 (w/w). 100 % CEWAF = 1:200 oil/dispersant:water (w/w). Sigmoidal concentration response curves were added, top and bottom variables were set to 100 and 0, respectively. Dotted lines indicate 95 % confidence band.

Table 5 Calculated lethal concentrations (LC) of CEWAFs on zebrafish embryos at 120 hpf under freshwater salinity conditions. Data are based on 3 valid and independent experiments (see Figure 5, Panel A**Figure 3**). A sigmoidal concentration-response curve was fitted to the data. Top and bottom were set to 100 and 0, respectively. Equation: $y = \frac{100}{1+10^{(\log EC50 - x)*hillslope}}$.

	Artificial freshwater
LC_{10} [%]	2.3
LC_{20} [%]	3.1
LC_{50} [%]	4.3

In general, the higher toxicity of CEWAF compared to WAF observed in the present study is in accordance with literature data. Ramachandran and colleagues exposed juvenile trouts to WAFs and CEWAFs of different oil types combined with the dispersant Corexit (1:20 DOR) (Ramachandran et al. 2004). The authors found a higher toxicity and a higher CYP1A induction

in CEWAF treatments than in WAF treatments. Even rainbow trout embryos that were exposed to WAFs of different crude oils and CEWAFs prepared with the dispersant Corexit were much more sensitive to CEWAFs, as the addition of dispersants increased the toxicity more than 35 to 300 fold (Wu et al. 2012).

Also, a study investigating the toxic effects of weathered Mesa light crude oil and the dispersant Corexit on marine mummichog larvae presented results with higher toxicity of the CEWAFs, even though the mortality data were not consistent across two experiments (e.g. experiment 1: 20 % mortality, experiment 2: 90 % mortality for the highest CEWAF test concentration) (Couillard et al. 2005).

Besides studies on the third generation dispersant Corexit, also some studies using the TOTAL product Finasol are available. Finasol OSR 51 was used in a study investigating the acute toxicity of a Maya crude oil and different spill treating agents on sea urchin embryo development (Rial et al. 2014). Finasol OSR 51 was the most toxic out of four tested spill treating agents. The CEWAF prepared from Maya crude oil and Finasol 51 (1:10 DOR) induced a higher toxicity compared to the WAF prepared form crude oil. Interestingly, the authors furthermore found a higher toxicity for Finasol OSR 51 compared to normal WAF. This demonstrates the embryo sensitivity towards dispersants and was also observed in the present study (see chapter 3.3.2). Moreover, other studies concluded that Finasol is a third generation dispersant with relatively high potential to induce acute toxicity compared to other third generation dispersants (Dussauze et al. 2015).

4. Summary and Conclusion

The aim of this study was to report suitable environmental parameters for the investigation of oil spill and oil spill response actions using zebrafish. Within this scope two main aspects needed to be addressed: Firstly, the tolerance of zebrafish embryos to the two environmental factors temperature and salinity mainly influencing the exposure conditions. Secondly, it had to be investigated whether zebrafish embryos adapted to specific environmental parameters outside their optimum conditions still react similar to the stressors of interest like chemicals, oil samples and dispersants.

Temperature conditions in zebrafish experiments cannot be changed towards the temperatures that are relevant for the study regions of the GRACE project. While zebrafish have a relatively broad thermal niche, temperatures 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 dramatically increase the toxicity (see chapter 3.3.2) as the embryos are no longer protected by the chorion. As a consequence, knowledge has to be produced on how the deviating temperature can affect experimental outcomes in terms of partitioning and availability of oil components.
Salinity was found to be a far better adjustable environmental parameter. Zebrafish embryos tolerated salinities up to 6 ‰ without any adverse effect. Thus, zebrafish embryo experiments can be conducted at salinities relevant for brackish water conditions of the study area in the Baltic Sea (5 ‰). This was verified with comparable results for all embryo toxicity experiments conducted simultaneously in artificial freshwater and brackish water conditions. However, we observed that salinity had an influence of Finasol OSR 51 toxicity. These results have to be verified with additional experiments on Finasol OSR 51 and additional model dispersants.

Besides the evaluation of suitable environmental parameters, this report presents the first results of WAF, dispersant and CEWAF toxicity on the early life stages of *D. rerio*. With the comparison to previous studies on crude oil induced fish toxicity we demonstrate that these data fit well in the available literature data on oil research.

Finally, from this study as well as from literature data that were discussed across the present report it can be concluded that the zebrafish is a suitable model organism in oil spill research. Nonetheless, some limitations have to be considered and integrated in the evaluation of study results.

Further investigations of crude oil induced fish toxicity will include the marine species *Oryzias melastigma* to cover a broader range of salinity and link the zebrafish freshwater/brackish water model to a marine fish model fish species. In addition, more mechanism-specific details underlying the observed morphological effects will be addressed. With this a more comprehensive ecotoxicological assessment of crude oil toxicity can be provided.

5. Literature

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