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Effects of oil and dispersant contaminated water on zebrafish and comparison to sticklebacks as a sentinel species

D3.6

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 D3.6 is to evaluate the effects of petroleum oils versus their chemically treated (with dispersant addition) counterparts on three-spined stickleback (*Gasterosteus aculeatus*), and in relation to effects in the freshwater model species zebrafish (*Danio rerio*). In addition, further investigations of crude oil toxicity included the marine species medaka (*Oryzias melastigma*).

A comparison between the effects could allow to understand how effects observed for zebrafish translate to effects on sticklebacks. This might enable laboratories to conduct a risk assessment of oil spill effects on endemic fish species by investigating the impact using laboratory zebrafish cultures. The experiments with the medaka, which is also a model species, serve here as a bridge from fresh water to marine systems. Knowledge of the transferability of zebrafish results to medaka could be a means to simplify comparisons to other, endemic marine species.

Due to massive, unexpected logistical and legal difficulties, less experiments to compare stickleback with zebrafish oil toxicity than intended were finalised. However, first results indicate that acute toxicity to high concentrations of petroleum components cause similar effects in both species. Higher toxicity was found for dispersed oil, in concordance with the vast majority of toxicity experiments within GRACE. Several former studies indicate some comparability of stickleback to zebrafish. However, comparisons between stickleback and other endemic fish species raise the question whether stickleback is a suitable sentinel species for oil spill toxicity in the study region. Nonetheless, results are still preliminary and to date no clear conclusion can be drawn. The suitability of the marine medaka as a bridging species between freshwater and marine systems also requires further investigation.

Comparison between zebrafish and medaka embryo toxicity will shed additional light on the possibility to conduct oil spill risk assessment based on laboratory fish toxicity tests. Crucial in this regards is the performance of embryo toxicity tests with stickleback, since embryo toxicity might greatly differ from effects on adults. In particular, embryo toxicity is much more relevant to the well-being of the population and thus provides very valuable data for risk assessment.

1. Introduction

Fish toxicity assessment of oil spills

Effect-based methods are valuable tools for oil spill risk assessment, as already shown in GRACE deliverables D3.9, D3.11-D3.18. The methods can be run to investigate water accomodated fractions (WAF) of oil samples under standardised conditions in the laboratory in a cost- and time-efficient higher throughput manner. Results are highly reproducible with sufficiently low variation, and comparison to former studies is supported by largely standardised procedures. However, relevance for the field is limited and difficult to predict from the data.

This holds especially true for the zebrafish as a model species for fish toxicity of petroleum components in the water phase. Being a tropical, Asian freshwater species, its habitats have little in common with the Arctic and Baltic Sea, in terms of temperature and salinity. To increase the value of zebrafish toxicity data for oil spill risk assessment in Northern, cold climates, knowledge and understanding would be required how effects on this model species would translate to effects on feral fish. A comparison between toxicity results for zebrafish with those for a feral fish species could allow to make that link and build a base for future risk assessment.

Hence, for this deliverable data on effects of WAF on zebrafish and a wild-caught population of three-spined stickleback as a species endemic to the Arctic Sea were collected. For building a bridge between freshwater and marine systems in terms of routine laboratory investigations of oil spill effects the marine medaka as a marine model species was included in this study.

Study species

Three-spined stickleback (*Gasterosteus aculeatus*), while not commonly used in the past for ecotoxicology, is gaining favor as an experimental model, in part because its genome has been sequenced. In the past much of the experimental work done with the stickleback had been with field-collected (wild) fish, but there are now viable approaches for culturing this species in the lab, thereby enhancing its value as a toxicological model (OECD 2012). For instance, three-spined stickleback is used for the androgenized female stickleback screen (AFSS) test. three-spined stickleback is a short-lived fish species with diverse salt-handling ecotypes, commonly found off the Atlantic and Pacific coasts of North America. The retreat of glaciers at the end of the last Ice Age resulted in a large number of new freshwater lakes and streams throughout the Northern hemisphere, and marine sticklebacks colonized and adapted to these newly formed freshwater habitats (Wang et al., 2014).

Danio rerio is a small tropical freshwater fish originating in the Ganges River and its tributaries in northern India. Currently, zebrafish are considered as a suitable model to investigate development, genetics, immunity, behavior, physiology, nutrition and it is widely used in toxicology and ecotoxicology (Teame, 2019). *Oryzias melastigma*, the marine medaka, is increasingly used for toxicity assessment as a marine counterpart to the well-established model zebrafish (Chen et al. 2011, Chen et al. 2009, Dong et al. 2014). The species originates from coastal waters of Pakistan, India or Thailand and is known to tolerate a broad range of salinity conditions. It has been shown that especially early life stages of fish are markedly sensitive towards petroleum compound exposure and hence acute toxicity and teratogenicity are important endpoints in the marine medaka. In compliance with the zebrafish model the embryonic development can be monitored easily due to a transparent chorion. With 9 - 11 days the embryonic development until hatching is elongated compared to the zebrafish, depending on temperature and salinity conditions. In general, species-specific characteristics have to be considered for establishing a marine model parallel to the zebrafish.

Figure 1 *O. melastigma* embryo (6 dpf) cultivated at 28 °C and 25 ‰ salinity conditions.

2. Materials and Methods

2.1 Oil and oil dispersant characterization

Naphthenic North Atlantic crude oil (NNA) was selected as the crude and untreated petroleum sample for the experiments. It is a light crude oil with low viscosity and characterized by a high proportion of low molecular weight saturates and aromatics. The commercially available dispersant FinaSol OSR® 52 (Total Fluids, Paris-La Defense, France) was selected to test its effect on oil toxicity. Following an oil spill, dispersants can be applied to combat the oil spill by alteration of the distribution of the oil in the water column. Dispersants lower surface tension and disperse oil into particulate-sized droplets. Smaller droplets of oil contain a higher surface area, allowing hydrocarbon-degrading bacteria to breakdown the oil more quickly. In spite the fact that the application of dispersants may reduce the overall impact of an oil spill, dispersing oil into water may result in an increase of chemical load of oil components into marine organisms. The dispersant used herein is relevant in the study region of the GRACE project and the treatment of the selected oil types. Details on the ingredients and composition of the dispersant are given in Deliverable 3.14.

2.2 Description of the experiments

In order to understand similarities and differences of crude oil toxicity in different fish species, multilevel responses in *D. rerio* and *G. aculeatus* were investigated in comprehensive experiments. Experimental setups including crude oil and dispersant samples, WAF preparation and exposure concentrations were largely consistent in the experiments, which is a major advantage of the GRACE project as the scientific community criticizes limited comparability of different studies from the past decades. Experiments on the brackish water/marine sticklebacks were conducted at The Plentzia Marine Station (PiE-UPV/EHU) of the University of the Basque Country (UPV/EHU) and on freshwater zebrafish at RWTH Aachen University, Germany. Further investigations of crude oil induced fish toxicity included the marine species *Oryzias melastigma.* These experiments were also performed at RWTH Aachen University, Germany. Hence, effects in the fresh- and brackish water species zebrafish and stickleback might directly be linked to effects in the marine medaka. Against the background of limited study comparability due to unique petroleum product characteristics and experimental setups the results of the present project provide useful information for crude oil risk assessment, as identical petroleum products using consistent WAF preparation methods were investigated.

The experiments were carried out according to the EU and Spanish regulation on Experimentation with animals in the EU. Positive evaluation of the Ethical Committee of the University of the Basque Country and Authorization of the Competent Authority for the project with animals (NoRefCEID: M20/2018/137: Exposure of *Gasterosteus aculeatus* fish to the water accommodated fraction of crude oil) were obtained before proceeding with the experimentation. All this documentation can be found in deliverable 7.5 (Orbea and Lekube, 2018).

2.2.1 Three-spined sticklebacks

To investigate biological effects of oil exposure on fish ca. 400 specimens of marine three-spined sticklebacks were caught in Oslofjord area and successfully shipped to UPV/EHU: Plentzia marine Station, Universidad del País Vasco/Euskal Herriko Unibertsitatea. It should be mentioned that due to legislation problems related to permit for fish transport, this sampling campaign was already a second attempt to collect the sticklebacks (collection of samples had to be repeated) and the sending of fish was substantially delayed.

Preparation of water-accommodated fractions (WAFs)

Low energy water-accommodated fractions (oil LEWAF) were obtained from Naphthenic North Atlantic crude oil (NNA) alone or after the addition of the Finasol OSR52 dispersant (oil+D LEWAF) and prepared at 10 °C using consensus procedures and conditions (details in deliverable D3.11). Oil or a dispersant/oil mixture (1:10) at an oil to 33 psu seawater (w:v) ratio of 1:200 were added to the surface of filtered sea water at 10 °C. 20 L glass Mariotte bottles were used to prepare the WAFs. The oil LEWAF and oil+D LEWAF were carefully stirred with low energy avoiding a vortex in the water phase (200 ± 20 rpm). After 40 h of incubation at 10 °C the LEWAFs were carefully drained off and dilutions were made as required for the toxicity bioassays.

Acute toxicity and sub-lethal effects in G. aculeatus

To estimate the acute toxicity of oil and mixture of oil and the dispersant to three-spined sticklebacks, the fish were exposed to 100 % WAF and a mixture of 100 % WAF and Finasol OSR52. The beakers were aerated and the animals were fed (with small red larvae of midge flies). However, already after 2 hours 100 % mortality was observed in the fish group exposed to the WAF and dispersant mixture. The fish exposed to 100 % WAF survived until day 5 and at this point the experiment was considered to be completed.

For sublethal toxicity (the adverse outcome link, AOL experiments), 14-day experiments were conducted with following exposure/control groups: control, 5%, 25% of NNA WAF; 5%, 25% of NNA and WAF mixture with dispersant. Twenty fish/experimental group under controlled laboratory conditions (photoperiod 12:8 h light:dark, 15 ºC and aerated constantly) were used. Fish were fed every day with frozen bloodworm and water was changed every three days with a new dosage of treatment. At day 3 and day 14, ten animals of each treatment were anaesthetized, weighed and measured. Gonads, liver, gills, muscle, brain and digestive tract were removed in order to perform histological, histochemical and molecular analysis. Gonads and liver were weighted separately in order to calculate gonadosomic index (GSI) and hepatosomatic index (GSI), Figure 2.

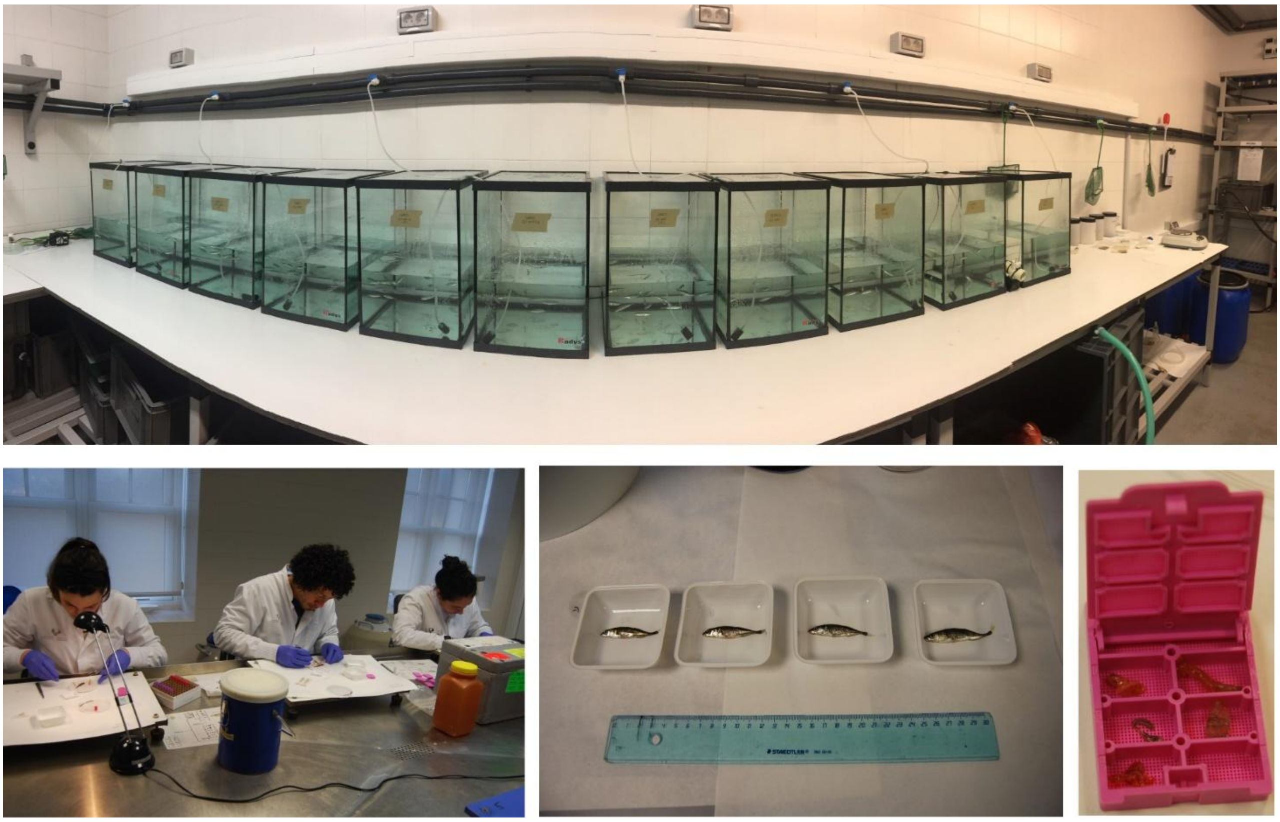

Figure 2 Experimental set-up and sampling of three-spined sticklebacks

2.2.2 Danio rerio

Preparation of water-accommodated fractions (WAFs)

For the *D. rerio* exposures, crude oil WAF was prepared according to the GRACE protocol and thus similar to the stickleback experiments. The different types of WAFs used for testing are low energy water-accommodated fractions (LEWAF) for oil exposure only and chemically enhanced water-accommodated fractions (CEWAF) for the combination of oil and dispersant exposure. Detailed description of the WAFs preparation is given in deliverable D3.12. Moreover, in the same deliverable a comprehensive chemical analysis profile for the naphthenic North Sea crude oil is available.

2.2.3 Medaka

In the framework of the GRACE project, an *O. melastigma* culture was established at the RWTH Aachen University. In a first step, the fish embryo acute toxicity test validated in the OECD guideline 236 was adapted to the marine species with respect to experimental setup, positive control substances and crude oil toxicity testing. All experiments were terminated at 9 dpf, which was shortly before hatching of larvae under the established laboratory conditions.

3. Results and discussion

3.1 G. aculeatus

In the acute toxicity test, exposure of adult stickleback to 100 % NNA+D LEWAF and equivalent concentrations of Finasol OSR52 caused 100 % mortality within 2-4 h, whereas survival on exposure to 100 % NNA LEWAF was 100 % after 24 h exposure.

On exposure to up to 25 % LEWAF (both NNA and NNA+D) for 7 d mortality was irrelevant both upon NNA LEWAF exposure and upon NNA+D LEWAF exposure, however, sublethal effects were recorded (ongoing analyses). Gonadosomatic index (GSI) and hepatosomatic index (HSI) remained unchanged (Figure 3). Biomarkers, histopathology and gene expression are being analysed but results are not expected until mid 2020. Some changes in gene expression of hsp90 (yellow); hsp70 (red) and elongf1 (green) have been envisaged in preliminary analyses (Figure 4).

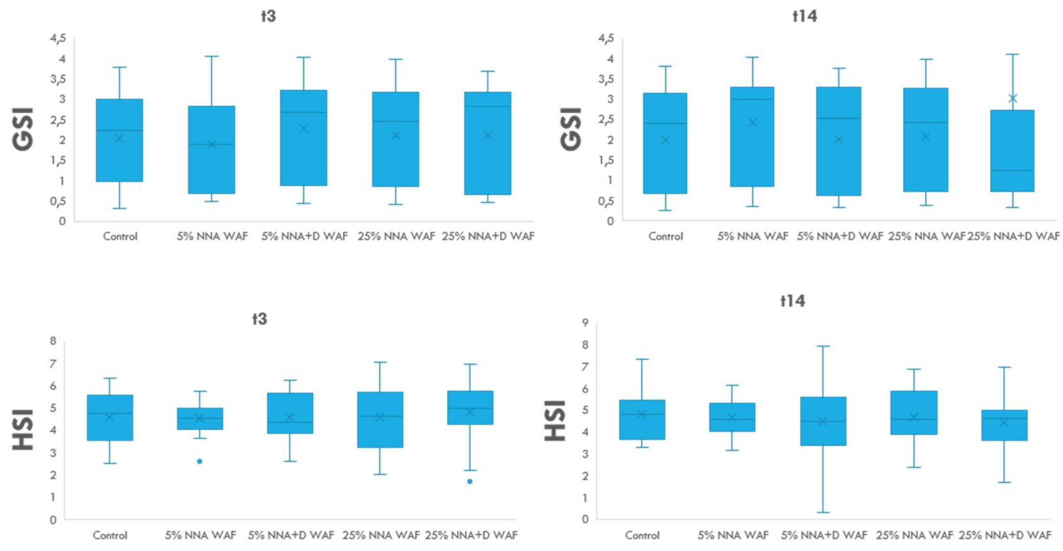

Figure 3 Gonadosomatic index (GSI) and hepatosomatic index (HSI) calculated for stickebacks exposure to NNA crude oil and its mixture with the dispersant.

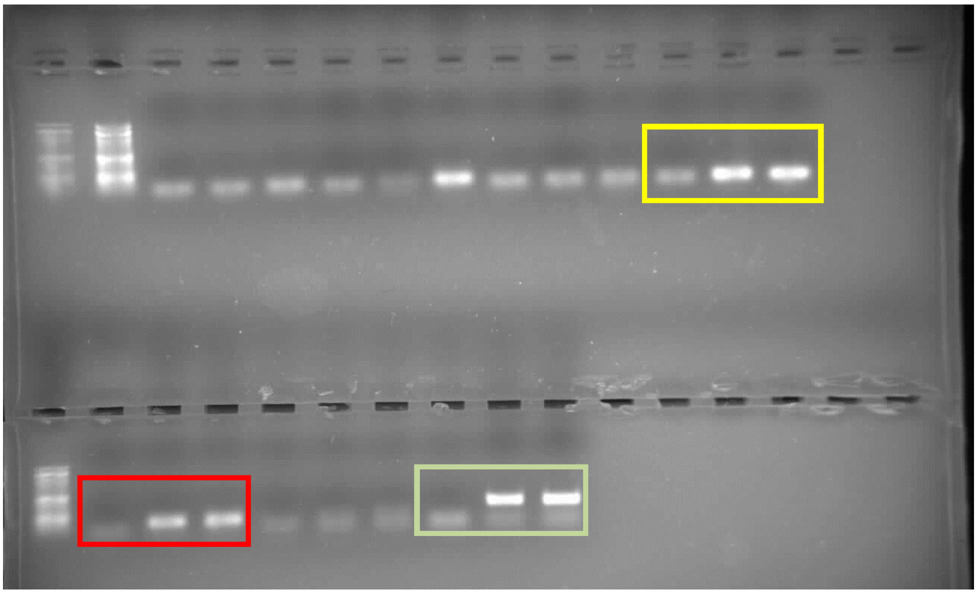

Figure 4 Gene expression of hsp90 (yellow); hsp70 (red) and elongf1 (green)

3.2 Zebra fish

Results of the adult zebrafish experiment can be found in D 3.12 on the adverse outcome links for zebrafish (Johann et al. 2019). Briefly, comparable to the first results of three-spined stickleback experiments, no mortality was observed in crude oil LEWAF exposed adult zebrafish. Furthermore, dispersed crude oil samples (LEWAF+D) induced mortality in both species during the semi-chronic exposure experiments. From biomarker activity measurements in zebrafish it was concluded that, overall, the dispersed crude oil LEWAF (5 % LEWAF+D) showed the highest effects on changes in biomarker levels. Furthermore, trends of increased (EROD, GST, CAT) or decreased (AChE) enzyme activity were consistent for 3 d and 21 d of exposure with the exception of catalase activity in gills. For the zebrafish experiments, also gene expression analysis has already been completed. Finally, results on biomarker levels, gene expression alterations and histopathology will be compared between the fresh- and brackish water species.

3.3 Medaka

First results on the acute and teratogenic effects of the naphthenic North Sea crude oil (NNA) in the marine medaka are available. The exposure towards the NNA WAFs resulted in a concentration-related increase of sublethal and lethal effects reaching 100 % in embryos exposed to the undiluted LEWAF stock (Figure 5). The marine medaka embryos chronically exposed to the LEWAF dilutions showed typical effects including heart deformation or edema described for other fish species in previous studies and the GRACE project (deliverable 3.12 on AOL in zebrafish). In a second step, additional endpoints focusing on possible mode of actions behind observed phenotypical malformations were established in the marine model. In particular, the endpoints xenobiotic biotransformation potential, neurotoxic potential and oxidative stress induction were focused on protein expression level. In this respect, protocols for 7-ethoxyresorufin-O-deethylase (EROD) activity, acetylcholinesterase (AChE) inhibition and catalase (CAT) activity were successfully adapted to investigate the biomarker responses in pre-hatching early life stages of the marine medaka. Future work will include the biomarker measurement in sublethal effect concentration of the naphthenic North Sea crude oil.

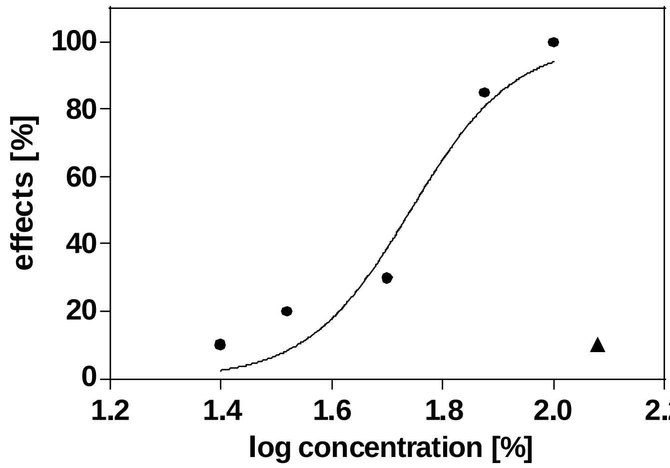

Figure 5 Sublethal and lethal effects in O. melastigma embryos (9dpf) exposed to LEWAF of a naphthenic North Sea crude oil. Points denote the effects in chronically exposed embryos (LEWAF stock: 1:50 (w/v)), while the triangle shows effects for untreated negative control embryos. Semi-static exposure conditions were used (medium exchange every 2 d). Sigmoidal concentration-response curves were added in GraphPad Prism 6 using the 4-parameter non-linear regression model, top and bottom variables were set to 100 and 0, respectively. Dotted lines indicate 95 % confidence band (Equation: Y= 1/(1+10^((LogEC50-X)*HillSlope).

3.4 *Comparison of toxicity of crude oil and its mixture with dispersant between fish*

No mortality was observed for crude oil LEWAF in both exposed adult zebrafish and sticklebacks. Furthermore, dispersed crude oil samples (LEWAF+D) induced mortality in both species during the semi-chronic exposure experiments. However, due to the lack of final data on sublethal effects on toxicity in sticklebacks it is difficult to draw final conclusion comparing sticklebacks and zebrafish.

In the literature there is a lack of data about oil exposure effects in three-spined sticklebacks (see reference list). Sensitivity of this species in comparison to other fish species can be approximated based on studies of other pollutants. Stickleback (*Gasterosteus aculeatus*) and killifish (*Fundulus heteroclitus*) have genomic regions under selection putatively related to pollution tolerance. Genetic variation of three-spined stickleback (*Gasterosteus aculeatus,* Gasterosteidae) exposed to kraft mill effluents that were known to impair reproduction, development and survival in fish in the Baltic Sea was found not to differ between polluted and cleaner reference sites (Lind and Grahn 2011), nor were there any differences in genetic separation between populations when comparing polluted and reference sites. However, pulp mill effluent acted as a selective agent on natural populations of stickleback, causing a convergence in genotype composition at multiple polluted sites in an open environment. The Japanese medaka (*Oryzias latipes*) is a suitable species for use in Fish Full Life-Cycle Test Guideline; however, other species such as fathead minnow (*Pimephalespromelas*), sheepshead minnow (*Cyprinodon variegatus*), three-spined stickleback (*Gasterosteusaculeatus*) and zebrafish (*Danio rerio*) are also suitable (OECD 2012). For endocrine disruption testing, against zebrafish, three-spined sticklebacks present the additional advantage that their genetic sex can be determined in addition to their phenotypic sex. In a study on pharmaceuticals, diclofenac (common non-steroidal anti-inflammatory drug (NSAID) widely used in both human and veterinary medicine to reduce inflammation and pain) caused histological changes in the three-spined stickleback at low µg/L concentrations (Näslund et al., 2017); however diclofenac has, with high probability, no adverse effect on zebrafish up to 320 µg/L (Memmert et al., 2013). The 96-hour median lethal concentration (96-hour LC50) values obtained from a study on the fish toxicity of nickel ranged from 19.3 to 61.2 mg Ni/l. According to nickel sensitivity, the species tested may be arranged in the following sensitivity order: rainbow trout > three-spined stickleback > perch = roach > dace (Svecevičius 2010). Arctic char has a lower capacity to metabolize PCBs compared to three-spined sticklebacks and zebrafish (Anderson et al., 2001). Responses to dietary PCBs showed correlation between three-spined sticklebacks and zebrafish, unlike Arctic char (Anderson et al., 2001). The sensitivity of various species and life stages of Alaskan freshwater and anadromous fish to benzene and the water-soluble fraction of Prudhoe Bay crude oil was determined with 96-hour toxicity tests (Moles et al., 1979): Three-spined sticklebacks were more tolerant than salmonids (stickleback crude-oil TLm: 10.4 mg/L; benzene TLm: 24.8 /L), however more sensitive than P. phoxinus to dispersants (Nagell et al., 1974). Geoghegan et al. (2008) observed up-regulation of CYP1A in both male and female three-spined stickleback following exposure to PAHs for 48 h. Overall the toxic effects of PAHs in this species seem to be strongly sex-dependent (Gao et al., 2008; Williams et al., 2009; Geoghegan et al., 2008). Sanchez et al. (2007) demonstrates the potential of stickleback as a sentinel fish species to assess sublethal stress in a multi-pollution context.

5. Conclusions

Due to massive, unexpected logistical and legal difficulties, less experiments to compare stickleback with zebrafish oil toxicity than intended were finalised. The dataset can be improved to gain a better picture. However, first results indicate that acute toxicity to high concentrations of petroleum components cause similar effects in both species. Effects are particularly severe with 100 % mortality only hours after the onset of exposure when the WAF represents oil dispersed using

Finasol OSR52. Higher toxicity of dispersed oil was found in the vast majority of toxicity experiments within GRACE, and hence these results are consistent with our previous knowledge.

Several former studies indicate some comparability of stickleback to zebrafish in terms of toxicity of a variety of different chemical classes. However, while this might point into the direction of the zebrafish as a suitable model for stickleback toxicity, comparisons between stickleback and other endemic fish species let assume that it might not be easy to establish sticklebacks as a sentinel species for oil spill toxicity in the study region. Nonetheless, since results are still preliminary and a large number of analyses are still going on, to date no clear conclusion can be drawn. The suitability of the marine medaka as a bridging species between freshwater and marine systems also requires further investigation.

All in all, the results are promising. Comparison between zebrafish and medaka embryo toxicity will shed additional light on whether and how it might be possible to conduct a reliable oil spill risk assessment regarding fish toxicity by means of laboratory experiments. A crucial step in this regards is the successful performance of embryo toxicity tests with stickleback. Embryo toxicity might greatly differ from effects on adults. Species could be less or more comparable than found for adults. In particular, embryo toxicity is much more relevant to the well-being of the population and thus provides very valuable data for risk assessment.

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