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Toxic impact of oil burning

D3.17

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

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Executive Summary

Herein, the toxic impact of crude oil burning residues was investigated using microscale assays with copepods and zebrafish embryos as test organisms. This investigation was aimed at contributing to the better understanding of the toxic impact of oil spills and oil spill responses as well as towards developing tools for their quantitative impact assessment in the Arctic.

As a part of a field experiment of burning (GRACE Deliverables D3.15 and D4.14), burning oil residues were used to produce WAF and evaluate its toxicity. Low Energy WAF (LEWAF) obtained from burning residues (IFO180-BR LEWAF) was less toxic than IFO180 LEWAF as for the two model toxicity test organisms employed herein, zebrafish embryos and copepods. Thus, *in situ* burning might seemingly be a promising alternative for oil spill response in iced seas; however, this deliverable only deals with a very preliminary study of the toxicity of burning oil residues and the risk assessment cannot be reliable without using more model test species and toxicological endpoints. Overall, more research on the toxicity and risk assessment of oil burning in iced seas would be pivotal to understand the environmental risk posed to Arctic, North Atlantic and Baltic ecosystems when burning is decided to be a part of the response in the case of oil spills.

1. Introduction

In situ burning is becoming a first option response to oil spills in iced seas. During an *in situ* burning operation the oil is ignited on the sea surface, and through this burning the oil volume is substantially reduced, which has been found to be effective for oil spills in Arctic ice-filled conditions (Sørstrøm et al. 2010).

In order to evaluate the toxic impact of oil burning, residues of the burned heavy fuel oil IFO180 were collected during the *in situ* burning field experiment along the Greenland coast in 2017. A detailed description of the experimental set up and the effects on the tidal community can be found in work package 4-associated deliverables of the GRACE project (D4.14; Wegeberg et al., 2018). In the present study the adverse effects of oil burning residues were investigated in different target organisms on different biological organisation levels. Furthermore, the toxicity induced by the oil burning residues was directly compared to the toxicity induced by the raw heavy fuel oil (D3.13& D3.15; Lehtonen et al., 2019; Lekube et al., 2019). The results contribute to the risk assessment of oil burning residues and further to the evaluation of *in situ* burning as a reliable, ecotoxicologically preferred alternative oil spill response strategy (D3.16; Marigomez et al., 2019).

These investigations altogether are aimed at providing a first approach towards the identification of toxicity associated to oil burning after oil spills in the Northern Atlantic Ocean and the Baltic Sea.

The following bioassays have been employed and constitute the backbone of this report:

- Embryo toxicity, enzyme activity,and behavioural tests with zebrafish, *Danio rerio*
- Survival assays with copepods

2. Material and Methods

2.1. Experimental design of the bioassays

2.1.1. Oils and oil burning

Burning oil residues were obtained from an off-shore *in situ* oil burning experiment (detailed in D3.15)that took place in a bay of the vicinity of Faeringehavn, south of Nuuk, Greenland, the 4th of July 2017. Approximately 1000 L of IFO180 (5 bbl of 200 L each) were released into a pyroboom in the bay and towed by two vessels. Fire was ignited and maintained for 1 h. Burning oil residues were sampled from the water surface with "collecting sheets" and stored in plastic bags at -20 ºC upon arrival to the lab.

2.1.2. Preparation of water-accommodated fractions for *D. rerio* exposure

The toxic effects of both raw oil and burned oil residues of IFO180 samples were investigated by using the water-accommodated fraction (WAF) in order to guarantee a direct comparability of generated results. A detailed description of the WAF preparation for zebrafish exposure can be found in previous deliverables of WP3 (e.g. D 3.12, D3.16). Briefly, low-energy water-accommodated fractions (LEWAFs) were prepared in aspirator glass flasks (500 mL) by application of oil or oil burning residue (1:10) on the surface of artificial sea water at an oil-to-water ratio of 1:50. The setup was carefully stirred with low energy avoiding a vortex in the water phase and incubated stirring at 10 °C to simulate cold climate conditions for 40 h 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, as a pre-requisite for zebrafish embryo testing.

2.1.2. Preparation of water-accommodated fractions for copepod exposure

LEWAFs of the IFO180burning residue samples and IFO180 samples were prepared at small scale after Singer et al. (2000) with modifications as detailed in deliverable D3.16, from oils alone or after the addition of a dispersant (oil+D LEWAF) and prepared at 10 °C using consensus procedures and conditions. Oil or a dispersant/oil mixture (1:10) at an oil-to-water (w:v) ratio of 1:200 were added to the surface of filtered sea water at 10 °C. 160 ml small bottles were used to prepare 100-140 ml solutions of the LEWAFs after 40 h stirring with low energy avoiding a vortex in the water phase at 10 °C. Further on, LEWAFs were carefully drained off and dilutions were made as required for the copepod toxicity bioassays.

2.2. Embryo toxicity tests with zebrafish, *Danio rerio*

Details on maintenance of the zebrafish culture as well as the fish embryo acute toxicity test can be found in GRACE deliverable D3.12. Briefly, the prolonged fish acute embryo toxicity test was performed up to a maximum of 120 h post fertilization (hpf). All experiments were terminated with the final measurement shortly before 120 hpf, so that no animal test authorization was required. The embryo toxicity assay was performed according to the OECD guideline 236(OECD, 2013) with minor modifications with respect to the sample type. Twenty embryos per sample concentration were investigated for lethal and sublehtal effects every 24 h.

2.3. Swimming behaviour of *D. rerio* in a light/dark transition test

At 96, hpf larval swimming behaviour alterations were investigated using a light/dark transition test. The swimming performance of LEWAF-treated larvae in sublethal effect concentrations (around EC_{10}) was compared to the behaviour of unexposed larvae. 16 to 20 zebrafish larvae per treatment were individually transferred into 96 well-plates and exposed to 2 cycles of alternating light (10 min) and dark (4 min) periods after an initial acclimatization time of 10 min in light conditions. The behavioral test was conducted using a DanioVision observation chamber and EthoVision tracking software (Noldus, The Netherlands).

2.4 Biomarker of xenobiotic metabolism and neurotoxicity in *D. rerio*

At 96 and 120 hpf the enzyme activity of 7-ethoxyresorufin-O-deethylase (EROD) and acetylcholinesterase (AChE) in unexposed and LEWAF-treated zebrafish larvae was investigated in order to evaluate the biotransformation phase I-inducing and the neurotoxic potential of the raw oil and the oil burning residues. Details on the material and methods can be found in D3.12 (Johann et al., 2019) of the GRACE project. Briefly, zebrafish embryos were exposed to sublethal effect concentrations of IFO180 LEWAF of raw oil and oil burning residues. A pool of 40 individuals per treatment concentration were anesthetized, inserted in buffer and immediately shock frozen in liquid nitrogen. EROD and AChE activity were measured according to established protocols as described in detail in D3.12 (Johann et al., 2019).

2.5. Survival assay with copepods

Copepods are considered as suitable model organisms to study the effects of toxicityin the marine environment. Thus, the effect of various oil WAFs has been well established on different copepod species in terms of mortality (Hansen et al., 2011, 2012, 2013).

Presently, survival assays with the copepod, *Tigriopus brevicornis* were carried out to assess the toxicity of the LEWAFs of IFO180 and IFO180 oil burning residue (IFO180-BR) (1:40 oil:water; 72 h low energy stirring at 10 ºC in the dark). LEWAF was produced using a modified protocol after Singer et al., *(*2000), as detailed above.

Lethality tests were conducted according to the international standard ISO14669. A total of 30 adult copepods were evenly distributed into 3 replicates, 10 copepods in each container. Copepods in each replicate were exposed to the contaminant for 72 h in 250 ml glass containers containing 50 ml of the LEWAF dilutions and water.

Each day of exposure, replicates were checked for mortality, and dead copepods were removed. 50 % of water was replaced every 48 h. A copepod was considered dead if it did notmove shortly after a gentle stimulation using a pipette. Copepods were not fed during the exposure period and no gender preference was considered when selecting adult copepods for the experiment. Median lethal concentration (LC50) was calculated after exposure of adults to the different LEWAF conditions for 72h.

Mortality at 72 h was analyzed using PROBIT analysis (SPSS) to calculate the LC50 of different LEWAF types.

3. Results and Discussion

3.1 Acute toxicity in *D. rerio*

In general, a concentration-related increase in sublethal and lethal effects was observed for the WAF of raw oil and of burning oil residues of IFO180. At the test end 50 % of the larvae were affected at LEWAF dilutions of 23.7 % of stock (raw oil) and 41.4 % of stock (oil burning residue), indicating a reduced toxicity of the LEWAF of IFO180 burning residue compared to the raw oil. On the sublethal effect level typical adverse effects like yolk sack or pericardial oedema as well as spinal deformations were observed. Exposure to the LEWAF of both raw oil and oil burning residues induced a reduced hatching success over the time window from 72 hpf until the test end (120 hpf).

Table 150 % effect concentration (EC_{50}) in the acute fish embryo toxicity test with D. rerio (120 hpf) exposed to LEWAF of rawIFO180 oil and burning IFO180 oil residues*. EC values were calculated by sigmoidal concentration-response curves fitted in GraphPad Prism 6 using the 4-parameter non-linear regression model with top and bottom variables set to 100 and 0, respectively (n=3).*

	EC50 (% LEWAF)
IFO 180	23.7%
IFO180-BR	41.4%

3.2 Swimming behaviour in *D. rerio*

Unexposed zebrafish larvae showed the expected swimming behavior under the light/dark transition of increased activity during the short dark (4 min) and less activity during the elongated light (10 min) phase (Figure 1). The swimming performance of petroleum product-exposed zebrafish larvae was mainly investigated at WAF dilutions inducing low levels of morphologically effects (EC_{10} - EC_{20}). Independent of the exposure concentration all zebrafish larvae exposed to the LEWAF of either raw oil or oil burning residues did not react

to the dark stimulus (Figure 1). No statistically significant difference was found between responses after exposure to LEWAF of raw IFO180 oil and LEWAF of oil burning residues. Hence, even identical exposure concentrations (12.5 and 25 % of LEWAF stocks) did not lead to an effect reduction in the oil burning residue treatment.

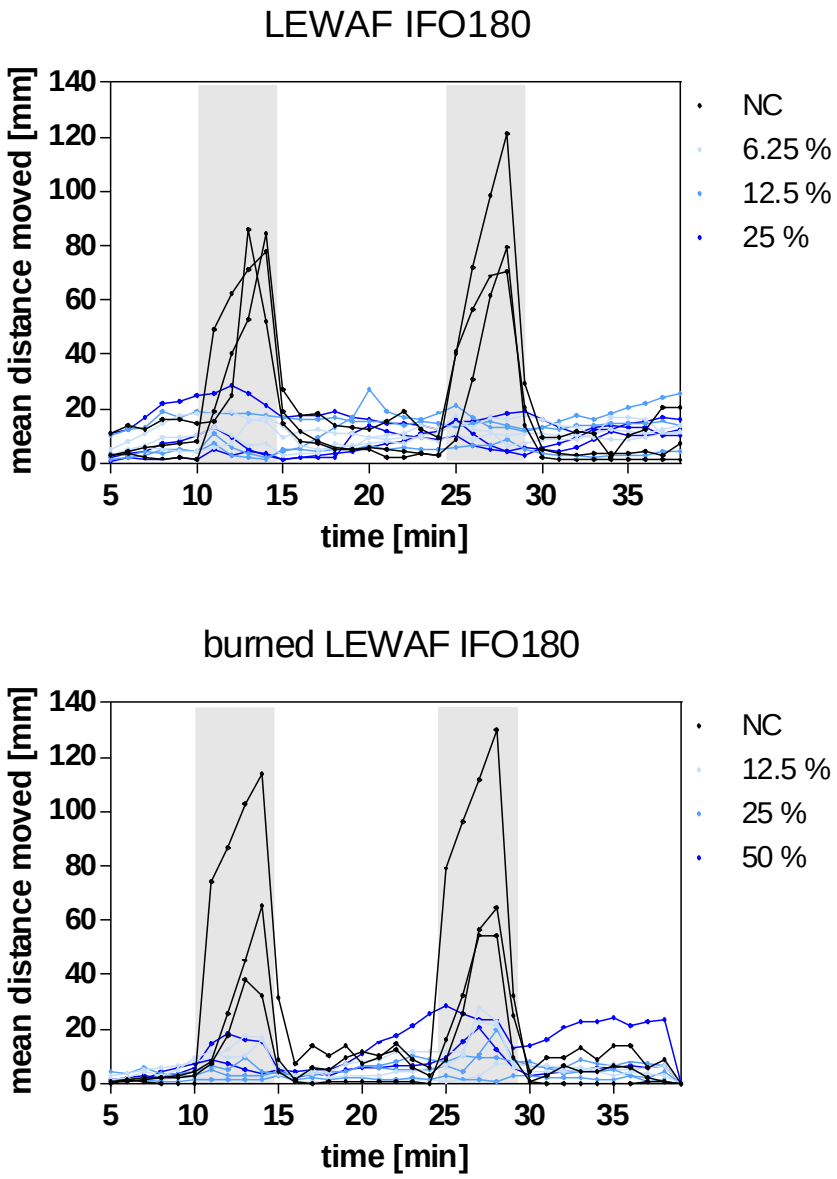

Figure 1 Alteration of zebrafish larvae (96 hpf) swimming behavior exposed to LEWAF of raw IFO180 oil and burning IFO180 oil residues in a light/dark transition test. *Independent experiments (3-4) are shown as individual lines with each line denoting the mean distance moved of 16 to 20 individual larvae.*

3.3 Biomarker activity in *D. rerio*

The biomarker activity was investigated at two early larval developmental stages of 96 and 120 hpf. Again, exposure concentrations were selected based on sublethal effect concentrations (below EC_{50}) and were almost identical to exposure conditions used for swimming behavior measurements.

The activity of CYP enzymes, representative for phase I biotransformation, was investigated using the fish embryo EROD assay (Schiwy et al 2015). In general, both raw and oil burning LEWAF exposure resulted in a concentration-dependent increase in EROD activity. Overall, CYP activity was stronger in 96 hpf zebrafish embryos than at 120 hpf. The raw LEWAF exposure induced a higher maximum EROD activity (3.5-fold) compared to oil burning LEWAF (2-fold), indicating a higher toxicity of raw IFO180 compared to oil burning residues.

As a biomarker of neurotoxicity, the potential of the petroleum product WAF to inhibit the acetylcholinesterase (AChE) was investigated. In contrast to clear effects in EROD induction, no concentration-related decrease in AChE activity was observed independent of the larval developmental stage or exposure concentration with relative activities around the untreated control. However, the highest exposure concentration of the raw IFO LEWAF led to a decrease in AChE activity with a mean normalized activity of 0.6-fold in 120 hpf larvae. Hence, a slight trend of a higher toxicity induced by the raw IFO180 can be concluded.

3.4. Survival assay with copepods

According to the obtained data shown below (Table 2), it seems that the oil burning residues of IFO180 are not toxic at all to this species of copepod; whose sensitivity to oil LEWAF is not markedly different from the one of the species, *Acartia tonsa* used in previous toxicity assays in which the 72 hr LC50 for IFO LEWAF was 44.8% LEWAF (D.3.16; Marigómez et al., 2019). Compared to the effects caused by the raw oil LEWAF a clear decrease in toxicity due to oil burning can be concluded also in terms of copepod exposure.

Table 2Median lethal concentration (LC_{50}) as % LEWAF after the copepod toxicity test of IFO 180 oil, and and burning oil residues of IFO180.

	72 hr LC50 (% LEWAF)
IFO 180	59%
IFO180-BR	100%

4. Concluding remarks

IFO180-BR LEWAF is less toxic than IFO180 LEWAF as for the two model toxicity test organisms employed herein, zebrafish embryos and copepods.

In situ burning is seemingly a promising alternative for oil spill response in iced seas; however, this deliverable only deals with a very preliminary study of the toxicity of burning oil

residues and the risk assessment cannot be reliable without using more model test species and toxicological endpoints. However, the applied zebrafish test battery with assays on mortality, teratogenicity, behavioural alterations, neurotoxicity and induction of xenobiotic resistance already represent a variety of possible effects and modes of action, and results clearly indicate a decrease in bioactivity of burned oil. Nevertheless, we need to understand the potential long-term toxic impact of these oil burning residues, as most likely they can persist for very long periods under the ice and even sink towards sediments. Moreover, in addition to oil burning residues, the toxicity of ashes and gases needs to be considered as well. Finally, the physical impact of residues and ashes cannot be neglected, especially in the framework of the raising concern of recently recognised environmental threats such as nanoparticles and microplastics.

For these purposes, microscale toxicity tests with copepods and zebrafish embryos can provide us with a sensitive, fast and reliable toolbox for assessing the toxic impact of burning oil after oil spills and oil spill responses; however, more model test systems and biological endpoints need to be thoroughly investigated. Likewise, new and advanced experimental designs and the combination of lab and field studies would be pivotal to understand the environmental risk posed to iced seas when burning is decided to be a part of the oil spill response in the case of accidental oil spills.

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