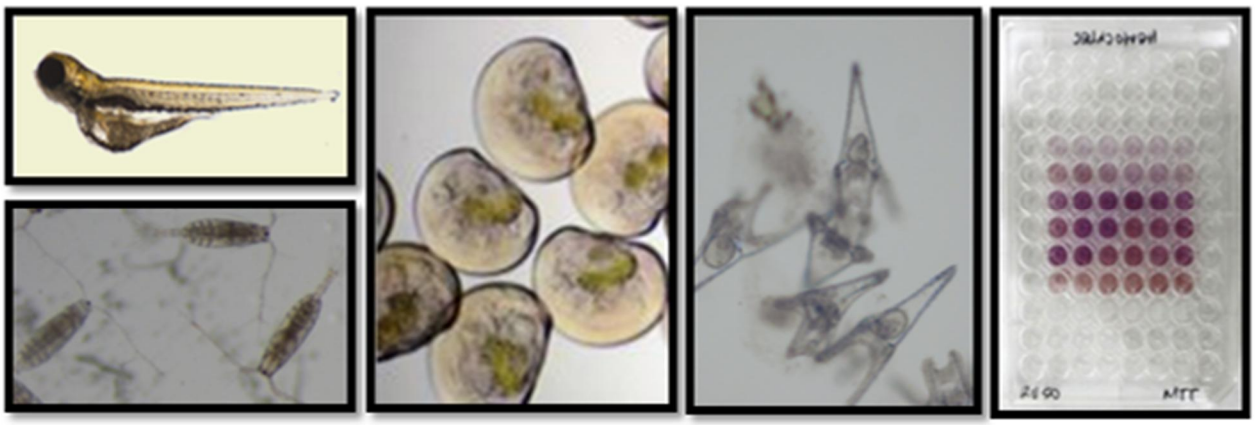
GRACE grant no 679266

Toxic impact of oil spills

Report and database on the toxic impact of oil spills and oil spill responses as determined using in vitro and microscale assays with invertebrate and fish larvae

D3.16

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



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Executive Summary

The water-accommodated fraction (WAF) simulates oil fractions that partition into the water column after an oil spill and are available as dissolved contaminants causing adverse effects on aquatic biota. Herein, the toxic impact of crude oil and dispersant samples was investigated using *in vitro* and microscale assays with invertebrates and fish larvae as test organisms together with chemical analyses of the waterborne PAHs in the WAF and of passive doser extracts. Copepods, mussels and sea-urchin and fish embryos were employed. 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. Toxicity tests were carried out to understand how toxicity to biota is affected by the physicochemical properties of the source oil and the application of dispersants under the natural variability in environmental conditions relevant in the Arctic ocean, the North Sea and the Baltic Sea (e.g., low temperatures and low salinities).

Three oils regionally relevant and with different physicochemical properties and chemical composition were used as model test chemicals: NNA, IFO 180 and MGO. The relative composition of their WAFs and the amount and stability of aromatic hydrocarbons were different and largely varied depending on how, and at which temperature, the WAF was prepared (LEWAF, CEWAF or HEWAF; using oil or oil+dispersant). Overall, the concentration of total PAHs was higher in oil+D LEWAF compared to oil LEWAF and at low seawater temperature (10ºC or lower). Toxicity (both lethal and sublethal) decreased from MGO>IFO180>NNA and the toxicity of oil+D LEWAF and dispersant alone (or as HEWAF) was higher than the toxicity of oil LEWAF. The results evidence that the use of the dispersants in ice seas could pose a clear risk for the marine organisms that should not be neglected to make decisions within the framework of an oil spill response. Likewise, oil+D LEWAF seems to be more toxic when produced at low temperatures. This might imply that toxicity data obtained for temperate environments would underestimate the risk of toxic impact of oils spills and oil spill responses in the Arctic ocean and the North Sea and the Baltic Sea.

It can be concluded that *in vitro* toxicity tests with mussel hemocytes and microscale toxicity tests with copepods, and sea urchin and zebrafish embryos can provide us with a sensitive, fast and reliable toolbox for assessing the toxic impact of oil spills and oil spill responses. Nevertheless, large research efforts are still required before an effective battery of toxicity tests can be selected and optimized to be regularly applied for risk and impact assessment of oil spills and oil spill responses in the Arctic ocean and the North Sea and the Baltic Sea.

1. Introduction

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 behaviour. Besides, the application of dispersants as a part of the oil spill response can modify their toxicity substantially. Dispersants reduce 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 (Chapman et al., 2007). After an accidental spill, crude and fuel oils will spread rapidly on open water and therefore the complete removal of oil by mechanical recovery systems can be difficult if not impossible, and applying dispersants is often a feasible alternative (Lee et al., 2015). Dispersants may reduce the overall impact of an oil spill dispersing oil into water (oil slicks are broken down and oil persistence in the millieu is reduced); however, their application may result in an increase of chemical load of oil components into marine organisms. On the one hand, dispersant application introduces extra chemicals into an already impacted environment. On the other hand, waterborne toxicity under recently dispersed oil slicks can be transiently more toxic to organism due to the higher concentration of oil droplets in combination with a higher bioavailability of oil and dissolved oil constituents (Couillard et al., 2005; Chapman et al., 2007). In addition, environmental conditions such as seawater temperature, salinity or radiation may influence the fate, behavior and toxicity of spilled oil; specifically, the composition and the amount of soluble compounds partitioning from crude oil into the water phase is altered under varying salinities and temperatures (Dupuis and Ucán-Marín, 2015).

One universal approach to evaluate crude oil impact on biota is to investigate the toxicity of the water-accommodated fraction (WAF), which simulates oil fractions that partition into the water column after an oil spill and are available as dissolved contaminants causing adverse effects on aquatic biota. On that basis, the toxic impact of crude oil and dispersant samples was investigated in GRACE WP3 using small-scale cell-based mechanism-specific *in vitro* bioassays[1] together with *in vitro* and microscale ecotoxicity assays with invertebrates and fish larvae as test organisms. Chemical analyses of the waterborne PAHs in the WAF and of passive dosers extracts were also carried out in order to complement the biological results and to better understand the toxicity of oil spills and oil spill response impacts.

[1] Cell-based mechanism-specific *in vitro* bioassays employed in deliverable D3.9 GRACE:

- Micro EROD on dioxin-like activity using the H4IIE cell line
- Mutagenicity using Ames fluctuation assay
- Endocrine disruption using ERα-CALUX® assay and AYES® assay
- Oxidative stress response in U2OS cells using Nrf2-CALUX® assay
- Toxic effects on the humpback whale cell-line HuWa1
- Cell Viability examination using MTT bioassay

These investigations altogether are aimed at providing a first approach towards the identification of toxicity profiles (Hamers et al., 2013) associated to oil spills and oil spill responses in the northern Atlantic ocean and the Baltic Sea, as well as towards the quantitative assessment of the predicted impact of oil spills and oil spill responses in the GRACE's target geographical region.

The results of the small-scale cell-based mechanism-specific *in vitro* bioassays are presented in Deliverable D3.9, together with toxicity fingerprinting and toxicity profiling. The present deliverable deals with the *in vitro* and microscale assays with invertebrates and fish larvae as test organisms, as a means to understand how toxicity to biota is affected by the physicochemical properties of the source oil and by the application of dispersants, always under the natural variability in environmental conditions that characterises the Arctic ocean, the North Sea and the Baltic Sea (e.g. low and changing temperatures and low salinities). Likewise, the use of multiple species testing can provide some insights into the understanding of the toxic impact from a wide and transversal perspective. Thus, copepods, mussels, sea-urchin and fish embryos were employed. Overall, the present outputs can be useful to derive a pattern for oil exposure (at best to be distinguished from other contamination) in order to, together with AOLs (deliverables D3.11, D3.12 and D3.14 GRACE) and selected MOA tests (deliverable D3.9 GRACE), provide scientifically-based support to the risk assessment strategy designed in GRACE WP5.

Thus, with a small deviation from the original plan due to technical or logistical reasons, the following bioassays have been employed and constitute the backbone of this report:

- *In vitro* multi-endpoint assays with mussel hemocytes
- Developmental assays with embryos of sea-urchins/mussels
- Embryo toxicity tests with zebrafish, *Danio rerio*
- Survival and behavioural assays with copepods
- Lethality test with adult stickleback fish, *Gasterosteus aculeatus*

2. Material and Methods

2.1. Experimental design of the bioassays

2.1.1. Oils and dispersants

Three different petroleum products were selected for the experiments: a naphthenic North Atlantic (NNA) crude oil, a commercially available marine gas oil (MGO) and an intermediate fuel oil (IFO 180). NNA crude oil was selected as the crude and untreated petroleum sample.

It is a light crude oil with low viscosity and characterized by a high proportion of low molecular weight saturates and aromatics (Table 1). It is rich in branched and cyclic saturated hydrocarbons. It has a low pour point, due to the low content of waxes, which might be relevant regarding to the temperature effect. This oil forms stable emulsions and was used in all the assays, which opens for the opportunity to compare results. Occasionally, other oils have been used for specific toxicity assays. MGO, used as a fuel in ships engines, is obtained from crude oils after a complex refining process involving atmospheric distillation and the refining of distillates. MGO is considered as light gas oil due to its high content (~60%) of aromatic hydrocarbons. IFO 180, which is a blend of heavy fuel oil and gas oil, was selected as an intermediate stage of petroleum products purity between crude and marine gas oil. IFO is characterized by a high viscosity (maximum viscosity = 180 centistokes) and a sulphur content of less than 3.5%.

Table 1. Summary of the most important properties of the oils used.
These parameters might vary depending on the specific oil sample and the refinery process.

	Density [g/mL)	Pour point [°C]	Viscosity	Wax [%]	Asphaltenes [%]
NNA	0.900	-36	299 cP at 2°C	0.9	0.04
IFO 180	>0.967	-10	180 cSt at 50°C	2%	10-15%
MGO	0.856	<-6	2-4 cSt at 40°C	-	-

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. The commercially available third-generation dispersants FinaSol OSR® 51 and FinaSol OSR® 52 (Total Fluides, Paris-La Defense, France) were selected to test their effect on oil toxicity. Both are suitable to be used in response to accidental spills of the selected oil types in the GRACE study region. These dispersants have slightly distinct chemical composition (Table 2) and a different performance depending on the salinity. Finasol OSR® 51 contains 15-30% non-ionic and 0.2-0.5 % anionic surfactants, while Finasol OSR® 52 contains >30 % non-ionic surfactants and 15-30 % anionic surfactants. Finasol® OSR 52 is compliant with all the three regulations on the market (EPA, MMO, CEDRE), while Finasol OSR 51 is compliant with two of them (MMO, CEDRE). Finasol OSR52 is used in more marine environments (sea water) whilst Finasol OSR51 is used at low salinites (brackish water).

Sealed oil drums (40-60 L) were delivered to partners by the oil supplier. Until they were opened the drums were stored at room temperature (e.g. in a chemical residues room). Once the drum was opened to retrieve (using a dispenser) 90 g aliquots in glass (borosilicate) bottles, it was sealed (with or without removing the dispenser) using aluminium foil, and stored in a cold room at <5ºC (see deliverable D3.11). Following the recommendation made

by Aurand & Coelho (2005), neat oil samples were stored in tightly sealed glass or metal containers with minimal headspace in the dark at <5ºC. Keeping the oil cold and dark appears to be more critical than the actual amount of headspace, provided this is minimal. With proper storage neat oil can be kept for several years (stored oil should be periodically tested against standard oil to check its composition).

Table 2. Ingredients and composition of the dispersants Finasol OSR® 51 and Finasol OSR® 52.

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

2.1.2. Preparation of water-accommodated fractions

Large scale WAF preparation: In general, all different WAFs were prepared according to Singer et al. (2000). The different types of WAFs used in the present study were low energy WAF (LEWAF) for oil exposure and for oil+dispersant exposure, and chemically enhanced WAF (CEWAF) for the combination of oil and dispersant (details in deliverables D3.9 and D3.11).

At RWTH, LEWAF was used only for oil exposure, whilst CEWAF was used for the combination of oil and dispersant exposure and HEWAF for dispersant exposure (Figure 1). In order to evaluate the possible changes in toxicodynamics or toxicokinetics of the dispersant in the presence of oil, Finasol OSR 51 and Miglyol 812® were also combined as an additional CEWAF. Miglyol 812® is an inert oil (mixture of medium chained triglycerides) that is commercially used in cosmetic industries. To proof the inertness of the Miglyol 812® a HEWAF of this sample only was prepared as well.

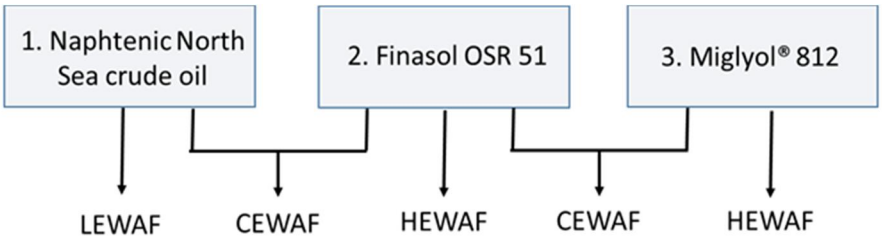


Figure 1: Schematically overview of different WAFs prepared at RWHT used in mechanism-specific *in vitro* based bioassays. The water accommodated fractions (WAF) can be distinguished for low energy WAF (LEWAF), chemically enhanced WAF (CEWAF) and high energy WAF (HEWAF).

Briefly, WAFs were prepared in aspirator glass flasks (500 mL) by application of oil or a dispersant-oil mixture (1:10) on the surface of 300 mL artificial medium (fish embryo exposure) at 10°C at an oil-to-water (w:v) ratio of 1:50 (LEWAF) or 1:200 (CEWAF), respectively. The LEWAF setup was carefully stirred with low energy avoiding a vortex in the water phase while the CEWAF was stirred at higher stirring speeds to create a 25% vortex in the water phase. The HEWAF of dispersant only and inert oil only was prepared as described for the CEWAF stock solution with dispersant loadings corresponding to the amounts added for the CEWAF production in order to ensure the comparability of the resulting stock solution. LEWAFs, CEWAFs and HEWAFs were incubated stirring at 10°C for 40 h and followed by 1 h settling time. Afterwards, water fractions were carefully drained off. After draining off the water phase dilution series prepared from the 100% stock solutions (1:50 LEWAF, 1:200 CEWAF, 1:200 HEWAF) were prepared for each bioassay. In general, the WAF samples were treated as water samples with specific modifications due to oil toxicity testing.

At UPV/EHU, low energy water-accommodated fractions (oil LEWAF) were obtained from oils alone or after the addition of a 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 water (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. After 40 h of incubation at 10°C the LEWAFs were carefully drained off and dilutions were made as required for the toxicity bioassays.

Small scale LEWAF preparation: for some *in vitro* toxicity assays WAF was prepared at small scale. Based on the agreed and operative procedure to prepare the LEWAF solution at 1:200 w/v ratio, this modified version tries to get small scale WAF solutions (100-140 ml). The aim of these small scale LEWAF solutions (both oil and oil+D LEWAFs) is to provide a friendly procedure to supply either small volume LEWAF or saturated PDMS sheets to carry

out *in vitro* tests or chemical measurements. The LEWAFs and the PDMS sheets were prepared following the next step-by-step procedure.

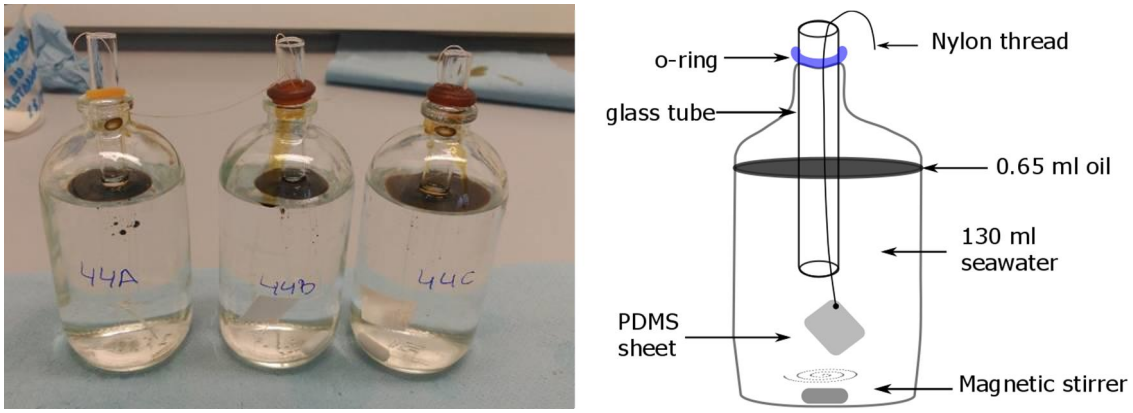


Figure 2. Scheme of the small (160 ml) LEWAF bottle with details about PDMS sheets.

Passive dosers: Several procedures were used to prepare passive dosers for *in vitro* assays. The first one consisted of loading silicone sheets (1–5 cm^2) with LEWAF organic chemicals by keeping the sheets immersed into the LEWAF under preparation. This procedure offered significant loadings of PAHs. However, the PAH dosed into water for the *in vitro* tests were too low because the kinetics of accumulation in the silicone sheets required more than 200 h of soft stirring. In a second attempt silicone o-rings (1.5 cm diameter and 1 mm thick) were loaded directly with source oil dissolved in methanol in order to assure a high loading. These o-rings proved to be successful to dose high concentrations of PAHs into small volumes (2-5 ml) of seawater) and, above all, the stability of these solutions lasted for 2 d. Thus, we could manage short-term (<48 h) *in-vitro* bioassays.

Polydimethylsiloxane (PDMS) sheets (AlteSil Industrial Sheet, Altair, UK; 0.5 mm thick) were employed as passive samplers for microscale toxicity tests that required larger volumes of test medium (e.g. seawater) or longer term exposures (e.g., beyond 2 d). PDMS sheets were thoroughly cleaned before their use (they are supplied protected with a talc cover)[2]. The clean PDMS sheets were then introduced in the bottles where the LEWAF was under preparation (Figure 2)[3] and stirred for 36 h at a low rate (without any appreciable vortex) at 10ºC and protected from light. Uptake of hydrocarbons in the WAF and the PDMS sheets

[2] They were sonicated (ultrasound bath) with acetonitrile (ACN) and methanol (MeOH) mixture (1:1) several times and renewing the solvent each time. As soon as the solvent mixture was free of suspended solid particles we assumed that the talc had been completely removed.

[3] The removal of the PDMS sheets without any contact with the superficial oil drop was facilitated by means of a glass tube (10 cm long, 10 mm i.d. and 1 mm thick) hanging with the help of an o-ring. This glass tube also enabled to retrieve the WAF without contact contamination with the remaining oil. The PDMS sheet (1-1.5 cm^2) was fixed with a nylon thread through the glass tube. Seawater and the stirrer were placed in the bottle. Afterwards, pre-weighted PDMS sheets were dangled through the glass tube with the aid of a nylon thread. Finally the volume of oil was added carefully avoiding any contact with the PDMS sheets.

from the oil requires a long equilibrium time. According to preliminary results obtained after incubating the PDMS sheets for 65 h under those conditions, it seems that PDMS sheets can reach equilibrium (saturation) after 24 h. PDMS sheets were retrieved through the glass tube, cleaned with MilliQ water and a linen free tissue to remove the seawater and suspended solids, and stored in the fridge in chromatographic vials until needed. LEWAF was also withdrawn through the glass tube using a Pasteur pipette.

2.1.3. Chemical analyses of the WAF and the PDMS extracts

The aliphatic and aromatic profiles of the raw target oils were analysed by GC-MS and GC-Q-TOF (still ongoing). PAH contents were quantified using the Norway mixture of 16 standards.

In addition, LEWAF stability was studied by monitoring the PAH profiles by solid phase microextraction (SPME) coupled to GC-MS. Preliminary data indicated that LEWAF preparation would require more than 200 h in order to achieve steady-state concentrations for most of the monitored PAHs. Seemingly, the initial concentration of PAHs achieved when applying the standard WAF preparation procedure (e.g., 40 h soft stirring) lasted less than 2 h and then quickly faded following a sharp declining profile.

Likewise, a gross estimate of the amount of total petroleum hydrocarbons present in the LEWAF obtained from different sources and at diferent temperatures was determined by fluorescence spectroscopy, using the Cytation 5, Gen5 software (Biotek). For this prupose, 250 µL of sample were added to a 96-well polystyrene plate and fluorescence was measured at 255 nm for excitation and 360 nm for emission (λ=50 nm). A calibration curve was obtained using serial dilutions of toluene (99.8% purity) from 100000 to 10 ppb, and the results were expressed as toluene equivalents (in ppt).

2.2. *In vitro* multi-endpoint assays with mussel hemocytes

The WAF of NNA crude oil was prepared according to Singer et al. (2000) with modifications (see 2.1.3) at three different temperatures representative of subarctic (10°C) and temperate (15 and 20°C) conditions, without and with the dispersant Finasol OSR52 (NNA LEWAF and NNA+D LEWAF, respectively). LEWAF produced at the three different temperatures were collected in glass vials for PAHs determination through GC–MS analysis, according to Prieto el al. (2007) with modifications.

Mussels (*Mytilus galloprovincialis*) were collected from Plentzia, Bay of Biscay, acclimatized for 2 d at 18ºC with daily food supply, constant aeration and a 12:12 h L:D photoperiod. After

acclimation, mussel hemocytes were isolated according to Katsumiti et al. (2017), seeded in glass coated 96-well plates and maintained for 24 h in pure culture media before performing the exposures. These primary cultures of hemocytes were exposed for 4 h in glass-coated microplates to different NNA LEWAF and NNA+D LEWAF dilutions (0.25, 2.5, 25, 50 and 100% LEWAF) and to the dispersant alone at the same concentrations used in the series of NNA+D LEWAF dilutions (1.25, 12.5, 125, 250 and 500 mg/L). Six replicates of each treatment were used in all tests, and the tests were repeated three times each.

Cell viability was assessed through the MTT assay (Sigma-Aldrich M5655) according to manufacturer's instructions. Plasma membrane integrity was assessed using the 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) assay (Lammel et al., 2013). ROS production was measured using the carboxy-H_2DCFDA assay (Invitrogen, C400) following manufacturer's instructions. MXR transport activity was assessed using the Vybrant™ multidrug resistance assay kit (Invitrogen, V13180), based on the intracellular conversion of the cell permeant non-fluorescent lipophilic calcein AM into a hydrophilic and highly fluorescent calceindye by endogenous esterases. Phagocytic activity was assessed in mussel hemocytes based on the capacity of the cells to phagocytize NR-stained zymosan (Pipe et al., 1995). Actin cytoskeleton integrity was assessed according to the protocol described in Chazotte et al. (1998). Significant differences ($p < 0.05$) between treatments were analyzed through Kruskal-Wallis test followed by Dunn's post hoc test using the R 3.5.0 statistical software (http://www.r-project.org/). LC50 values were calculated through probit analysis using the SPSS 23.0 software (Chicago, USA).

2.3. Developmental assays with embryos of sea-urchins/mussels

Mussel larvae bioassays will be completed next year season as a part of Laura de Miguel's PhD thesis at PiE-UPV/EHU. We experienced technical problems to obtain viable gametes: *M. edulis* gametes were not fully mature and *M. trossulus* adults spawned regularly in the tank, always out of any controlled conditions. In contrast, sea urchin bioassays were successfully accomplished.

Bioassays with larvae and embryos of sea urchin *Paracentrotus lividus* have been already used to determine the toxicity of crude oil and dispersants (Beirás and Saco-Alvárez, 2006). Presently, the objective was to assess the effects of dispersants on the toxicity of various oil types at a wide range of temperatures from cold seawater (under ice) to worm (sub-tropical) seawater including a whole range of temperatures from temperate seas in beween.

Thus, several series of bioassays were carried out to assess the toxicity of IFO180 LEWAF and the joint toxicity of mixtures of IFO180 and Finasol OSR52 dispersant (IFO+D LEWAF) after having produced the LEWAF at different temperatures (5, 10, 15, 20 and 25ºC) using a modified protocol after Singer *et al., (*2000), as detailed above. Furthermore, a comparative study of the toxicity of NNA, IFO180 and MGO of the oil LEWAF and oil+D LEWAF (using Finasol OSR52) was also carried out (1:40 oil:water and 1:400 dispersant:water; eq. 1:10 dispersant:oil; 72 h low energy stirring at 10ºC in the dark).

Standardized protocols of sea urchin embryo toxicity tests (including measures of viability, developmental progression and abnormalities). Briefly, gametes were obtained from sea urchin *Paracentrotus lividus* by injecting 1mL KCl into the coelomic cavity to stimulate the spawning. Once the sperm and eggs are obtained, the next step was fertilization. Later these eggs were transferred to glass vials containing the experimental solutions (sequential dilutions of different LEWAFs) to be incubated during 48 h at 20ºC in darkness. After 48 h, larvae were fixed with formalin and examined at the stereo microscope. The maximum length of 35 individuals per vial was measured as a parameter to determine growth inhibition relative (%) to the control and to calculate its median effective concentration (EC50). Besides, specific abnormalities and malformations were recorded after examining 100 larvae per treatment and the toxicity index (TI) was quantified by integrating the frequency of abnormalities detected (Carballeira et al., 2012).

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

Besides regional relevant species, the zebrafish (*Danio rerio*) is integrated herein as a universal laboratory model species. This fish species has been established as a popular model in a variety of scientific fields including biomedical research and (eco)toxicology (Strähle et al., 2012).

At RWTH, wildtype zebrafish of the WestAquarium strain (Bad Lauterburg, Germany) from the facilities of the RWTH Aachen University were used for embryonic endpoints. Details on maintenance of the zebrafish culture can be found in deliverable D3.12 GRACE (section III.3). Briefly, breeding groups of 100 to 150 adult zebrafish from 1 to 2 years of age were kept in 170 L tanks of a flow-through system. Fishes were fed twice a day with dry flakes and larvae of *Artemia* sp. 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. Zebrafish embryos and larvae below 120 hpf are not protected animal stages according to

EU Directive 2010/63/EU (EU, 2010)[4]. After termination, larvae were euthanized by prolonged immersion in a benzocaine ethanol solution.

The embryo toxicity assay was performed according to the OECD guidelines 236 (OECD, 2013) with minor modifications in respect to the sample type. Details about the experimental setup can be found in deliverable 3.12 section III.4.1. 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). Embryonic development was investigated for lethal and sublethal effects every 24 h. Concentration-response curves were established using the software GraphPad Prism version 6 (GraphPad, San Diego, USA) and LCx (concentrations inducing x % mortality) were calculated.

Regarding zebrafish embryo tests conducted at the BCTA RG Zebrafish Experimental Facilities in UPV/EHU, these were performed according to the OECD guidelines 236 (OECD, 2013) with minor modifications. Thus, embryos were treated with NNA LEWAF and NNA+D LEWAF (FINASOL OSR52) produced by different means (Table 3) at different temperatures from 5 to 28ºC, and to the dispersant FINASOL OSR52.

Breafly, newly fertilized embryos were exposed to test chemicals through different means for 5 d in standard conditions (embryo medium without methylene blue; 28ºC):

- NNA LEWAF and NNA+D LEWAF were produced at small scale (as above detailed) in deionized water used for the preparation of the embryo medium.
-
- NNA LEWAF and NNA+D LEWAF were produced in marine water at either small or large scale (as above detailed) and PDMS sheets were incubated in the LEWAF for 24 h and used as passive dossers. For some conditions, herein two different approaches were tested: (1) PDMS sheets of 1 cm^2 were allowed to desorb in embryo medium for 48 h and, after removing the sheets, the resulting medium was used for exposure; and (2) PDMS sheets were allowed to desorb in embryo medium for 48 h and, then, the embryos were exposed in presence of the PDMS sheets.

[4] See also: Strähle et al. (2012), TierSchG (Tierschutzgesetz) and the respective regulation TierSchVerV (Tierschutz-Versuchstierverordnung).

Table 3: Summary of the FET tests performed in this study.

TEST COMPOUND	EXPOSURE CONCENTRATION
FINASOL OSR52	0.01-1000 mg/L
PDMS sheets in clean MW and EM	Blank
NNA and D NNA+D LEWAF produced at large scale (20 L) in MW at 10°C	With and without desorbed PDMS sheets
NNA and NNA+D LEWAF produced at small scale (160 mL) in MW at 5, 10, 15 and 20 °C	With desorbed PDMS sheets
NNA and NNA+D LEWAF produced produced in EM at 28°C	- 0.25 %, 2.5%, 25%, 50%, 100% - With desorbed PDMS sheets

EM: embryo medium; MW: marine water.

2.5. Survival and behavioral assays with copepods

Copepods are considered as suitable model organisms to study the effects of toxicity on the marine environment. Thus, the effect of various oil WAFs has been well established on different copepods species in terms of mortality, development, egg production or motility (Hansen et al., 2011, 2012, 2013). Particularly, the calanoid copepod, *Acartia tonsa,* further fulfils the practical criteria as a suitable toxicological model species and is one test species recommended by ISO for the evaluation of lethality and toxicity of several contaminants (Gorbi et al 2012). Presently, the objective was to assess the effects of dispersants on the toxicity of various oil types at a wide range of temperatures from cold seawater (under ice) to worm (sub-tropical) seawater including a whole range of temperatures from temperate seas in between.

Thus, survival and behavioral assays with the copepod, *Acartia tonsa*, were carried out to assess the toxicity of NNA, IFO180 and MGO LEWAF and NNA+D LEWAF (using Finasol OSR52) (1:40 oil:water and 1:400 dispersant:water; eq. 1:10 dispersant:oil; 72 h low energy stirring at 10ºC in the dark). LEWAF was produced using a modified protocol after Singer *et al., (*2000), as detailed above.

Several generations of *Acartia tonsa* were intensively cultured in PiE-UPV/EHU facilities for 6 mo. Copepods were maintained in 10 L tanks filled with 0.2 µ-filtered seawater at 18ºC, 30 psu and constant photoperiod (16:8 h L:D cycle). Copepods were fed every second day using a 1:1 mixture of the brown microalgae *Isochrysis galbana* and the green microalgae

Tetraselmis chuii. To prevent potential cannibalism, recently hatched naupli were collected from the main tanks and transferred into smaller 5 L tanks until reaching adulthood.

Lethality test were conducted according to the international standard ISO14669. A total of 30 adult copepods were evenly distributed into 6 replicates, 5 copepods in each container. Copepods in each replicate were exposed to the contaminant for 96 hours 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 hours. A copepod was considered dead if it didn't move 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) were calculated after exposure of adults to the different LEWAF conditions for 96 h.

The reproductive capability was studied by investigating egg production capabilities and egg hatching success. Twenty adult females were selected under a dissecting microscope. Additionally, 5 adult males were selected to be included in each treatment to assist in maintaining female fertility throughout the whole experiment (Holste and Peck, 2006). The fecundity assay was divided into three consecutive stages: exposure stage, recovery stage and individual female fecundity test stage. In exposure stage, copepods were placed into 300 ml glass containers filled with 150 ml mixture of 0.2 micro mesh filtered water and WAF. Copepods were exposed for the semichronic period of seven days and every two days,50% of the water was replaced, dead copepods were removed and containers were cleaned from fecal pellets and dead algae. Throughout the whole experiments, copepods were fed daily with the algae *Tetraselmis chuii.* During the recovery phase, the surviving copepods were gently pipetted to clean filtered seawater for four days. Water was replaced every two days. In the single fecundity experimental phase, the surviving females were transferred to incubating chambers containing clean filtered water for three days. Each day, eggs were counted and copepods were fed daily. Each day, recently laid eggs from each chamber were pipetted to individual vials containing seawater to allow to hatch. Hatched eggs were counted and unhatched eggs after 48 hours were considered unviable. In order to further study the effects of WAF on copepod fecundity, lipid quantification on fecund females were conducted using the lipophilic dye Nile Red. Copepod staining was based on the protocol by Tingaud-Sequeira et al. (2011). In parallel, histological analysis was used in order to determine internal morphology inside productive females and to investigate the lipid accumulating regions.

Mortality at different timeframes (48 ,72, 96 h) from lethal toxicity assays were analyzed using PROBIT analysis (SPSS) to calculate the LC50 of different LEWAF types. Data from egg production rate, egg hatching success, Nile red fluorescence were initially checked for normal distribution using Kolmogrov-Smirnov test (SPSS). Data showing normal distribution were analyzed by one way ANOVA. Significant differences were further analyzed using Dunnett Post-Hoc test to identify the significance from control. However, data showing non normal distribution were analyzed using the non parametric test Kruskal-Wallis one way ANOVA. The association between the percentage of reproductive females and LEWAF doses was analyzed by Chi square test using SPSS software. Egg hatching success during individual fecundity test was calculated as follows: (number of hatched per females/total number produced per female) x100.

2.6. Lethality test with adult stickleback fish, *Gasterosteus aculeatus*

As reported to revise the deliverables during the running of the project, we experienced difficulties to obtain marine/brackish water stickleback (it is relatively easy for freshwater but not for the ones we need) and (most relevantly) difficulties for import/export (transportation) of non-commercial fish; which was unexpected as a risk.

Finally, only a limited number of adults was obtained and these were prioritized for AOL experiments. Nevertheles, preliminary "trial and error" lethality tests were conducted with a few individuals in order to decide the concentrations to be used in the AOL experiments.

3. Results and Discussion

3.1. Experimental exposure levels

GC/MS revealed that the relative compositon of PAHs in the LEWAF obtained from the three test oils (NNA, IFO 180 and MGO) was different (Table 4). The three types of LEWAF contained essentially low molecular weight PAHs, consisting of 2 or 3 rings, and naphthalene was the dominant compound. However, the concentration of naphthalene in LEWAF was markedly higher in NNA than in the other two test oils whist the lowest one was found in MGO.

In addition, our results indicated that the amount of total aromatic hydrocarbons (toluene equivalents) present in LEWAF largely varied depending on the source oil and the preparation conditions (Figure 3). Thus, the concentration of total PAHs in seawater were much higher in NNA+D LEWAF compared to NNA LEWAF preparations (Table 5).

Table 4. Concentrations of most prevalent PAHs in the three different types of LEWAF prepared at 10ºC (NNA, IFO 180, MGO). All concentrations presented at (ng/L) (mean values±SD) (n=3)

PAH	NNA	IFO 180	MGO
Naphthalene	19295 ± 76	8441±66	347±25
Acenaphthene	347±25	965±30	362±37
Fluorene	1008±30	909±115	1161±95
Phenanthrene	1115±15	1968±63	1953±71
Anthracene	LDL	132±18	LDL
Fluoranthene	16±0.70	23±2.30	25±3
Pyrene	141±33	56±20	37±30
Benzo[A]Anthracene	10±0.60	29±30	7±0.50
Total PAHs	21934	12524	14759

Likewise, LEWAF obtained from IFO 180 presented higher total aromatic hydrocarbons at all temperatures, except at 25 ºC, than that obtained from NNA and MGO. In contrast, MGO+D LEWAF presented higher total aromatic hydrocarbons at 5 and 15 ºC than in any other experimental group. Similar results were reported by Cohen et al. (2001) and Couillard et al. (2005) where the addition of oil dispersant for the preparation of WAF caused a two to five-fold increase in the total PAH concentration in these samples compared to WAF without dispersant. Additionally, chemical analysis showed that WAF produced at 10, 15 and 20 ºC showed similar PAH concentrations, whereas in WAFD samples, PAH concentrations increased with decreasing temperatures.

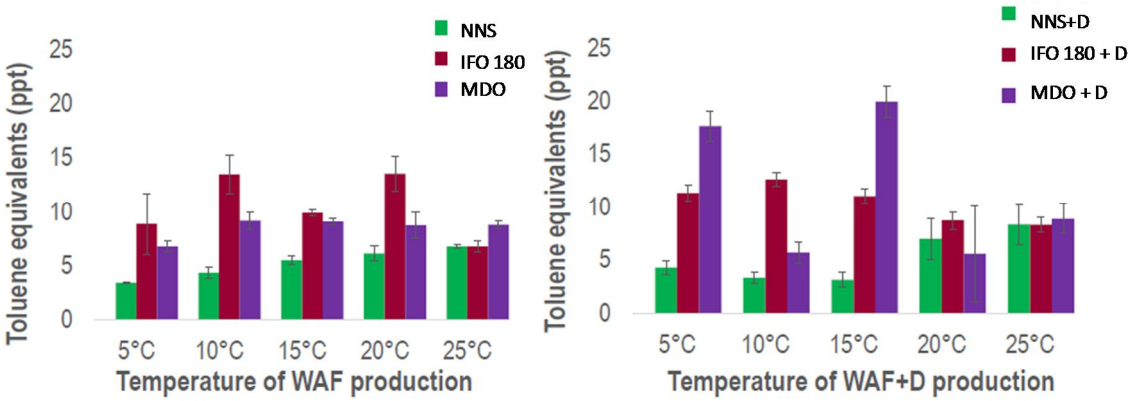


Figure 3: Equivalents of toluene (ppt) in NNA, IFO 180 and MGO LEWAF (left) and NNA+D, IFO 180+D and MGO+D produced at different temperatures (5-25ºC).

Table 5. GC-MS analysis of total PAHs present in LEWAF samples produced at 10, 15 and 20°C. Values are given in ng/L (means ± SD) (Katsumiti et al., 2019)

	10ºC	15ºC	20ºC
NNA LEWAF	2030±36	2087±36	2246±27
NNA+D LEWAF	24475±365	13327±67	7545±37

3.2. *In vitro* multi-endpoint assays with mussel hemocytes

NNA+D LEWAF and dispersant alone were more cytotoxic than NNA LEWAF; NNA and NNA+D LEWAF and dispersant increased ROS production and decreased intracellular calcein in hemocytes; NNA+D LEWAF and dispersant damaged plasma membrane and actin cytoskeleton and decreased phagocytosis. LEWAF exposure caused a decrease in hemocytes viability only at the highest dilution whereas NNA+D LEWAF and the dispersant alone were cytotoxic at the three highest concentrations. Temperature of production of NNA LEWAF, NNA+D LEWAF and dispersant did not influence their cytotoxicity to hemocytes. LEWAF increased ROS production and MXR transport activity in hemocytes. Exposure to NNA+D LEWAF and dispersant increased ROS production, provoked plasma membrane and actin cytoskeleton disruption and decreased phagocytic activity. In conclusion, the dispersant tested was toxic to mussel hemocytes and it greatly increased the toxicity of NNA+D LEWAF.

In vitro techniques with mussel hemocytes (Katsumiti et al., 2019) could represent an useful tool to rapidly screen the toxicity of oil compounds in the marine environment and to decipher mechanisms of toxicity at cellular level (Cajaraville et al., 1996). In addition, *in vitro* assays may also provide important data on the toxicity of oil dispersants thus supporting the selection of better oil spill remediation strategies.

Table 6. 4 hr LC50 values (MTT assays) obtained in mussel hemocytes exposed to NNA and NNA+D LEWAF produced at different temperatures and to the dispersant (Finasol OSR52) alone.

	10ºC	15ºC	20ºC
NNA LEWAF	>100%	>100%	>100%
NNA+D LEWAF	97.93%	>100	>100%
Finasol OSR52	265.58 mg/l	276.94 mg/l (4 hr)	232.74 mg/l

The oil dispersants Corexit 9500 and Corexit 9527, used on the Deepwater Horizon oil spill, caused cytotoxic effects, increased oxidative stress and genotoxicity in a variety of cell lines including sperm whale skin fibroblasts (Wise et al., 2014; Daussauze et al., 2015). To the best of our knowledge, cytotoxicity of the dispersant Finasol OSR52 has not been reported yet but *in vivo* studies have shown it to be more toxic than Corexit 9500 (Daussauze et al., 2015). In *M. galloprovincialis* exposed *in vivo* to Finasol OSR52, EC50 value was 13.4 mg/L (www.oilspillresponse.com). In the present study, Finasol OSR52 was toxic to mussel hemocytes after 4 h in vitro exposure (Table 6); cytotoxicity starting at a exposure concentration of 125 mg/L of dispersant. NNA+D LEWAF was cytotoxic to hemocytes in a dose dependent manner starting at a 25% dilution. Overall, in agreement with previous data

(Dussauze et al., 2015), the ranking of toxicity was: Finasol OSR52 > NNA+D LEWAF > NNA LEWAF.

LC50 values in hemocytes exposed to NAN+D LEWAF produced at 10ºC was lower than when exposed to LEWAF produced at higher temperatures (Table 6). These results are in agreement with chemical analysis that showed that concentrations of PAHs were between 3 to 12 times higher in NNA+D LEWAF compared to NNA LEWAF samples, thus explaining the higher toxicity of the former. A decrease in plasma membrane integrity was observed in hemocytes exposed to NNA+D LEWAF and the dispersant alone but not in NNA LEWAF exposures. The decrease in plasma membrane integrity may be due to the high affinity (lipophilicity) of surfactants present in dispersant formulation to lipid surfaces (e.g. plasma membrane), promoting lipid peroxidation and increasing ROS production (De Lorenzo et al., 2016). Indeed, ROS production increased in hemocytes exposed to LEWAF and to the dispersant alone, the effect being more marked in cells exposed to NNA+D LEWAF and dispersant alone. PAHs are known to enhance ROS generation in mussels in mussel hemocytes exposed *in vitro* (Gómez-Mendikute et al., 2002).

In mussels, phagocytosis in hemocytes represents the main cell-mediated immune response to pathogens and xenobiotics. Therefore, phagocytic activity has been used to assess immunocompetence of mussel hemocytes exposed to environmental pollutants (Cajaraville et al., 1996; Katsumiti et al., 2014, 2019). Presently, phagocytic activity decreased in hemocytes treated with NNA+D LEWAF and dispersant but not in those treated with NNA WAF, which again can be related to the lower levels fo PAHs in NNA WAF in comparison with the other treatments (Figure 4). Likewise, previous studies have shown that PAHs can damage hemocytes actin cytoskeleton (Gómez-Mendikute et al., 2002). Presently, also NNA+D LEWAF and the dispersant had significant effects on hemocytes actin cytoskeleton while no effect was found in cells treated with NNA LEWAF.

Taken together, results showed differences in the toxicity of NNA LEWAF, NNA+D LEWAF and dispersant alone to mussel hemocytes. These differences could be partly attributed to the different PAH concentrations in LEWAF samples and due to the inherent toxicity of the dispersant present in NAN+D LEWAF. NNA WAF provoked low cytotoxicity to hemocytes but elicited significant sublethal responses. NNA+D LEWAF and the dispersant showed a higher cytotoxicity than NNA LEWAF and both affected the same cellular processes with different magnitude of response. In all the treatments, increased ROS production seems to be a main driver of toxicity.

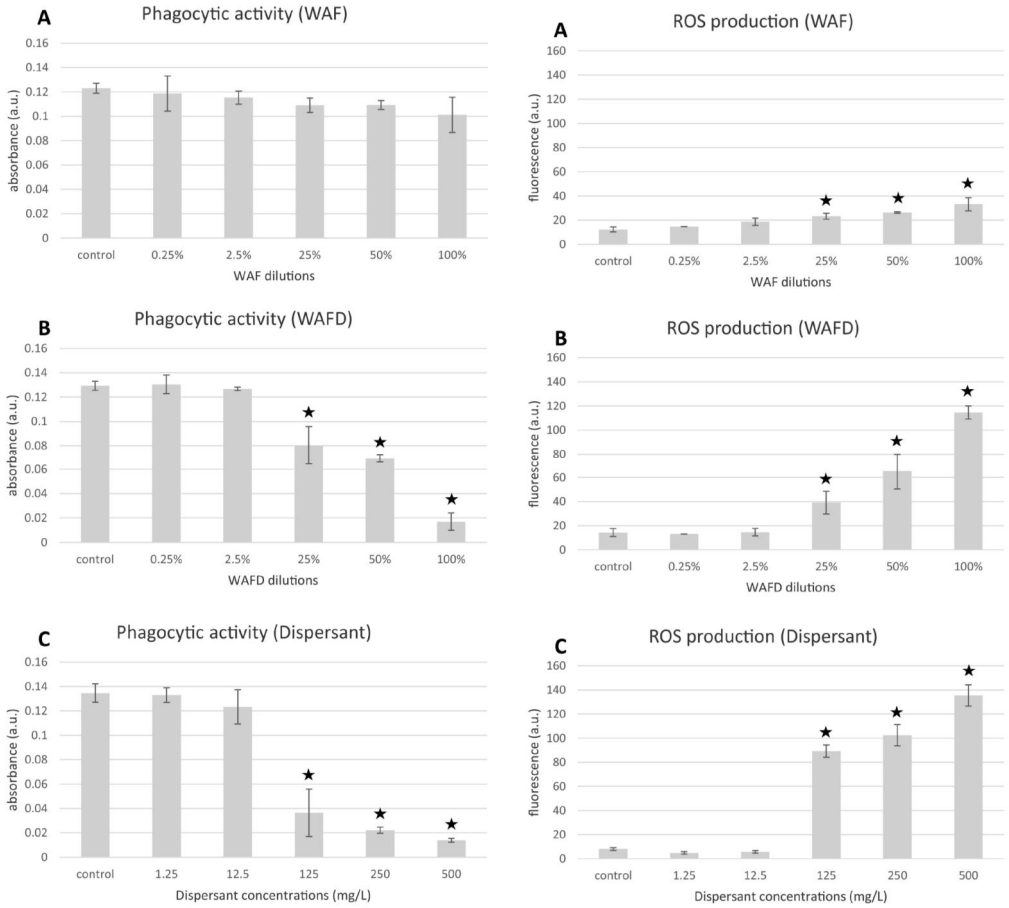


Figure 4. (left column) Phagocytic activity in mussel hemocytes exposed to NNA LEWAF (A), NND+D LEWAF (B) and dispersant (C) for 4 h. Data are given as absorbance arbitrary units (means ± SD). Stars indicate significant differences ($p<0.05$) in treated cells with respect to controls according to the Kruskal-Wallis test followed by Dunn's post hoc test. *n* = 6 replicates per treatment. Each exposure experiment was repeated three times.
(right column) ROS production in mussel hemocytes exposed to NNA LEAF (A), NNA+D LEWAF (B) and dispersant (C) for 4 h. Data are given as fluorescence arbitrary units (means ±SD). Stars indicate significant differences ($p<0.05$) in treated cells with respect tocontrols according to the Kruskal-Wallis test followed by Dunn's post hoc test. *n* = 6 replicates per treatment. Each exposure experiment was repeated three times.

Overall, *in vitro* tests with mussel hemocytes represent sensitive and fast tools for the evaluation of the toxic impact of oil spill and oil spill response and clearly evidence that the use of the dispersant Finasol OSR52 can pose clear risks for marine organisms such as mussels by causing severe alterations in their immune defence system. Likewise, we have obtained preliminary evidences indicating that NAN+D LEWAF would be more toxic when produced at low temperatures (10 ºC) than at higher ones (15-20 ºC), which might suppose an added risk for oils spills and oil spill responses in ice seas in the GRACE target study region.

3.3. Developmental assays with embryos of sea-urchins

Irrespective of the temperature of LEWAF production, LC50 values were higher (lower toxicity) upon exposure to oil LEWAF than to oil+D LEWAF, for all the oils tested; however these differed in toxicity, being NNA the least toxic and IFO180 the most toxic, with MGO inbetween, at all temperatures tested (Table 7). Dispersion (oil + dispersant) seemingly increases WAF toxicity, but the degree of effect varied with the temperature at which the WAFs were produced. Indeed, it was also found that Finasol OSR52 LEWAF can be toxic for larval early life stages

Regarding effects on larval growth, at 25ºC NNA LEWAF provoked less growth inhibition (reduced larvae length) than IFO or MGO LEWAF. Growth inhibition was more marked on oil+D LEWAF exposure than on oil LEWAF exposure for all the studied oils. Dispersion seemingly increases oil toxicity, but the degree of effect varied with the temperature at which the LEWAF was produced.; however, no differences in growth inhibition were found between crude oils alone or mixed with dispersant at other LEWAF preparation temperatures.

Table 7. EC_{50} (%) values on exposure to NNA, IFO 180 and MGO LEWAF and LEWAF+D (with Finasol OSR52) produced at different temperatures from 5 to 25ºC. (EC_{50} based on Probit analysis)

Temp.	NNA	NNA+D L	IFO180	IFO180+D	MGO	MGO+D	OSR52
5°C	67	70	29	11	30	18	13
10°C	76	41	39	10	53	34	6
15°C	86	38	25	10	51	16	9
20°C	63	33	43	25	59	57	22
25°C	86	84	58	29	57	36	19

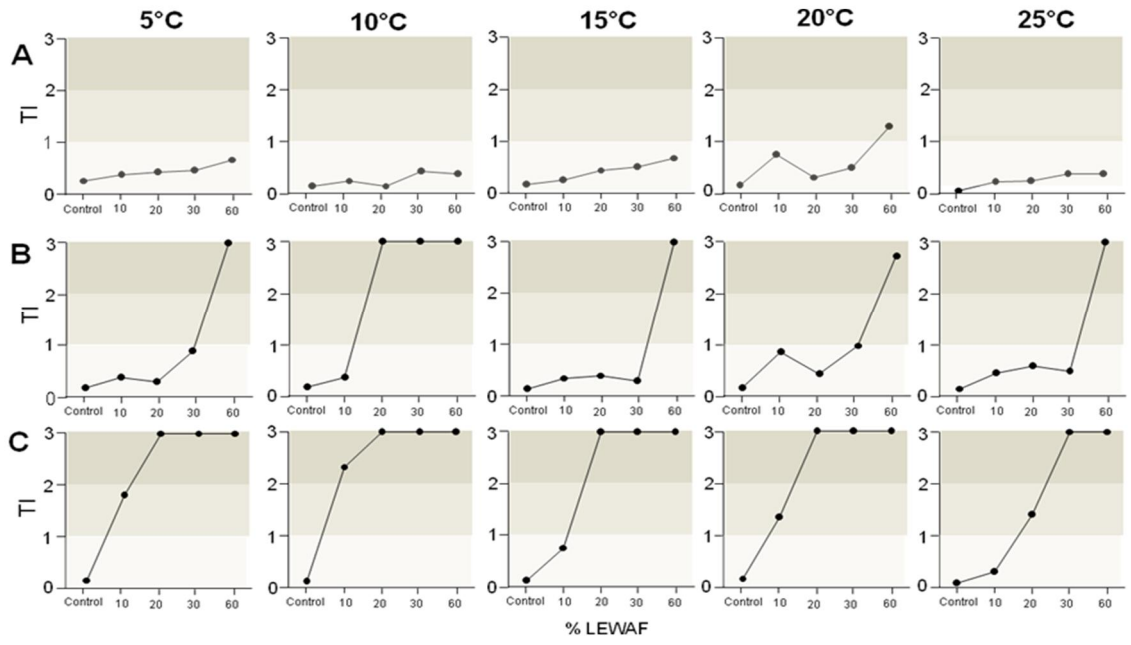


Figure 5. Toxicity Index (TI) at different concentrations (%) of exposure to NNA WAF (A), NNA+D WAF (B) and Finasol OSR52 WAF (C) produced at different temperatures (5-25ºC).

Regarding abnormalities, low TI values were found in sea urchin larvae exposed to NNA LEWAF and NNA+D LEWAF in comparison to IFO and MGO LEWAF and MGO+D LEWAF exposed ones (Figure 5). In addition, alterations in catalase activity (CAT) were observed at the lowest temperature (5 ºC) in NNA LEWAF and NNA+D LEWAF. In the case of IFO180, CAT was altered in all temperatures except at 25 ºC, suggesting that IOF180 causes oxidative stress in sea urchin larvae.

Overall, if the toxicity of the three types of oil is compared, it has been shown that toxicity decreases from MGO < IFO180 < NNA when sea urchin larvae are exposed to oil LEWAF or oil+D LEWAF. Oil spill toxicity clearly depends on the type of spilled oil, the weather conditions (e.g. seawater temperature) and the application of dispersants. Indeed, dispersants can exert toxic effects and also make oil chemical compounds more available to biota thus resulting in enhanced toxicity.

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

The acute toxicity of petroleum products towards zebrafish early life stages has already been addressed in deliverable D3.12 reporting on the adverse outcome links in zebrafish. D3.12 focuses on petroleum toxicity induced links of molecular/cellular events with adverse outcomes in zebrafish, for which the establishment of sublethal effects concentrations in zebrafish embryonic development from the acute fish embryo toxicity test is the critical first

step. However, as additional data on other oil types have been completed after D3.12 submission, contributing to a better understanding of oil type specific similarities and differences, the completed data are included in the present report. Furthermore, D3.12 aimed to understand and link the effects on different biological organization levels while the present report mainly focuses on the toxic impact on different study region-relevant and laboratory model species. Additionally, oil spill response actions such as chemical dispersion were investigated in detail for the present report. Hence, acute toxicity data in zebrafish early life stages are discussed from a different perspective.

As shown in Figure 6 a concentration-related increase in mortality was observed for all oil types exposure scenarios with concentration-response curves spanning a range between no effects and 100 % mortality determined for undiluted stocks (1:50). With the exception of the LEWAF IFO180 treatment all exposure scenarios resulted in high reproducible effects within the 3 independent experiments denoted as individual points in the panels. Based on the calculated LC50 values (Table 8), indicating WAF dilutions resulting in 50 % mortality, the individual petroleum products showed differences in their toxic potential. While the NNA crude oil was the less toxic (LC50 = 48 % of stock), the marine gas oil (36 %) was the most toxic closely followed by the intermediate fuel oil (36.8 %).

Table 8. Acute toxicity in zebrafish larvae (120 hpf) exposed to WAF dilutions of crude oil (NNA), refined petroleum products (MGO, IFO180) and dispersant (Finasol OSR 51). *Acute toxicity is expressed as WAF dilution inducing 50 % lethal effects determined according to OECD 236 (LC_{50}). LC_{50} values were calculated using the 4-parameter non-linear regression model in GraphPad Prism 6 out of 3 independent experiments.*

	LEWAF	CEWAF
NNA	48.0%	4.2%
IFO 180	36.0%	2.2%
MGO	36.8%	5.2%
Finasol OSR51	0.0035 mg/L	

The types of phenotypic adverse effects that have been induced by the different oil types were mainly consistent. All oil types induced malformations referred as blue-sac disease in the literature. Especially during the embryonic development, the cardiovascular system or the development of the craniofacial structures are affected at low aqueous concentration of PAHs originating from the crude oil (De Soysa et al. 2012, Perrichon et al. 2016). Most prominent, heart deformation and yolk sac or pericardial edema have been observed in all oil type exposure dilutions down to 16.7 % of stock (MGO LEWAF) or 0.78 % of stock (MGO CEWAF), respectively. Several spinal deformations occurred especially in MGO and IFO180 treated embryos. A delayed hatching was observed only in NNA exposed embryos (graphs for hatching success see D3.12).

Compared to LEWAF exposure, dispersed petroleum samples (CEWAFs) were extremely toxic to the early life stages of zebrafish with exposure concentrations around 2 -5 % of stock still inducing 50 % mortality (LC50, Table 8). The role of dispersant in CEWAF toxicity is controversially discussed in the scientific community. Hence, additional experiments with the dispersant Finasol OSR 51 using stock solutions of corresponding dispersant amounts to CEWAF stock preparation have been performed. Based on the calculated LC50 value (7 % HEWAF), the dispersant contributes to the toxicity of NNA CEWAF. However, it cannot exclusively explain the observed high toxicity of CEWAF as the dispersant induced toxicity in zebrafish embryos was below CEWAF induced toxicity. Hence, a combination of the dispersant toxicity and a higher availability of crude oil compounds in the exposure solutions due to dispersion effects of kinetically partitioning is most likely to be responsible for the high CEWAF toxicity. Furthermore, also a higher availability of oil droplets has to be considered as additional route of exposure.

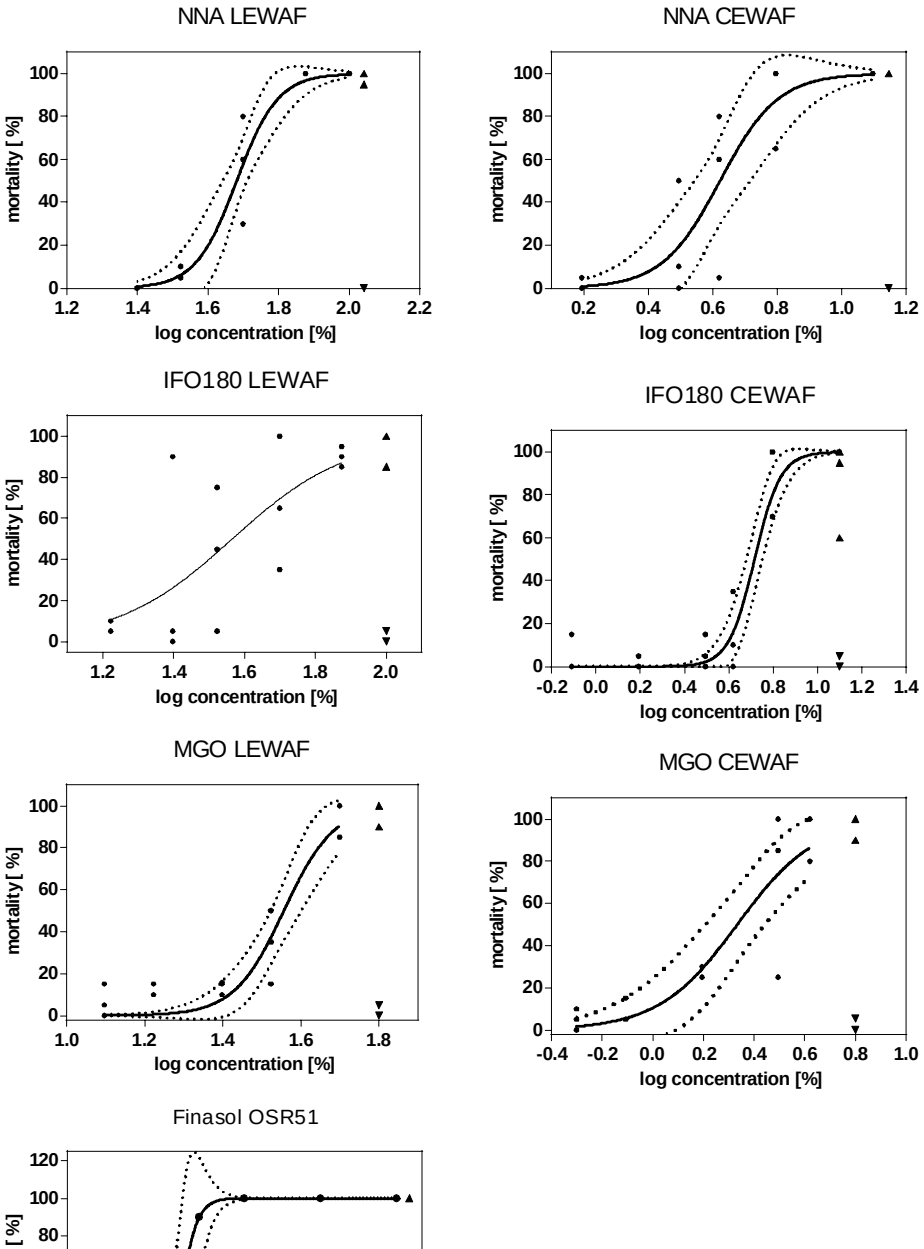


Figure 6. Mortality of zebrafish larvae (120 hpf) exposed to WAF dilutions of crude oil (NNA), refined petroleum products (MGO, IFO180) and dispersant (Finasol OSR 51). Exposure solutions of low energy water-accommodated fractions (LEWAF, oil only) and chemically-enhanced water-accommodated fractions (CEWAF, oil + dispersant Finasol OSR 51 (DOR:1:10 (w/w)) have been prepared form stocks of 1:50 (w/v) and 1:200 (w/v), respectively. Dispersant exposure concentrations are expressed corresponding to proportion used for CEWAF preparation. Points denote the mortality of chronically exposed embryos (each point: 1 out of 3 independent replicates). Triangles show the negative (artificial medium, pointing downwards) and positive controls (3,4-dichloraniline). Sigmoidal concentration-response curves were added in GraphPad Prism 6 using the 4-parameter non-linear regression model with top and bottom variables set to 100 and 0, respectively. Dotted lines indicate 95 % confidence band (Equation: Y= 1/(1+10^((LogEC50-X)*HillSlope).

However, it has to be considered that Finasol OSR 51 has only been used in the combination with the NNA crude oil while the other petroleum products have been combined with the dispersant Finasol OSR 52. Even though previous studies at RWTH Aachen University (unpublished data) indicated similar biological effects of both third generation dispersants divergent toxic responses in zebrafish embryos cannot be excluded as the composition in respect to compounds and percentage portion of these complex mixtures are different (see deliverable D3.12 GRACE).

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. 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 unaffected embryos died within the following 24 h of exposure (Figure 6). 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. 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.

From the present study it can be concluded that dispersed NNA (as CEWAF) was far more toxic than untreated NNA (NNA LEWAF). In accordance with other experiments conducted in the GRACE project, a 1:10 dispersant:oil ratio (DOR) was used. However, 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. In general, the higher toxicity of CEWAF compared to LEWAF 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 LEWAF 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 (Couillard et al., 2005): e.g. Experiment 1: 20 % mortality; Experiment 2: 90 % mortality for the highest CEWAF test concentration. 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 (Dussauze et al. 2015).

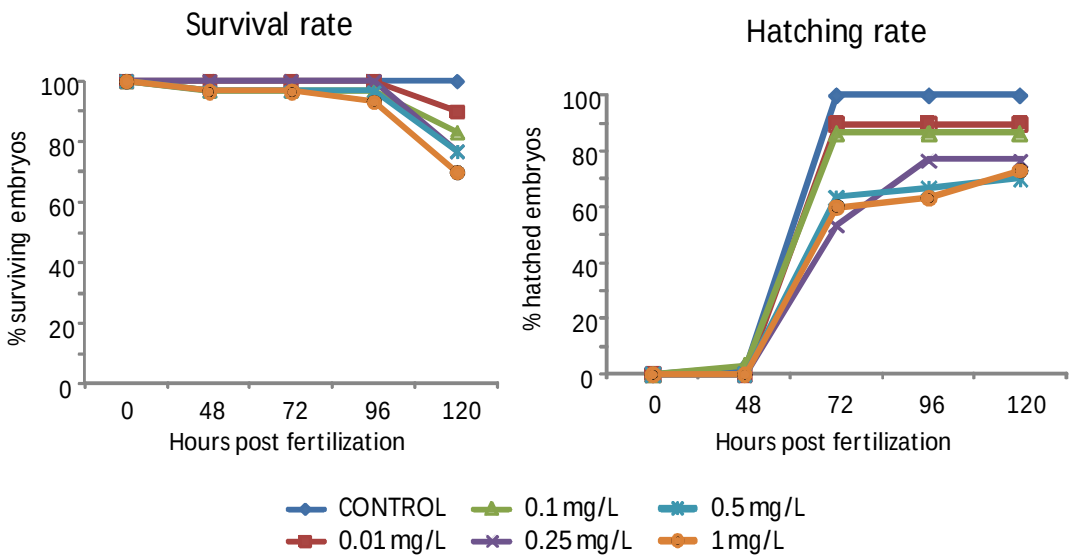
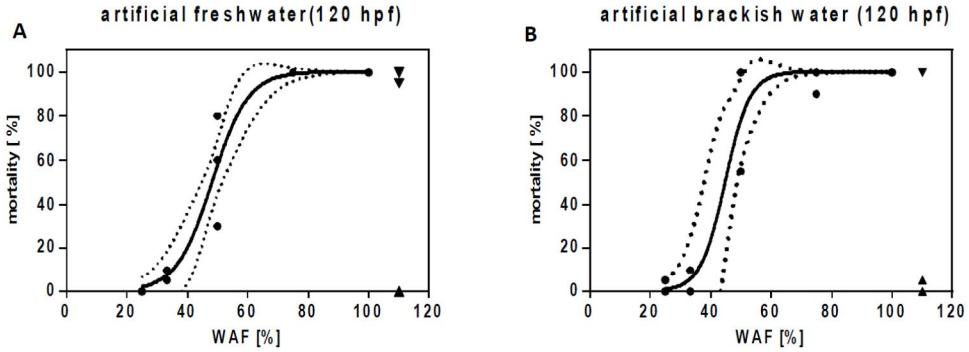


Figure 7. Survival and harching rate of zebrafish embryos exposed to Finasol OSR 52.

A second set of experiments dealt with exposure to NNA+D LEWAF (instead of CEWAF) in comparison with NNA LEWAF and used FInasol OSR52). It is worth noting that exposure of zebrafish embryos to the dispersant Finasol OSR52 only caused 100 % mortality at concentrations as high as ≥ 500 mg/L. However, it caused an increase in the prevalence of malformations at concentrations as low as 0.01 mg/L and decreased hatching rate at around 0.25 mg/L (Fig. 7). Direct waterborne exposure to NNS LEWAF and NNS+D LEWAF (produced in embryo medium) resulted in a greater embryo mortality than the exposure through PDMS sheets. Regarding sublethal toxicity, significant differences were observed in hatching rate and in the prevalence of malformations of embryos exposed to NNS LEWAF and NNS+D LEWAF, irrespective of the temperature at which these LEWAFs were produced. In contrast, no clear differences were observed in relation with the temperature of LEWAF preparation. Overall, greater sublethal effects were observed in the case of embryos exposed to NNS+D LEWAF than to NNS LEWAF.



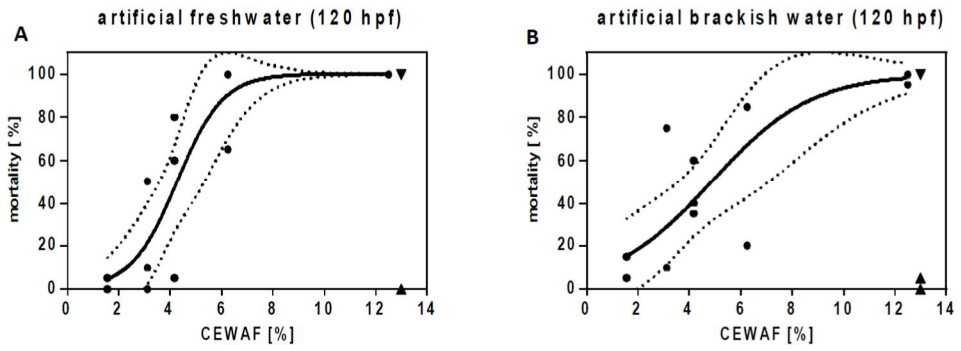


Figure 8. Mortality of zebrafish larvae (120 hpf) exposed to NNA LEWAF (top graphs), NNA CEWAF (middel garphs) and Finasol OSR 51 (bottom graphs) at two different medium salinities. Panel A shows the larval mortality induced by test chemicals prepared in artificial freshwater medium (0.5 ‰), while panel B shows larval mortality induced by chemical 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.

Finally, salinity changes can be a major driver of the toxic impact of oil spills and oils spill response in ice seas, especially in the Arctic ocean and the Baltic Sea. 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 at two different salinities (freshwater and brackish water), At both salinities concentration-dependent increase of mortality with increasing LEWAF concentration was observed (Figure 8). 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. Furthermore, morphological effects and altered locomotor behaviour were also observed, the toxic effects largely varying depending on the salinity (deliverables D1.4 and D3.2 of the GRACE project; WP1 and WP3).

It can be concluded that zebrafish embryos appear to be a suitable model test organism to study the toxic impact of WAF and how this toxicity is influenced by the temperature and by the application of chemical dispersants.

3.5. Survival and behavioral assays with copepods

96 hours LC50 results showed that MGO LEWAF was the most toxic of the three types of WAF, followed by IFO 180 , whereas NNA was the least toxic of the three test oils (Table 9). Hansen et al (2013) exposed copepods *Calanus finmarchicus* and *Calanus glacialis to*

marine diesel WAF and suggested that toxicity depended on species specific lipid content and size. *Acartia tonsa* is much smaller in size and has less lipid content than *Calanus* and therefore its toxicological response to LEWAF exposure is more rapid (related aspects were presented and discussed in deliverable D3.14 GRACE).

Table 9: 48, 72 and 96 h LC50 of the adult *Acartia* on exposure to NNA, IFO 180 and MGO LEWAF and NNA+D LEWAF (with Finasol OSR52) produced at 10ºC. (LC_{50} based on Probit analysis)

PAH	48hr LC50	72hr LC50	96hr LC50
NNA LEWAF	>100%	>100%	>100%
NNA+D LEWAF	34%	21%	21%
IFO 180	70.21%	44.8%	34%
MGO	34%	10%	8%

Sublethal concentrations were used to determine the effects of exposure on reproductive outputs. Adult females were exposed to LEWAF for 7 d followed by a recovery period of 4 d, then the surviving females were individually assessed for reproductive outputs. 96-h LC50 results assisted in determination the sublethal concentrations to be used for fecundity and egg hatching assays. For the NNA LEWAF reproductive assay 10 %, 30 % and 50 % LEWAF, for IFO 180 LEWAF and MGO LEWAF 10 %, 25 % and 40 % LEWAF, and 1 %, 5 % and 7 % LEWAF, respectively.

After recovery period ended, adult females were individually placed in incubation chambers for 3 d to check the female reproductive capability and the individual egg production rates. Females produced 2 and more eggs during the 3 d experimentation period were considered reproductive females. The most remarkable effects were recorded on exposure to IFO 180 and MGO LEWAF (Figure 9). Likewise, lipid storage in females was seemingly only compromised on exposure to MGO LEWAF, with significant effects at a exposure so low as 7 % MGO LEWAF.

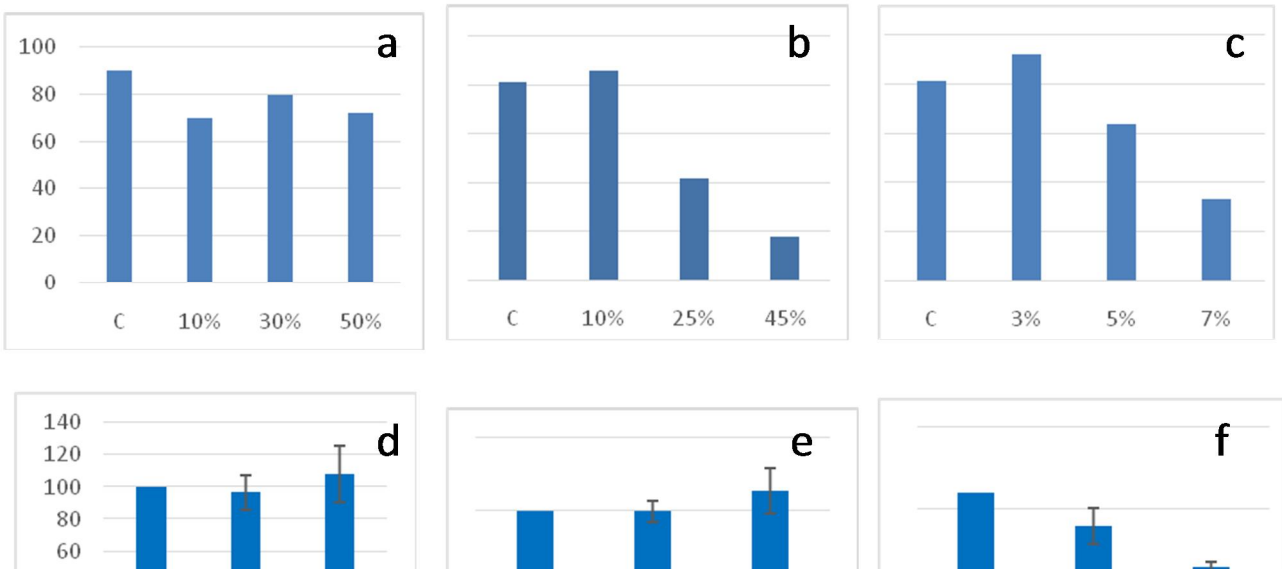


Figure 9: The Percentage of egg producing females exposed to a) NNA LEWAF, b) IFO 180 LEWAF, c) MGO LEWAF. Percentage of fluorescence intensity compared to control (100%) after 96 h exposure in d) NNA LEWAF, e) IFO 180 LEWAF, f) MGO LEWAF. Error bars denote Standard error

3.6. Lethality test with adult stickleback fish, *Gasterosteus aculeatus*

Exposure of adult stickleback to 100 % NNA+D LEWAF and equivalent concentrations of Finasol OSR52 caused death within 2-4 hr whereas survival on exposure to 100 % NNA LEWAF was 100 % after 24 h exposure. Based on these trials the maximum experimental concentration for the AOL experiment was arbitrarily decided to be 25 % LEWAF (both NAN and NAN+D). Further on, after 7 d exposure the mortality was irrelevant both upon NNA LEWAF exposure and upon NNA+D LEWAF exposure. However, sublethal effects are being recorded (currently in progress).

4. Concluding remarks

The relative composition of PAHs in WAFs was different in the 3 tested oils (NNA, IFO 180 and MGO), the amount and stability of total aromatic hydrocarbons (toluene equivalents) largely varying depending on the preparation method (LEWAF, CEWAF, HEWAF of oil or of oil+D) and conditions (e.g. temperature for WAF preparation). Thus, total aromatic hydrocarbons were higher in IFO 180 LEWAF than in NNA and MGO LEWAFs (except at high preparation temperatures: 25 ºC) and in MGO+D LEWAF than in NNA and IFO180 LEWAFs (at 5-15 ºC). Overall, the concentration of total PAHs in seawater was higher in oil+D LEWAF compared to oil LEWAF and at low seawater temperature (10 ºC) compared to higher ones (15 ºC and higher); thus, whereas for a given oil the PAH concentrations in oil LEWAF produced at 10-20 ºC were similar, the PAH concentrations in oil+D LEWAF increased with decreasing temperatures.

Overall, if the toxic impact of the three types of oil (NNA, IFO 180, MGO) is compared it can be concluded that toxicity (both lethal and sublethal and within a multiple test species context) decreases from MGO>IFO180>NNA. Moreover, the results indicate that the toxicity

of oil+D LEWAF and dispersant alone (or as HEWAF) is higher than the toxicity of oil LEWAF, also for diverse endpoints and species. These different toxic impact could be partly attributed to the different PAH concentrations in LEWAF samples and partly attributed to the inherent toxicity of the dispersant present in oil+D LEWAF. In any case, the present results clearly evidence that the use of the third generation dispersants in ice seas could pose a clear risk for the marine organisms that should not be neglected to make decisions within the framework of an oil spill response[5].

Likewise, in agreement with the aforementioned chemical results, it seems that oil+D LEWAF would be more toxic when produced at low temperatures (10 ºC) than at higher ones (15-20 ºC). This last output is relevant because it implies that the use of toxicity data obtained for temperate and subtropical environments would underestimate the risk of toxic impact of oils spills and oil spill responses in the Arctic ocean and the North Sea and the Baltic Sea. Likewise, salinity can be a major driver of the toxic impact of oil spills and oils spill response, especially in the Baltic Sea conditions; both lethal and sublethal toxic effects of oil LEWAF, CEWAF and HWAF largely varying depending on the salinity.

Finally, we can conclude that *in vitro* toxicity tests with mussel hemocytes and microscale toxicity tests with copepods, and sea urchin and zebrafish embryos can provide us with a sensitive, fast and reliable toolbox for assessing the toxic impact of oil spills and oil spill responses. Nevertheless, great research efforts are still required before an effective battery of toxicity tests can be selected and optimized to be regularly applied for risk and impact assessment of oil spills and oil spill responses in the Arctic ocean and the North Sea and the Baltic Sea.

5. References

Aurand D, Coelho G. 2005. Cooperative aquatic toxicity testing of dispersed oil and the "Chemical Response to Oil Spills: Ecological Effects Research Forum" (CROSERF). Ecosystem Management and Associates Inc., Lusby, MD. Tech Rpt 07-03, 105 pp + Appendices.

Avila, T.R., Bersano, J.G.F., Fillmann, G. 2010. Lethal and sub-lethal effects of the water-soluble fraction of a light crude oil on the planktonic copepod *Acartia tonsa*. J. Braz. Soc. Ecotoxicol., 5, 19-25.

Beiras, R., Saco-Alvarez, L. 2006. Toxicity of seawater and sand affected by the Prestige fuel-oil spill using bivalve and sea urchin embryogenesis bioassays. Wat. Air Soil Pollut., 177: 457–466.

[5] NOTE: We must be cautious when using results of toxicity tests to extrapolate to consequences in the field (especially when comparing chemically dispersed *vs.* naturally dispersed oil and using non-native species tested individually under optimal lab conditions). Therefore, it is worth mentioning that any recommendation for or against using dispersants must reflect the high degree of uncertainty and the limitations of the methodological approach and of the current state of the art regarding our knowledge of the marine environment in the Arctic ocean, the North sea and the Baltic sea.

Cajaraville, M.P., Olabarrieta, I., Marigómez, I. 1996. In vitro activities in mussel hemocytes as biomarkers of environmental quality: a case study in the Abra estuary (Biscay Bay). Ecotoxicol. Environ. Saf. 35, 253–260.

Carballeira, C., Ramos-Gomez, J., Martın-Dıaz, L., DelValls TA. 2012. Identification of specific malformations of sea urchin larvae for toxicity assessment: application to marine pisciculture effluents. Mar. Envir. Res. 77, 12-22.

Chapman, H., Purnell, K., Law, R.J., Kirby, M.F. 2007. The use of chemical dispersants to combat oil spills at sea: a review of practice and research needs in Europe. Mar. Pollut. Bull., 54, 827-838

Chazotte, B. 1998. Nonimmunological fluorescent labelling of cellular structures. S. Schaefer (Ed.), Cells: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York.

Couillard, C. M., Lee, K., Légaré, B. & King, T. L. (2005). Effect of dispersant on the composition of the water-accommodated fraction of crude oil and its toxicity to larval marine fish. Environ. Toxicol. Chem., 24, 1496-1504.

DeLorenzo, M.E., Eckmann, C.A., Chung, K.W., Key, P.B., Fulton, M.H. 2016. Effects of salinity on oil dispersant toxicity in the grass shrimp, *Palaemonetes pugio*. Ecotoxicol. Environ. Saf. 134, 256–263.

de Soysa TY, Ulrich A, Friedrich T, Pite D, Compton SL, Ok D, et al. Macondo crude oil from the Deepwater Horizon oil spill disrupts specific developmental processes during zebrafish embryogenesis. BMC Biol. 2012;10:40.

Dupuis A, Ucán-Marín F. A literature review on the aquatic toxicology of petroleum oil: an overview of oil properties and effects to aquatic biota. DFO Can Sci Advis Sec Res Doc. 2015;007:52.

Dussauze, M., Pichavant-Rafini, K., Le Floch, S., Lemaire, P. and Theron, M. (2015) Acute toxicity of chemically and mechanically dispersed crude oil to juvenile sea bass (*Dicentrarchus labrax*): Absence of synergistic effects between oil and dispersants. Environ. Toxicol. Chem., 34, 1543-1551.

Gómez-Mendikute, A., Etxeberria, A., Olabarrieta, I., Cajaraville, M.P. 2002. Oxygen radical production and actin filament disruption in bivalve haemocytes treated with benzo[a]pyrene. Mar. Environ. Res. 54, 431–436.

González-Doncel, M., González, L., Fernández-Torija, C., Navas, J.M:, Tarazona, J.V. 2008. Toxic effects of an oil spill on fish early life stages may not be exclusively associated to PAHs: studies with Prestige oil and medaka (Oryzias latipes). Aquat. Toxicol., 87, 280-288

Gorbi,G., Invidia, M., Savorelli, F., Faraponova, O., Giacco, E., Cigar, M., Buttino, I., Leoni, T., Prato, E., Lacchetti, I., Sei, S. 2012. Standardized methods for acute and semichronic toxicity tests with the copepod *Acartia tonsa*. Environ. Toxicol. Chem. 31, 2023–2028.

Hamers T, Legler J, Blaha L, Hylland K, Marigomez I, Schipper CA, Segner H, Vethaak AD, Witters H, De Zwart D, Leonards P. 2013. Expert opinion on toxicity profiling–report from a NORMAN expert group meeting Integ Environ. Assess. Mngmnt., 9: 185-191.

Hansen, B. H., Altin, D., Rørvik, S. F., Øverjordet, I. B., Olsen, A. J., & Nordtug, T. (2011). Comparative study on acute effects of water accommodated fractions of an artificially weathered crude oil on *Calanus finmarchicus* and *Calanus glacialis* (Crustacea: Copepoda). Sci. Total Environ., 409, 704–709.

Hansen, B. H., Altin, D., Olsen, A. J., & Nordtug, T. (2012). Acute toxicity of naturally and chemically dispersed oil on the filter-feeding copepod *Calanus finmarchicus*. Ecotox. Environ. Safety, 86, 38–46.

Hansen, B. H., Altin, D., Øverjordet, I. B., Jager, T., & Nordtug, T. (2013). Acute exposure of water soluble fractions of marine diesel on Arctic *Calanus glacialis* and boreal *Calanus finmarchicus*: Effects on survival and biomarker response. Scie. Total Environ., 449, 276–284

Holste, L., Peck, M.A. 2006. The effects of temperature and salinity on egg production and hatching success of Baltic *Acartia tonsa* (Copepoda: Calanoida): A laboratory investigation. Mar. Biol. 148: 341–350.

Katsumiti, A., Gilliland, D., Arostegui, I., Cajaraville, M.P. 2014. Cytotoxicity and cellular mechanisms involved in the toxicity of CdS quantum dots in hemocytes and gill cells of the mussel *Mytilus galloprovincialis*. Aquat. Toxicol. 153, 39–52.

Katsumiti, A., Nicolussi, G., Bilbao, D., Prieto, A., Etxebarria, N., Cajaraville, M.P. 2019. *In vitro* toxicity testing in hemocytes of the marine mussel *Mytilus galloprovincialis* (L.) to uncover mechanisms of action of the water accommodated fraction (WAF) of a naphthenic North Sea crude oil without and with dispersant. Sci. Total Environ., 670: 1084-1094.

Lammel, T., Boisseaux, P-. Fernandez-Cruz, M.L., Navas, J.M. 2013. Internalization and cytotoxicity of graphene oxide and carboxyl graphene nanoplatelets in the human hepatocellular carcinoma cell line Hep G2. Part. Fibre Toxicol., 10, p. 27

Lee, K., Boufadel, M., Chen, B., Foght, J., Hodson, P., Swanson, S., Venosa, A. 2015. Expert Panel Report on the Behaviour and Environmental Impacts of Crude Oil Released into Aqueous Environments. Royal Society of Canada, Ottawa, ON. ISBN: 978-1-928140-02-3

Neff, J.M., Ostazeski, S., Gardiner, W., Stejskal, I., 2000.Effects of weathering on the toxicity of three offshore Australian crude oils and a diesel fuel to marine animals. Environ. Toxicol. Chem. 19, 1809–1821.

OECD. 2013. Test No. 236: Fish Embryo Acute Toxicity (FET) Test, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264203709-en.

Perrichon, P., Le Menach, K., Akcha, F., Cachot, J., Budzinski, H. and Bustamante, P. (2016) Toxicity assessment of water-accommodated fractions from two different oils using a zebrafish (*Danio rerio*) embryo-larval bioassay with a multilevel approach. Sci. Total Environ., 568, 952-966.

Pipe, R.K., Coles, J.A., Farley, S.R.1995. Assays for measuring immune response in the mussel Mytilus edulis. Tech. Fish Immunol., 4, 93-100

Prieto, A., Zuloaga, O., Usobiaga, A., Etxebarria, N., Fernández, L.A. 2007. Development of a stir bar sorptive extraction and thermal desorption–gas chromatography–mass spectrometry method for the simultaneous determination of several persistent organic pollutants in water samples. J. Chromatogr. A. 1174, 40–49.

Ramachandran SD, Sweezey MJ, Hodson PV, Boudreau M, Courtenay SC, Lee K, et al. 2006. Influence of salinity and fish species on PAH uptake from dispersed crude oil. Mar Pollut Bull, 52:1182–9.

Rial, D., Vazquez, J.A:, Murado, M.A. 2014. Toxicity of spill-treating agents and oil to sea urchin embryos Sci. Total Environ., 472, 302-308

Rodrigues, R.V., Miranda-Filho, K.C.,Gusmão, E.P:, Moreira, C.B:, Romano, L.A., Sampaio, L.A. 2010. Deleterious effects of water-soluble fraction of petroleum, diesel and gasoline on marine pejerrey Odontesthes argentinensis larvae. Sci. Total Environ., 408, 2054-2059

Singer MM, Aurand D, Bragin GE, Clark JR, Coelho GM, Sowby ML, Tjeerdema R. 2000. Standardization of the preparation and quantitation of water-accommodated fractions of petroleum for toxicity testing. Mar Pollut Bull, 40: 1007-1016.

Strähle, U., Scholz, S., Geisler, R., Greiner, P., Hollert, H., Rastegar, S., Schumacher, A., Selderslaghs, I., Weiss, C. and Witters, H. (2012) Zebrafish embryos as an alternative to animal experiments—a commentary on the definition of the onset of protected life stages in animal welfare regulations. Reprod. Toxicol. 33, 128-132.

Tingaud-Sequeira, A, Ouadah, N And Babin, PJ. 2011. Zebrafish obesogenic test: a tool for screening molecules that target adiposity. J Lipid Res 52: 1765-1772.

Wise, C.F., Wise, J.T.F., Wise, S.S., Thompson W.D., Wise Jr., J.P., Wise Sr. J.P. 2014. Chemical dispersants used in the Gulf of Mexico oil crisis are cytotoxic and genotoxic to sperm whale skin cells. Aquat. Toxicol. 152, 335–340.

Wu, D., Wang, Z., Hollebone, B., McIntosh, S., King, T. and Hodson, P.V. (2012) Comparative toxicity of four chemically dispersed and undispersed crude oils to rainbow trout embryos. Environ.Toxicol. Chem. 31, 754-765.

6. Appendix: Toxicity database outline

Gross estimates (preliminary approach) of critical toxicity values obtained after in vitro toxicity tests with mussel hemocytes and microscale toxicity tests with sea urchin, zebrafish embryos and copepods.

LC50 or EC50 (%WAF) * mg dispersant/L		IN VITRO TESTS WITH MUSSEL HEMOCYTES		SEA URCHIN EMBRYO TESTS		ZEBRAFISH EMBRYO TESTS				COPEPOD TESTS		
		Survival (MTT)	Phagoc. Activity	Viability	Toxicity Index	Acute Toxicity 120 hpf - PDMS in SW	Hatching rate - PDMS in SW	Acute Toxicity 120 hpf - Artificial FW	Acute Toxicity 120 hpf - Artificial BW	Acute Toxicity 96 h	Reproductive Capability	Lipid Storage in Females
5ºC	NNA LEWAF			67	>60	100	100					
	NNA+D LEWAF			70	<40	93	93					
	IFO LEWAF			28								
	IFO+D LEWAF			11								
	MGO LEWAF			30								
	MGO+D LEWAF			18								
	Finasol OSR2 *			13	<15							
10ºC	NNA LEWAF	>100	>100	76	>60	97	97	>50	>40	>100	>50	>50
	NNA+D LEWAF	98	<50	41	<15	80	80			21	>15	>15
	NNA CEWAF							>4	>5			
	IFO LEWAF			39				>40		34	>15	>40
	IFO+D LEWAF			10				>4				
	MGO LEWAF			53				>30		8	>5	>5
	MGO+D LEWAF			34				>2				
	Finasol OSR1 *							>0.004	>0.015			
	Finasol OSR2 *	266	<50	6	<5							
15ºC	NNA LEWAF	>100		86	>60	97	97					
	NNA+D LEWAF	>100		38	<50	87	97					
	IFO LEWAF			25								
	IFO+D LEWAF			10								
	MGO LEWAF			51								
	MGO+D LEWAF			16								
	Finasol OSR2 *	277		9	<15							
20ºC	NNA LEWAF	>100		63	>60	97	97					
	NNA+D LEWAF	>100		33	<50	77	77					
	IFO LEWAF			43								
	IFO+D LEWAF			25								
	MGO LEWAF			59								
	MGO+D LEWAF			57								
	Finasol OSR2 *	232		22	<15							
28ºC	NNA LEWAF			86	>60	90	90	>100				
	NNA+D LEWAF			84	<50	97	97	45				
	IFO LEWAF			58								
	IFO+D LEWAF			29								
	MGO LEWAF			57								
	MGO+D LEWAF			36								
	Finasol OSR2 *			19	<25	37	77	16				

NOTE: For the tests using PDMS incubated in NNA LEWAF and NNA+D LEWAF produced in artificial sea water at different temperatures statistical significan differenceS in zebrafish embryo survival respect to the control group only at NNA+D LEWAF producet at 15ºC and 20ºC