Influence of dispersant application on the toxicity to sea urchin embryos of crude and bunker oils representative of prospective oil spill threats in Arctic and Sub-Arctic seas

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ABSTRACT

This study deals with the toxicity assessment of crude and bunker oils representative of prospective oil spill threats in Arctic and Sub-Arctic seas (NNA: Naphthenic North-Atlantic crude oil; MGO: Marine Gas Oil; IFO: Intermediate Fuel Oil 180), alone or in combination with a third-generation dispersant (Finasol OSR52®). Early life stages of sea urchin, Paracentrotus lividus, were selected for toxicity testing of oil low-energy water accommodated fractions. A multi-index approach, including larval size increase and malformation, and developmental disruption as endpoints, was sensitive to discriminate from slight to severe toxicity caused by the tested aqueous fractions. IFO (heavy bunker oil) was more toxic than NNA (light crude oil), with MGO (light bunker oil) in between. The dispersant was toxic and further on it enhanced oil toxicity. Toxic units revealed that identified PAHs were not the main cause for toxicity, most likely exerted by individual or combined toxic action of non-measured compounds.

1. Introduction

Maritime traffic and oil platforms are potential sources of oil pollution of growing interest in the North Atlantic and the Baltic Sea, where oil production and transportation are blossoming economic activities (OSPAR, 2009; HELCOM, 2010). Moreover, climate change driven unparallelled ice retreat is giving rise to new maritime trade routes through the Arctic region, thus increasing the threat for large oil spills in cold seas (Yang et al., 2018). Naphthenic North Atlantic crude oil (NNA) is a regionally relevant light crude oil extracted in the North Sea and largely used worldwide for industrial applications and gasoline production. Likewise, some bunker oils are of special interest in the region: marine gas oil (MGO) is a light distillate used in fishing boats, ferries or tugs and intermediate fuel oil IFO 180 (IFO) is a heavy fuel increasingly being used in shipping vessels and tankers. These three petroleum compounds could be considered as ad hoc research cases representative of potential threats regarding marine oil spills in the North Atlantic, the Baltic Sea and the Arctic region.

Preparedness to respond to oil spills is crucial to counteract their environmental and socio-economic impact. Oil spill response includes a variety of alternative measures and actions for the removal and cleaning up of the spilled oil and its oiled residues including mechanical recovery (manually or using, skimmers, mechanical barriers or sorbents), in-situ burning, chemical dispersion, bioremediation and natural attenuation (Chen et al., 2019). The biological impact of the oil spill and the efficiency of the oil spill response vary depending on prevailing environmental conditions during the spill, properties of the spilled oil, accessibility, preparedness and socio-economic impact, amongst other factors (Beyer et al., 2016; Wenning et al., 2018). Mechanical recovery and in-situ burning can be hampered by remoteness.
and extreme weather conditions in oil spills that might occur in boreal iced seas, and bioremediation and natural attenuation can be too slow at extreme low temperatures; thus, the use of dispersants has been suggested as a useful alternative solution (Prince et al., 2016).

Chemical dispersants contain surfactants that allow breaking down the oil slick into small droplets that enter the water column to be removed through biodegradation, thus facilitating the removal of hydrocarbons from seawater at shorter times than in the case of non-dispersed oil (Prince et al., 2016). However, this action leads, at least transiently, to increased concentration and bioavailability of waterborne hydrocarbons, which could enhance the toxic effects of the oil spill (Ramachandran et al., 2004; Schein et al., 2009; Rico-Martínez et al., 2013; Dussauze et al., 2015; Alexander et al., 2017; Li et al., 2018; Echols et al., 2019). Moreover, dispersants are made of a mixture of chemicals that can be toxic as well, although earlier formulations were replaced by newly developed third generation concentrates, claimed to be less toxic (Epstein et al., 2005; George-Ares and Clark, 2006; Alameda et al., 2014; Dussauze et al., 2015). Finasol OSR52® (Total Special Fluids, France) is a third-generation concentrate marine dispersant designed to treat spills in salt water, which has been recommended for Arctic environments (Steffek et al., 2016). Based on dilutions of the dispersant in culture media, in vitro studies showed that Finasol OSR52® is toxic to zebrafish embryos (Johann et al., 2020b) and mussel haemocytes (Katsumiti et al., 2019) but little is known about its toxicity to marine organisms upon waterborne exposure (Wise and Wise, 2011). Exceptionally, Finasol OSR52® was found to be very toxic for juvenile sea bass, Dicentrarchus labrax (Dussauze et al., 2015). Other dispersants of the Finasol family, such as Finasol OSR51®, have been shown to be toxic to sea urchin embryos; indeed, the dispersant and the chemically dispersed oil were more toxic than the oil water accommodated fraction (Rial et al., 2014).

Sea urchins are ecologically relevant marine organisms with planktonic and benthic life stages, frequently used as model organism in developmental and cancer research (Ernst, 2011; McClay, 2011; Buckley and Rast, 2012), regulatory toxicity testing (USEPA, 2002; Standard Canada, 2011; ASTM, 2012), and environmental impact and risk assessment (Beiras et al., 2003, 2012; Saco-Alvarez et al., 2008; Carballeira et al., 2012). Toxicity testing using early life stages (e.g., embryos and larvae) of sea urchin is a sensitive and low-cost tool to evaluate the toxicity of petroleum compounds and hydrocarbons (Bellas et al., 2008, 2013; Saco-Alvarez et al., 2008; Beiras et al., 2012). The so-called “sea urchin embryo toxicity test” (SET test) provides a general view of the toxicity exerted by chemicals bioavailable in seawater by quantifying (in terms of pluteus larvae size: ΔL) the degree of developmental completion achieved by the embryos (Beiras et al., 2012). The toxicity index (TI) is calculated after scoring the frequencies of target abnormalities recorded at the pluteus larva stage (Carballeira et al., 2012). Skeletogenesis plays a relevant role in long-term maintenance (e.g. g. body density, sinking rate and orientation of plutei) and development of larval forms (Pennington and Strathmann, 1990). Other tests deal with the evaluation of toxic effects in early developmental stages such as the first cleavage 90-min post-fertilisation (Kobayashi, 1990) or the gastrula stage 24-h post-fertilisation (Morroni et al., 2009), which provides toxicologically relevant data. The sea urchin developmental disruption (SED) assay merges both the ecological and toxicological perspectives. The SEDD assay includes the calculation of various indices. The inhibition of pluteus larvae formation index (IPLFD) reveals the competence of pluteus larvae to successfully settle and progress to become a benthic adult. Regarding its ecological significance, it is comparable to the inhibition of fruiting formation index proposed for soil toxicity testing using the slime mould Dystostelium discoides as test organism (Balbo and Bozzaro, 2008). In addition, as stated for the D. discoides approach (Rodriquez-Ruiz et al., 2013), clues about possible modes of action of the tested chemicals can be envisaged depending on the stage in which the development progression is arrested. In sea urchins, the developmental program includes the progression throughout various main stages (e.g., egg, morula, blastula, gastrula and pluteus larva), which may be arrested or delayed in presence of waterborne contaminants (Quiniou et al., 1999). Arrest of cleavage, gastrulation and metamorphosis may be related, amongst others, to impairment of cell cycle and membrane function, cell signalling, adhesion and differentiation, and cytoskeleton depending on the timeline of each stage (Lyons et al., 2012). Thus, the sensitivity of sea urchin embryos and larvae to pollutant exposure varies depending on the developmental stage (Alexander et al., 2017). In the SEDD assay, a high cleavage disruption index (CDI) indicates arrest at the morula stage or earlier, a high gastrulation disruption index (GDI) indicates arrest before entering the pluteus larva stage, and a high metamorphosis disruption index (MDI) indicates arrest before the pluteus larvae reach a fully viable stage.

Carried out within the framework of the UE-funded project GRACE (Jørgensen et al., 2019), the present study aims at contributing to the toxicological characterisation of the aqueous fractions of NNA, MGO and IFO oils and how this is influenced by the application of Finasol OSR52® dispersant. These aqueous fractions are complex mixtures of PAHs and other compounds (Meador and Nahrchang, 2019; Wheeler et al., 2020) and therefore the contribution of individual PAHs and their mixture to their toxicity was assessed upon estimates of toxic units (Sprague, 1970). Although the conditions for production of oil aqueous fractions (Singer et al., 2000) and for sea urchin embryo toxicity testing (Beiras et al., 2012) were of limited regional relevance for cold seas, they were selected to interpret the toxicity of the tested chemicals against existing data on oil toxicity, which mainly deal with temperate seas. This understanding was deemed worthy to properly design and interpret further research about the influence of low temperatures and the presence of ice cover on oil spill toxicity. Likewise, using a well-known toxicity testing model such as P. lividus (Bellas et al., 2008, 2013; Saco-Alvarez et al., 2008; Beiras et al., 2012) was seen as a genuine approach to provide data to advance in risk assessment of oil spills in the Arctic and Sub-Arctic seas before the use of regionally relevant autochthonous test species is feasible.

2. Materials and methods

2.1. Test chemicals

Three oils and one dispersant were selected as representative of prospective oil spill threats in Arctic and Sub-Arctic seas (ESM 1): (a) a Naphthenic North Atlantic crude oil (NNA), a very light crude oil of low viscosity, rich in branched and cyclic saturated hydrocarbons; (b) a distillate marine gas oil (MGO), supplemented with the dye DyeGuard Green MC25 (John Hogg Technical Solutions; UK); (c) the intermediate viscosity, rich in branched and cyclic saturated hydrocarbons; (b) a fuel oil IFO 180 (IFO), a heavy bunker oil of high viscosity with low amounts of volatile hydrocarbons (Polaroil, Greenland); and (d) the third-generation dispersant Finasol OSR52® (D) containing >30% non-ionic and 15–30% anionic surfactants (Total Special Fluids, France; SDS no. 30034 2015). Test chemicals were stored in a cool room (4-6 °C).

2.2. LEWAF production and chemical analysis

The Low Energy Water Accommodated Fraction (LEWAF) in filtered seawater (FSW) of the three oils (NNA LEWAF, MGO LEWAF and IFO LEWAF), the dispersant (D LEWAF) and the combination of the oils with the dispersant (NNA-D LEWAF, MGO-D LEWAF, IFO-D LEWAF) was produced in the darkness at 10 °C according to Katsumiti et al. (2019), modified after Singer et al. (2000) (ESM 2). Briefly, oils (1:200; w oil/v FSW), dispersant (1:2000; w D/v FSW) and their mixtures (1:10 w D/w oil in 1:200; w oil/v FSW) were poured into filtered seawater in 200 mL glass bottles and stirred at 200 ± 20 rpm (no vortex; low energy) for 40 h. This procedure was used for a general screening of toxicity (Experiment-1) in which successive dilutions of LEWAF in FSW (1, 2, 3, 5, 8, 13, 21, 34, 55, 89 and 100%) were prepared following a Fibonacci dose escalation between 0 and 100% LEWAF. In addition, in order to characterise a wider range of toxic effects a second experiment (Experiment-2) was carried out, in which a higher oil/FSW ratio (1:40 instead...
of 1:200) and a longer stirring time (72 h) were used to obtain the LEWAF. Successive dilutions of LEWAF in FSW (8, 21, 34, 55, 89, 100% LEWAF) were also prepared following a Fibonacci dose escalation, after excluding some of the lower doses from the dilution series in order to optimize the experimental set up.

The specific PAH composition of each LEWAF (ESM3) was determined by gas chromatography–mass spectrometry (GC–MS) after Prieto et al. (2007). A mix standard solution of 18 PAHs (CRM47543; Supelco, Bellefonte, USA) was used for calibration in the GC–MS analysis. A mixture of 5 deuterated compounds (Chiron, Trondheim, Norway) was used as internal standard. Stir-bars (10 mm length; 0.5 mm film thick; Gerstel GmbH & Co, Mülheim an der Ruhr, Germany) were introduced in aqueous samples (35 mL) during 315 min. Once the extraction step was over, stir-bars were rinsed in Milli-Q water to eliminate seawater and dried with paper tissue. Then, they were desorbed using a TDS-2 unit connected to a CIS-4 injector (Gerstel) with the following conditions: desorption time (10 min), desorption temperature (300°C), desorption flow (23 mL/min), cryo-focusing temperature (~50°C) and vent pressure (7 psi). The chromatographic conditions were setup as described in Prieto et al. (2007). Detection limits are given in ESM 3.

2.3. Sea urchin embryo toxicity test (SET)

Both in Experiment-1 and in Experiment-2, the sea urchin 48 h embryo toxicity assay was carried out according to ICES (International Council for the Exploration of the Sea; Beiras et al., 2012). Gametes were obtained from sexually mature sea urchins (Paracentrotus lividus) collected from a rocky shore in Armintza (43°26′01″N 2°53′56.1″W; Bay of Biscay) in spring (March-May) 2017. Spawning was induced by injecting 1 mL 0.5 M KCl through the perioral membrane into the coelom. Females were individually placed in 100 mL beakers containing FSW (32 psu; 0.2 μm sieve). After they spawn, the medium was sieved through a nylon mesh (100 μm pore size) to collect the eggs, which were suspended into FSW in a 50 mL falcon tube. Sperm was pipetted directly from the aboral body surface of males, avoiding contact with seawater to prevent sperm activation. Fertilisation was achieved by adding a few drops of sperm to the egg suspension. Quality assurance was carried out by checking gamete viability (egg roundness and sperm motility) and fertilisation rate (~90% fertilised eggs) upon examination in an inverted light microscope (Nikon Eclipse Ti-2) at 10× magnification. Within 30 min after fertilisation, the successfully fertilised eggs were transferred to glass vials containing 10 mL of the test solutions (50 embryos/mL), capped with Teflon lids. Toxicity assays were conducted in completely darkness at 20°C. After 48 h exposure to the LEWAFs, larvae were fixed by adding two drops of 40% formaldehyde. The longest dimension of larvae (L in μm; sample size: n = 35 larvae per vial × 3 replicates) and the egg size at t0 (L0 in μm; sample size: n = 35 egg per vial × 3 exposure replicates) were measured using a Nikon Di-Qi2 camera attached to an inverted microscope (Nikon Eclipse Ti-2) and NIS-Elements Imaging Software v4.30 (Nikon Instruments BV). Size increase (∆L = L-L0) and its EC50 were calculated (Beiras et al., 2012).

In Experiment-2, specific abnormalities of the pluteus larvae were recorded (n = 100 larvae per vial × 3 replicates per experimental group) and integrated in the Toxicity Index (TI, in a 0-100 range; after Carballeira et al., 2012). Briefly, the counts of larvae with incorrect arrangement of skeletal rods (L1), larvae with no skeleton or in which skeletal rods were absent, incomplete, or in which the shape was anomalous (L2), and blastula and gastrula stages and prepluteus larvae, indicative of development blockage (L3) were determined upon examination at 10× magnification in an inverted light microscope (Nikon Eclipse Ti-2). The TI value for each replicate was calculated as follows:

\[
TI = \frac{(1 \times L1) + (2 \times L2) + (3 \times L3)}{3}
\]

where 1, 2 and 3 are the severity factors arbitrarily allocated to L1, L2 and L3 degrees of alteration, respectively (Carballeira et al., 2012).

In Experiment-2, sublethal toxicity was also evaluated as the capacity of sea urchin to undergo its developmental program. Sea urchin embryo developmental disruption (SEDD) was measured in terms of inhibition of pluteus larvae formation index (IPLFI) and alterations in developmental progression (cleavage disruption index (CDI) and gastrulation disruption index (GDI) during embryo development; and metamorphosis disruption index (MDI) during larval development). The longest dimension of each larvae (L in μm; n = 35 larvae per vial × 3 replicates) was measured as described above. The frequency of embryos undergoing cleavage (C) or differentiation at blastula or gastrula stages (G) and the frequency of normally developed larvae (N) or larvae with pathological alterations (P1) were determined upon examination in an inverted light microscope (Nikon Eclipse Ti-2) at 10× magnification (n = 100 larvae per vial × 3 replicates) to calculate the following indices:

\[
\text{IPLFI} = \frac{1}{3} \sum_{i=1}^{3} \frac{(L_{\text{max}} - L_i) \times 100}{(0.5 \times L_{\text{max}})}
\]

\[
\text{CDI} = \frac{100}{2 \log(C + G + N + P1)} \times \log \left( \frac{C + G + N + P1}{G + N + P1} \right)
\]

\[
\text{GDI} = \frac{100}{2 \log(G + N + P1)} \times \log \left( \frac{G + N + P1}{N + P1} \right)
\]

\[
\text{MDI} = \frac{L_{\text{max}} - L_0}{0.5 \times L_{\text{max}}} \times \frac{100}{2 \log(C + G + N + P1)} \times \log \left( \frac{C + G + N + P1}{N + P1} \right)
\]

where L_{\text{max}} is the average longest dimension of the larvae for the experimental control group.

2.4. Toxic units

The concentrations in a mixture of individual pollutants expressed as fractions of the EC50 of each pollutant (toxic units –TU−1; Sprague, 1970), the relative contribution of each individual PAH to the TUs of LEWAFs (RTI) and the relative concentration of each PAH in the mixtures (RCi) were calculated. For this purpose, EC50 values for various individual PAHS (Naph, Flu, Pyr, and Flu) were available for P. lividus embryos (Bellas et al., 2008). For other individual PAHs, the mean of the EC50 values reported in the literature for marine organisms (corals, mysids, copepods, brachiopods, decapods, mollusc larvae, echinoderm larvae and fish juveniles) were used as consensus EC50 to calculate the TUs (ESM 4; Ott et al., 1978; Ward et al., 1981; Holcombe et al., 1983; Trucco et al., 1983; Spehar et al., 1999; Pillai et al., 2003; Barata et al., 2005; Calbet et al., 2006; Bellas et al., 2008; Olsen et al., 2011; Frantzen et al., 2012; Lyons et al., 2012; Renegar et al., 2017; Knap et al., 2017). EC50 for the mixture of PAHS (EC50_{\sum PAHs}) was estimated as the mean of the EC50s of the measured individual PAHs in each LEWAF; and the TUs of the mixture (TU_{\sum PAHs}) were calculated as the ratio between \sum PAHs and EC50_{\sum PAHs}. Then, the relative contribution of each individual PAH to the TUs of the mixture was determined as RTI = TU_{PAH} / TU_{\sum PAHs} where TU_{PAH} means the TU estimated for this individual PAH. In parallel, the relative concentration of each PAH in the mixture was calculated as RCi = C_{PAH}/\sum PAHs; where C_{PAH} stands for the individual concentration of each PAH. Thus, the ratio RTI/RCi was calculated as indicative of whether the toxicity of this individual PAH (‘i’) in the
mixture was, or not, the one expected due to its proportion in the composition of the mixture (assuming the Concentration Addition (CA) model; Altenburger et al., 2003).

2.5. Statistical analysis

Statistical analyses were carried out using SPSS statistical package (IBM SPSS Statistics 24.0). Shapiro-Wilk’s test and Levene’s test were performed to study normality and equality of variances of the datasets, respectively. EC50 values were calculated through Probit analysis. For normal data, differences between control and each exposure group were tested using the parametric one-way ANOVA test followed by post hoc procedures (T Dunnett if the variances were homogenous and T3 Dunnett if they were not). For non-normal data set, the non-parametric Kruskal-Wallis test was used. Linear regressions were compared using nett if they were not). For non-normal data, differences between control and each exposure group were performed to study normality and equality of variances of the datasets, tested using the parametric one-way ANOVA test followed by post hoc procedures (T Dunnett if the variances were homogenous and T3 Dunnett if they were not). For non-normal data set, the non-parametric Kruskal-Wallis test was used. Linear regressions were compared using the ANCOVA test. Significant differences in chemical data were tested with the Z-score test. Level of significance for all analyses was p < 0.05.

3. Results

3.1. LEWAF chemical composition

PAH concentration and PAH composition profile were found to be comparable amongst NNA, MGO and IFO LEWAFs, without and with dispersant, with major differences only for the cases of Naph, 2-MN and Ace; which, on the other hand, were very variable (Table 1; ESM 5).

The concentrations of Naph and 2-MN were higher in NNA LEWAF than in MGO and IFO LEWAF in both experiments, especially in Experiment-1. In contrast, the concentration of 1-MN was comparable in all the LEWAFs in both experiments. The concentration of 2-MN in the three oils and the concentrations of Naph and 1-MN in MGO were higher after addition of dispersant than in the case of oil LEWAFs, only in Experiment-2. The concentrations of Ace, Flu and Phe were high in NNA LEWAF, MGO LEWAF and IFO LEWAF, and the concentration of Acy was relatively high in IFO LEWAF in both experiments. Moreover, the concentrations of Ace, Flu, Phe, Pyr and B[a]A + Chr were higher in NNA+D LEWAF than in NNA LEWAF in Experiment-1 but not in Experiment-2. Conversely, in the case of MGO, the addition of dispersant did not exert any effect on the LEWAF PAH composition in Experiment-1; however, in Experiment-2 the concentrations of Ace, Flu, Acy, Pyr, B[a]A + Chr and, most remarkably, Flu and Phe, were much higher in MGO+D LEWAF than in MGO LEWAF. In the case of IFO, the concentrations of Acy, Ace, Flu, Phe, Fluoro, and, especially, Ant, Pyr and B[a]A + Chr were higher in IFO+D LEWAF than in IFO LEWAF in Experiment-1; and like in the case of MGO, the effect was even more marked in Experiment-2 than in Experiment-1.

Overall, the ∑PAHs (without Naph) was higher than in any other group in IFO+D LEWAF in Experiment-1 and in MGO+D LEWAF in Experiment-2. Asterisks indicate significant differences in each oil LEWAF type (Z-score). (UDL: under detection limits; 16PAHs: low molecular weight PAHs (those with superscript were used to calculate ∑16PAHs); 16HMPAHs: high molecular weight PAHs (those with superscript were used to calculate ∑16HMPAHs); #: without naphthalenes.

<table>
<thead>
<tr>
<th>Experiment-1</th>
<th>NNA LEWAF</th>
<th>NNA+D LEWAF</th>
<th>MGO LEWAF</th>
<th>MGO+D LEWAF</th>
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<td>528</td>
<td>383</td>
<td>256</td>
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<tr>
<td>Ace (1)</td>
<td>492</td>
<td>469</td>
<td>709</td>
<td>2046</td>
<td>1619</td>
<td>4821*</td>
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<tr>
<td>Flu (1)</td>
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<td>1887</td>
<td>28895*</td>
<td>1372</td>
<td>5412*</td>
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<tr>
<td>Am (1)</td>
<td>UDL</td>
<td>UDL</td>
<td>UDL</td>
<td>136</td>
<td>136</td>
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<tr>
<td>Phe (1)</td>
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<td>1884</td>
<td>1877</td>
<td>76949*</td>
<td>1999</td>
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<tr>
<td>Pyr (1)</td>
<td>87</td>
<td>104</td>
<td>34</td>
<td>4580</td>
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<td>Fluor (2)</td>
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<td>204</td>
<td>20</td>
<td>8941</td>
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<tr>
<td>B[a]A + Chr (2)</td>
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<td>24</td>
<td>UDLD</td>
<td>662</td>
<td>14</td>
<td>4834*</td>
</tr>
<tr>
<td>B[ghiP] (2)</td>
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<td>UDLD</td>
<td>UDLD</td>
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<td>578</td>
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<tr>
<td>B[ghiPj] (2)</td>
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<td>UDLD</td>
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<td>33</td>
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<td>550</td>
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<tr>
<td>I1,2,3-edjP (2)</td>
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<td>UDLD</td>
<td>UDLD</td>
<td>20</td>
<td>UDLD</td>
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<td>∑PAHs (2)</td>
<td>3854</td>
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<td>4684</td>
<td>122708*</td>
<td>5509</td>
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<td>4061</td>
<td>4630</td>
<td>108419*</td>
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</tr>
<tr>
<td>∑16HMPAHs (2)</td>
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<td>332</td>
<td>54</td>
<td>14,289</td>
<td>92</td>
<td>18,249</td>
</tr>
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</table>
Experiment-2 (Table 1). The $\sum_{\text{LMW}}$PAHs was higher in IFO+D LEWAF than in any other group in Experiment-1; meanwhile, in Experiment-2 $\sum_{\text{LMW}}$PAHs was high in MGO+D LEWAF compared to the other groups (Table 1).

### 3.2. Toxicity of oils alone and combined with dispersant

After exposure to the dispersant, the $\text{EC}_{50(D)}$ for $\Delta L$ in sea urchin larvae was 13% LEWAF (63 ± 3 mg Finasol OSR52/L FSW) in Experiment-1 and 5% LEWAF (118 ± 9 mg Finasol OSR52/L FSW) in Experiment-2.

On exposure to NNA alone or in combination with the dispersant, there was a progressive reduction in $\Delta L$ at increasing concentrations of both NNA and NNA+D LEWAF, more markedly in Experiment-2 than in Experiment-1, especially in NNA+D LEWAF (Fig. 1A and B; ESM 6). Additional toxicity endpoints were only measured in Experiment-2. Thus, TI and GDI increased linearly at increasing concentrations of both NNA and NNA+D LEWAF, with a steeper slope in the former case (ANCOVA; $p < 0.05$; Figs. 2A and 3B; ESM 6). In contrast, CDI increased linearly at increasing concentrations of MGO+D LEWAF but remained around 0% on exposure to MGO LEWAF (Fig. 3D; ESM 6).

At increasing concentrations of both IFO and IFO+D LEWAF, there was a progressive reduction in $\Delta L$ in both experiments, more markedly in Experiment-2 than in Experiment-1 and more prominently in IFO+D LEWAF than in IFO LEWAF (Fig. 1E and F; ESM 6). In Experiment-2, TI and IPLFI reached a value of 100 on exposure to 34% IFO LEWAF and 21% IFO+D LEWAF, with higher toxicity critical threshold values in the

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**Fig. 1.** Size increase ($\Delta L$ in $\mu$m) of sea urchin larvae exposed to oil LEWAF and oil+D LEWAF produced in (A, C, E) Experiment-1 (A: NNA and NNA+D LEWAFs; C: MGO and MGO+D LEWAFs; E: IFO and IFO+D LEWAFs and in (B, D, F) Experiment-2 (B: NNA and NNA+D LEWAFs; D: MGO and MGO+D LEWAFs; F: IFO and IFO+D LEWAFs. Dots: mean values per treatment in $\mu$m ($N = 105$). Segments: standard deviation. Asterisks indicate significant differences between each exposure concentration and its respective control group (ANOVA; $p < 0.05$). Median effective concentrations ($\text{EC}_{50}$; confidence intervals in ESM 6) were calculated after probit analysis.
related to the LAH values higher than 1 were calculated for several individual PAHs (Table 2); (a) 2-MN, Pyr and Flu in NNA \+ D LEWAF; (b) 2-MN, Pyr, Flu and B[a]P in MGO \+ D LEWAF; (c) 2-MN, Pyr and B[a]P but most remarkably Ant in IFO \+ D LEWAF; and (d) B[a]A + Chr in the three oil \+ D LEWAFs.

4. Discussion

Different PAH profiles were found depending on the oil-loading ratio and stirring time. These might be explained because the LEWAFs might not be in a stable state of equilibrium after 40-72 h of mixing (Johann et al., 2020b), as expected when the experiments were designed following existing guidelines (Singer et al., 2000). It is conceivable that the conditions to reach the equilibrium vary depending on e.g. the oil characteristics and the temperature of LEWAF production, amongst other factors (Curl and O’Donnell, 1977; Faksness et al., 2008). Under the present conditions, the steady state for NNA was achieved after 50-100 h of stirring (Bilbao et al., submitted), which would justify the slight differences presently found between Experiment-1 (40 h) and Experiment-2 (72 h). Although different oil types may differ in their partitioning kinetic due to different physical chemical characteristics, a comparable timing cannot be disregarded for MGO and IFO. Moreover, upon dispersant addition, the sum of PAHs was the highest in MGO \+ D LEWAF, with Flu, Phe and 4-ring PAHs as major contributors. Accordingly, MGO has lower viscosity than NNA (reduced dispersability: 3000-7000 cP) and IFO (poor/slow dispersibility: >7000 cP) which makes it easy dispersable (<3000 cP), resulting in high waterborne concentrations of substituted naphthalenes, aromatic hydrocarbons and other toxic compounds (EMSA, 2010).

The PAH profiles of LEWAFs were always dominated by conspicuous concentrations of Naph, 1-MN, 2-MN, Ace, Flu and Phe (n-C18), light 2-3 ring PAHs quite common in oil LEWAFs (Brown et al., 2016). Yet, there were some differences amongst the PAH profiles of the three oil LEWAFs and these profiles were dramatically modified upon dispersant addition. The concentration of Naph and 2-MN was higher, 2-4 and 3-6 times respectively, in NNA LEWAF than in the other two oil LEWAFs; in contrast, the concentration of Phe was almost one half than in MGO and IFO LEWAFs. Likewise, the concentration of Ace was 2-3 times higher in IFO LEWAF than in the other oil LEWAFs, whilst the concentration of Flu deviated from the CA joint action. RT/RC values higher than 1 were always found to be significant for MGO and IFO LEWAF, in which an all-or-nothing response was observed (p < 0.05). Asterisks indicate significant differences between linear regression coefficients of oil and oil \+ D LEWAF for each tested oil (n=100); 3 replicates per treatment. Median effective concentrations (EC50; confidence intervals in ESM 6) calculated after probit analysis, and non-observed effect concentration (NOEC) values are shown for each case. Only significant regression models are represented (p < 0.05). Former case (Figs. 2C and 4C; ESM 6), CDI increased linearly at increasing concentrations of IFO \+ D LEWAF, whereas it remained around 0% on exposure to IFO LEWAF (Fig. 3G; ESM 6). Likewise, GDI increased linearly at increasing concentrations of IFO LEWAF but reached a value of 100 on exposure to 21% IFO \+ D LEWAF (Fig. 3H; ESM 6). In contrast, no linear regression was found to be significant for MDI neither for IFO \+ D LEWAF, in which an all-or-nothing response was observed (Fig. 3E; ESM 6).

Regarding NNA, MGO and IFO LEWAFs, the sum of TUs was below “1” for all the toxicity endpoints investigated after exposure to NNA \+ D and IFO \+ D LEWAFs, except for GDI in the IFO \+ D LEWAF treatment; however, the sum of TUs exceeded the value of “1” for all the endpoints on exposure to MGO \+ D LEWAF (Table 2). RT was always higher than “1”, revealing that some individual PAHs deviated from the CA joint action. RT/RC values higher than “1” were calculated for several individual PAHs (Table 2); (a) 2-MN, Pyr and Flu in NNA \+ D LEWAF; (b) 2-MN, Pyr, Flu and B[a]P in MGO \+ D LEWAF; (c) 2-MN, Pyr and B[a]P but most remarkably Ant in IFO \+ D LEWAF; and (d) B[a]A + Chr in the three oil \+ D LEWAFs.

4.1. Twitchiness of oil LEWAF

The size of larvae decreased, and the TI and IPLFI increased on exposure to the LEWAF of the three tested oils. In agreement, exposure to the WAF of diverse oils (Angola crude oil, Heavy Fuel Oil API 11.47, IFO 380 and diesel N. 0) is known to cause length reduction and abnormalities in pluteus larvae of various sea urchin species (Fernández et al., 2006; Lv and Xiong, 2009; Bellas et al., 2013; Rial et al., 2013; Pereira...
et al., 2018). On the other hand, NNA LEWAF was less toxic than the LEWAF of the other two oils. Accordingly, Naph, which exerts no or slight adverse effects in the case of fish embryos (Black et al., 1983; Incardona et al., 2004; Adams et al., 2014), was the dominant individual PAH in NNA LEWAF, together with 1-MN and 2-MN. These light PAHs may cause non-polar narcotic toxicity (De Hoop et al., 2011) and effects on eggs through irregularities in cleavage pattern and formation of blastula embryos (Falk-Petersen et al., 1982). Yet, in the present study, early embryo stages seem to be less sensitive to WAF exposure than pluteus larvae, like in the Antarctic sea urchin *Sterechinus neumayeri* (Alexander et al., 2017). Thus, LEWAF exposure had no effect on early embryo development (cleavage disruption) as revealed by low CDI values but elicited a dose-dependent response in GDI (gastrulation disruption). In agreement, PAHs affect axial development and patterning in sea urchin embryos by disrupting the regulation of β-catenin in the blastula/gastrula transition (Pillai et al., 2003). β-catenin is a multi-functional protein involved in cell/cell adhesion and cell fate specification during embryo development (Oliver et al., 2008); and PAH exposure causes β-catenin accumulation in cell nuclei of sea urchin embryos, *Lytechinus anemesis* (Pillai et al., 2003). Further on, only concentrations close to 100% LEWAF of NNA and IFO caused, in an all-or-nothing fashion, metamorphosis disruption (high MDI values), which on exposure to MGO LEWAF occurred in a dose-dependent manner. Changes in MDI may reflect alterations in spicule formation, which is inhibited in sea urchin larvae exposed to oil LEWAF (Sekiguchi et al., 2018). The present results confirm that the responsiveness of the various developmental stages is different.

Overall, IFO LEWAF was more toxic than MGO LEWAF, which can be attributed to the dominance of 3-ring PAHs, which cause acute embryo toxicity (Incardona et al., 2004, 2005; Hodson et al., 2007; Lee et al., 2011; Le Bihan et al., 2014). Thus, in weathered Alaska North Slope crude oil the toxicity of the mixture depended on the proportion of 3-ring compounds (Incardona et al., 2004). Indeed, 3-ring PAHs can be nearly 3 times more toxic than Naph (Black et al., 1983). Moreover, due to their lipophilicity and effects on K$^+$ and Ca$^{2+}$ channels, the 3-ring PAHs such as Phe can cause non-polar narcosis, like 2-ring PAHs such as Naph, 1-MN and 2-MN do as well (Incardona et al., 2004). IFO 180, which is rich in 2-MN and Phe (Johann et al., 2020b), also induces strong adverse effects in fish embryo development (Adams et al., 2014; Bornstein et al., 2014; Johann et al., 2020b). Interestingly, *P. lividus* embryos seem to be particularly sensitive to heavy oil LEWAF in comparison with copepods, fish embryos and mussel larvae (Saco-Alvarez et al., 2008).

In the three oil LEWAFs, the concentrations of several individual PAHs (Naph, Flu, Phe, Pyr and Fluo) were below critical threshold values of toxicity reported for *P. lividus* (Bellas et al., 2008; Fernández et al., 2006). 2-MN is more toxic than 1-MN, and this is more toxic than Naph to sea urchin embryos (Falk-Petersen et al., 1982). Exposure to 0.25 mg Phe/L causes gastrulation disruption in sea urchin embryos (Pillai et al., 2003). Toxicity data for other identified PAHs are not reported for sea urchin *P. lividus* embryos but are available for other aquatic test organisms (ESM 4). These toxicity critical values are much higher than the <0.1 μg/L recorded for individual 3-ring PAHs in oil.
LEWAFs but of comparable magnitude to the ones corresponding to total PAH values, which ranged between 0.04 and 0.31 mg PAH/L depending on the oil and the endpoint. These values are within the range of the 96-h concentration of dispersed oil (Bejarano et al., 2014). Nevertheless, often believed to be the primary causative agents for toxicity of oil aqueous fractions, PAHs have never been shown to cause toxic effects at so low concentrations (0.1 – 5 µg/L) except when they are part of a complex crude oil mixture. This has been commonly reported and could be explained by synergistic effects amongst PAHs, or by the presence of other compounds with far greater toxicity (Meador and Nahrgang, 2019). According to these authors, characterizing the toxicity of the oil LEWAFs in terms of the ΣPAHs has a great degree of uncertainty and may be misleading and of limited value for decision making in environmental protection. Thus, the ΣPAHs may not be the most appropriate dose metric for crude oil toxicity, whereas the nominal loading proportion might provide a more reliable integration of the mixture toxicity, as recommended by the European Chemicals Agency (Wheeler et al., 2020).

Presently, the identified individual PAHs seem not to be the main cause for toxicity of the oil LEWAFs because TUs were lower than 1 for all the toxicity endpoints. However, RT values higher than 1 suggest that one or more individual PAHs exhibited more toxicity than the one that could be predicted for the mixture toxicity. Thus, 2-MN in all the oil LEWAFs and Phe in NNA and MGO LEWAFs could be responsible for a part of the toxicity (RT/RC > 1). Interestingly, both PAHs cause non-polar narcosis (Irwin et al., 1997; Incardona et al., 2005); and mixtures of compounds exerting only one mode of action can be modelled satisfactorily by assuming CA joint activity (Altenburger et al., 2003), the basis of the TU approach.

Nevertheless, whilst the mixture toxicity assessment is limited to a few identified PAHs (Meador and Nahrgang, 2019), aqueous fractions of oils are a cocktail of PAHs (many not identified) combined with other chemicals so that individual PAHs are not necessarily the major determinant of toxicity (Hokstad et al., 2000; Neff et al., 2000; Evans and Nipper, 2007; Bellas et al., 2008; Engraff et al., 2011; Barron et al., 1999; Barron et al., 2020). PAHs are just a small fraction of the organic compounds found in this complex mixture; hence, the toxic potential of the non-PAH fraction cannot be neglected. Usually, PAHs constitute less than 1% of the total petroleum hydrocarbons of crude oils (Sammarco et al., 2013) and most of the compounds are unidentified and commonly known as the unresolved complex mixture (UCM; Farrington and Quinn, 2015). Uncharacterized aqueous phase compounds are likely important contributors to the toxic response that act by specific or non-specific modes of action, especially the polar fraction (Meador and Nahrgang, 2019). Crude oil has many polar compounds containing sulfur, nitrogen, and oxygen that can constitute a major portion of its aqueous fraction (Molby et al., 2009). Moreover, BTEX (benzene, toluene, ethylbenzene, and xylene) is a volatile fraction of oil WAFs (Bejarano et al., 2014) known to cause acute toxic effects on aquatic organisms (Barron et al., 2018), and therefore its potential toxicity to sea urchin embryos cannot be disregarded. Unfortunately, BTEX was not measured in the present study. Overall, with the exception of a certain degree of narcosis caused by 2-MN and Phe in NNA and MGO LEWAFs, identification of specific individual toxic compounds as main contributors to overall toxicity may not be possible.
by 2-MN in all the oil LEWAFs and also by Phe in NNA and MGO LEWAF, it seems that the toxicity of the oil LEWAFs can be only partially attributed to individual PAHs or the CA action of the mixture. Yet, it would rather be exerted by individual or combined toxic action of other compounds present in the LEWAFs that were not identified, in agreement with previous studies (Barron et al., 1999; Melbye et al., 2009; Johann et al., 2020b). Unfortunately, presently only a few representative PAHs (USEPA 16 list) were analysed assuming the widespread practice in environmental monitoring that the oil toxicity to marine organisms is due to the aromatic hydrocarbon fraction (Pelletier et al., 1997). Extensive chemical analysis of the WAF including UCM compounds and other oil chemicals would be required, which is not a realistic option at present (Wheeler et al., 2020). On the other hand, microdroplets, which may be present at high concentrations in WAF produced by diverse ways (e.g., loadings, sampling times, media), might also contribute to toxicity (Di Toro et al., 2007; Redman et al., 2012).

The expression of EC50 and NOEC in terms of nominal LEWAF dilutions (% LEWAF) would integrate the effects of measured PAHs, non-measured compounds and microdroplets. Therefore, it seems to be a reasonable alternative, already used to compare the toxicity of the WAF of Macando crude light oil to various target marine species (Stefansson et al., 2016).

4.2. Toxicity of the dispersant

Except for the dispersant Slickgome NS, which was shown not to be toxic to seaurchin embryos (Alexander et al., 2017), a wide variety of dispersants is toxic to diverse marine invertebrates (Verriopopoulos et al., 1986, 1987; Epstein et al., 2000; Lee et al., 2013; DeLeo et al., 2016). Presently, Finasol OSR52® was toxic for sea urchin larvae, with EC50(%) values in the range of 63-118 mg Finasol OSR52/L, with slightly higher toxicity when LEWAF was produced at low dispersant-loading ratio and short stirring times. In agreement, Finasol dispersants had been shown to be toxic for sea urchin larvae in previous studies. The 48-h EC50 for Δ1 in P. lividus was 1.2 mg Finasol OSR51/L (Rial et al., 2014) and the 72-h EC50 for larval abnormalities in Arbacia punctulata was 14 mg Finasol OSR52/L (Barron et al., 2020). Overall, the EC50 values recorded in the present study are comparable to the median toxicity values reported for Finasol OSR52® in various marine species. The 48-h LC50 was 9 mg Finasol OSR52/L for the mytid Americanamysis bahia and the 96-h LC50 was 12 mg Finasol OSR52/L for the Atlantic silveryside Menidia beryllina (USEPA, 2019). Nevertheless, the sensitivity of marine organisms to Finasol OSR52® is very diverse and the LC50 values can vary in the range of 4-105 mg/L (Delorenzo et al., 2017). On the other hand, Finasol OSR52® seems to be more toxic than other dispersants. For instance, the Finasol OSR52® LC50 for various marine species was lower than the Corexit 9500A LC50 values, which in some cases were as high as 700 mg/L (Delorenzo et al., 2017). Likewise, the 96-h LC50 of Finasol OSR52® for seabass juveniles (77 mg/L) was also lower than the LC50 values of other dispersants such as Corexite9500A, Slickgome NS and Inpol IP90 (Dussauze et al., 2015). Alike, the 48-h LC50 values for A. bahia were 28 mg Finasol OSR52/L and 120 mg Corexit 9500A/L, and the 96-h LC50 values for M. beryllina were 113 mg Finasol OSR 52/L and 201 mg Corexit 9500A/L (Barron et al., 2020). In contrast, other dispersants such as Accel seem to be more toxic than Finasol OSR52®, with a LC50 of 5 mg/L for mysids and 8 mg/L for fish (Barron et al., 2020).

4.3. The influence of dispersant on oil toxicity

The LEWAFs obtained after adding Finasol OSR52® to the oils were more toxic than the LEWAFs obtained from the pure oils. Consequently, dispersant application decreased further the size of larvae, and increased TI and IPLFI values, especially on exposure to NNA-D and IFO-D LEWAF, and less markedly on exposure to MGO-D LEWAF. Based on in vitro toxicity assays with mussel haemocytes, Finasol OSR52® was found to be more toxic than NNA-D LEWAF, which was more toxic than NNA LEWAF (Katsumi et al., 2019). Similarly, other studies also showed that dispersant addition enhances the toxicity of the water-accommodated fraction (WAF) of crude oils (Epstein et al., 2000; Lar amore et al., 2014; Lee et al., 2013; Rial et al., 2014; DeLeo et al., 2016). Exceptionally, Slickgome seems to be non-toxic to embryos of the Antarctic sea urchin and hence its addition did not increase IFO 180 toxicity (Alexander et al., 2017). Overall, the higher toxicity of oil-D LEWAF in comparison with oil LEWAF might be caused by increased concentrations of oil components in the water fraction or by direct toxicity of the dispersant rather than by the consequence of a synergistic action of the oil and the dispersant (Rial et al., 2014; Dussauze et al., 2015).

Unlike for the case of oil LEWAF, exposure to oil-D LEWAF of the three tested oils altered early embryo development (CDI). Likewise, it caused disruption in gastrulation (GDI) and metamorphosis (MDI), which occurred in a dose dependent manner for NNA-D and MGO-D LEWAF treatments, and as an all-or-nothing early response upon IFO-D LEWAF exposure. The influence of dispersant addition on oil toxicity could be explained because it may increase the amount of PAHs and alter the PAH profile in the LEWAFs (Yamada et al., 2003; Delorenzo et al., 2017). Thus, a greater bioavailability of oil components enhanced acute toxicity of oil mixed with Finasol OSR52® in zebrafish embryos (Johann et al., 2020a). Presently, the PAH levels and composition in the LEWAFs changed upon dispersant addition. Thus, together with n-C18, n-C18 PAHs found in pure oil LEWAFs (Naph, 1-MN, 2-MN, Acy, Flu and Phe) other major LEWAF components were found after dispersant application. These included n-C18-C28 PAHs such as Pyr and B[a] + Chr that were identified in the LEWAFs of the three tested oils, and Acy (n-C18-C24), and Fluo (n-C20-C28) that were identified in MGO-D and IFO-D LEWAFs, and Ant (n-C18-C18) and 5- and 6-ring PAHs (n-C14-C24) in IFO-D LEWAF. In quantitative terms, dispersant addition caused a 2-3 times increase in the concentration of Naph in the three oil-D LEWAFs, and resulted in a twice higher concentration of 1-MN in NNA-D LEWAF. In parallel, the concentration of various 3-ring PAHs, was much higher upon application of the dispersant to MGO and IFO 180, most especially for Flu in MGO-D LEWAF (1-3 fold increase) and Phe in both cases (>-20-30×). Likewise, the concentration of 4-ring PAHs was at least 2-fold higher upon application of the dispersant to MGO and IFO 180 than in the corresponding pure oil LEWAFs. Moreover, the proportion of individual 5- and 6-ring PAHs in IFO LEWAF increased upon dispersant addition. A comparable enrichment in high molecular weight PAHs in oil WAF was observed also after application of Corexite 9500 to weathered Mesa light crude oil (Coulard et al., 2005). Similarly, the concentration of total PAHs and saturated hydrocarbons was one order of magnitude higher in WAF oil upon Corexite 9500A addition (Stefansson et al., 2016).

As discussed for oil LEWAFs, oil-D LEWAF toxicity could be partially attributed to narcosis caused by 2- and 3-ring PAHs (Irwin et al., 1997; Incardona et al., 2005), dominant PAHs identified in oil-D LEWAF, say: (a) 2-ring PAHs (Naph, 1-MN and 2-MN) in the three oil-D LEWAFs; and (b) 3-ring PAHs in MGO-D LEWAF (Acy, Ace, Flu and Phe) and IFO-D LEWAF (Ace, Flu, Ant and Phe). Accordingly, the proportion of these PAHs in the mixture relative to the total concentration of identified PAHs was greater than 99% in NNA-D LEWAF and 87% in the other two oil-D LEWAFs. In addition, the augmented toxicity observed (especially for MGO and IFO 180) upon dispersant application might be attributed also to the higher concentration of 4- to 6-ring PAHs recorded in comparison with the LEWAF of pure oils. These included: (a) 4-ring PAHs (Pyr, Phe, B[b] + Chr) in MGO + D and IFO + D LEWAF; and (b) 5- and 6-ring PAHs (B[a] + P, B[b] + F, B[a]H[P], D[a]A and Ind[1,2,3-cd]P) in IFO-D LEWAF. Certainly, 4-ring PAHs (e.g., B[a]A) influence gastrulation in sea urchin embryos through disturbance of the vascular endothelial growth factor signalling pathway, and suppress spicule formation through down-regulation of sm 50, E26 t-s, and Al homeobox genes (Suzuki et al., 2015; Sekiguchi et al., 2018; Honda and Suzuki, 2020). Moreover, 4-ring PAHs (e.g., Pyr) are toxic to fish embryos acting via the AHR-CYP1A pathway (Incardona et al., 2004). This pathway,
altered upon PAH exposure, is present in sea urchins, in which it plays a crucial role in gastrulation and spiculogenesis regulation (Goldstone et al., 2006; Puga et al., 2009). Its alteration up to the morula stage be less relevant, and evidence of toxicity would be recorded only after advances through gastrulation and larval metamorphosis. In contrast, in early interactions between PAHs and factors governing cleavage stage but they would result in increasing toxicity and stronger evidences of development disruption as the developmental process advances through gastrulation and larval metamorphosis. In contrast, in absence of dispersant, the effect of high molecular weight PAHs would be less relevant, and evidence of toxicity would be recorded only after gastrulation, as shown by the lower responsiveness of CDI on exposure to oil LEWAFs in comparison with exposure to oil-d LEWAFs.

Toxicity data available for individual PAHs in *P. lividus* embryos indicate that 4-ring PAHs (Pyr and Flu) are more toxic than 3-ring PAHs (Flu and Phe) and these are more toxic than 2-ring PAHs (Naph), with EC50 values in the range of 0.12-5 mg/L for each individual PAH (Bellas et al., 2008). *P. lividus* embryos are as sensitive as bivalves to Pyr, which is much less toxic to fish (Lyons et al., 2002; Bellas et al., 2008; Frantzen et al., 2012). Flu is highly toxic for *P. lividus* embryos but these are less sensitive than clam embryos (Spehar et al., 1999; Bellas et al., 2008). Overall, *P. lividus* embryos are less sensitive to exposure to individual PAHs than other sea urchin species (Spehar et al., 1999; Pillai et al., 2003; Bellas et al., 2008). On the other hand, toxicity data for identified key high molecular weight PAHs (Ant, B[a]A, Chr, B[a]P) are practically lacking for sea urchin embryos. Fortunately, they are available for other aquatic organisms in which EC50 values vary in the range of 0.01-0.1 mg/L depending on the species (ESM 4). These critical thresholds values are much higher than the <0.3 µg/L recorded in the present study for individual 3- to 6-ring PAHs in oil-d LEWAFs but of comparable magnitude to the ones corresponding to total PAH values, which ranged between 0.03 and 0.47 mg PAH/L depending on the oil and the endpoint. Thus, for instance, the ECSVNolv was 0.26 mg PAH/L (45% LEWAF) for NNA-d, 0.45 mg PAH/L (44% LEWAF) for MGO-d and 0.03 mg PAH/L (6% LEWAF) for IFO-d, and the ECSVIFS was 0.06 mg PAH/L (11% LEWAF) for IFO-d. In agreement, LC50 values for bivalve and echinoderm larvae in the range of 9.6-32.2% LEWAF were reported upon addition of Corexit 9500A to Macondo oil (Stefansson et al., 2016). Nevertheless, EC50s based on total PAHs can lead to wrong conclusions because for the same total PAHs the toxicity may vary with changes in the PAH fingerprint of the mixture that may occur after addition of dispersant. Alternatively, the use of TUs allows an intuitive understanding of the toxicity of a changing mixture of PAHs (Di Torro et al., 2007).

The sum of TUs was higher than in the case of oil LEWAFs but still below 1 for most toxicity endpoints investigated after exposure to NNA-d and IFO-d LEWAFs. In contrast, the sum of TUs exceeded the value of 1 for all the endpoints investigated in the case of MGO-d LEWAF. Thus, although in MGO-d LEWAF there might be a synergistic effect of the mixture, in the majority of the cases the identified individual PAHs would explain partially the toxicity of the oil-d LEWAFs, as above discussed for the case of pure oil LEWAFs. One or more individual PAHs exhibited more toxicity than predicted for the mixture toxicity (RT < 1), revealing that some individual PAHs deviated from the CA joint action. In addition, RT/RC values higher than 1 recorded in several individual PAHs in the oil-d LEWAFs (2-MN, Ant, Pyr, Flu, B[a]P, and B[a]A + Chr) indicate that these were responsible for a part of the toxicity. These PAH and TU profiles fit well with aspects above discussed about the toxicity of oil-d LEWAFs being mediated by at least three potential modes of action (non-polar narcosis, AHR-CYP1A pathway and direct interactions with DNA). Paradoxically, the validity of the TU approach can be limited if more than one mode of actions is elicited because the approach is based on the assumptions of a CA joint action (Altenburger et al., 2003). Nevertheless, it has been suggested that the toxicity of binary mixtures of oil WAFs and dispersants to sea urchin embryos can be described according to conventional CA and IA models (Rial et al., 2014).

5. Concluding remarks

A multi-index approach of the SET test, including larval size increase, larval malformation and developmental disruption as endpoints, was sensitive to discriminate from slight to severe toxicity caused by aqueous fractions of crude and bunker oils representative of prospective oil spill threats in Arctic and Sub-Arctic seas. In addition, dispersant application enhanced the toxicity of the aqueous fractions of the oils and therefore the use of dispersants in the aftermath of oil spills involving NNA, MGO and IFO oils is not without risk.

The sensitivity of ecologically relevant endpoints such as ∆L, TI and IPIFLI varied. IPIFLI and TI revealed toxic effects at exposure levels below EC50 values whilst in the case of ∆L the LOEC and the EC50 were always in the range of the same experimental LEWAF dilution. GDI and MDI were more sensitive than CDI, and for oil LEWAFs they showed EC50 values comparable to those obtained from ∆L, TI and IPIFLI, which suggests that gastrulation was the most affected developmental stage, and that the effects persisted during metamorphosis. In contrast, EC50 values for CDI were similarly low in the case of oil-d LEWAFs, suggesting that for these mixtures the toxic action commenced before gastrulation, during cleavage. Overall, GDI was the most sensitive of the six endpoints studied herein regarding the toxicity of IFO-d LEWAF; and MDI was the most sensitive upon MGO, NNA-d and MGO-d LEWAF exposures. This multi-index approach of the SET test revealed different levels of toxicity caused by the LEWAF of the studied oils, alone or in combination with dispersants. Thus, the heavy bunker oil IFO was more toxic than the light crude oil NNA with the light bunker oil MGO in between. Finasol OSR52® LEWAF was toxic for sea urchin larvae. The LEWAFs obtained after adding Finasol OSR52® to the oils were more toxic than the LEWAFs obtained from the pure oils. Based on the toxic units approach, the identified individual PAHs were not the main cause for toxicity of the oil LEWAFs, and toxicity of oil-d LEWAFs seemed to be mediated by at least three potential modes of action (non-polar narcosis, AHR-CYP1A pathway and direct interactions with DNA).

Although the sea urchin, *P. lividus*, is a marine invertebrate model commonly used in toxicity testing, it is not representative of cold seas. Likewise, the environmental conditions employed herein for LEWAF production and exposure experiments are borderline regarding the Arctic and Sub-Arctic region. Obviously, we are aware that the use of autochthonous species to conduct exposure experiments under regionally relevant environmental realistic conditions should be a research priority to gain reliability in the risk assessment of oil spills in Arctic and Sub-Arctic seas. In the meantime, the present investigation provides useful results on the toxicity of crude and bunker oils representative of prospective oil spill threats in the region and on the influence of dispersant application on their toxicity.

CRediT authorship contribution statement

Laura DeMiguel-Jiménez: Methodology, Investigation, Visualization, Writing original draft, Writing, review and editing. Nestor Etxebarria: Methodology, Formal analysis. Xabier Lekube: Methodology, Investigation. Urzti Izagirre: Conceptualization, Investigation, Writing original draft, Supervision. Ionan Marigómez: Conceptualization, Funding acquisition, Formal analysis, Supervision, Writing original draft, Writing, review and editing.
Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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