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Influence of oil spill response strategies 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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TABLE OF CONTENTS

I. INTRODUCTION

1. Oil: origin and uses

Crude oil is originated underground from fossilized organic materials (zooplankton and phytoplankton) incorporated into sedimentary rocks after millions of years by geochemical processes (Fig. 1). Oil is extracted and it is transported to oil refineries for production of diverse petrochemicals (fuel oils, diesel fuel, kerosene, lubricants, hydrocarbon gas liquids such as propylene or propane and other petroleum products (waxes, residual fuel oils; EIA, 2019)).

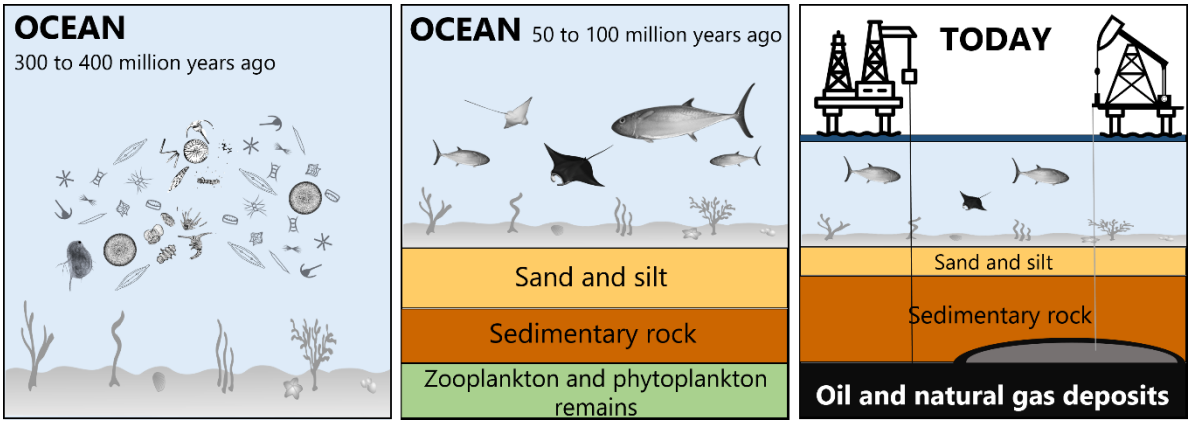

Figure 1. Origin and formation of oil. Adapted from the U.S. Energy Information Administration (EIA, 2019).

Nowadays, despite the increasing use of alternative sources of energy as renewables, oil and its refined components are the most used fuels in the energy sector along with natural gas and coal, being essential resources for the economy of countries around the world. Global oil production and consumption increased in 2019, being heavily concentrated in the United States and China, despite being less than half the growth rate in 2018 (IEA, 2019; IEEJ, 2019; BP, 2020). However, in Europe, a different panorama has been observed since 2008 with a decrease in fossil fuel utilization (BP, 2020). Currently, the Covid-19 pandemic disaster greatly affected global oil demand derived from lockdowns and a plunge in international air transport demand (Koyama and Suehiro, 2021). Despite in 2020 oil demand had a decreased, according to scenarios predicted by various agencies, it will restore the pre-pandemic level in 2022, increasing the demand of all petroleum products (e.g. fuel oil and gasoline sales for transportation and industrial use, as well as, crude oil demand will increase respective to previous years in all regions (IEEJ 2021)).

1.1. Oil composition, classification and toxicity

Petroleum oil is a complex mixture that contains a vast quantity of chemical compounds with a broad range of chemical, physical and toxicological properties (Singer et al., 2000; Marshall and Rodgers, 2008). More than 75% of these compounds are hydrocarbons, which range from small, volatile compounds to very large, non-volatile compounds; and can be classified according to their properties (Fig. 2):

- Aliphatic hydrocarbons are non-cyclic open chain compounds divided into three structurally different groups based on saturation and bonds between carbon molecules: (a) alkanes – saturated hydrocarbons with single carbon bonds, (b) alkenes – unsaturated hydrocarbons double carbon bond, and (c) alkynes – unsaturated hydrocarbons containing a triple carbon bond; (Flowers et al., 2015).
- Aromatic hydrocarbons are formed by six-carbon benzene rings divided into two different groups based on the number of benzene rings that possess: (a) monocyclic aromatic hydrocarbons (MAHs) which are composed of a single aromatic ring. MAHs include benzene, toluene, ethylbenzene and xylene (BTEX) which are the most volatile and water-soluble aromatic hydrocarbons, and (b) polycyclic aromatic compounds (PAHs) which contain two or more benzene rings. PAHs are classified in:
    - Light molecular weight (LMW) PAHs with two or three rings. E.g. naphthalene, fluorene, phenanthrene.
    - High molecular weight (HMW) PAHs with four or more benzene rings. E.g. fluoranthene, pyrene, benzo[a]pyrene.

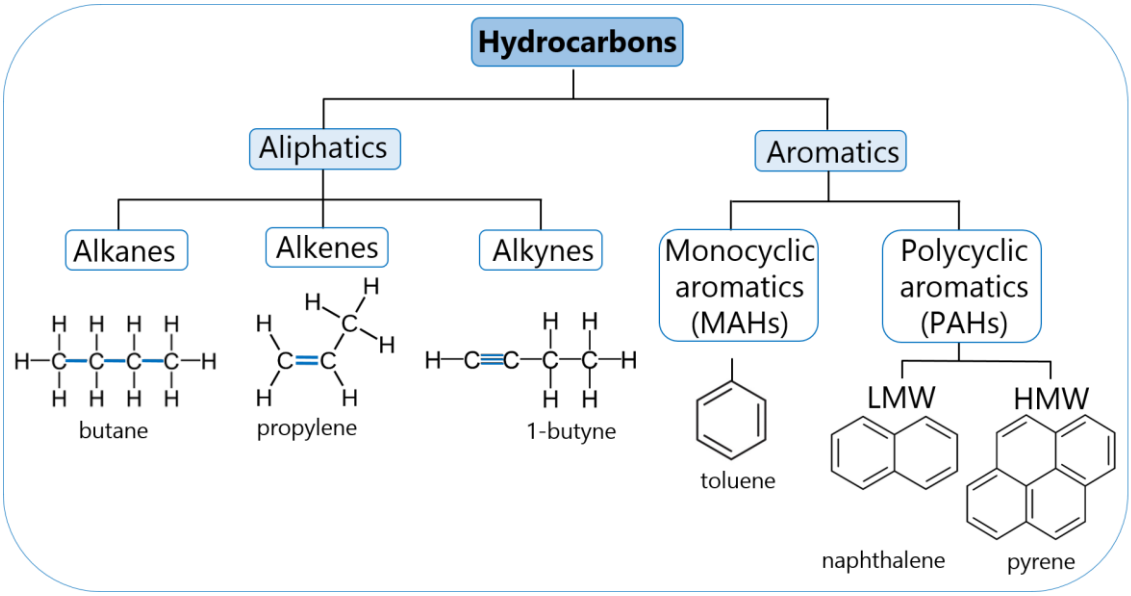

Figure 2. Hydrocarbon classification: aliphatics that are divided into alkanes (only single bonds), alkenes (carbon-carbon double bond) and alkynes (carbon-carbon triple bond), and aromatic hydrocarbons that are divided into monocyclic and polycyclic aromatic compounds (LMW: light molecular weight; HMW: high molecular weight).

The relative proportion of these chemical compounds varies between petroleum oils influencing physical and chemical properties (NASEM, 2016). Oil composition varies widely among oil types and it can be classified by both physical and chemical characteristics, such as specific gravity (API gravity[1]), viscosity[2], density[3] and sulphur content[4] to obtain:

- Light oil: Oil with API gravity higher than 31.1° (less than 870 kg/m^3)
- Intermediate or medium oil: Oil with API gravity between 22.3° and 31.1° (870 to 920 kg/m^3)
- Heavy oil: Oil with API gravity below 22.3° (920 to 1000 kg/m^3)
- Extra heavy oil: Oil with API gravity below 10.0° (greater than 1000 kg/m^3)

[1] The American Petroleum Institute gravity (API gravity) is a measure of how heavy or light an oil liquid is compared to water.

[2] Viscosity is the measure of any liquid's resistance to flow. Oil viscosity refers to how easily oil pours at specified temperatures and is inversely proportional to the API gravity.

[3] Oil density is the ratio of the mass of oil to its volume and it varies with API gravity of oil and the temperature conditions.

[4] Sulphur content refers to the amount of sulphur and sulphur compounds present in oil.

This classification is complemented with the property of sour oil (high sulphur content) or sweet oil (low sulphur content). However, despite the above general classification of oils based in their physicochemical properties, those properties may also vary depending on the place where the oil is extracted and the refinery process followed.

The hydrocarbons present in oils are known to be toxic to aquatic organisms. For that reason, there has been a great demand on analyse their toxicity. Among the hydrocarbons present in oil, alkanes are the predominant but they are not fully related with toxicity to aquatic organisms because have low water solubility. Conversely, aromatic hydrocarbons are compounds highly soluble in water being bioavailable to aquatic organisms. Thus, the relative content of aromatic hydrocarbons are typically considered to be the major responsible of the oil spill toxicity affecting marine organisms (Anderson, 1974; Eisler, 1987; Engraff et al., 2011). Among aromatic hydrocarbons, MAHs are highly volatile and rapidly evaporated from the surface of oil spills (Neff et al., 2000). Thus, PAHs are typically postulated to be the main contributors to oil spills toxicity. The toxic mechanism of PAHs is related with interfering cellular membrane function and enzyme systems associated with the membrane (Neff et al., 2000). The $_{LMW}$PAHs have higher solubility being more bioavailable and hence, contributing to acute toxicity by a process known as narcosis (Barata et al., 2005). The narcosis action results in disruption of the membrane surface involving lack of mobility at sub-lethal concentration, and mortality with prolonged or greater exposure. Yet, $_{HMW}$PAHs have lower solubility and are known to persist in the environment contributing to chronic toxicity (Eisler 1987) and carcinogenicity (Baird et al., 2005). Moreover, mixture of hydrocarbon compounds can have an additive or synergic effect eliciting enhanced toxicity. Therefore, in order to measure environmental quality, a list of 16 PAHs, ranging from 2-ring to 5-ring compounds (Fig. 3), has been proposed by the U.S. Environmental Protection Agency (USEPA) to be representative for all the PAHs present in petroleum oils to assess oil spill toxicity using toxicity assays and to evaluate the environmental health status.

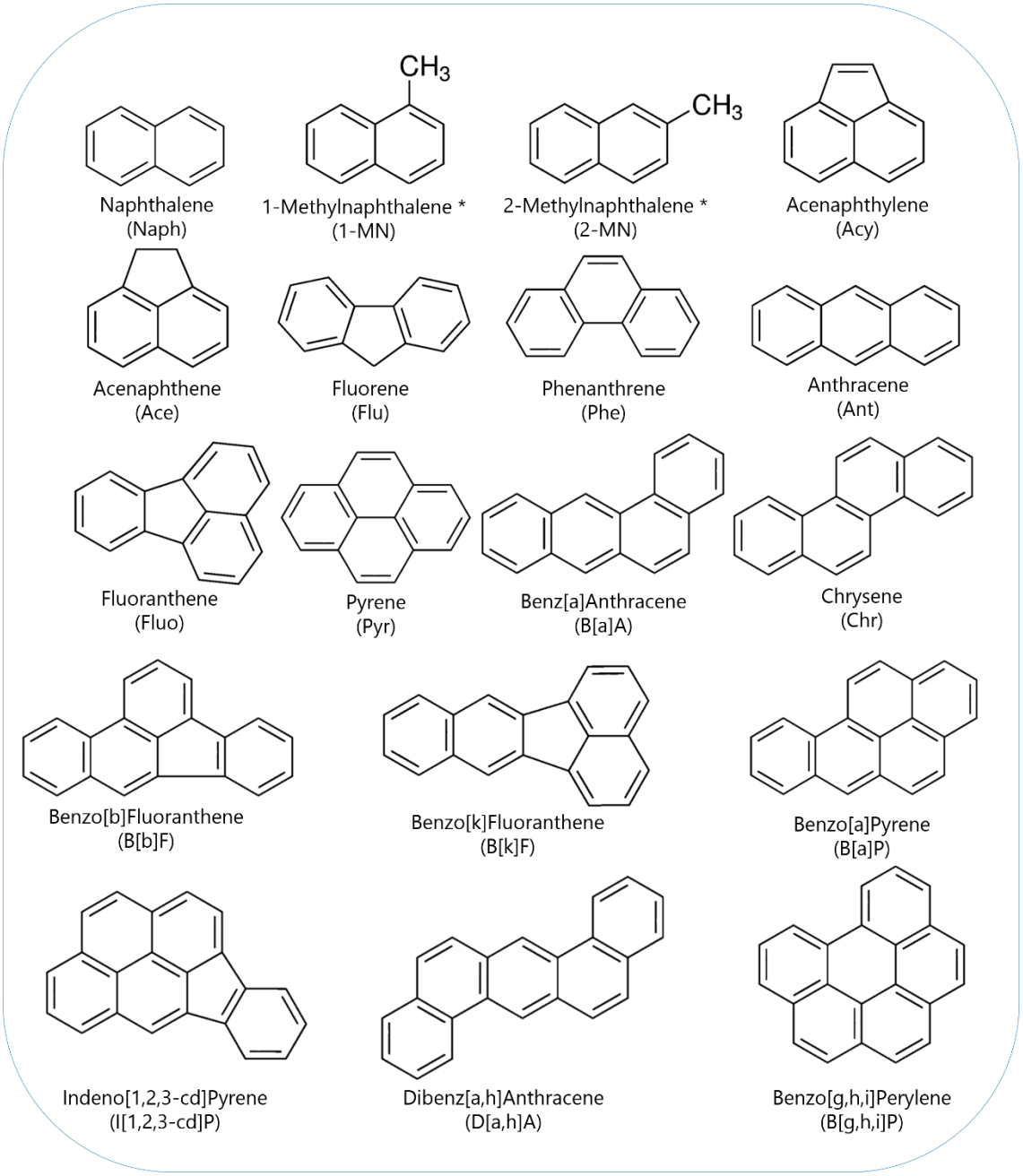

Figure 3. Structure of 16 USEPA priority pollutant PAH compounds (*Not included in U.S. EPA list but included in chemical analysis of the present Ph.D. Thesis).

1.2. Release of oil into the environment

Since oils have multiple uses relevant in our society and oil remains the most used fuel in the energy mix, they are also the most likely substance to enter into the environment. Oil platforms, as well as, maritime traffic, have been growing during the last years in response to oil demand. Approximately, oil tankers transport the 90% of all the oil produced around the world, resulting maritime shipping the principal transportation mode (Walker et al., 2019). Consequently, oil spills after tanker accidents account for 10-15% marine oil pollution world-wide (Tornero and Hanke, 2016). For instance, last year 2020 approximately 1,000 tonnes of oil was spilled as a result of tanker incidents (ITOPF, 2020). Some examples are given in Table 2, a review of oil spills occurred in the last decade around the world, including the name of the incident, the localization, the year and the type of crude oil spilled (ITOPF 2019). Since 2002 spills of light, intermediate, heavy and diesel oil have been occurred around the world (Table 2). In the last decade, the Asiatic continent has witnesses the major number of oil spills derived of shipping incidents (twelve). However, America and Africa, despite having less shipping incidents, five in each continent, the amount of oil spilled has been greater than in Asia (0.25 Mt) with 1440 Mt and 652 Mt, respectively. In Europe, conversely, with seven incidents, the amount of oil spilled has been 0.07 Mt (Table 2). Despite in Europe the number of incidents resulting in oil pollution has declined in the last decades (ITOPF 2016), it is still very likely that they will occur once and again. Particularly, enhanced by globalization and aided by climate change driven ice retreat, new maritime trade routes constitute an emerging threat in the Arctic and Subarctic region (Arctic Council, 2009; Pirotta et al., 2019), where meteorological and environmental conditions can be extreme and accessibility very limited due to remoteness, thus jeopardising oil spill response.

Table 2. Review of shipping incidents derived in oil spills occurred in the last decade (ITOPF 2019).

Incident (Buke name, place and year)	Type of oil spilled	Vessel type
Prestige (Galicia, Spain, 13th November 2002)	63000 t[1] of heavy oil	Oil tanker
Ushuaia (Antarctic Peninsula, 4th December 2008)	440 t of diesel oil	Cruise ship
Gulsar Ana (Madagascar, 26th August 2009)	586 Mt[2] of heavy oil 66 Mt of diesel oil	Bulk carrier
Agios Dimitrios 1 (China, 15th September 2009)	859 t of heavy oil	Container ship
Eagle Otome (United States, 23rd January 2010)	1440 Mt of light oil	Oil tanker
Bunga Kelana 3 (Singapore/Malaysia, 25th May 2010)	2500 t of light oil	Bulk carrier
Fu Ping Yuan (Republic of Korea, 15th June 2010)	60 t of heavy oil 18 t of diesel oil	General cargo ship
Gdansk (Venezuela, 28th January 2011)	520 t of heavy oil	Bulk carrier
Oliva (Tristan da Cunha, 16th March 2011)	1400 t of intermediate oil 70 t of diesel oil	Bulk carrier
Golden Trader (Denmark, 10th September 2011)	205 t of heavy oilil	Bulk carrier
Rena (New Zealand, 5th October 2011)	1700 t of heavy oil 61 t of diesel oil	Container ship
Tk Bremen (France, 16th December 2011)	70 t of heavy oil	Bulk carrier
Alfa 1 (Greece, 5th March 2012)	1499 t heavy oil 300 t intermediate oil 250 t diesel oil	Oil tanker
Stolt Valor (Kingdom of Saudi Arabia, 15th March 2012)	430 t of heavy oil 13000 t of MTBE[3] 1300 t of IBAL[4]	Chemical tanker
St Thomas de Aquinas (Philippines, 16th August 2013)	120 t of intermediate oil	Roll-on/roll off (RORO[5])
Silver (Morocco, 23rd December 2013)	4940 t of heavy oil 190 t of heavy oil	Product tanker
Flinterstar (Belgium, 6th October 2015)	100-300 t of fuel oil	General cargo ship

[1] t (tonne)

[2] Mt (mega tonnes)

[3] MTBE (methyl tertiary-butyl ether)

[4] IBAL (isobutylaldehyde)

[5] RORO ship: ferries designed to carry wheeled cargo such as cars, trucks, semi-trailer trucks, trailers and railroad cars

Table 2. Continuation.

Incident (Buke name, place and year)	Type of oil spilled	Vessel type
Ennore (Chenai, India, 28th January 2017)	160 t[1] of heavy oil	Oil tanker
Sanchi (Shangai, 6th January 20198	2000 t of heavy oil and burn for over a week	Oil and bulk carrier
MV Solomon Trader (Rennell Island, 4th February 2019)	600 t of heavy oil	Bulk carrier
MV Wakashio (Pointe d'Esny, Mauritius, 25th July 2020)	1000 t of diesel oil	Bulk carrier
MT New Diamond (Sri Lanka,3rd September 2020)	230,000 t, burn intermittently for over a week	Large crude carrier
Nendo (Nendo Island, 20th January 2021)	1000 t of heavy oil	Bulk carrier

[1] t (tonne)

1.3. Oil spill response strategies

To counteract oil spills a variety of oil spill responses have been used depending on environmental conditions, properties of the spilled oil, accessibility, available resources and cost considerations (Chen et al., 2019; Prendergast and Gschwend, 2014):

- Manual recovery that consists in remove oil from shorelines physically. Sometimes this technique is not possible to perform due to spill area remoteness (Ventikos et al., 2004), and a large number of people is necessary to be performed being in direct contact with the oil.
- Booms that are mechanical barriers to contain the oil spill and enable further cleaning steps, and skimmers that are mechanical devices designed to remove oil from the water surface. The use of both methods is limited when rough weather and winds inducing strong currents and breaking waves are present (USEPA 1999; Ventikos et al., 2004).
- Sorbents are a passive technique of clean up, made of natural (e.g. wood products or clay) or synthetic materials that can remove the oil from the water by either adsorption (adhesion to a surface) or absorption (incorporate into another substance). However, oil recovery and disposal can need extra procedures (e.g. be burned or be disposed in a landfill), their use can difficult

the posterior use of mechanical skimmers or to be harmful to the environment due to sorbent sinking (USEPA 1999; Ventikos et al., 2004).

- *In-situ* burning is a clean-up method that comprises controlled burning of the oil (Fig. 4). Burning is a low-cost technique with a simple execution and small amount of burn residues (Allen and Ferek, 1993; Fritt-Rasmussen et al., 2015). However, burn residues can sink and affect marine organisms and the smoke plume contain airborne toxicants that can cause potential environmental and health problems (Buist et al., 1977; Sartz PP, 2017).

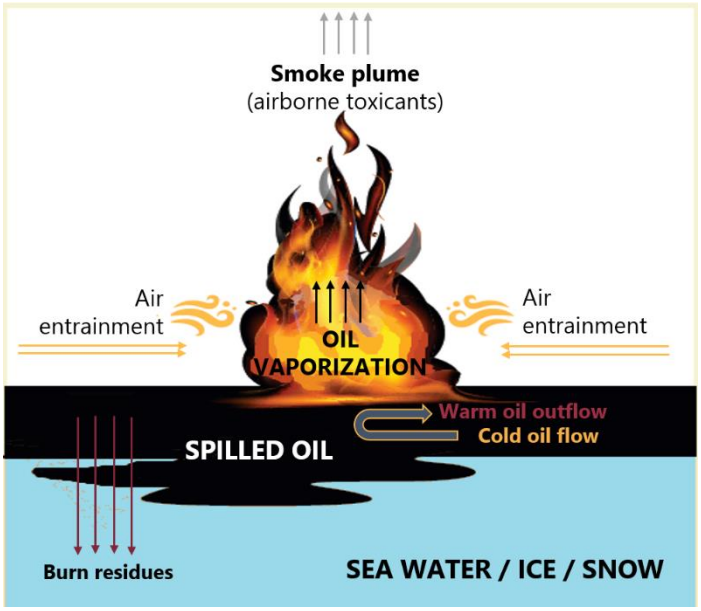

Figure 4. Conceptual scheme of an *in-situ* oil burning on water, on burn residues and smoke plume are created. (Source: adapted from Fritt-Rasmussen, 2010).

- Bioremediation is a remediation option enhancing the ability of microorganisms (bacteria, fungi and algae) to metabolize petroleum hydrocarbons in order to be removed from the marine environment. The most common microorganisms involved in oil degradation are bacteria of *Acinetobacter* spp., *Alcanivorax* spp., *Cycloclasticus* spp., *Oleiphilus* spp., *Oleispira* spp., *Pseudomonas* spp., *Rhodococcus* spp. and *Thalassolituus* spp. (Head et al., 2006; Ghosal et al., 2016; Yakimov et al., 2017; Varjani, 2017a; Wang et al., 2018). It is important to differentiate between bioremediation and biodegradation. The former is a human intervention and the latter is a natural characteristic of microorganisms. Hence, bioremediation involves:

- Bioaugmentation: the addition of exogenous oil-degrading microorganisms to the spill to increase the existing microbial communities and speed up biodegradation.
- Biostimulation: the addition of nutrients or electron acceptors to speed up biodegradation by existing microbial populations.

However, bioremediation and natural attenuation can be too slow depending on different environmental factors, such as temperature, salinity, pressure, substrate, electron acceptors or the availability of pollutants and nutrients (Varjani and Upaani, 2017b); thus, the use of dispersants to enhance biodegradation has been suggested as a useful alternative solution (Coolbaugh and McElroy, 2011; Prince et al., 2016).

- Dispersants are chemical agents, commonly applied by aerial spraying, containing surfactants that allow breaking up the oil slick into small droplets. These droplets are available for microbes which facilitates the removal of hydrocarbons from seawater at shorter times (Prince et al., 2016), and also, aid by natural processes such as wind, waves and currents, break droplets down further helping to clear oil from the water surface (USEPA 1999). Surfactants present in dispersant products have both a hydrophilic (water-soluble) and lipophilic (oil-soluble) constituents. The lipophilic part attaches to the oil phase and the hydrophilic part extends into the water phase, reducing the surface tension and allowing the oil to mix (Chen et al., 2019; Fig. 5). 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. In fact, a wide variety of dispersants are toxic to diverse marine invertebrates such as corals (Epstein et al., 2000; DeLeo et al., 2016), branchiopods (Verriopoulos et al., 1986; 1987), copepods (Lee et al., 2013), mussels (Katsumiti et al., 2019), sea urchins (Rial et al., 2014) and fish (Dussauze et al., 2015; Johann et al. 2020).

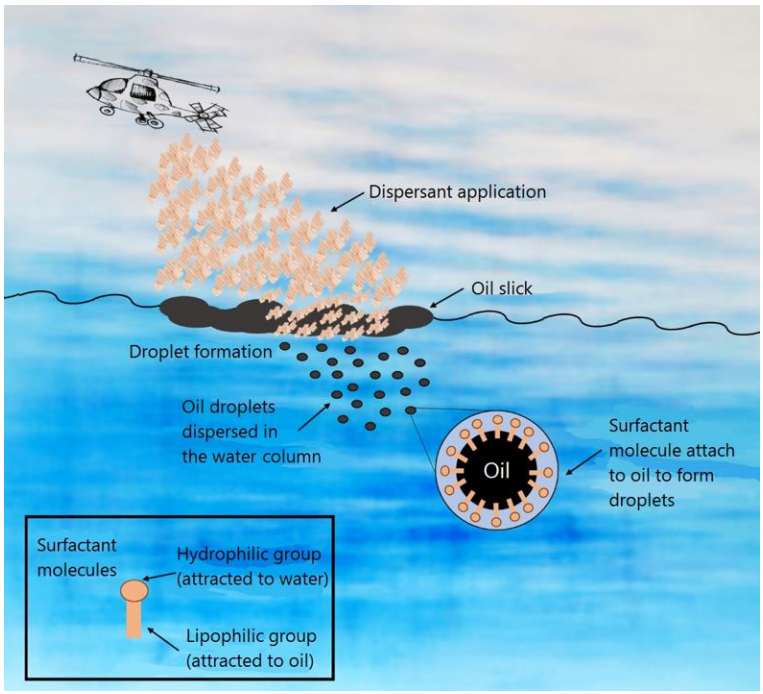

Figure 5. Mechanism of chemical dispersion: surfactant attaches at the oil-water interface promoting formation of small oil droplets reducing the surface tension and allowing oil to mix in the water column. Scheme modified from Graham et al. (2016).

Oil response techniques after an oil spill should be well planned and balanced, to avoid causing more damage than the pollution itself. Therefore, preparedness to respond to oil spills is crucial to counteract their environmental and socio-economic impact.

1.4. Oil spill behaviour in diverse environmental scenarios

The impact of an oil spill will depend on different factors, such as the type of oil spilled or in which environmental conditions the spill will take place. Weather conditions imply variations in physicochemical composition modifying oil behaviour and oil persistence at sea, as well as their toxicity (NAP, 2003). In fact, oil will not undergo the same weathering[5], spreading or natural attenuation processes in temperate, cold or ice-covered regions and therefore spilled oil will behavior differently. In that sense, the main environmental factors affecting oil spilled in temperate-open water conditions are wind and wave energy (Fig. 6A). However, the low temperature of seawater and, more

[5] Weathering is the change of physical and chemical properties of the spilled oil due to different natural processes (e.g. evaporation, water-in-oil emulsification, oil-in-water dispersion, spreading, sedimentation, oxidation and biodegradation; Sørstrøm et al., 2010)

remarkably, the presence of ice-cover are crucial factors in cold waters (Figs. 6B-6C; Wilkinson et al., 2017). The sea ice retreat driven by climate change is improving easier petroleum exploration and maritime transport, thus increasing the threat for oil spills in Arctic seas (Afenyo et al., 2016). Arctic regions present an ice-cover that change with the season influencing differently oil behavior in summer than in winter. On the one hand, summer season have up to 24-hour of solar input in some regions and seawater will have warmer temperatures promoting the melting of ice and snow making sea ice more mobile, facilitating oil migration. (Fig. 6B). However, in winter due to will not be solar input, seawater will have colder temperatures with slow rate of water renewal and water stratification promoting the ice growing and making sea ice less mobile, limiting oil spreading and promoting oil encapsulation (Fig. 6C). In the Arctic and Subarctic region the sea surface temperature may vary in the range of around 5-25ºC along the year, depending on the season and the geographical area (Stigebrandt and Gustafsson, 2003; Siegel and Gerth, 2018; Carvalho and Wang, 2020). Changes in seawater temperature are shown to determine differently the type of hydrocarbons entering in the water column (Perkins et al., 2003; 2005) which could modify the toxicity of the oil. However, there is a lack of studies assessing the influence of temperature on oil spill toxicity. Those diverse scenarios where an oil spill can occur and the prevailing environmental conditions during the spill, as well as the properties of the spilled oil and the accessibility to the spill location will influence the severity of the biological impact and the efficiency of the oil spill response (USEPA, 1999; Beyer et al., 2016; Wenning et al., 2018).

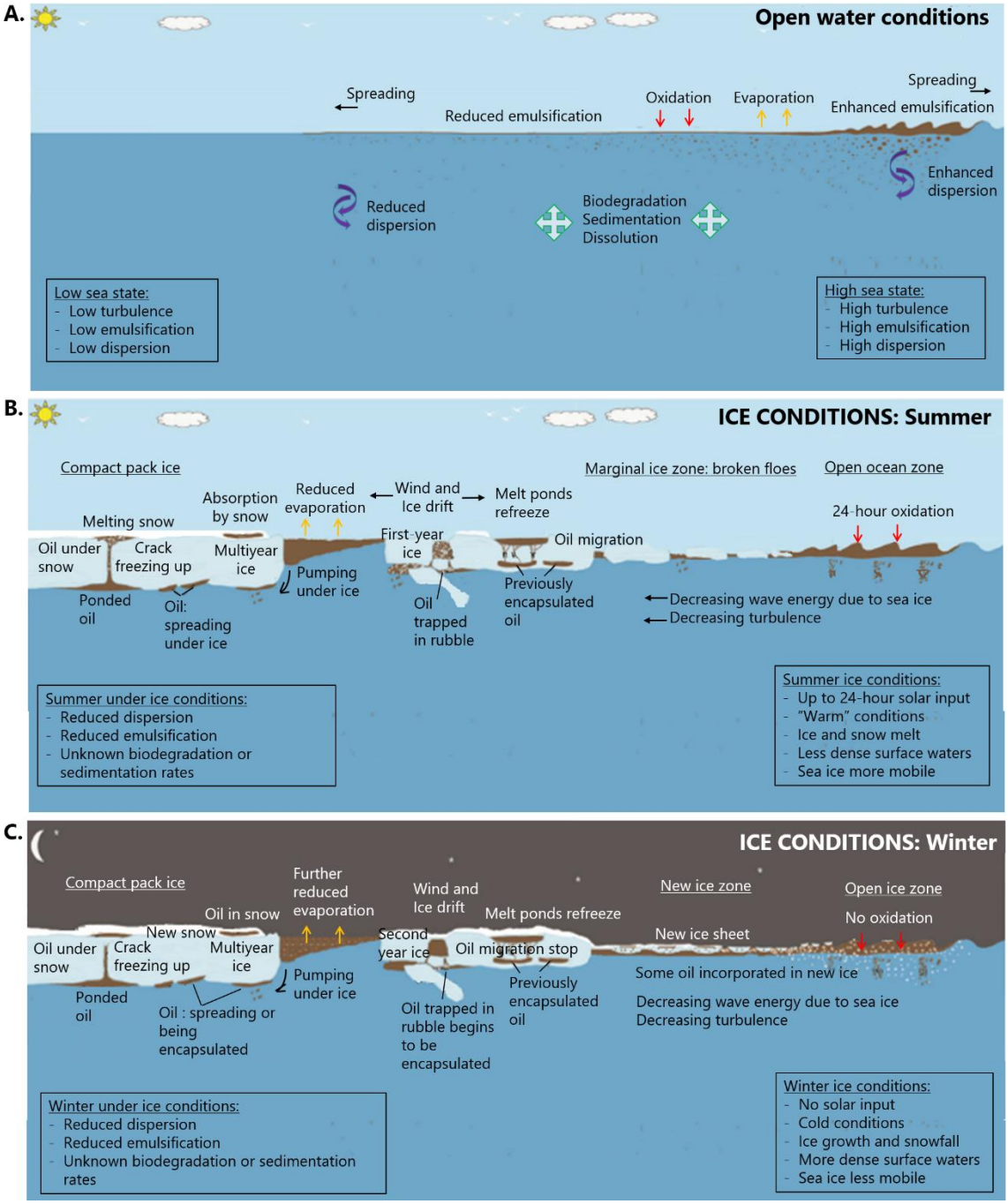

Figure 6. A schematic representation of oil-ice interaction and weathering processes in Arctic waters for: **A)** open water conditions, **B)** summer ice conditions and **C)** winter ice conditions. Modified from Wilkinson et al. (2017).

1.5. Testing oil toxicity

Wildlife is susceptible to the effects of oil spills in a number of ways. A direct oil-contact, an inhalation of vapors or oil ingestion can affect larger animals, such as marine mammals, birds and fish. Microscopic plants and animals such as algae, crustaceans, bivalves and echinoderms can be also negatively affected because are present in the water column saturated with dissolved petroleum compounds, which are more available to them. Thus, to mimic this environmental scenario and test compounds that have low solubility, such as oil, a laboratory prepared mixtures known as water-accommodated fraction (WAF) are recommended and commonly used. WAF is the aqueous medium saturated with petroleum products, mainly hydrocarbons, without particles of bulk material derived from low energy mixing (non-vortexed mixing; Singer et al., 2000). WAF approach is the only currently known method for testing the toxicity of the whole of a complex substance, even when some of its constituents remain unknown (Wheeler et al., 2020). However, the chemical composition of a WAF varies depending on oil type, and minor differences in WAF preparation, such as the oil-loading ratio or the mixing time, can modify its composition (Faksness et al., 2008). Different WAF solutions can be produced depending on the situation that is aimed to reflect that might be encountered in the field (e.g., presence or waves, application of chemical dispersant, a combination of both; Table 3). For instance, LEWAF accounts for low-energy mixing WAF without identifiable dispersion or emulsification of the oil (no vortex, stirring speed of 200±20 rpm, no settling time; Singer et al., 2000). Nevertheless, HEWAF (high-energy or mechanically enhanced WAF) and CEWAF (chemically enhanced WAF where oil and dispersant are combined in the vessel) are heavily influenced by the presence of bulk oil droplets in these solutions (higher stirring speeds with 20-25% vortex and need of settling time; Singer et al., 2000). Thus, in order to interpret any observed effects to use WAF data for toxicological assessment and to minimize and avoid hamper extrapolation amongst studies and laboratories is needed to use standardized protocols, or at least, to clearly describe WAF preparation procedures.

WAF has been largely used in oil toxicity testing because its simplicity to simulate environmental realistic scenarios and to have a wide applicability. Diverse marine species

of algae, bivalves, crustaceans, echinoderms, polychaetes and fish have been used to test oil toxicity as is shown in Table 3, since are continually exposed to stresses both chronic and acute oil toxicity. Algae are usually included in many risk assessment representing primary producers and because are ubiquitous (Ceschin et al., 2020). Invertebrates, which have a potential role in ecosystems through food chains as primary consumers and secondary producers, and vertebrates (e.g., fish, representing secondary consumers) show a wide range of responses to oil from mortality to sublethal impacts manifested by physiological, cytogenic or carcinogenic effects (Table 3). Indeed, the use of invertebrates in research is widespread since supports the replacement of one animal species for non-mammalian animal models and are useful for seeking mechanistic links between individual and population or community levels (Lagadic and Caquet, 1998). Among invertebrates, sea urchins present features that make them a valuable model organism in diverse areas, such as oil toxicity testing (Esposito et al. 1986):

- are commonly found in most marine environments (are distributed from the Arctic to tropical regions occupying keystone positions in those ecosystems (Byrne 1994)
- are easy to handle after short training
- have a low costs
- involve short-term tests
- allow to test different pollutants simultaneously due to each female own a huge number of eggs
- are metazoan organisms (multi-cellular animals)
- exhibit high sensitivity and reproducibility (are susceptible and sensitive to local and large-scale perturbations such as oil spills)
- proportionate relevant data on action mechanisms

Table 3. Review of oil WAF toxicity assays using aquatic organisms. (WAF: water-accommodated fraction, LEWAF: low-energy mixing WAF, CEWAF: chemically enhanced WAF, HEWAF: mechanically enhanced WAF, FSW: filtered seawater, FW: freshwater, D: dispersant, stir: stirring, AChE: Acetylcholinesterase, EROD: 7-ethoxy-resorufin-O-deethylase, LPX: lipid peroxidation activity).

Species used	Compound	WAF	Endpoint measured	References
Algae				
Tetraselmis tetrathele	Heavy oil and dispersant	LEWAF 1:9 (v oil/v FSW) 23 hr stir CEWAF 1:10 (v D/v FSW), 23 hr stir	Algal growth	Santander-Avanceña et al., 2016
Invertebrates				
Amphipod				
Leptocheirus plumulosus	Light oil and dispersant Corexit and Finasol	CEWAF 1:40 (w oil/v FSW), 18 hr stir 1:20 (w D/v oil+D), 18 hr stir	Survival analysis and sublethal effects (Cytochrome P450, AChE, EROD, LPX)	DeLorenzo et al., 2017
Clam				
Mercenaria mercenaria	Light oil and dispersant Corexit and Finasol	CEWAF 1:40 (w oil/v FSW), 18 hr stir 1:20 (w D/v oil+D), 18 hr stir	Survival analysis and sublethal effects (Cytochrome P450, AChE, EROD, LPX)	DeLorenzo et al., 2017
Copepods				
15 different species	Light oil	WAF 1:9 (w oil: v FSW), 24 hr stir	Survival analysis, activity, impaired swimming ability, loss of balance.	Jiang et al., 2012
Corals				
Paramuricea type B3, Callogorgia delta and *Leiopathes glaberrima*	Light and dispersant Corexit 9500A	WAF 1:4000 (v oil: v FSW), 24 hr stir	Survival analysis	DeLeo et al., 2016

Table 3. Continuation.

Species used	Compound	WAF	Endpoint measured	References
Mussels				
Mytilus galloprovincialis	Petroleum hydrocarbons	LEWAF 1:12 (v oil: v FSW), 12 hr stir	Lysosomal structure alterations in oocytes	Cajaraville et al., 1991
M. galloprovincialis	A light and heavy oil and a commercial lubricant oil	LEWAF 1:12 (v oil: v FSW), 12 hr stir	Survival, growth and gonad development	Cajaraville et al., 1992
M. galloprovincialis	Heavy oil	LEWAF 1:10 (w oil: v FSW), 24 hr stir	Lysosomal biomarkers, tissue-level biomarkers, enzyme activity and metabolites	Blanco-Rayón et al., 2019
Mysid				
Americamysis bahia	Light oil and dispersant (Corexit and Finasol)	CEWAF 1:40 (w oil/v FSW), 18 hr stir 1:20 (w D/v oil+D), 18 hr stir	Survival analysis and sublethal effects (Cytochrome P450, AChE, EROD, LPX)	DeLorenzo et al., 2017
A. bahia	3 light oils and dispersants (Corexit 9500, Finasol, Accell Clean® DWD, CytoSol®, solidifier Gelco 200®)	LEWAF 1:20 (w oil/v FSW), 18 hr stir 1:40 (w oil/v FSW), 18 hr stir	Short-term development (72 hr static non-renewal), 7 d growth and survival (static renewal)	Barron et al., 2020
A. bahia	Light oil and Corexit 9500A dispersant	WAF 1:40 (w oil: v FSW), 18 hr stir CEWAF 1:10 (w D/v oil+D), 18 hr stir	Survival analysis	Hemmer et al. 2011

Table 3. Continuation.

Species used	Compound	WAF	Endpoint measured	References
Oyster				
Crassostrea gigas	*Deepwater Horizon* slick oil and dispersant Corexit 9500A	HEWAF 1:500 (w oil/v FSW), 30 s stir CEWAF 1:10 (w D/v oil+D), 18 hr stir	Fertilization success, embryogenesis progress, larval development, larval size	Vigner et al., 2015
Polychaete				
Neanthes arenaceodentata	Light oil and dispersant Corexit and Finasol	CEWAF 1:40 (w oil/v FSW), 18 hr stir 1:20 (w D/v oil+D), 18 hr stir	Survival analysis and sublethal effects (Cytochrome P450, AChE, EROD, LPX)	DeLorenzo et al., 2017
Shrimp				
Litopenaeus vannamei	Medium oil	LEWAF 1:10 (v oil/v FSW), 24 hr stir	Survival analysis and activity: impaired swimming ability, loss of balance, comatose.	Asadi and Khoiruddin, 2017
Palaemonetes pugio	Light oil and dispersant Corexit and Finasol	CEWAF 1:40 (w oil/v FSW), 18 hr stir 1:20 (w D/v oil+D), 18 hr stir	Survival analysis and sublethal effects (Cytochrome P450, AChE, EROD, LPX)	DeLorenzo et al., 2017
Snail				
Ilyanassa obsoleta	Light oil and dispersant Corexit and Finasol	CEWAF 1:40 (w oil/v FSW), 18 hr stir 1:20 (w D/v oil+D), 18 hr stir	Survival analysis and sublethal effects (Cytochrome P450, AChE, EROD, LPX)	DeLorenzo et al., 2017

Table 3. Continuation.

Species used	Compound	WAF	Endpoint measured	References
Vertebrates				
Fish				
Cyprinodon variegatus	Light oil and dispersant Corexit and Finasol	CEWAF 1:40 (w oil/v FSW), 18 hr stir 1:20 (w D/v oil+D), 18 hr stir	Survival analysis and sublethal effects (Cytochrome P450, AChE, EROD, LPX)	DeLorenzo et al., 2017
Danio rerio	Light and heavy oil	LEWAF 1:10000 (w oil BAL 110: v FW) 1:1200 (w oil HFO: v FW)	Morphological analyses, swimming ability, cardiac activity, in vivo EROD activity, DNA integrity.	Perrichon et al. 2016
Epinephelus coicoides	Light oil	LEWAF 1:10 (w oil: v FSW), 24 hr stir	Egg hatching, survival larvae, morphological abnormalities.	Karam et al. 2014
Menidia beryllina	Light oil and dispersant Corexit 9500A	WAF 1:40 (w oil: v FSW), 18 hr stir CEWAF[3] 1:10 (w D/v oil+D), 18 hr stir	Survival analysis	Hemmer et al. 2011
Pimephales promelas	3 light oils and dispersants (Corexit 9500, Finasol, Accell Clean® DWD, CytoSol®, solidifier Gelco 200®)	LEWAF 1:20 (w oil/v FSW), 18 hr stir 1:40 (w oil/v FSW), 18 hr stir	Short-term development (72 hr static non-renewal), 7 d growth and survival (static renewal)	Barron et al., 2020

2. Sea urchin as model species

The Echinodermata phylum comprises sea stars (Asteroidea), sea urchins and sand dollars (Echinoidea), brittle stars (Ophiuroidea), sea cucumbers (Holothuroidea) and sea lilies (Crinoidea). Echinoderms, e.g., sea urchins, and specifically their early-life stages, offer an excellent opportunity for studies in diverse research areas due to its field accessibility, its quick synchronous development, its optical transparency and because are easy to manipulate. For instance, the European research community used sea urchins for the discovery of many principles of early development (McClay, 2011). The extensive knowledge available of sea urchin development confers many opportunities on other research areas. Thus, they are also commonly used in cancer research (McClay 2011), in regulatory toxicity testing (Canada Standard, 2011), in DNA damage and repair studies (Hose J, 1985; Le Bouffant et al., 2007, Le Bouffant et al., 2008; El-Bibany et al., 2014; Reinardy and Bodnar, 2015), and in environmental impact and risk assessment (Beiras et al., 2003, Saco-Álvarez et al., 2008; Beiras et al., 2012; Carballeira et al., 2012).

Among sea urchin species, *Paracentrotus lividus* (Lamarck, 1816) is the echinoderm species of choice for most research communities since is a regular edible echinoid, its populations are relatively stable for several years and its spread throughout the Mediterranean coast, the north eastern Atlantic from Scotland and Ireland to southern Morocco and the Canary Islands (Boudouresque and Verlaque 2013).

Phylum Echinodermata
Class Echinoidea
Order Camarodonta
Family Parechinidae
Species *Paracentrotus lividus* (Lamarck, 1816)

2.1. Biological cycle

Sea urchins present a pelagic larva that after metamorphosis the juvenile settles and grows into the adult.

P. lividus is a gonochoric species with only a single annual gametogenic cycle in temperate echinoids, though some individuals can present mature gonads year-round (Sánchez-España et al., 2004). Male and female sea urchins simultaneously release their gametes to the marine environment (Hardin J, 1994) usually from spring to early summer, but the period of spawning differs among locations (Boudouresque and Verlaque 2013).

Once the first sperm attaches to the egg surface, a progressive increase of the cytosolic level of calcium ions (Ca^{2+}) throughout the egg occur and the change in the NADPH redox state trigger the elevation of the fertilization membrane that acts as a permanent block to polyspermy (Eisen et al., 1984; Galione et al., 1993). The unfertilized eggs are haploid cells blocked in interphase (G1 phase). When the fertilisation is achieved and the Ca^{2+} is released, the intracellular pH increases activating the egg (entering into S-phase) which leads to the initiation of protein and DNA synthesis and the completion of the first mitotic division of the embryonic development (Moundoyi et al., 2018). The first early cell divisions of sea urchins are rapid, radial and synchronous. Within 1 hour after fertilization, cleavage start and three successive divisions every 30 minutes occur rising from one to two cells, from two to four cells and from four to eight equivalent cells called blastomeres (Fig. 7). The fourth (16-cell stage embryo) and fifth (32-cell stage embryo) cleavages produce cells of different sizes (mesomeres, micromeres, and macromeres) which are related to future embryo structures. Mesomeres are the future ectoderm, small micromeres are related to coelomic pouches (a mesodermal structure that in adult is called coelom), large micromeres produce primary mesenchyme cells (PMCs) which are the future skeletogenic cells and macromeres are the future endoderm (Fig. 7). By the sixth cleavage (64-cell stage embryo) the blastocel is originated; and after the seventh division (128-cell stage embryo) begins the blastula stage, where all the cells are the same size remaining in one layer of epithelial cells, which is able to swim free after hatching from the fertilization membrane (Matranga V, 2005; McClay, 2011). Later,

gastrulation occurs in distinct temporal phases. First, some PMCs migrate towards two ventro-lateral sites to form a subequatorial ring (Fig. 7; Matranga V, 2005; McClay 2011; Katow H, 2015).

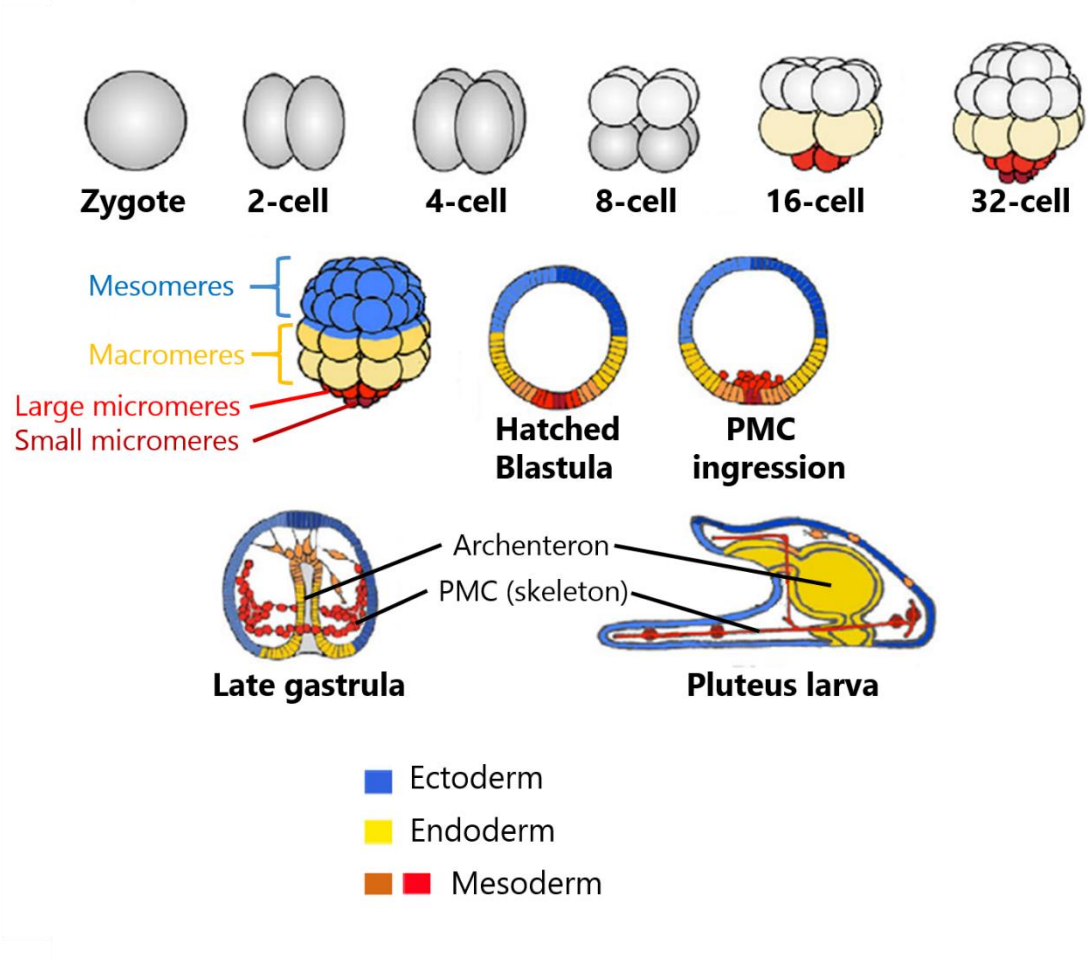

Figure 7. Scheme explaining sea urchin development from the zygote to the pluteus stage. At 32-cell stage there are mesomeres (blue), macromeres (yellow) and micromeres (red). PMCs (primary mesenchyme cells; skeletogenic cells). (Scheme modified from McClay 2011).

Secondly, the epithelium, derived from macromeres, invaginates to form archenteron that is the future intestine. At that point, the gastrula stage is achieved. To form pluteus larvae after 48 hours, the ectoderm, derived from mesomeres, changes sea urchin gastrula shape. PMCs uptake calcium from seawater as the source of calcium in the endoskeleton. Calcium accumulation by the embryo has been shown to increase ten-fold during gastrulation (with 70% of the calcium being deposited in spicules) to form larvae skeleton through biomineralization (Decker and Lennarz, 1988; Wilt FH, 2002, Ettensohn et al., 2003; Wilt and Ettensohn, 2007; McIntyre et al., 2014; Hu et al., 2018).

Multiple compounds can alter sea urchin biological cycle and development, such as heavy metals, nanoparticles, sediment elutriates, wastewater-treatment plant effluents and, petroleum compounds including from light to heavy oils, diesel oils and chemical dispersants that are relevant in the present work (Table 4). Exposure to those oil compounds is known to cause length reduction and abnormalities in pluteus larvae (Fernandez et al., 2006; Lv and Xiong, 2009; Bellas et al., 2013; Rial et al., 2013; Alexander et al., 2017; Pereira et al., 2018). For instance, light PAHs are shown to exhibit a non-polar narcotic toxicity mode of action (De Hoop et al., 2011) causing alterations in cleavage pattern and blastula formation (Falk-Petersen et al., 1982). Indeed, PAHs are known to affect axial development and patterning by disrupting the regulation of β–catenin (involved in skeletogenesis; Röttinger et al., 2004; Oliveri et al., 2008) in the blastula/gastrula transition (Pillai et al., 2003), and oil WAF exposure alters spicule formation (Sekiguchi et al., 2018). Disturbance in the sea urchin developmental program (e.g., egg, morula, blastula, gastrula and pluteus larva) will limit the competence of pluteus larvae to successfully settle and progress to become a benthic adult having an ecological repercussion since are central organisms in structuring benthic communities (Pearse, 2006).

Table 4. Review of toxicity assays where sea urchin species have been used.

Species of sea urchin	Compound	Endpoint measured	References
Anthocidaris crassispina	Mn, Pb, Cd, Zn, Cr, Ni, Fe, and Cu	Development and abnormalities	Kobayashi and Okamura, 2005
Arbacia lixula, Paracentrotus lividus and *Sphaerechinus granularis*	AgNP	Development and abnormalities	Burić et al., 2015
Arbacia punctulata	Three light oils and dispersants (Corexit 9500, Finasol, Accell Clean® DWD, CytoSol®, solidifier Gelco 200 ®)	Development	Barron et al., 2020
A. punctulata and *Strongylocentrotus purpuratus*	A light, an intermediate oil, and a diesel oil	Development and abnormalities	Neff et al., 2000
Hemicentrotus pulcherrimus	PAHs	Larval growth and morphological features	Honda and Suzuki, 2020
Lytechinus anemesis	PAH (Phe, Flu, Pyr, Fluo) and quinolone	Development and patterning	Pillai et al., 2003
P. lividus	$PbCl_2$	Development and abnormalities	Warnau et al., 1996
P. lividus	Cd, Cu, Pb and Zn	Development and abnormalities	Radenac et al., 2001
P. lividus	Heavy oil	Larval growth	Fernández et al., 2006
P. lividus	Heavy oil and diesel oil	Larval growth	Saco-Álvarez et al., 2008
P. lividus	PAHs (Naph, Phe, Flu, Pyr, Fluo)	Larval growth	Bellas et al., 2008
P. lividus	Cd, Pb, Cu and Zn	Development and abnormalities	Dermeche et al., 2012
P. lividus	Diesel oil	Larval growth	Bellas et al., 2013
P. lividus	Light and heavy oil	Larval growth	Rial et al., 2013
P. lividus	Dispersants: CytoSol, Finasol OSR51, Agma OSD 569 and OD4000	Development and larval growth	Rial et al., 2014

Table 4. Continuation.

Species of sea urchin	Compound	Endpoint measured	References
P. lividus	Polystyrene NPs	Development and gene expression	Della Torre et al., 2014
P. lividus	TiO_2 NP, Co NPs Ag NP	Morphological abnormalities (skeleton bio-mineralization)	Gambardella et al., 2015a
P. lividus	SiO_2 NPs	Development, abnormalities and enzymes (acetylcholinesterase; AChE)	Gambardella et al., 2015b
P. lividus	Carbon NPs	Abnormalities and enzymes (AChE)	Mesarič et al., 2015
P. lividus	Metallic Nickel NPs	Development	Kanold et al., 2016
P. lividus	Sediment elutriates	Abnormalities	Vethaak et al., 2017
P. lividus	Diesel oil	Fertilization success and development	Pereira et al., 2018
P. lividus	Wastewater treatment plant effluents	Larval growth and abnormalities	Mijangos et al., 2021
S. granularis	Quality water of the Bay of Brest (Heavy metals, pesticides, TBT and PAH)	Development	Quiniou et al., 1999
Sterechinus neumayeri	Intermediate oil and Slickgone NS dispersant	Development and abnormalities	Alexander et al., 2017
Strongylocentrotus droebachiensis	Light oil	Mortality, abnormalities, larvae growth	Arnberg et al., 2018
S. purpuratus	Pb and Zn	Development	Tellis et al., 2014
S. purpuratus	Heavy oil	Development and abnormalities	Duan et al., 2018

2.2. Sea urchins for toxicity assessment in seawater

As it has been mentioned before, sea urchins are frequently used as model organism in regulatory toxicity testing (USEPA, 2002; Canada Standard, 2011; ASTM, 2012), in environmental impact and risk assessment (Beiras et al., 2003, Saco-Álvarez et al., 2008; Beiras et al., 2012; Carballeira et al., 2012). Most commonly, the endpoints of sea urchin embryo toxicity assays refers to analyses at the pluteus stage, without recording earlier alterations such as embryo malformation and arrest or delay of embryogenesis and metamorphosis (Morroni et al., 2016). The simple observation of normal *vs.* abnormal embryos is rapid and easy to evaluate, commonly used in regulatory toxicity (Canada Standard, 2011), in toxicity testing (Pagano et al., 1996) or in environmental quality assessment (Quiniou et al., 1999), but is restricted to a qualitative criteria. In an alternative approach to improve the sensitivity of sea urchin embryo assays with a semi-quantitative criteria (grading of abnormalities), Carballeira et al. (2012) recorded the percentage of abnormal larvae including four categories of skeletal malformation that included pre-larva non-developed stages, which were used to calculate a toxicity index (Fig. 8A). Moreover, measuring size increase (quantitative criteria) provide an efficient, objective and sensitive endpoint essential in risk assessment and toxicological studies (Fig. 8B; Beiras et al., 2012).

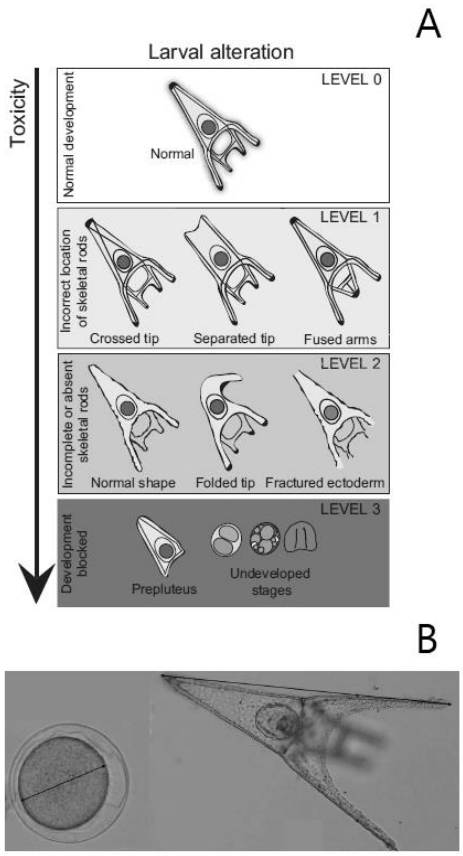

Figure 8. A) Classification of larval malformations according to degree of alteration (Carballeira et al. 2012). **B)** Measurement of maximum dimension in fertilized egg and pluteus larvae (Beiras et al. 2012).

However, the toxic effects elicited in early embryo stages, such as the first cleavage after 90 min of fertilization (Kobayashi, 1990) or the gastrula stage after 24 hr of fertilization (Morroni et al., 2016) are only rarely used as endpoints. Exceptionally, Morroni et al. (2016) recorded the percentage of normally developed embryos at two different stages, gastrula and pluteus, to calculate an integrative index of toxicity that weighted the severity at each stage, which was a relevant approach to enhance the sensitivity of the *P. lividus* embryo-toxicity assay in case of moderate toxicity (Fig. 9). Quiniou et al. (1999), Warnau and Pagano (1994), Pagano et al. (1996) and Guillou et al. (2000) used a qualitative criteria to classify larval development into four categories: dead embryos or larvae, retarded larvae, gastrula or blastula stages and pluteus larvae with anomalies of skeleton or digestive tract (Fig. 9). Similarly, Lavtizar and Okamura (2019) assess larval development classifying stages into malformed skeleton or gut, reduced larval size or observed pre-pluteus larval stages (fertilized egg, blastula, gastrula, prism; Fig. 9). Martino et al. (2017) also used a qualitative criteria but categorized abnormal

embryos into five morphotypes: larvae with a regular skeleton, larvae with no skeleton, larvae with shorter skeleton, larvae with asymmetry in skeleton and larvae with an incorrect pattern of skeleton (Fig. 9). Conversely, Chiarore et al. (2020) used both semi-qualitative and quantitative criteria classifying larvae into five main classes: undeveloped embryos, gastrulae, prism, early plutei and echinoplutei (known as pluteus larvae). Furthermore, for pluteus larvae, Chiarore et al. (2020) evaluated the frequency of abnormal larvae and the length of spicules (Fig. 9). A wide variety of nomenclature is frequently used among toxicity studies hindering direct comparisons despite assessing similar endpoints and developmental stages. Thus, the use of a unified nomenclature is necessary and crucial to compare and integrate toxicity data.

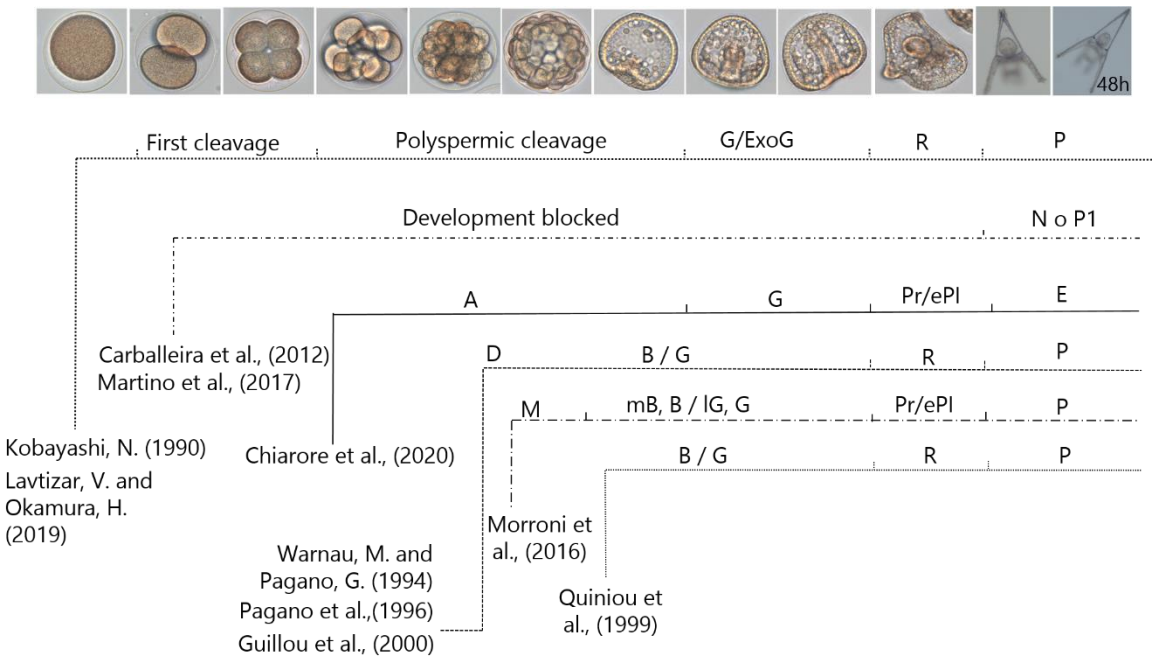

Figure 9. Nomenclature of stages evaluated in each study along 48 hr of the sea urchin *P. lividus* development. A: arrest, B: blastula, D: dead, E: echinoplutei; ePl: early pluteus, ExoG: exogastrula, G: gastrula, lG: late gastrula, M: morula, mB: mesenchyme blastula; Bl: blastula; N: normal pluteus larvae, P: pluteus larvae, P1: pluteus larvae with developmental abnormalities, Pr: prism, R: pluteus larvae with <50% normal size.

Moreover, their single annual gametogenic cycle narrow down the experimentation period highlighting the importance to benefit from the huge number of eggs that own each female allowing to test different pollutants simultaneously, but also to attain the most complete information possible in each bioassay. Thus, the present Ph.D. thesis has work on that issue to provide a unified toxicity-testing tool to obtain robust toxicity information in a limited period of time and from relatively low number of animals.

3. The GRACE project

The present Ph.D. thesis was framed inside the European Union 2020 Horizon project GRACE (Fig. 10): "Integrated oil spill response actions and environmental effects" (2016-2019; http://www.grace-oil-project.eu).

Figure 10. Logo of the GRACE project.

This project was a trans-disciplinary consortium constituted by 13 partners (work-groups and scientists from Europe and Canada: Finnish Environment Institute, Aarhus University, University of Tartu, Tallin Technical University, RWTH Aachen University, University of the Basque Country, Norwegian University of Science and Technology, Greenland oil spill response A/S, LAMOR corporate Ab, Meritaito Oy, SSPA Sweden AB, Norut Narvik AS and the University of Manitoba).

Since the Baltic Sea receive high maritime traffic pressure (Lavrova et al., 2014; Fig. 12) and the Arctic regions of the North Atlantic contains noteworthy amount of undiscovered oil and gas reserves (Gautier et al., 2009), the threat of oil spills is blooming and their hazardous ecological and socio-economical consequences are relevant to be studied. Moreover, Arctic ecosystems are highly vulnerable to oil spills due to their peculiar environmental conditions (low temperature of seawater and, more remarkably, the presence of ice-cover) and remoteness. These features could modify the chemical composition of the WAFs of the spill products and intensify the toxicity to marine biota (Atlas and Bartha, 1972; Word et al., 2014; Nordam et al., 2017) and they can be major factors hampering clean-up operations after oil spills.

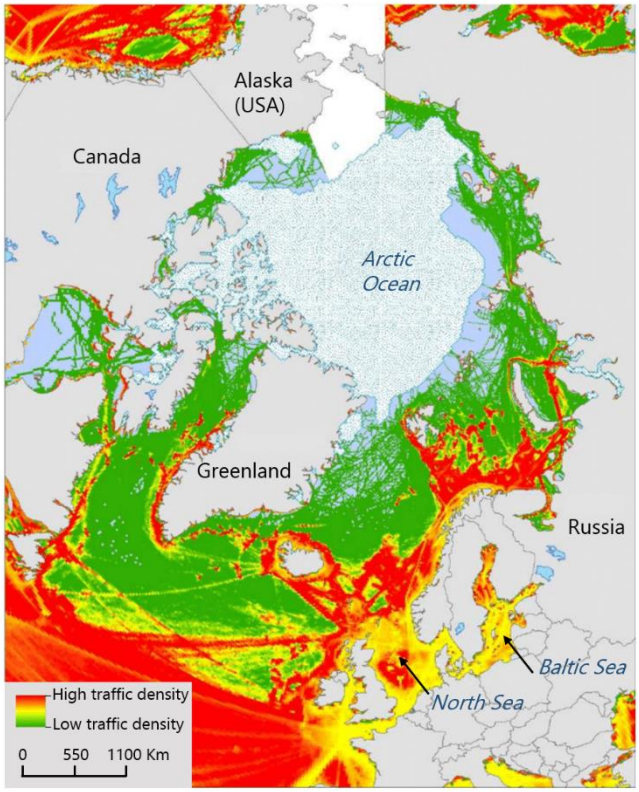

Figure 12. Maritime traffic density in the North Sea and the Baltic Sea (Modified from Lasserre and Têtu, 2020).

Thus, the main aim in the GRACE project was to achieve a holistic approach of the impact of oil spills in cold climate conditions, and evaluate the effectiveness and environmental effects of diverse marine oil spill response technologies in ice-infested areas in the northern Atlantic Ocean and in the Baltic Sea (Jørgensen et al., 2019). Likewise, GRACE aimed to develop and provide the knowledge to counteract the environmental and socio-economic impacts of oil spills as an applicable decision-support tool (Jørgensen et al., 2019). In order to achieve the main goal, the project was divided into five work packages each of them focused in a specific topic:

- Oil spill detection, monitoring, fate and distribution
- Oil biodegradation and bioremediation
- **Oil impacts on biota using biomarkers and ecological risk assessment**
- Combating oil spill in coastal Arctic waters – effectiveness and environmental effects
- Develop and launch a strategic net environment benefit analysis (sNEBA) for decision-making

The present Ph.D. thesis was included in the work package focused in assess the biological impacts of oils spills occurring in different environmental conditions (e.g., testing oil WAF in a wide range of temperatures and oil WAF weathered under ice), and the impact of oil spill response strategies commonly used in these cold regions (e.g., chemical dispersion and *in-situ* burning operations). In fact, this present work was a key cornerstone in the European project GRACE inside the work package "Oil impacts on biota using biomarkers and ecological risk assessment" because sea urchin bioassays allow to test different pollutants simultaneously in a short period of time giving a great amount of robust toxicity information.

The work obtained in the different work packages was strongly interlinked and available in a large number of reports (http://www.grace-oil-project.eu) achieving successfully the objectives of the project. Finally, the expected influences of GRACE project were and are several (Jørgensen et al., 2019) contributing to alleviate the negative effects of oil pollution and response methodologies on the marine environment, coastal economies and communities. Proportionate a better decision support tools for oil spill response strategy in different conditions. Improve the communication and trading between the scientific community and governments, and offering knowledge for companies producing oil response equipment and monitoring services.

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II. STATE OF THE ART, HYPOTHESIS AND OBJECTIVES

STATE OF THE ART

In the last years, despite the increasing use of alternative sources of energy as renewables, oil and its refined components are indispensable in the energy sector, as well as, for transportation and industrial use. Maritime shipping is the principal transportation mode of all the oil produced around the world. Specially, the Baltic Sea, the North Sea and Arctic regions receive high maritime traffic pressure in response to oil demand. Therefore, the threat of oil spills due to transportation activities is blooming, being likely to enter into the environment and affect marine organisms. 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 (e.g. mechanical recovery, *in-situ* burning, chemical dispersion, bioremediation or natural attenuation). However, the efficiency and consequences of those countermeasures will vary depending on prevailing environmental conditions during the spill, type and properties of the spilled oil (crude or bunker oils), accessibility to the spill and the toxicity that can exert on biota.

Wildlife, such as algae, crustaceans, bivalves and echinoderms, are susceptible to the effects of oil spills because are present in the water column saturated with dissolved petroleum compounds which are known to produce toxic effects. From a chemistry point of view, oil toxicity is commonly studied based in the PAH (polycyclic aromatic hydrocarbon) fraction present in the aqueous phase, as recommended by the USEPA, to be postulated as the main contributors to oil spills toxicity. From a biological point of view, the use of early life stages of marine organisms, such as invertebrates, is widespread since supports the replacement of one animal species for non-mammalian animal models, and are sensitive and useful for seeking mechanistic links between individual and population or community levels. Among invertebrates, the sea urchin embryo has been frequently used to assess toxicity due to it is a sensitive, reliable and inexpensive tool. Diverse endpoints with a qualitative, semi-quantitative or quantitative criteria are often used to assess toxicity, but are frequently focused on the final point of the development (pluteus stage) leaving behind other developmental stages that may be relevant to discriminate among levels of toxicity. In spite of assessing similar endpoints and

developmental stages, frequently a unified nomenclature in a standardized test is missing hindering direct comparisons among toxicity studies. However, the sea urchin embryo bioassay is an essential tool in toxicity due to allows doing a high throughput screening of a wide set of conditions (e.g. diverse environmental conditions, type and properties of oils and the impact of the oil spill response selected).

To assess the influence of oil spill response strategies (such as, dispersant application, under ice weathering and oil burning) on the toxicity of crude and bunker oils representative of cold seas is needed to perform a great amount of tests involving a wide set of conditions. Sea urchin embryos are a suitable tool to perform a massive and robust toxicity profiles that provide essential information for oil spill management.

HIPOTHESIS

Oil response strategies envisaged to respond against prospective oil spills in cold seas of the Arctic and Sub-Arctic regions (dispersant application, under ice weathering and oil burning) do not necessarily reduce the toxicity of the aqueous fraction of regionally relevant crude and bunker oils, which depends on the waterborne cocktail of PAHs, represented by the 16 in the USEPA list. Unfortunately, reliable toxicity testing approaches using autochthonous species are not currently available; therefore, toxicity tests with allochthonous sea urchin embryos, for which substantial supporting information is available, may provide a suitable tool for reliable assessment of oil toxicity in cold seas.

OBJECTIVES

The **main objective** of the present work is to gain deeper knowledge on oil spill toxicity and its countermeasures in diverse environmental conditions using the sea urchin *Paracentrotus lividus* embryo after building up a set of indices to evaluate the disturbance in sea urchin development progression.

This general objective has been divided into the four **specific objectives** described below which are addressed in the different chapters of the Results section:

- To assess the influence of the application of dispersant upon the toxicity of crude and bunker oils with a new designed bioassay using different concentrations of oil-water and oil-dispersant.
- To evaluate the influence of a wide range of temperature upon the toxicity of crude and bunker oils, and how this toxicity is modified by the application of dispersant.
- To characterise the toxicity of burned bunker oil residues obtained in a large-scale field experimental oil spill.
- To determine how under ice weathering modifies the toxicity of crude and bunker oils, and how this toxicity is influenced by the application of dispersant.

III. RESULTS AND DISCUSSION

CHAPTER 1

Influence of dispersant application on the toxicity to sea urchin embryos of crude and bunker oils of interest in boreal iced seas

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ABSTRACT

There is an increasing concern regarding the risks of oil spills in iced seas and the use of dispersants as a part of the oil spill response. Dispersants decrease the residence time of the oil in the environment but increase hydrocarbon concentrations in the water column, which can enhance their toxicity. In the present study, the influence of the application of a third generation dispersant (Finasol OSR52®) upon the toxicity of crude and bunker oils of interest in boreal iced seas was investigated using sea urchin embryotoxicity tests. The LEWAFs (Low-Energy Water Accommodated Fraction) of three types of oil (Naphthenic North Atlantic crude oil (NNA); Marine Gas Oil (MGO); Intermediate Fuel Oil 180 (IFO)) were produced at 10°C. Toxicity tests with *Paracentrotus lividus* embryos included the conventional sea urchin embryo test, the determination of the toxicity index and a novel sea urchin developmental disruption assay. 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, it was concluded that the identified individual PAHs were not the main cause for toxicity of the LEWAFs. The responsiveness of the various developmental stages to oil toxicity was different. Thus, sea urchin embryo toxicity assays that include various developmental stages in their analyses provide us with improved sensitivity to discriminate from slight to severe levels of toxicity. This is particularly relevant for moderately toxic but environmentally realistic mixtures such as the LEWAFs of the studied oils alone or in combination with dispersant.

Keywords: oil, dispersant, iced seas, sea urchin embryo, toxicity, mixtures.

RESUMEN

La preocupación por los riesgos que conlleva un vertido de petróleo y el uso de dispersantes como respuesta a dichos eventos en los mares helados ha ido aumentando. Los dispersantes disminuyen el tiempo de residencia del petróleo en el medio ambiente, pero aumentan las concentraciones de hidrocarburos en la columna de agua, lo que puede aumentar su toxicidad. En el presente estudio, se investigó la influencia de la aplicación de un dispersante de tercera generación (Finasol OSR52®) sobre la toxicidad de distintos petróleos de interés en los mares boreales mediante experimentos de toxicidad con embriones de erizos de mar. Los LEWAF (Low-Energy Water Accommodated Fraction) de tres tipos de petróleo (el crudo ligero: Naphthenic North Atlantic (NNA); el diésel: Marine Gas Oil (MGO); y el fueloil: Intermediate Fuel Oil 180 (IFO)) se produjeron a 10°C. En las pruebas de toxicidad utilizando embriones de erizo de mar de la especie *Paracentrotus lividus* se incluyeron: el test convencional con estos embriones, la determinación del índice de toxicidad y un ensayo novedoso de la alteración del desarrollo de dicho organismo. El fueloil IFO fue más tóxico que el diésel MGO y este, más tóxico que el crudo ligero NNA. El Finasol OSR52® LEWAF fue tóxico para las larvas de erizo de mar. Los LEWAF obtenidos después de agregar el dispersante Finasol OSR52® a los distintos petróleos fueron más tóxicos que los LEWAF de los petróleos puros. Sobre la base de enfoque de unidades tóxicas, se concluyó que los hidrocarburos policíclicos aromáticos (PAH) individuales identificados no eran la principal causa de toxicidad de los LEWAF. La respuesta a la toxicidad del petróleo fue diferente en las diversas etapas del desarrollo. Por lo tanto, los ensayos de toxicidad utilizando embriones de erizos de mar que incluyen varias etapas del desarrollo en sus análisis proporcionan una mejora en la sensibilidad para discriminar desde niveles leves a niveles graves de toxicidad. Esto es particularmente relevante para mezclas moderadamente tóxicas, pero ambientalmente realistas, como los LEWAF de los petróleos estudiados ya sean solos o en combinación con dispersantes.

Palabras clave: petróleo, dispersante, mares helados, embriones de erizo de mar, toxicidad, mezclas.

RESUM

La preocupació pel que fa als riscos de vessaments de petroli en mars glaçats i l'ús de dispersants com a part de la resposta a aquests vessaments està en augment. Els dispersants disminueixen el temps de residència del petroli al medi ambient, però augmenten les concentracions d'hidrocarburs a la columna d'aigua, fet que pot fer incrementar la seva toxicitat. En el present estudi, es va investigar la influència de l'aplicació d'un dispersant de tercera generació (Finasol OSR52®) sobre la toxicitat de diferents tipus de petrolis d'interès en els mars glaçats boreals mitjançant proves de toxicitat amb embrions d'eriçons de mar. Els LEWAF (Low-Energy Water Accommodated Fraction) de tres tipus de petroli (un petroli lleuger: Naphthenic North Atlantic (NNA); un diesel marí: Marine Gas Oil (MGO); i un fueloil pesat: Intermediate Fuel Oil 180 (IFO)) es van produir a 10°C. Les proves de toxicitat amb embrions d'eriçó de mar de l'espècie *Paracentrotus lividus* van incloure: el test convencional amb aquests embrions, la determinació de l'índex de toxicitat i un nou assaig d'alteració del cicle de desenvolupament d'aquest organisme. El fueloil pesat IFO va ser més tòxic que el diesel marí MGO, i aquest més tòxic que el petroli lleuger NNA. El Finasol OSR52® LEWAF va ser tòxic per a les larves d'eriçons de mar. Els LEWAF obtinguts després d'afegir Finasol OSR52® als petrolis eren més tòxics que els LEWAF dels petrolis purs. Basant-se en l'enfocament de les unitats tòxiques, es va concloure que els hidrocarburs aromàtics policíclics (PAH) individuals identificats no eren la principal causa de toxicitat dels LEWAF. La resposta a la toxicitat del petroli va ser diferent en les diverses etapes del desenvolupament. Per tant, els assajos de toxicitat amb embrions d'eriçó de mar que inclouen diverses etapes de desenvolupament en els seus anàlisis ens proporcionen una millora en la sensibilitat per discriminar des de nivells de toxicitat més baixos a més alts. Això és particularment rellevant per a mescles moderadament tòxiques, però realistes mediambientalment, com els LEWAF dels petrolis estudiats ja siguin sols o en combinació amb dispersants.

Paraules clau: petroli, dispersant, mars glaçats, embrions d'eriçó de mar, toxicitat, mescles.

LABURPENA

Itsaso izoztuetan petrolio isuriek sortzen dituzten arriskuek eta isuri horiei aurre egiteko sakabanatzaileen erabilerak gero eta kezka handiagoa sortzen dute. Sakabanatzaileek gutxitu egiten dute petrolioa uretan egoten den denbora, baina handitu egiten dituzte hidrokarbono kontzentrazioak ur-zutabean, eta horrek toxikotasuna areagotu dezake. Ikerketa honetan, hirugarren belaunaldiko sakabanatzaile batek (Finasol OSR52®) itsaso boreal izoztuetan olio gordin eta bunker olioen toxikotasunean duen eragina aztertu da, itsasoko itsas-triku enbrioien toxikotasun probak erabiliz. Hiru olio moten (Naphthenic North Atlantic crude petrolio (NNA), Marine Gas Oil (MGO) eta Intermediate Fuel Oil 180 (IFO)) LEWAFak (Low-Energy water Accommodated Fraction-ak) ekozitu dira 10º-C tan. *Paracentrotus lividus* enbrioiekin egindako toxikotasun probak egin dira, besteak beste, ohikoa den itsas- triku enbrioien testa, toxikotasun indizearen kalkulua eta organismo horren garapenaren aldaketak neurtzeko proba berritzaile bat. Emaitzen arabera, IFO fuel-oila MGO diesela baino toxikoagoa da eta hau NNA petrolio gordina baino toxikoagoa. Finasol OSR52® LEWAFa, itsas-trikuen larbentzat toxikoa dela ikusi ahal izan da. Era berean, Finasol OSR52® olioak gehitu ondoren lortutako LEWAF-ak olio hutsetatik lortutakoak baino toxikoagoak dira. Unitate toxikoen hurbilketan oinarrituta, ondorioztatu zen identifikatutako PAHak ez zirela LEWAFen toxikotasun arrazoi nagusia. Garapen fasearen arabera petrolioaren toxikotasuna ezberdina izan dela atzeman da. Azkenik, itsas-trikuak erabiltzen dituzten toxikotasun entseguek garapenaren fase ezberdinak kontuan hartzen badituzte, toxikotasunarekiko sentsibilitate hobea dutela ondorioztatu da, toxikotasun maila txikiak eta larriak bereizi daitezkeelarik. Hau bereziki aipagarria da erdi mailako toxikotasuna duten baina ingurumenarekiko errealistak diren nahasketetan, adibidez, ikertutako petrolioen LEWAFetan, izan hutsik edo izan sakabanatzaileekin batera.

Hitz gakoak: olioa, sakabanatzailea, itsaso izoztuak, itsas-triku enbrioiak, toxikotasuna, nahasketak.

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 unparalleled 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 oil spills in boreal iced seas 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 (Coolbaugh and McElroy, 2013; 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 (Epstein et al., 2000; George-Ares and Clark, 2000; 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 for 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).

Toxicity testing using early life stages of marine organisms (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; Saco-Álvarez et al., 2008; Beiras et al., 2012; Bellas et al., 2013). Some tests deals with observations at larval stages and have therefore ecological significance regarding the impact of chemicals on the output of viable larvae after the processes of embryogenesis and larval metamorphosis. Thus, the so-called "sea urchin embryo toxicity test" (SET) provides a general view of the toxicity exerted by chemicals bioavailable in seawater by quantifying (in terms of pluteus larvae size) the degree of developmental completion achieved by the embryos (Beiras et al., 2012). Likewise, a comparable assay was designed to calculate the toxicity index (TI) 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. 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-hr post-fertilisation (Morroni et al., 2016), which provides toxicologically relevant data but cannot be interpreted in terms of environmental impact. The ecological and toxicological perspectives can be merged by an integrated approach, as it was done to design the *Dyctiostelium discoideum* developmental cycle assay for soil toxicity testing (Balbo and Bozzaro 2008; Rodriguez-Ruiz et al., 2013).

Sea urchins are ecologically relevant marine organisms with planktonic and benthic life stages, frequently used as model organism in developmental and cancer research (McClay 2011), in regulatory toxicity testing (USEPA 2002; Canada Standard, 2011; ASTM 2012) and in environmental impact and risk assessment (Beiras et al., 2003, Saco-Álvarez et al., 2008; Beiras et al., 2012; Carballeira et al., 2012). As a result, there is extensive knowledge of its cellular and molecular biology and its complex genome (Ernst, 2011; Buckely and Rast, 2012); which offers a unique opportunity to achieve an integrated sea urchin developmental disruption (SEDD) assay. The SEDD assay includes an index of ecological relevance inspired in the inhibition of fruiting formation index proposed for soil toxicity testing using the slime mould *D. discoideum* as test organism (Balbo and Bozzaro, 2008). In sea urchins, the inhibition of pluteus larvae formation index (IPLFI) would reveal the competence of pluteus larvae to successfully settle and progress to become a benthic adult. On the other hand, as stated for *D. discoideum* approach (Rodriguez-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). The sensitivity of sea urchin embryos and larvae to pollutant exposure varies depending on the developmental stage (Alexander et al., 2017). Disturbance in development progression can be quantified upon application of a

set of indices. Thus, a high cleavage disruption index (CDI) would indicate arrest at the morula stage or earlier, a high gastrulation disruption index (GDI) would indicate arrest before entering the pluteus larva stage, and a high metamorphosis disruption index (MDI) would indicate arrest before the pluteus larvae reach a fully viable stage. 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).

In the present study, the influence of the application of Finasol OSR52® dispersant upon the toxicity of NNA, MGO and IFO was investigated using the SET test to calculate ΔL (Beiras et al., 2012), TI (Carballeira et al., 2012) and the four indices (CDI, GDI, MDI, IPLFI) of the SEDD assay developed herein.

2. MATERIALS AND METHODS

2.1. LEWAF production and chemical analyses

Three oils and one dispersant were selected as relevant regarding potential oil spills in boreal ices seas (Appendix I):

- *Naphthenic North Atlantic crude oil (NNA),* a very light crude oil of low viscosity, rich in branched and cyclic saturated hydrocarbons.
- *Marine Gas Oil (MGO),* a distillate marine gas oil, supplemented with the dye Dyeguard Green MC25 (John Hogg Technical Solutions; UK).
- *Intermediate Fuel Oil IFO 180 (IFO),* a heavy bunker oil of high viscosity with low amounts of volatile hydrocarbons (Polaroil, Greenland).
- *Finasol OSR52® dispersant (D)*, a third-generation dispersant containing >30% non-ionic and 15–30% anionic surfactants (Total Special Fluids, France; SDS no. 30034 2015).

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 and IFO+D LEWAF) was produced in the darkness at 10°C according to Katsumiti et al. (2019), modified after Singer et al. (2000). Briefly, oils (1:200; w oil/v FSW), dispersant (1:2000; w D/v FSW) and their mixtures (1:10 w D/w oil+D 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 hr. 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 hr) 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. A schematic representation of the experimental design is showed in Fig. 1.

The specific PAH composition of each LEWAF was determined by gas chromatography-mass spectrometry (GC-MS) after Prieto et al. (2007). A mix standard solution of 18 PAHs[1] (CRM47543; Supelco, Bellefonte, USA) was used for calibration in the GC-MS analysis. A mixture of 5 deuterated compounds[2] (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-

[1] Naphthalene (Naph), 1-methylnaphthalene (1-MN), 2-methylnaphthalene (2-MN), acenaphthylene (Acy), acenaphthene (Ace), fluorene (Flu), anthracene (Ant), phenanthrene (Phe), pyrene (Pyr), fluoranthene (Fluo), benz[a]anthracene (B[a]A), chrysene (Chr), benzo[a]pyrene (B[a]P), benzo[b]fluoranthene (B[b]F), benzo[k]fluoranthene (B[k]F), benzo[g,h,i]perylene (B[g,h,i]P), dibenz[a,h]anthracene (D[a,h]A), indeno[1,2,3-cd]pyrene (I[1,2,3-cd]P).

[2] Norwegian Standard (S-4124-200-T): naphthalene-d^8, byphenyl-d^{10}, phenanthrene-d^{10}, pyrene-d^{10}, benzo[a]anthracene-d^{12}, benzo[a]pyrene-d^{10}, benzo[g,h,i]perylene-d^{12}.

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 limit values are given in Appendix II.

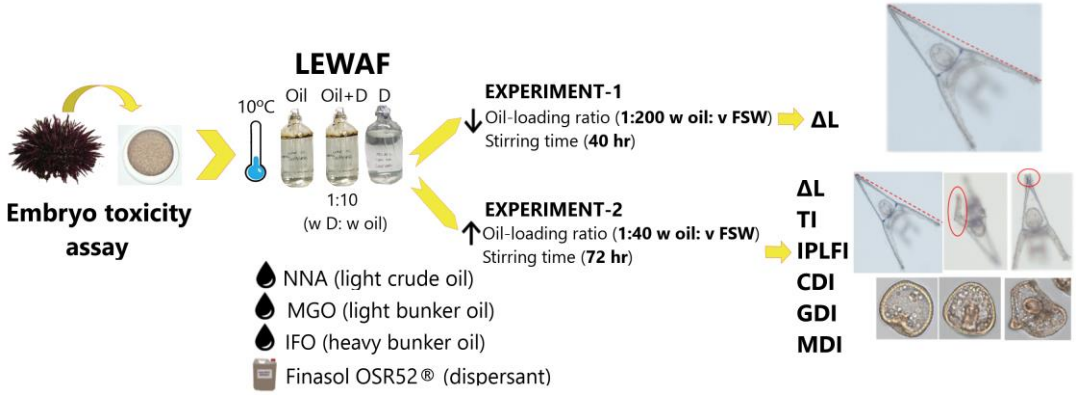

Figure 1. Schematic representation of the experimental design followed in the present study. The LEWAF of the three oils (NNA, MGO and IFO) alone or in combination with Finasol OSR52® dispersant (D) were produced in two different conditions (Experiment-1 with low oil-loading ratio and shorter stirring time, and Experiment-2 with high oil-loading ratio and longer stirring time). In both experiments, toxicity was assessed using the sea urchin embryo toxicity assay. In Experiment-1 only the size increase (ΔL) was recorded; in Experiment-2 the size increase (ΔL) was recorded, the toxicity index (TI) and the indices included in the sea urchin embryo developmental disruption (SEDD) assay (the inhibition pluteus larvae formation index (IPLFI), the cleavage disruption index (CDI), the gastrulation disruption index (GDI) and the metamorphosis disruption index (MDI) were calculated.

2.2. Sea urchin embryo toxicity test (SET)

Both in Experiment-1 and in Experiment-2, the sea urchin 48 hr 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 (43º26'01.1"N 2º53'56.1"W; Bay of Biscay) in spring (March-May) 2017. Spawning was induced by injecting 1 mL 0.5M 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 capped containing 10 mL of the test solutions (50 embryos/mL) to conduct toxicity assays (in completely darkness at 20ºC).

After 48 hr exposure, 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 exposure replicates) and the egg size at t_0 (L_0 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; Appendix III). Images were taken with NIS-Elements Imaging Software v4.30 (Nikon Instruments BV). Size increase ($\Delta L=L-L_0$) and its EC_{50} were calculated (Beiras et al., 2012).

2.3. Toxicity Index (TI) in pluteus larvae

In Experiment-2, specific abnormalities of the pluteus larvae were recorded (n=100 larvae per vial × 3 exposure replicates) and integrated in the Toxicity Index (TI, in a 0-100 range; after Carballeira et al., 2012; Appendix IV). Briefly, the counts of larvae with incorrect arrangement of skeletal rods (L1) and larvae with no skeleton or in which skeletal rods were absent, incomplete or in which the shape was anomalous (L2), and the counts of 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) to calulate the TI value for each replicate, 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).

2.4. Sea urchin embryo developmental disruption (SEDD) assay

In Experiment-2, sublethal toxicity was evaluated as the capacity of sea urchin to undergo its developmental program (Appendix V); this was measured in terms of inhibition of pluteus larvae formation index (IPLFI), and potential mechanisms of toxic action on developmental processes were identified by examining main stages of 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_i in µm; n=35 larvae per vial × 3 exposure replicates) was measured as detailed 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 exposure replicates) to calculate the following indices:

$$IPLFI = \frac{1}{r}\sum_{i=1}^{r}\frac{(L_{max} - L_i) \times 100}{(0.5 \times L_{max})};$$

$$CDI = \frac{100}{2\log(C+G+N+P1)} \times \log\frac{(C+G+N+P1) \times C}{G+N+P1};$$

$$GDI = \frac{100}{2\log(G+N+P1)} \times \log\frac{(G+N+P1) \times G}{N+P1}; \text{ and}$$

$$MDI = \frac{L_{max} - L_i}{0.5 \times L_{max}} \times \frac{100}{2\log(C+G+N+P1)} \times \log\frac{(C+G+N+P1) \times (C+G)}{(N+P1)};$$

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

2.5. Data treatment and 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 sets, 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 with major differences only for the cases of Naph, 2-MN and Ace; which, on the other hand, were very variable (Fig. 2). The Naph concentration in NNA LEWAF was in the range between 200 µg/L (Experiment-2) and 350 µg/L (Experiment-1), with values of 50-100 µg/L for MGO and IFO LEWAF in both experiments. The concentration of 1-MN varied between 50-100 µg/L for the LEWAFs of the three oils in both experiments; and the concentration of 2-MN in NNA LEWAF was in the range between 100 µg/L (Experiment-2) and 300 µg/L (Experiment-1), with values of 50-100 µg/L for MGO and IFO LEWAF in both experiments.

The addition of dispersant seemed to alter the PAH distribution in particular in MGO+D LEWAF (Fig. 2). Thus, the Naph concentration in NNA+D LEWAF was found to be between 300 µg/L (Experiment-2) and 400 µg/L (Experiment-1), with values of 100 µg/L (Experiment-2) and 400 µg/L (Experiment-1) for MGO+D and values of 100 µg/L for IFO+D in both experiments (Table 3). The 1-MN concentration in NNA+D

LEWAFs was 200 µg/L in Experiment-1 and 50 µg/L in Experiment-2, with values varying between 50-100 µg/L for MGO+D and IFO+D LEWAF in both experiments (Table 3). The concentration of 2-MN varied between 150-350 µg/L for the LEWAFs of the three tested oils in both experiments (Table 3).

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 (Table 3). 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 (Table 3). 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, Fluo, Acy, Pyr, B[a]A+Chr and, most remarkably, Flu and Phe, were much higher in MGO+D LEWAF than in MGO LEWAF (Table 3). In the case of IFO, the concentrations of Acy, Ace, Flu, Phe, Fluo, 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 (Table 3). 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 (Table 3). The $\sum_{HMW}$PAHs was higher in IFO+D LEWAF than in any other group in Experiment-1; meanwhile, in Experiment-2 $\sum_{LMW}$PAHs was high in MGO+D LEWAF compared to the other groups (Table 3).

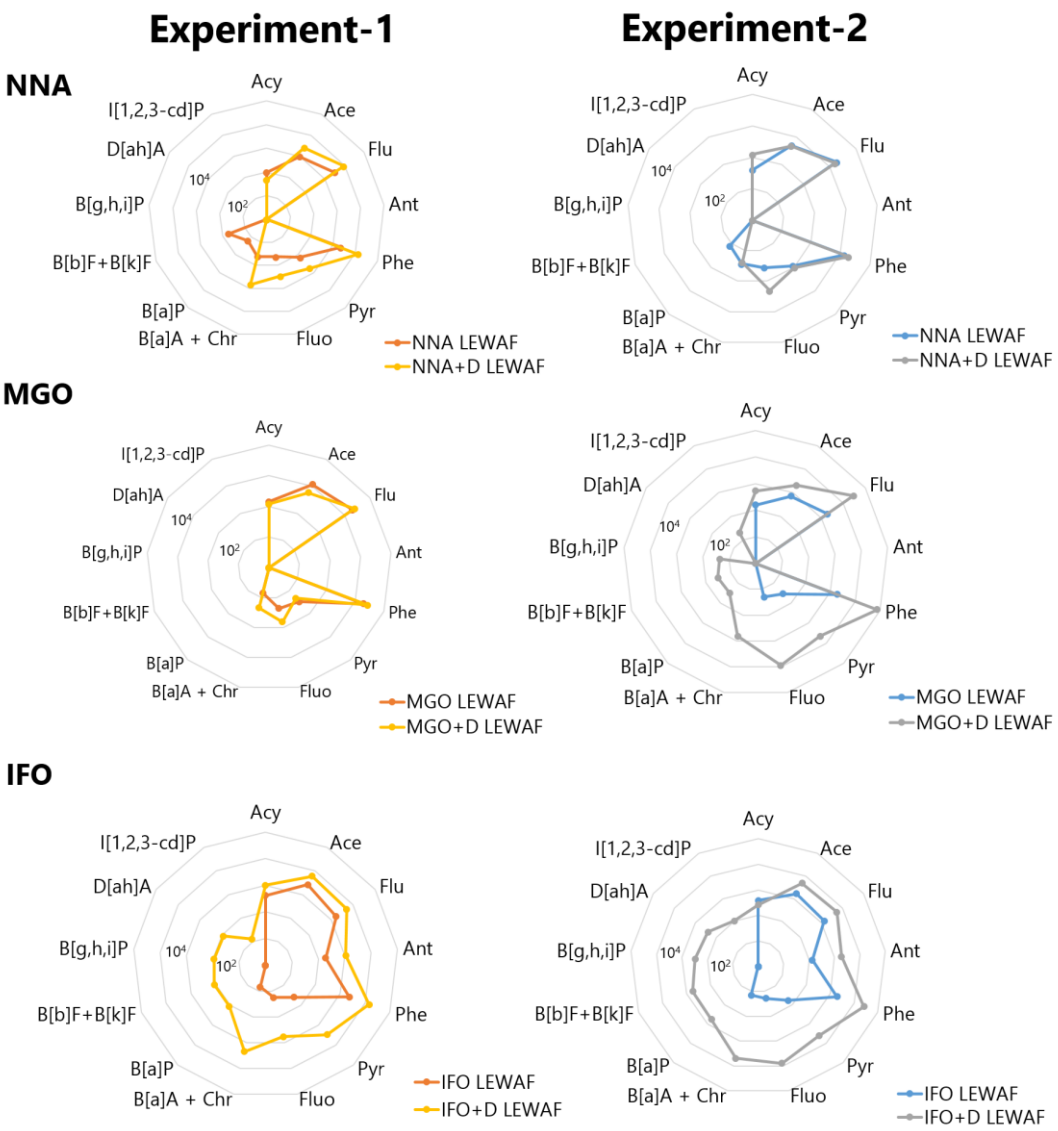

Figure 2. Profile of PAHs, represented in logarithmic scale, in oil LEWAF and oil+D LEWAF samples of NNA, MGO and IFO produced in Experiment-1 and Experiment-2.

Table 3. GC-MS analysis of PAHs (ng/L) in oil LEWAF and oil+D LEWAF samples of NNA, MGO and IFO produced in Experiment-1 and Experiment-2. Asterisks indicate significant differences in each oil LEWAF type (Z-score). (UDL: under detection limits; $_{LMW}$PAHs: Low molecular weight polycyclic aromatic hydrocarbons; $_{HMW}$PAHs: High molecular weight polycyclic aromatic hydrocarbons; #: Total of PAHs without Naphthalene).

Experiment-1	NNA LEWAF	NNA+D LEWAF	MGO LEWAF	MGO+D LEWAF	IFO LEWAF	IFO+D LEWAF
Naph	351221	439059	112311	71814	92285	73614
1-MN	72842	**173919***	32904	27011	77566	92047
2-MN	306563	365838	42043	26763	91365	107758
Acy (1)	98	46	142	120	419	**1009***
Ace (1)	996	2649	1144	585	2607	**5999***
Flu (1)	3158	**9111***	2043	2436	1673	5066
Ant (1)	UDL	UDL	UDL	UDL	188	1088
Phe (1)	2269	13569	1992	2737	2337	14774
Pyr (2)	139	575	30	21	40	**3056***
Fluo (2)	42	294	23	65	18	**588***
B[a]A + Chr (2)	41	681	7	22	7	**2176***
B[a]P (2)	16	UDL	UDL	UDL	UDL	115
B[b]F + B[k]F (2)	53	UDL	UDL	UDL	UDL	115
B[g,h,i]P (2)	UDL	UDL	UDL	UDL	UDL	89
D[ah]A (2)	UDL	UDL	UDL	UDL	UDL	84
I[1,2,3-cd]P (2)	UDL	UDL	UDL	UDL	UDL	13
∑PAHs	737439	**1005741***	268504	307590	192638	131576
$\sum_{LMW}$PAHs Σ(1)	6251	25376	5321	5879	7223	27946
$\sum_{HMW}$PAHs Σ(2)	292	1549	60	108	65	**6235***
$\sum_{Naph}$PAHs	730626	**978816***	187258	125589	261216	273419
∑PAHs#	6813	26925	5380	5987	7288	34171

Table 3. Continuation.

Experiment-2	NNA LEWAF	NNA+D LEWAF	MGO LEWAF	MGO+D LEWAF	IFO LEWAF	IFO+D LEWAF
Naph	183967	309377	87088	382091	51738	123021
1-MN	32912	21707	29058	**84287***	54884	48832
2-MN	88769	234417	31106	423605	52881	296789
Acy (1)	40	124	157	528	383	256
Ace (1)	492	469	709	2046	1619	**4821***
Flu (1)	1787	1584	1887	**28895***	1372	5412
Ant (1)	UDL	UDL	UDL	UDL	136	1923
Phe (1)	1373	1884	1877	**76949***	1999	26221
Pyr (2)	87	104	34	4580	59	3944
Fluo (2)	36	204	20	8941	19	7699
B[a]A + Chr (2)	27	24	UDL	662	14	**4834***
B[a]P (2)	12	UDL	UDL	30	UDL	578
B[b]F + B[k]F (2)	UDL	UDL	UDL	33	UDL	550
B[g,h,i]P (2)	UDL	UDL	UDL	23	UDL	301
D[ah]A (2)	UDL	UDL	UDL	UDL	UDL	242
I[1,2,3-cd]P (2)	UDL	UDL	UDL	20	UDL	102
∑PAHs	309502	569893	165105	525524	151936	**1012162***
$\sum_{LMW}$PAHs Σ(1)	3692	4061	4630	**108419***	5509	38632
$\sum_{HMW}$PAHs Σ(2)	162	332	54	14289	92	18249
$\sum_{Naph}$PAHs	305648	565500	147252	**889983***	159503	468642
∑PAHs#	3854	4393	4684	**122708***	5601	56882

3.2. Toxicity of oils alone and combined with dispersant

3.2.1. Dispersant (D)

The $EC50_{(D)}$ for the size increase (ΔL) 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. Photographs of sea urchin larvae exposed to Finasol OSR52® dispersant are shown in Fig. 3.

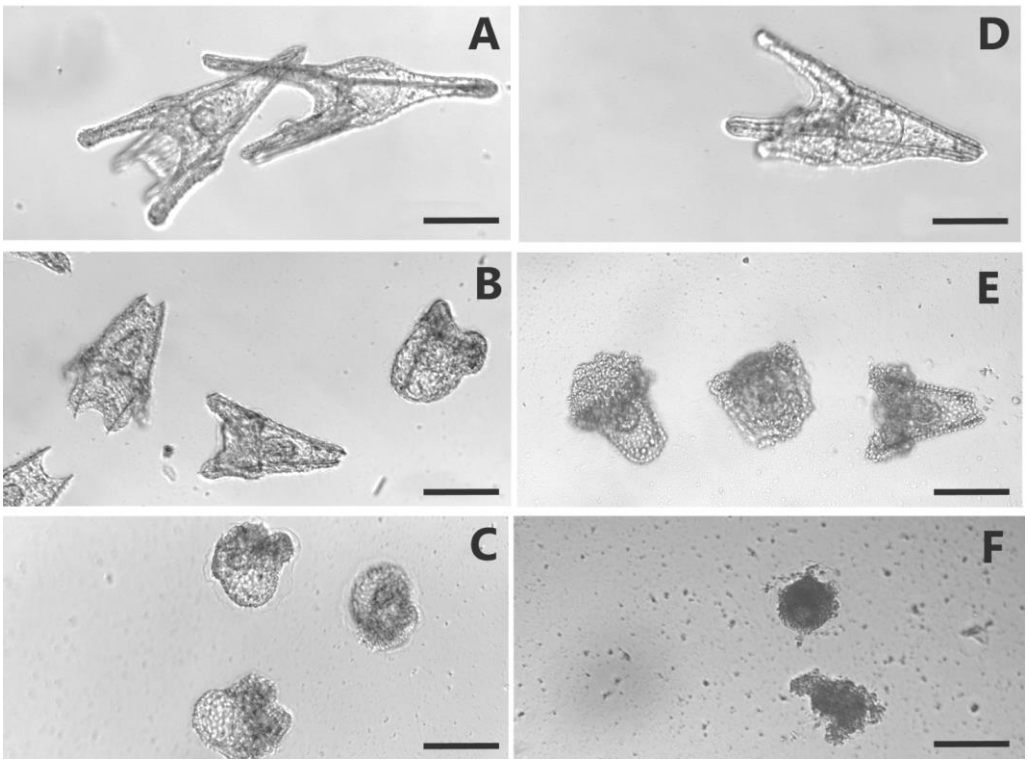

Figure 3. Photographs of sea urchin *P. lividus* larvae observed after 48 hr of exposure to Finasol OSR52® dispersant exposure in both experiments. Experiment-1 (low oil-loading ratio a shorter stirring time): **A)** Normal pluteus larvae; **B)** 8% D LEWAF; **C)** 34% D LEWAF; and in Experiment-2 (high oil-loading ratio and longer stirring time): **D)** Normal pluteus larvae; **E)** 8% D LEWAF; **F)** 21% D LEWAF.

3.2.2. NNA and NNA+D

There was a progressive reduction in ΔL at increasing concentrations of both NNA and NNA+D LEWAF in both experiments, with a more marked decrease in Experiment-2 than in Experiment-1 (Figs. 4A and 4B). Thus, whilst in Experiment-1 $EC50_{(NNA)}$ could not be calculated (>100% LEWAF) and $NOEC_{(NNA)}$ was 89% LEWAF, in Experiment-2 the $EC50_{(NNA)}$ was 71% LEWAF and $NOEC_{(NNA)}$ was 55% LEWAF. Moreover, the reduction was more marked in NNA+D LEWAF than in NNA LEWAF in

both experiments; the $EC50_{(NNA+D)}$ values were lower than 21% LEWAF in Experiment-1 and 42% LEWAF in Experiment-2, and $NOEC_{(NNA+D)}$ values were 8% and 21% LEWAF in Experiment-1 and Experiment-2, respectively (Figs. 4A, 4B, 8A, 8B, 8G and 8H).

In Experiment-2, both larval abnormalities (TI) and development disruption (CDI, GDI, MDI and IPLFI) were quantified. TI increased linearly at increasing concentrations of both NNA and NNA+D LEWAF (Fig. 5A). The slope of TI against exposure concentration in NNA+D LEWAF was significantly higher than in NNA LEWAF (ANCOVA; $p<0.05$; Fig. 5A). Thus, whilst $EC50_{(NNA)}$ was 77% LEWAF and $NOEC_{(NNA)}$ was 34% LEWAF, the $EC50_{(NNA+D)}$ was 46% LEWAF and $NOEC_{(NNA+D)}$ was 21% LEWAF. CDI increased linearly at increasing concentrations of NNA+D LEWAF whereas it remained around 0% on exposure to NNA LEWAF (Fig. 6A). $EC50_{(NNA+D)}$ was 43% LEWAF and $NOEC_{(NNA+D)}$ was 34% LEWAF (Fig. 6A). GDI increased linearly at increasing concentrations of NNA and NNA+D LEWAF (Fig. 6B). The slope of GDI against exposure concentration in NNA+D LEWAF was significantly higher than in NNA LEWAF (ANCOVA; $p<0.05$; Fig. 6B). Thus, whilst $EC50_{(NNA)}$ was 78% LEWAF and $NOEC_{(NNA)}$ was 34% LEWAF, the $EC50_{(NNA+D)}$ was 45% LEWAF and $NOEC_{(NNA+D)}$ was 34% LEWAF. MDI increased linearly at increasing concentrations of NNA+D LEWAF, whereas it remained around 0% on exposure up to 100% NNA LEWAF, when it suddenly rose to 89% LEWAF (Fig. 6C). $EC50_{(NNA)}$ was 93% LEWAF, $NOEC_{(NNA)}$ was 34%, $EC50_{(NNA+D)}$ was 42% LEWAF and $NOEC_{(NNA+D)}$ was 34% (Fig. 6C). IPLFI increased linearly at increasing concentrations of NNA+D LEWAF, whereas it remained around 0% on exposure up to 100% NNA LEWAF when it suddenly rose to 96% LEWAF (Fig. 7A). Thus, $EC50_{(NNA)}$ was 80% LEWAF, $NOEC_{(NNA)}$ was 34%, $EC50_{(NNA+D)}$ was 44% LEWAF and $NOEC_{(NNA+D)}$ was 21% LEWAF (Fig. 7A).

3.2.3. MGO and MGO+D

There was a progressive reduction in ΔL at increasing concentrations of both MGO and MGO+D LEWAF in both experiments (Figs. 4C and 4D). Thus, in Experiment-1 $EC50_{(MGO)}$ was 46% LEWAF and $NOEC_{(MGO)}$ was 8% LEWAF and in Experiment-2 the $EC50_{(MGO)}$ was 54% LEWAF and $NOEC_{(MGO)}$ was 34% LEWAF. The reduction was similar in MGO LEWAF and MGO+D LEWAF; the $EC50_{(MGO+D)}$ values

were lower than 33% LEWAF in Experiment-1 and 44% LEWAF in Experiment-2, and $NOEC_{(MGO+D)}$ values were 21% and 34% LEWAF in Experiment-1 and Experiment-2, respectively (Figs. 4C, 4D, 8C, 8D, 8I and 8J).

In Experiment-2, MGO and MGO+D LEWAF treatments also rendered a linear increase in TI (ANCOVA; $p>0.05$; Fig. 5B). Thus, toxicity critical threshold values were similar in MGO LEWAF and in MGO+D LEWAF (Fig. 5B): $EC50_{(MGO)}$ was 51% LEWAF, $NOEC_{(MGO)}$ was 55% LEWAF, $EC50_{(MGO+D)}$ was 45% LEWAF and $NOEC_{(MGO+D)}$ was 34% LEWAF. CDI increased linearly at increasing concentrations of MGO+D LEWAF, whereas it remained around 0% on exposure to MGO LEWAF (Fig. 6D). $EC50_{(MGO+D)}$ was 46% LEWAF and $NOEC_{(MGO+D)}$ was 55% LEWAF (Fig. 6D). After both MGO and MGO+D LEWAF treatments the same linear increase in GDI and MDI was found at increasing LEWAF concentrations (ANCOVA; $p>0.05$; Fig. 6E-6F). Toxicity critical threshold values were similar in MGO LEWAF and in MGO+D LEWAF (Fig. 6E-6F). For GDI, $EC50_{(MGO)}$ was 50% LEWAF, $NOEC_{(MGO)}$ was 34% LEWAF, $EC50_{(MGO+D)}$ was 44% LEWAF and $NOEC_{(MGO+D)}$ was 34% LEWAF. For MDI, $EC50_{(MGO)}$ was 47% LEWAF, $NOEC_{(MGO)}$ was 34% LEWAF, $EC50_{(MGO+D)}$ was 42% LEWAF and $NOEC_{(MGO+D)}$ was 34% LEWAF. After both MGO and MGO+D LEWAF treatments the same linear increase in IPLFI was found at increasing LEWAF concentrations (ANCOVA; $p>0.05$; Fig. 7B). Toxicity critical threshold values were similar in MGO LEWAF and in MGO+D LEWAF (Fig. 7B): $EC50_{(MGO)}$ was 49% LEWAF, $NOEC_{(MGO)}$ was 8% LEWAF, $EC50_{(MGO+D)}$ was 44% LEWAF and $NOEC_{(MGO+D)}$ was 34% LEWAF.

3.2.4. IFO and IFO+D

There was a progressive reduction in ΔL at increasing concentrations of both IFO and IFO+D LEWAF in both experiments, with a more marked decrease in Experiment-2 than in Experiment-1 (Figs. 4E and 4F). Thus, whilst in Experiment-1 $EC50_{(IFO)}$ was 44% LEWAF and $NOEC_{(IFO)}$ was 1% LEWAF, in Experiment-2 the $EC50_{(IFO)}$ was 28% LEWAF and $NOEC_{(IFO)}$ was 21% LEWAF. The reduction was more marked in IFO+D LEWAF than in IFO LEWAF, especially in Experiment-1; the $EC50_{(IFO+D)}$ values were lower than 13% LEWAF in Experiment-1 and 8% LEWAF in Experiment-2, and

$NOEC_{(IFO+D)}$ values were 2% and 8% LEWAF in Experiment-1 and Experiment-2, respectively (Figs. 4E, 4F, 8E, 8F, 8K and 8L).

In Experiment-2, after both IFO and IFO+D LEWAF treatments, TI reached a value of 100 on exposure to 34% IFO LEWAF and to 21% IFO+D LEWAF (Fig. 5C). Toxicity critical threshold values were higher in IFO LEWAF than in IFO+D LEWAF (Fig. 5C): $EC50_{(IFO)}$ was 22% LEWAF, $NOEC_{(IFO)}$ was 21% LEWAF, $EC50_{(IFO+D)}$ was 7% LEWAF and $NOEC_{(IFO+D)}$ was <8% LEWAF. CDI increased linearly at increasing concentrations of IFO+D LEWAF, whereas it remained around 0% on exposure to IFO LEWAF (Fig. 6G). Thus, $EC50_{(IFO+D)}$ was 45% LEWAF and $NOEC_{(IFO+D)}$ was 21% LEWAF (Fig. 6G). GDI increased linearly at increasing concentrations of IFO LEWAF whereas it already reached a value of 100 on exposure to 21% IFO+D LEWAF (Fig. 6H). Thus, $EC50_{(IFO)}$ was 52% LEWAF, $NOEC_{(IFO)}$ was 21% LEWAF, $EC50_{(IFO+D)}$ was 6% LEWAF and $NOEC_{(IFO+D)}$ was <8% LEWAF (Fig. 6H). No linear regression was found to be significant for MDI neither for IFO LEWAF nor for IFO+D LEWAF (Fig. 6I). In both cases an all-or-nothing response was observed, with the following toxicity critical threshold values: $EC50_{(IFO)}$ was 48% LEWAF, $NOEC_{(IFO)}$ was 21%, $EC50_{(IFO+D)}$ was 11% LEWAF and $NOEC_{(IFO+D)}$ was 8% LEWAF (Fig. 6I). IPLFI reached a value of 100 on exposure to 34% IFO LEWAF and to 21% IFO+D LEWAF (Fig. 7C). Thus, toxicity critical threshold values were higher in IFO LEWAF than in IFO+D LEWAF (Fig. 7C): $EC50_{(IFO)}$ was 27% LEWAF, $NOEC_{(IFO)}$ was 21% LEWAF, $EC50_{(IFO+D)}$ was 9% LEWAF and $NOEC_{(IFO+D)}$ was <8% LEWAF

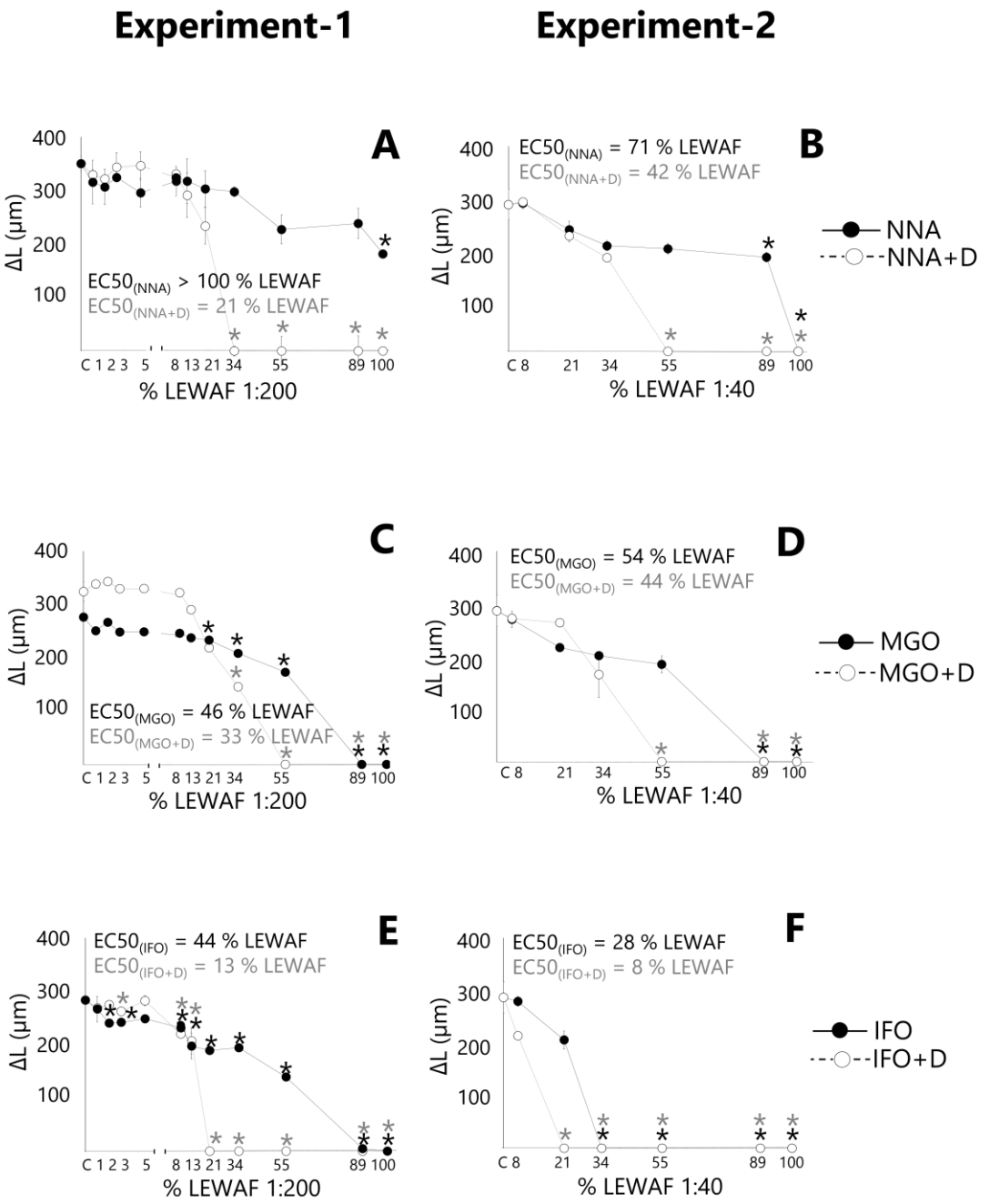

Figure 4. Size increase (ΔL in μm) of sea urchin larvae exposed to oil LEWAF and oil+D LEWAF produced in Experiment-1: **A)** NNA and NNA+D LEWAFs; **C**) MGO and MGO+D LEWAFs; **E)** IFO and IFO+D LEWAFs; and in Experiment-2: **B)** NNA and NNA+D LEWAFs; **D)** MGO and MGO+D LEWAFs; **F)** IFO and IFO+D LEWAFs. Values are given in μm (mean ± SD). Asterisks indicate significant differences between each exposure concentration and its respective control group (ANOVA; $p<0.05$). Median effective concentrations (EC50) were calculated after probit analysis.

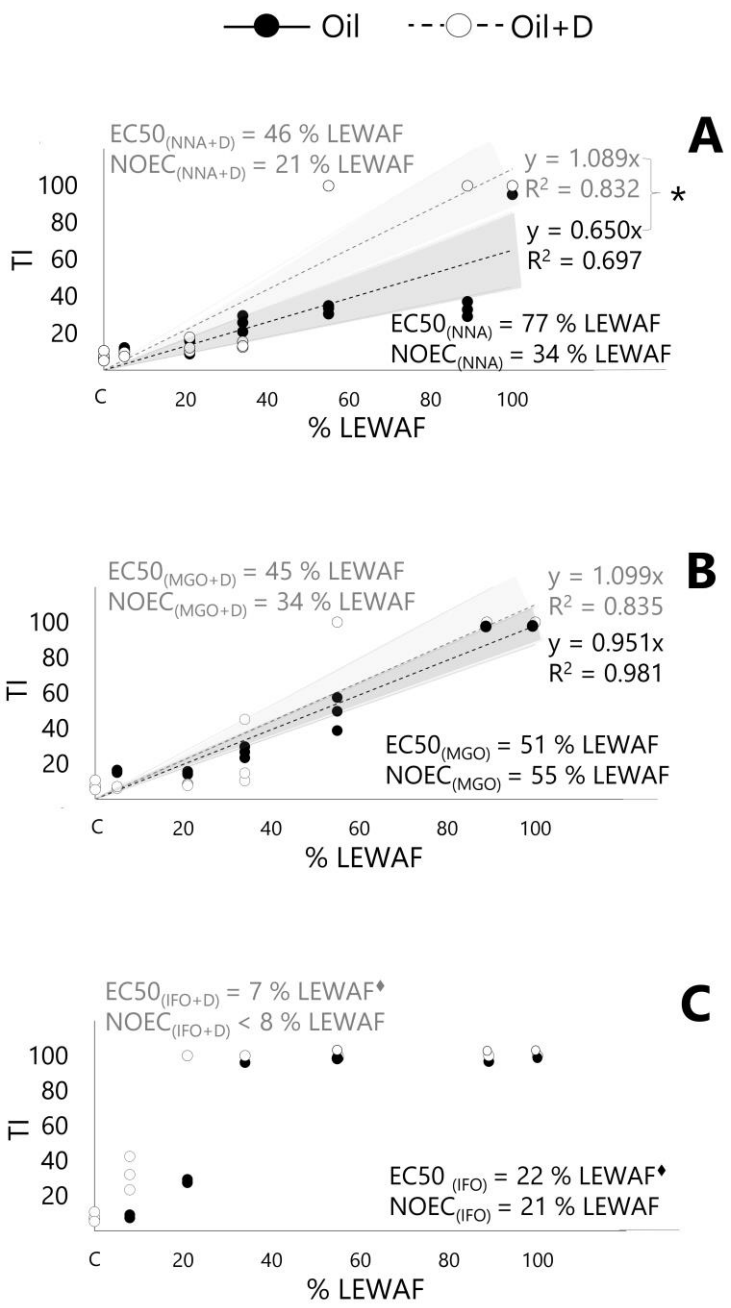

Figure 5. TI (Toxicity Index) recorded in sea urchin embryos exposed to oil and oil+D LEWAFs: **A)** NNA and NNA+D LEWAFs; **B)** MGO and MGO+D LEWAFs; and **C)** IFO and IFO+D LEWAFs. LEWAF production: 1:40 w/v oil:FSW; 1:10 w/w oil:dispersant; 72 hr stirring; 10ºC. Median effective concentrations calculated upon linear regression models (EC50) or after probit analysis (EC50♦), and non-observed effect concentration (NOEC) values are shown for each case. Only significant regression models are represented (p<0.05). Asterisks indicate significant differences between linear regression coefficients of oil and oil+D LEWAF for each tested oil (ANCOVA; p<0.05).

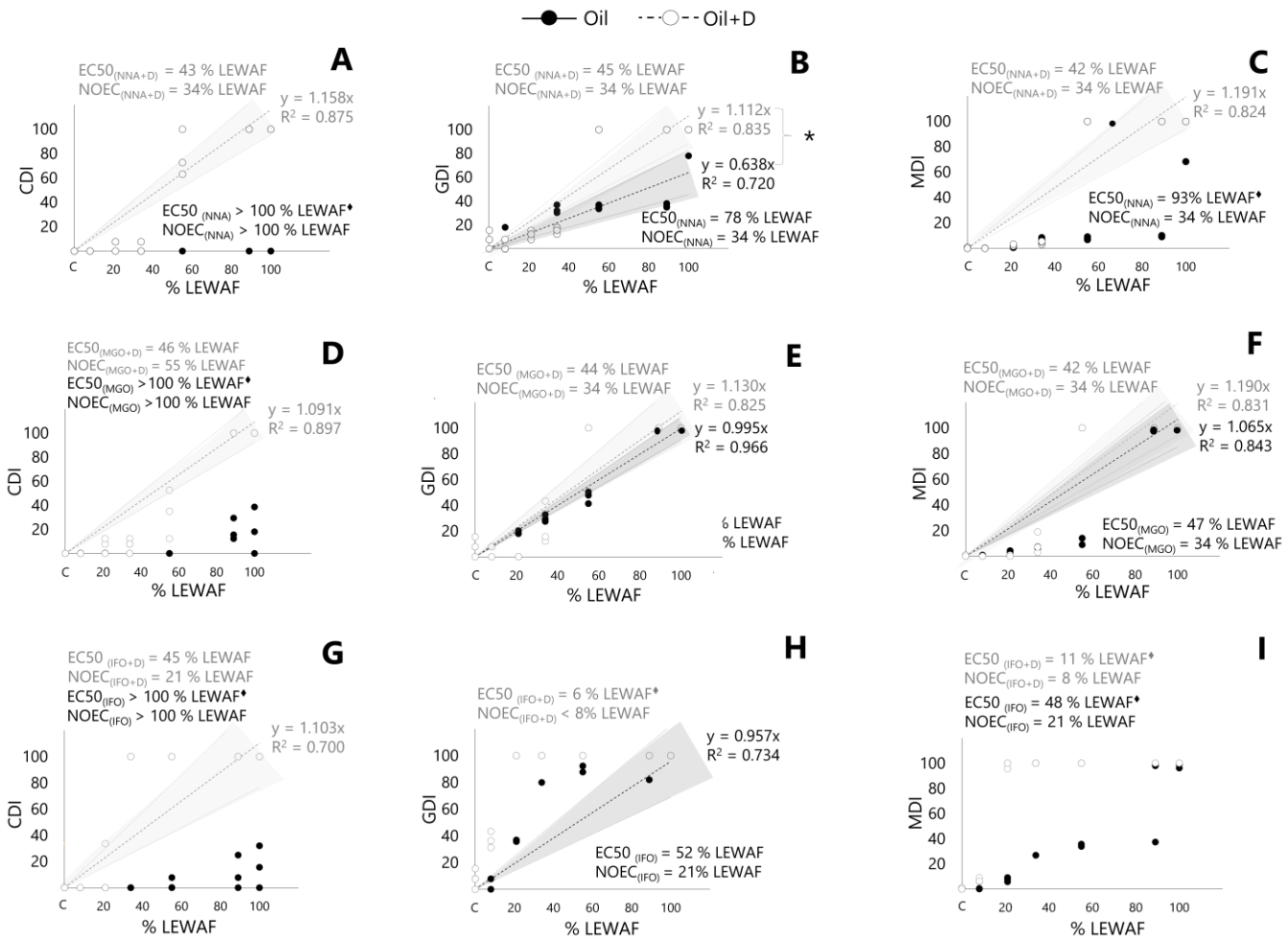

Figure 6. CDI (Cleavage Disruption Index), GDI (Gastrulation Disruption Index), MDI (Metamorphosis Disruption Index) recorded in sea urchin embryos exposed to oil and oil+D LEWAFs: **A-C)** NNA and NNA+D LEWAFs; **D-F)** MGO and MGO+D LEWAFs; and **G-I)** IFO and IFO+D LEWAFs. LEWAF production: 1:40 w/v oil:FSW; 1:10 w/w oil:dispersant; 72 hr stirring; 10ºC. Median effective concentrations calculated upon linear regression models (EC50) or after probit analysis (EC50♦), and non-observed effect concentration (NOEC) values are shown for each case. Only significant regression models are represented *(p*<0.05). Asterisks indicate significant differences between linear regression coefficients of oil and oil+D LEWAF for each tested oil (ANCOVA; *p*<0.05).

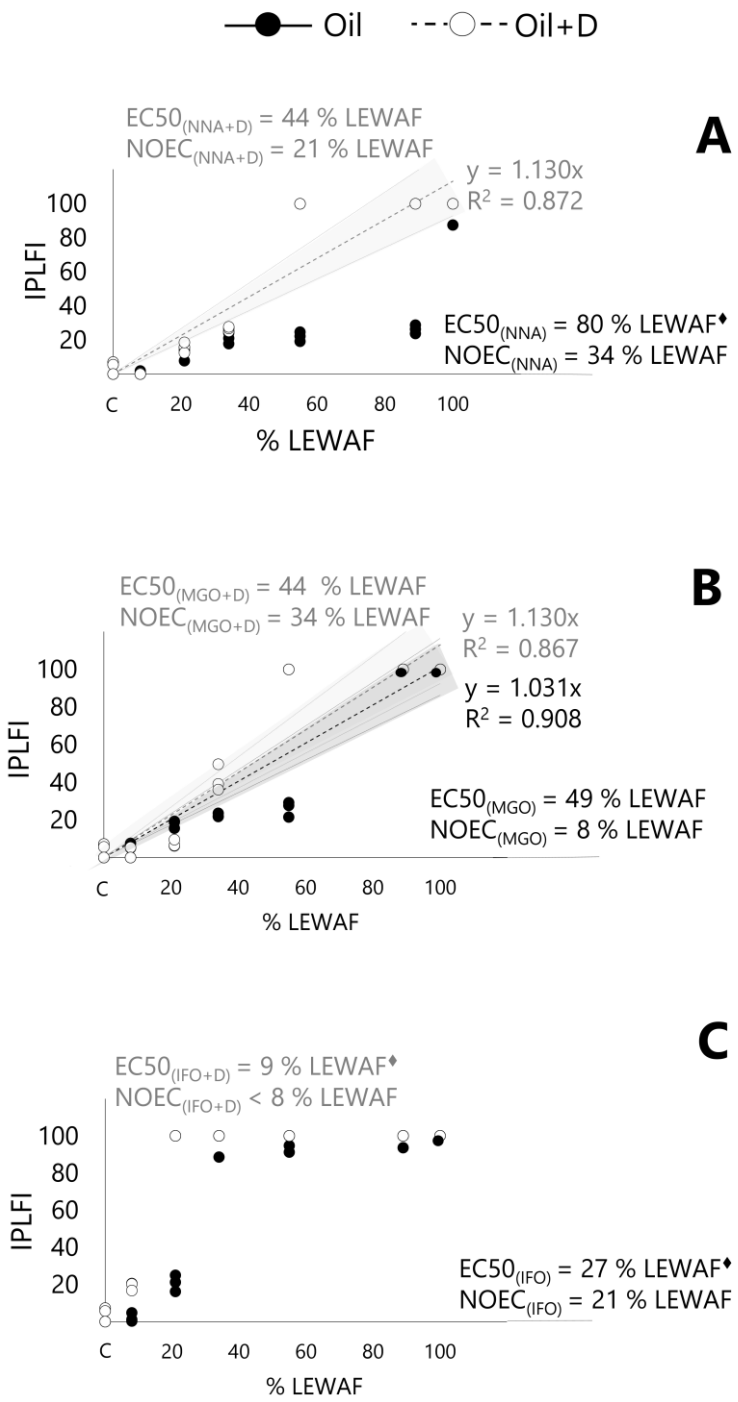

Figure 7. IPLFI (Inhibition Pluteus Larvae Formation Index) recorded in sea urchin embryos exposed to oil and oil+D LEWAFs: **A)** NNA and NNA+D; **B)** MGO and MGO+D; **C)** IFO and IFO+D LEWAF production: 1:40 w/v oil:FSW; 1:10 w/w oil:dispersant; 72 hr stirring; 10ºC. Median effective concentrations calculated upon linear regression models (EC50) or after probit analysis (EC50♦), and non-observed effect concentration (NOEC) values are shown for each case. No significant differences were found between linear regression coefficients of oil and oil+D LEWAF for each tested oil (ANCOVA; $p>0.05$).

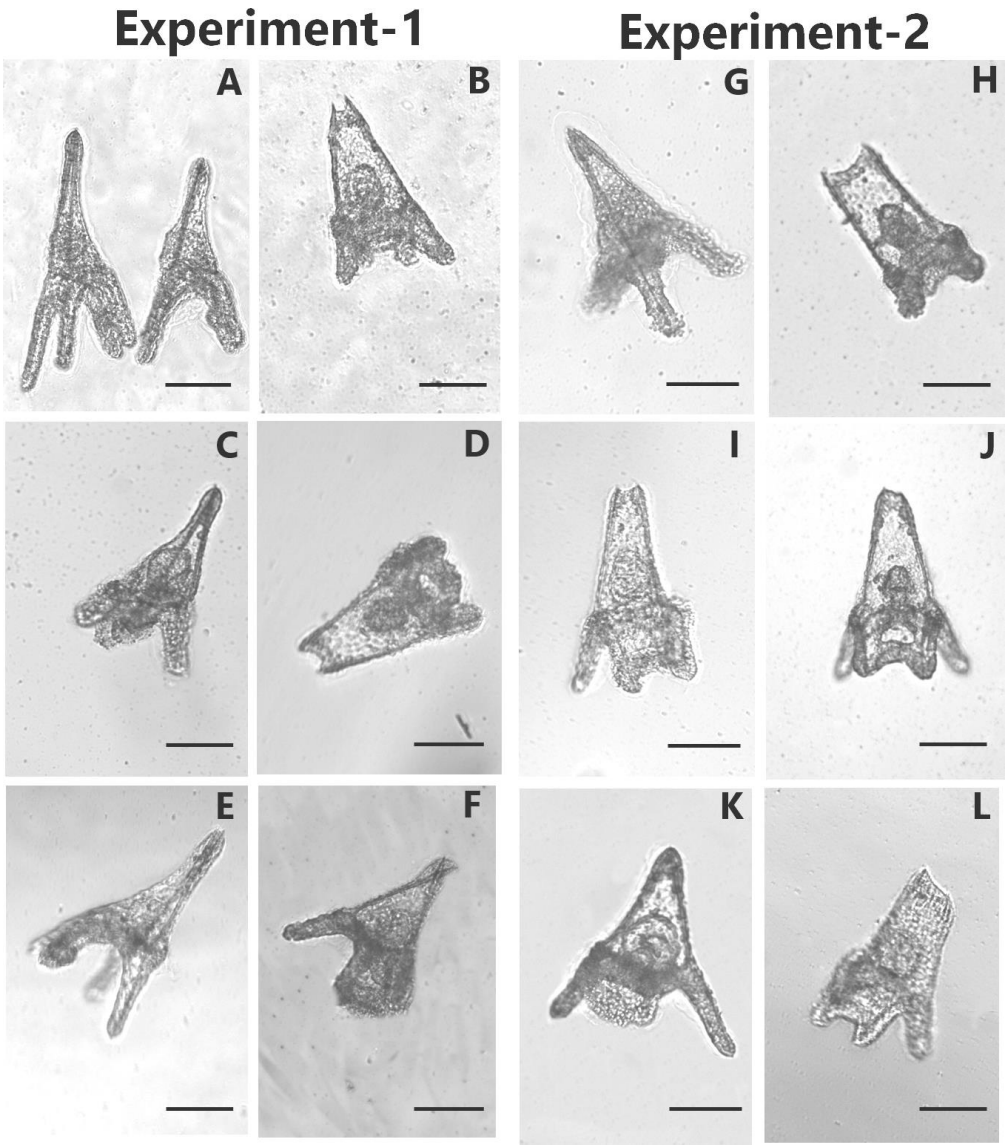

Figure 8. Photographs of sea urchin *P. lividus* larvae observed after 48 hr of exposure to oil and oil+D in Experiment-1 (A-F) and Experiment-2 (G-L). Pictures corresponding to EC50 values in size increase (ΔL) or near to them. **A)** 100% NNA LEWAF; **B)** 21% NNA+D LEWAF; **C)** 55% MGO LEWAF; **D)** 34% MGO+D LEWAF; **E)** 34% IFO LEWAF; **F)** 8% IFO+D LEWAF; **G)** 89% NNA LEWAF; **H)** 34% NNA+D LEWAF; **I)** 55% MGO LEWAF; **J)** 34% MGO+D LEWAF; **K)** 21% IFO LEWAF; **L)** 8% IFO+D LEWAF. Scale bars 100 µm.

4. DISCUSSION

Differences in the PAH profile were found depending on the oil-loading ratio and stirring time. These might be explained because the LEWAFs of the present study might not be in a stable state of equilibrium after 40-72 hr of mixing (Johann et al., 2020b), as it was expected when the experiments were designed following existing guidelines (Singer et al., 2000). It is conceivable that the conditions to reach the stable state of equilibrium vary depending on e.g. the oil characteristics and the temperature of LEWAF production, among other factors (Curl and O'Donnell, 1977; Faksness et al., 2008). Under the present conditions, the steady state for NNA (produced at 10ºC, 1:200 w oil: w FSW) is achieved after 50-100 hr of stirring (Bilbao et al*., submitted),* which would justify the slight differences presently found between Experiment-1 (40 hr) and Experiment-2 (72 hr). 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 addition of dispersant, the sum of PAHs was the highest in MGO+D LEWAF, with Flu, Phe and 4-ring PAHs as major contributors. This can be explained because MGO has lower viscosity than NNA (reduced dispersability: 3000-7000 cP) and IFO (poor/slow dispersability: >7000 cP) which makes it easy dispersable (<3000 cP), resulting in high concentrations of substituted naphthalenes, aromatic hydrocarbons and other toxic compounds in the water column (EMSA, 2010).

As a whole, the PAH profiles of oil LEWAFs and oil+D LEWAFs were dominated by conspicuous concentrations of Naph, 1-MN, 2-MN, Ace, Flu and Phe (n-C_9-C_{18}) in the three tested oils. These light 2-3 ring PAHs seem to be quite common in oil LEWAFs. For instance, the 99% of hydrocarbons also belong to the n-C_9-C_{18} fraction in diesel LEWAF, with peaks of Naph and 2-MN (Brown et al., 2016). Nevertheless, there were some differences amongst the PAH profiles of the three oil LEWAFs and, moreover, 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 the half of the concentrations measured in MGO and IFO LEWAFs. Likewise, the concentration of

Ace was 2-3 times higher in IFO LEWAF than in the other two oil LEWAFs, whilst the concentration of Flu was practically one half of the others. As result, the toxicity levels and the toxicological profile of the three LEWAFs can be expected to be different. On the other hand, the addition of Finasol OSR52® caused:

- A large increase in the Naph concentration in the three oil+D LEWAFs, less markedly in IFO+D LEWAF;
- A slight increase in the concentration of 1-MN, Phe, Pyr and Fluo in NNA+D LEWAF;
- A remarkable increase in 3-4 ring PAHs, especially Flu and Phe, and a subtle increase in 5-6 ring PAHs in MGO+D LEWAF;
- A notable increase in 3-ring PAHs, including Ant, but most remarkably in 4-ring (e.g., B[a]A+Chr) and 5- and 6-ring (e.g. B[a]P) PAHs in IFO+D LEWAF.

Overall, together with a different level of toxicity upon addition of dispersant in comparison with the pure tested oils, different modes of action can be expected, as these depend on the number of rings and molecular weight of the PAHs, amongst other factors (Black et al., 1993; Incardona et al. 2004; Adams et al., 2014).

4.1. Toxicity of oil LEWAF

In the present study, 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. This can be explained because Naph, which has been shown to exert 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 exhibit a non-polar narcotic toxicity mode of action (De Hoop et al., 2011) and cause effects on eggs through irregularities in cleavage pattern and formation of blastula embryos (Falk-Petersen et al., 1982). However, it seems that in the present study early embryo stages are less sensitive to WAF exposure than pluteus larvae, as also seen in the Antartic sea urchin *Sterechinus neumayeri* (Alexander et al., 2017). In agreement with the latter, exposure to the LEWAF of the three oils had no effect on early embryo developmental stages (cleavage disruption) as revealed by low CDI values, whereas in GDI, the parameter indicative of gastrulation disruption, a dose dependent response was recorded. In agreement, PAHs are known to 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 known to be involved in cell/cell adhesion and cell fate specification during embryo development (Oliveri et al., 2008); and exposure to PAHs causes β-catenin accumulation in cell nuclei of sea urchin *Lytechinus anemesis* embryos (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 known to be 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, known to be strong drivers at least for acute embryo toxicity (Incardona et al. 2004, 2005; Hodson et al., 2007; Lee et al 2011; Le Bihanic et al. 2014). For instance, in weathered Alaska North Slope crude oil the toxicity of the mixture depended on the proportion of 3-ring compounds, particularly Phe or the total 3-ring PAH fraction (Incardona et al., 2004). Indeed, the toxicity of 3-ring PAHs can be nearly 3 times greater than the toxicity of Naph (Black et al., 1983). On the one hand, due to their lipophilicity and effects on K^+ and Ca^{++} 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). The heavy oil IFO 180 is rich in 2-MN and Phe (Johann et al., 2020b), which are known to induce strong adverse developmental effects also in fish embryos (Adams et al., 2014; Bornstein et al., 2014; Johann et al., 2020b). Interestingly, sea urchin *(P. lividus)* embryos seem to be particularly sensitive to the LEWAF of heavy oils. They are more sensitive to IFO 380 and fuel N.6 LEWAF exposure than copepods and fish embryos, and as sensitive as mussel larvae (Saco-Álvarez et al., 2008).

Despite of the differences in the PAH profile among the 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). Toxicity data for other identified PAHs are not reported for sea urchin *P. lividus* embryos but are available for other aquatic test organisms. The 96-hr LC50 of Ace for fish juveniles *(C. variegatus*; *O. mykiss* and *Salmo trutta)* is in the range of 0.6-3 mg Ace/L (Ward et al., 1981; Holcombe et al., 1983). Mysids *(Americamysis bahia)* and decapods *(Janicellas pinicauda)* are more sensitive than fish *(Oithona davisae)* to 1-MN exposure, to which corals *(Porites divaricata)* are not very sensitive with a 48-hr LC50 of 12 mg 1-MN/L (Barata et al., 2005; Renegar et al., 2017). Conversely, corals *(P. divaricata)* are very sensitive to 2-MN (48-hr LC50=0.2 mg 2-MN/L) to which crustaceans *(Cancer magister)* are less sensitive (Renegar et al., 2017). The range of LC50 values obtained for various Arctic species including arthropods, molluscs and fish was 0.4-5.4 mg 2-MN/L (Olsen et al., 2011). 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 including vegetalization and archenteron evagination (Pillai et al., 2003). In any case, these toxicity critical values are much higher than the ones recorded in the present study for individual 3-ring PAHs in oil LEWAFs (<0.1 µg/L) 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. Thus, for instance, the $EC50_{GDI}$ was 0.24 mg PAH/L (78% LEWAF) for NNA and 0.08 mg PAH/L for MGO and IFO (50-52% LEWAF), and the $EC50_{MDI}$ was 0.07 mg PAH/L (47% LEWAF) for MGO. These values are within the range of the 96-hr consensus

value (0.5 mg PAH/L) used for ecological risk assessments 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 among 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).

Mixtures of compounds exerting only one (narcotic or specific) mode of action can be modelled satisfactorily by assuming Concentration Addition (CA) joint activity (Altenburger et al., 2003). Presently, toxic units (TUs), the concentrations in a mixture of individual pollutants expressed as fractions of the EC50 of each pollutant (Sprague, 1970), were calculated to visualize the toxic power of each individual PAH and give an idea of the individual contribution to the toxicity of the LEWAFs (Table 4). For this purpose, EC50 values for various individual PAHs (Naph, Fluo, 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, braquiopods, decapods, mollusc larvae, echinoderm larvae and fish juveniles) were used as consensus EC50 to calculate the TUs (Appendix VI; Ward et al., 1981; Holcombe et al., 1983; Trucco et al., 1983; Spehar, 1999; Lyons et al., 2002; Pillai et al., 2003; Calbet et al., 2007; Bellas et al., 2008; Frantzen et al., 2012; Renegar et al., 2017; Knap et al., 2017). Further on, the relative contribution of each individual PAH to the TUs of the mixture ($\sum TU_{\sum PAHs}$) was determined as $RT_i = TU_{PAHi} / \sum TU_{\sum PAHs}$; where TU_{PAHi} means the TU estimated for this individual PAH. In parallel, the relative concentration of each PAH in the mixture was calculated as $RC_i = C_{PAHi} / \sum PAHs$; where C_{PAHi} stands for the individual concentration of

each PAH. Thus, the ratio RT_i/RC_i was interpreted 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 CA model). Regarding NNA, MGO and IFO LEWAFs, the sum of TUs was below "1" for all the toxicity endpoints investigated (Table 4), indicating that the identified individual PAHs were not the main cause for toxicity of the oil LEWAFs. However, the RT_i was always higher than "1", especially for IFO LEWAF, suggesting that one or more individual PAHs exhibited more toxicity than the one that could be predicted for the mixture toxicity. Indeed, 2-MN in all the oil LEWAFs and Phe in NNA and MGO LEWAFs could be responsible for a part of the toxicity, as suggested by RT_i/RC_i values higher than "1" (Table 4). This is interesting because both PAHs have the same mode of action: 2-MN is an alkylated naphthalene belonging to the group of highly persistent low molecular weight PAHs that causes narcosis (Irwin et al., 1997) and Phe is a 3-ring PAH also causing non-polar anaesthetic effects (Incardona et al., 2005).

Table 4. Summary of the TU analysis of the toxicity of LEWAFs based on the mixture of identified PAHs. The sum of TUs (∑TU) for each toxicity endpoint (ΔL, TI, IPLFI, CDI, GDI and MDI) is "1" if there is additive toxicity, ">1" if there is synergistic effect and "<1" if the toxicity is not caused by the mixture assuming the CA joint action. The sum of the TUs of individual PAHs vs. the TUs of the sum of PAHs ($\sum TU_{PAHi}/TU_{\sum PAHs}$) is "1" if all the PAHs in the mixture exert the same toxicity, ">1" if there are one or more individual PAHs with more toxicity than expected from its contribution to the mixture according to the CA model; and "<1" otherwise. The balance between the relative contribution of an individual PAH to the toxicity of the mixture and its relative contribution to the chemical composition of the mixture (RT_i/RC_i) is "1" if the individual toxicity of this PAH is the one expected due to its proportion in the mixture (CA model); "<1" if it is not a contributor to the mixture toxicity; and ">1" if there this PAH exerts toxicity beyond the one expected as a part of the mixture.

	NNA LEWAF	**NNA+D LEWAF**	**MGO LEWAF**	**MGO+D LEWAF**	**IFO LEWAF**	**IFO+D LEWAF**
$\sum TU_{\Delta L}$	0.44	0.14	0.12	0.63	1.34	0.21
$\sum TU_{TI}$	0.48	0.13	0.09	0.68	1.37	0.18
$\sum TU_{IPLFI}$	0.49	0.12	0.11	0.65	1.34	0.23
$\sum TU_{CDI}$	0.62	0.25	0.42	0.64	1.40	1.15
$\sum TU_{GDI}$	0.48	0.13	0.22	0.67	1.34	0.15
$\sum TU_{MDI}$	0.58	0.12	0.20	0.63	1.28	0.28
$\sum TU_{PAHi}/TU_{\sum PAHs}$	1.69	1.42	2.15	2.21	2.55	4.15
RT/RC_{Naph}	0.10	0.12	0.08	0.08	0.07	0.04
$RT/RC_{1\text{-}MN}$	0.81	0.96	0.63	0.62	0.54	0.33
$RT/RC_{2\text{-}MN}$	2.94	3.51	2.32	2.25	1.96	1.20
RT/RC_{Acy}	-	-	-	0.45	0.39	0.24
RT/RC_{Ace}	0.65	0.77	0.51	0.50	0.43	0.27
RT/RC_{Flu}	0.25	0.30	0.20	0.19	0.17	0.10
RT/RC_{Ant}	-	-	-	-	-	10.22
RT/RC_{Phe}	1.17	1.39	0.92	0.89	0.78	0.48
RT/RC_{Pyr}	-	-	-	2.97	2.58	1.58
RT/RC_{Fluo}	-	-	-	1.51	1.32	0.81
$RT/RC_{B[a]A + Chr}$	-	-	-	38.28	33.28	20.44
$RT/RC_{B[a]P}$	-	-	-	-	2.22	1.36

The TU approach has been often applied to evaluate the toxicity of aqueous fractions of oils; however, it limits the characterization of the mixture toxicity only in terms of the sum of identified PAHs (Meador and Nahrgang, 2019). In contrast, oil aqueous fractions 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 et al. 2007; Bellas et al., 2008; Engraff et al., 2011; Barron et al., 1999; Wheeler et al., 2020). PAHs are just a small fraction of the aqueous concentration of organic compounds found in this complex mixture; hence, the toxic potential of the non-PAH fraction cannot be neglected. In most crude oils, PAHs constitute less than 1% of the total petroleum hydrocarbons (Sammarco et al., 2013) and most of the compounds are unidentified and commonly known as the unresolved complex mixture (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 sulphur, nitrogen, and oxygen that can constitute a major portion of its aqueous fraction (Melbye et al., 2009). Presently, with the exception of a certain degree of narcosis caused 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 as it would rather be exerted by individual or combined toxic action of other compounds present in the LEWAFs that have not been identified in this study.

4.2. Toxicity of the dispersant

Except for the dispersant Slickgone NS, which was shown not to be toxic to sea urchin embryos (Alexander et al., 2017), a wide variety of dispersants is toxic to diverse marine invertebrates (Verriopoulos 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 $EC50_{(D)}$ 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-hr EC50 for ΔL in *P. lividus* was 1.2 mg

Finasol OSR51/L (Rial et al., 2014) and the 72-hr 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-hr LC50 was 9 mg Finasol OSR52/L for the mysid *Americamysis bahia* and the 96-hr LC50 was 12 mg Finasol OSR52/L for the Atlantic silverside *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-hr LC50 of Finasol OSR52® for seabass juveniles (77 mg/L) was also lower than the LC50 values of other dispersants such as Corexit9500A, Slickgone NS and Inipol IP90 (Dussauze et al., 2015). Alike, the 48-hr LC50 values for *A. bahia* were 28 mg Finasol OSR52/L and 120 mg Corexit 9500A/L, and the 96-hr 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 Accell 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. Similarly, other studies also showed that adding dispersant to crude oils enhances the toxicity of the water-accommodated fraction (WAF) of the oils. 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 (Katsumiti et al., 2019). Dispersant (Finasol OSR51®) and oil+dispersant LEWAF were shown to be more toxic than oil LEWAF for sea urchin embryos (Rial et al., 2014). Augmented toxicity was reported in coral larvae exposed to the WAF of Egyptian light crude oil

combined with various dispersants (Inipol IP90, Bioreico R93, Emulgal C100, Biosolve and Petrotech PTI25; Epstein et al., 2000), and in cold water corals exposed to MASS light oil spiked with Corexit 9500A (DeLeo et al., 2016). Finally, enhanced toxicity was reported also in copepods exposed to Iranian heavy crude oil mixed with Corexit 9500 and Hiclean (Lee et al., 2013). Exceptionally, Slickgone seems to be non-toxic to embryos of the Antarctic sea urchin, *Sterechinus neumayeri*, and hence the addition of this dispersant 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, early embryo developmental stages (CDI) were altered on exposure to oil+D LEWAF of the three tested oils. Likewise, dispersant application 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 on oil toxicity could be explained because dispersant addition may increase the amount of PAHs and alter the PAH profile in the LEWAFs (Yamada et al., 2003; DeLorenzo et al., 2017). Similarly, a greater bioavailability of oil components was found to be the cause for enhanced acute toxicity of oil mixed with Finasol OSR52® in zebrafish embryos (Johann et al 2020a). Indeed, in the present study, the PAH levels and composition in the LEWAFs changed upon the addition of dispersant in comparison with the LEWAFs of oils without dispersant. Thus, together with n-C_9-C_{18} PAHs found in oils without dispersant (Naph, 1-MN, 2-MN, Ace, Flu and Phe) other major components of the LEWAF were found after dispersant application. These included n-C_{19}-C_{28} PAHs such as Pyr and B[a]A+Chr that were identified in the LEWAFs of the three tested oils, and Acy (n-C_9-C_{18}), and Fluo (n-C_{19}-C_{28}) that were identified in MGO+D and IFO+D LEWAFs, and Ant (n-C_9-C_{18}) and 5- and 6-ring PAHs (n-C_{19}-C_{28}) in IFO+D LEWAF. In quantitative terms, the addition of dispersant 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-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, there was an increase in the proportion of individual 5- and 6-ring PAHs in LEWAF of IFO 180 combined with Finasol OSR52® in comparison with the LEWAF of the oil without dispersant. A comparable enrichment in high molecular weight PAHs in oil WAF was observed also after application of Corexit 9500 to weathered Mesa light crude oil (Couillard et al., 2005).

As discussed above for the case of pure oil LEWAFs, the toxicity of mixtures of oil and dispersant could be partially attributed to the narcosis caused by the 2- and 3-ring PAHs (Irwin et al., 1997; Incardona et al., 2005), which were dominant PAH compounds identified in oil+D LEWAF:

- 2-ring PAHs such as Naph, 1-MN and 2-MN in the three oil+D LEWAFs;
- 3-ring PAHs such as Acy, Ace, Flu and Phe in MGO+D LEWAF, and Ace, Flu, Ant and Phe in IFO+D LEWAF.

Indeed, the relative proportion of these PAHs in the mixture in comparison with the total concentration of identified PAHs was greater than 99% in NNA+D LEWAF and 87% in the other two oil+D LEWAFs. However, the augmented toxicity observed (especially for MGO and more markedly for IFO 180) upon de application of dispersant might be also attributed to the higher concentration of 4- to 6-ring PAHs recorded in comparison with the LEWAF of pure oils:

- 4-ring PAHs such as Pyr, Fluo and B[a]A+Chr in MOG+D and IFO+D LEWAF;
- 5- and 6-ring PAHs such as B[a]P, B[b]F+B[k]F, B[g,h,i]P, D[ah]A and Ind[1,2,3-cd]P in IFO+D LEWAF.

Certainly, 4-ring PAHs (e.g., B[a]A) are known to influence gastrulation in sea urchin embryos, *Hemicentrotus pulcherrimus* through disturbance of the vascular endothelial growth factor signaling pathway and to 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) were shown to be toxic to fish embryos acting via the AHR-CYP1A pathway (Aryl Hydrocarbon Receptor-Cytochrome P450 1A; Incardona et al., 2004). AHR, known to be altered upon PAH exposure, is present in sea urchins (Goldstone et al., 2006). The kinase p38 MAPK is a signaling molecule regulated by the AHR that plays a crucial role in gastrulation and spiculogenesis regulation (Puga et al., 2009). The inhibition of this kinase up to the morula stage would cause anomalies in gastrulation whereas at the morula/blastula transition it would cause impairment in skeleton formation and morphogenesis (Caterina et al., 2008). Therefore, it is conceivable that altered gastrulation (GDI) and spiculogenesis (MDI) could be the result, at least partially, of AHR mediated changes in p38 MAPK upon PAH exposure. In addition, the 5-ring PAHs identified in IFO+D LEWAF are known to cause genotoxicity and carcinogenesis, as well as endocrine disruption, through direct interaction with the DNA (Baird et al., 2005). Thus, it is feasible that, together with the non-polar narcosis that 2- and 3-ring PAHs could cause, enhanced toxicity upon application of the dispersant could result from alterations caused by 4-ring PAHs in AHR functioning, as well as from genotoxicity, carcinogenicity and endocrine disruption caused by 5-ring PAHs. As a whole, early interactions between PAHs and factor governing embryo development could be already initiated and evident at the 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 in the present study 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 Fluo) 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; Appendix VI). *P. lividus* embryos are as sensitive as bivalves (mussels, oysters) to Pyr, which is much less toxic to fish, *Mallotus villosus* (Lyons et al., 2002; Bellas et al., 2008; Frantzen et al 2012). Fluo is highly toxic for *P. lividus* embryos but these are less sensitive than clam *(Mulinea lateralis)* 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 such as *Arbacia lixula* (Fluo) and *Lytechinus anemesis* (Flu) (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. Ant is more toxic for clams *(M. lateralis)* than for brachiopods *(Artemia salina)*, for which Chr is less toxic than Ant (Kagan et al., 1987; Pelletier et al., 1997; Appendix VI). B[a]A and B[a]P are highly toxic to brachiopods *(Daphnia pulex)* and molluscs *(Crassostrea gigas),* although B[a]P is less toxic for the latter (Trucco et al., 1983, Lyons et al., 2002). In any case, these critical thresholds values are much lower than the ones recorded in the present study for individual 3- to 6-ring PAHs in oil+D LEWAFs (<0.3 µg/L) 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 $EC50_{GDI}$ 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 $EC50_{MDI}$ was 0.06 mg PAH/L (11% LEWAF) for IFO+D.

The sum of TUs was higher than in the case of oil LEWAFs but still 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 4). Thus, although in MGO+D LEWAF and in the particular case of cleavage disturbance on exposure to IFO+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.

Overall, one or more individual PAHs exhibited more toxicity than predicted for the mixture toxicity. On the one hand, the sum of the TUs of individual PAHs was always higher than the TUs corresponding to the sum of PAHs ($\Sigma TU_{PAHi}/TU_{\Sigma PAHs} > 1$), revealing that some individual PAHs deviated from the CA joint action. On the other hand, RT_i/RC_i values were greater than "1" for several individual PAHs (Table 4), and therefore it is conceivable that these were responsible for a part of the toxicity, say:

- 2-MN, Pyr and Fluo in NNA+D LEWAF;
- 2-MN, Pyr, Fluo and B[a]P in MGO+D LEWAF;
- 2-MN, Pyr and B[a]P but most remarkably Ant in IFO+D LEWAF;
- B[a]A+Chr in the three oil+D LEWAFs.

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).

4.4. Sea urchin embryo assays in oil spill risk assessment

The sea urchin embryo toxicity test is a reliable, sensitive and inexpensive tool to assess the toxicity of pollutants including metals, pharmaceuticals, biocides and complex mixtures such as e.g. the WAF of oils (Fernández et al., 2006; Saco-Alvarez et al., 2008; Bellas et al., 2013; Morroni et al., 2016). Most commonly, the endpoints of sea urchin embryo toxicity assays are restricted to analyses at the pluteus stage, without recording earlier alterations such as embryo malformation and arrest or delay of embryogenesis and metamorphosis (Morroni et al., 2016). The simple observation of normal vs. abnormal embryos is certainly rapid and easy to evaluate (Beiras et al., 2003). The toxicity criteria can be qualitative (normal *vs.* abnormal

larvae; Pagano et al., 1996; Quiniou et al., 1999, Canada Standard, 2011), semi-quantitative (grading of abnormalities; Carballeira et al., 2012) or quantitative (length increase; Saco-Alvarez et al., 2010; Beiras et al., 2012). Measuring length increase is efficient, objective and sensitive compared to classical qualitative grading/scoring of abnormal larvae (Saco-Alvarez et al., 2010). Yet, rather than as sign of altered growth (which can be understood from its name), this endpoint should be interpreted in terms of skeletal alterations during metamorphosis. In this transition, there is no feeding or growth but reshaping, leading to elongated and hollow differentiated larvae that would be able to swim, feed and settle; which are ecologically relevant traits (O'Donnell et al., 2010).

In an alternative approach to improve the sensitivity of sea urchin embryo toxicity assays, Carballeira et al. (2012) recorded the percentage of abnormal larvae including four categories of skeletal malformation that included pre-larva non-developed stages, which were used to calculate the toxicity index. The formation of the larval skeleton was conceived as a central event in sea urchin morphogenesis because the skeleton supports the larval body and determines its shape playing a role in orientation and swimming (Pennington and Strathmann, 1990; Ettensohn and Malinda, 1993). Thus, early skeletal alterations may decrease the ability of larvae to swim, feed, avoid predators and settle (O'Donnell et al., 2010) and compromise larval survival and continuity of the sea urchin population, which is ecologically relevant (Carballeira et al., 2012). On the other hand, the most common effects observed in the development of sea urchin embryos toxicants are a delay in developmental progression and the impairment of embryo differentiation and larvae metamorphosis (Fernández et al., 2006; Saco-Alvarez et al., 2008; Bellas et al., 2013; Sekiguchi et al., 2018). In sand dollar *(Peronella japonica)* and sea urchin *(Heliocidaris erythrogramma)*, the sensitivity to chemicals varied from cleavage to metamorphosis, with gastrulation and larval metamorphosis being more sensitive than early cleavage and pluteus formation (Kobayashi, 1980). Yet, the toxic effects elicited in early embryo stages, such as the first cleavage after 90 min of fertilization (Kobayashi, 1990) or the gastrula stage after 24 hr of fertilization (Morroni et al., 2016), are only rarely used as the endpoints. Exceptionally, Morroni et al. (2016)

recorded the percentage of normally developed embryos at two different stages, gastrula and pluteus, to calculate an integrative index of toxicity that weighted the severity at each stage, which was a relevant approach to enhance the sensitivity of the *P. lividus* embryo-toxicity assay in case of moderate toxicity. Presently, together with the SET and the TI assays, the SEDD assay was applied to test the toxicity of crude and bunker oils of interest in boreal iced seas.

The sensitivity of ecologically relevant endpoints such as ΔL, TI and IPLFI varied (Table 5). For instance, IPLFI and TI revealed toxic effects at exposure levels below EC50 values whilst in the case of ΔL 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 ΔL, TI and IPLFI, which suggest 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 was originated before gastrulation, during cleavage. Nevertheless, 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. Overall, the sea urchin embryo toxicity assays that include various developmental stages in their analyses provide us with improved sensitivity to discriminate from slight to severe levels of toxicity. This appears to be particularly relevant for moderately toxic but environmentally realistic mixtures such as e.g. sediment elutriates (Morroni et al., 2016), effluents (Carballeira et al., 2012) or the LEWAF of oils alone or in combination with dispersants (present study).

Table 5. Schematic representation of toxicity critical values recorded in sea urchin embryos for CDI, GDI, MDI, IPLFI, TI and ΔL on exposure to NNA, NNA+D, MGO, MGO+D, IFO and IFO+D LEWAFs. **Green**: NOEC or lower concentration; **yellow**: concentration between NOEC and EC50; **orange:** EC50 or higher concentration; **red**: 100% effect (EC100).

TOXICITY ENDPOINT	TESTED OIL(+D)	% LEWAF						
		0	8	21	34	55	89	100
CDI	NNA							
	NNA+D							
	MGO							
	MGO+D							
	IFO							
	IFO+D							
GDI	NNA							
	NNA+D							
	MGO							
	MGO+D							
	IFO							
	IFO+D							
MDI	NNA							
	NNA+D							
	MGO							
	MGO+D							
	IFO							
	IFO+D							
IPLFI	NNA							
	NNA+D							
	MGO							
	MGO+D							
	IFO							
	IFO+D							
TI	NNA							
	NNA+D							
	MGO							
	MGO+D							
	IFO							
	IFO+D							
ΔL	NNA							
	NNA+D							
	MGO							
	MGO+D							
	IFO							
	IFO+D							

5. CONCLUDING REMARKS

In the present study, the influence of the application of a third generation dispersant (Finasol OSR52®) upon the toxicity of crude and bunker oils of interest in boral iced seas was investigated using sea urchin embryotoxicity tests. 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, it was concluded that the identified individual PAHs were not the main cause for toxicity of the oil LEWAFs, and toxicity of oil+D LEWAFs might be mediated by at least three potential modes of action (non-polar narcosis, AHR-CYP1A pathway and direct interactions with DNA). Whereas no effect on early sea urchin embryo developmental stages was shown for any of the LEWAF of the three oils tested, disruption in gastrulation and metamorphosis was observed confirming that the responsiveness of the various developmental stages to oil toxicity is different. Thus, sea urchin embryo toxicity assays that include various developmental stages in their analyses provide us with improved sensitivity to discriminate from slight to severe levels of toxicity, being particularly relevant for moderately toxic but environmentally realistic mixtures such as the LEWAFs of the studied oils alone and in combination with dispersant.

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CHAPTER 2

Influence of temperature in sea urchin embryo toxicity of crude and bunker oils alone and mixed with dispersant

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De Miguel L, Izagirre U, Lekube X, Marigómez I. Toxicity to sea urchin embryos of WAFs produced at different temperatures with crude oils alone and mixed with dispersant. Platform presentation.

29th ANNUAL MEETING OF THE SOCIETY OF ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY (SETAC)-EUROPE, Helsinki, 26-30 May 2019.

De Miguel L, Bilbao D, Etxebarria N, Lekube X, Izagirre U, Marigómez I. Comparative toxicity of light oil, intermediate oil and diesel oil WAFs alone and mixed with dispersant: sea urchin bioassays. Poster and spotlight presentation.

ABSTRACT

New maritime trade routes, enhanced by globalization and aided by climate change-driven ice retreat, constitute an emerging threat of oil spills after tanker accidents in the Arctic and Subarctic region. The severity of the impact of an oil spill and the efficiency of the oil spill response depends on environmental conditions, such as water temperature. In the Arctic and Subarctic region the seawater surface temperature (SST) may vary in the range of around 5-25ºC along the year, depending on the season and the geographical area. Thus, the oil spill properties, dispersant efficiency and oil toxicity can be very different depending on the SST and the type of oil, influencing oil spill impact and response. Therefore, the present investigation aims at determining how temperature modifies the toxicity of crude and bunker oils of interest in iced seas (Naphthenic North Atlantic crude oil (NNA), marine gas oil (MGO), and an intermediate fuel oil IFO 180 (IFO)) and how this toxicity is influenced by the application of a commonly used dispersant (Finasol OSR52®). For these purposes, a multi-index approach including larval size increase, larval malformation, developmental disruption and genotoxicity as endpoints was applied. The polycyclic aromatic hydrocarbons (PAH) profiles were found to be different depending on the oil and the temperature of LEWAF (Low-Energy Water Accommodated Fraction) production. However, the sum of PAHs only showed minor variations amongst the LEWAFs of the three oils produced at different temperatures. Upon dispersant application, the sum of PAHs in LEWAFs was lower at high production temperatures (20-25ºC) in the cases of NNA and MGO, and unrelated to production temperature in IFO. Moreover, the values were overall much higher than in the case of the corresponding oil LEWAFs without dispersant. The level of DNA damage caused by exposure to oil and oil+D LEWAFs varied depending on the LEWAF production temperature in a different way for each oil. Moreover, the degree of genotoxicity was augmented after dispersant application. Likewise, oil LEWAF of the three tested oils caused length reduction, abnormalities and development impairment in pluteus larvae of *Paracentrotus lividus*, irrespective of the temperature of LEWAF production, although the severity of the effects varied with the oil type, dispersant application and LEWAF production temperature. The toxicity of the LEWAFs of the three studied oils, alone or in combination with dispersant, could be only partially attributed to individual PAHs or to the mixture; thus, a large part of the toxicity is conceived to be due to the unresolved complex mixture (UCM) and polar compounds present in those LEWAFs. Nevertheless, the use of nominal proportion loading (% LEWAF) values seems to be a useful best available practice for toxicity assessment of oil aqueous fractions produced at different temperatures in the 5-25ºC range.

Keywords: oil, temperature, dispersant, sea urchin embryo, toxicity, mixtures.

RESUMEN

La aparición de nuevas rutas marítimas de índole comercial, potenciadas por la globalización y favorecidas por el retroceso de hielo provocado por el cambio climático, constituyen una amenaza emergente, en la región ártica y subártica, de los vertidos de petróleo tras accidentes de barcos y petroleros. La gravedad del impacto que tiene un vertido de petróleo y la eficacia de la respuesta al mismo dependen de las condiciones ambientales como, por ejemplo, la temperatura del agua. En la región ártica y subártica, la temperatura de la superficie del agua del mar (SST) puede variar en un rango de unos 5-25ºC a lo largo del año, dependiendo de la estación y de la zona geográfica. Por lo tanto, las propiedades de los vertidos de petróleo, la eficacia de los dispersantes y la toxicidad del petróleo pueden ser muy diferentes en función de la SST y del tipo de petróleo, lo que influye en el impacto y la respuesta a dichos vertidos. Así, la presente investigación tuvo el objetivo de determinar cómo la temperatura modifica la toxicidad del LEWAF (Low-Energy Water Accommodated Fraction) de tres tipos de petróleo (el crudo ligero: Naphthenic North Atlantic (NNA)); el diésel: Marine Gas Oil (MGO); y el fueloil: Intermediate Fuel Oil 180 (IFO)), de interés en mares boreales mediante y cómo esta toxicidad se ve influenciada por la aplicación de un dispersante comúnmente utilizado (Finasol OSR52®). Para estos propósitos se aplicó una aproximación utilizando multi-índices entre los que se incluyeron los indicadores de longitud larvaria, malformación larvaria, alteración del desarrollo y genotoxicidad. Los perfiles de hidrocarburos policíclicos aromáticos (PAH) eran diferentes según el tipo de petróleo y la temperatura de producción de los LEWAFs. Sin embargo, la suma de PAHs solamente mostró mínimas variaciones entre los LEWAFs de los tres petróleos producidos a las distintas temperaturas. Tras la aplicación del dispersante, la suma de PAHs en los LEWAFs fue menor a altas temperaturas de producción (20-25ºC) en los casos de NNA y MGO, y en cambio, en el caso de IFO no tuvo relación con la temperatura de producción. Además, los valores de PAHs fueron mucho más altos en el caso de los LEWAFs de los petróleos mezclados con dispersante que los correspondientes LEWAFs sin dispersante. El daño al ADN producido por la exposición al LEWAF del petróleo solo y mezclado con dispersante varió en función de la temperatura de producción de dichos LEWAFs de manera distinta para cada uno de los petróleos. El grado de genotoxicidad aumentó tras la aplicación del dispersante. Así mismo, los LEWAF de los tres petróleos provocaron una reducción de la longitud larvaria, anormalidades y una deficiencia en el desarrollo de los embriones de erizo de mar *Paracentrotus lividus*, independientemente de la temperatura de producción de los LEWAF; aunque la gravedad de dichos efectos varió con el tipo de petróleo, la aplicación de dispersante y la temperatura de producción de los LEWAF. La toxicidad de los LEWAF de los tres petróleos, solos o en combinación con el dispersante, sólo pudo atribuirse parcialmente a los PAH individuales o a la mezcla; así, se concibe que una gran parte de la toxicidad se debe a las concentraciones de UCM y a los compuestos polares presentes en esos LEWAF. No obstante, el uso de concentraciones nominales (% LEWAF) parece ser una buena práctica disponible para la evaluación de la toxicidad de las fracciones acuosas del petróleo producidas a diferentes temperaturas en el rango de 5-25ºC.

Palabras clave: petróleo, temperatura, dispersante, embrión de erizo de mar, toxicidad, mezclas.

RESUM

L'aparició de noves rutes marítimes d'índole comercial, potenciades per la globalització i afavorides pel retrocés de gel produït pel canvi climàtic, constitueixen una amenaça emergent, en la regió àrtica i subàrtica, dels vessaments de petroli produïts després d'accidents de vaixells i petroliers. La gravetat de l'impacte que té un vessament de petroli i l'eficàcia de la resposta al mateix depenen de les condicions ambientals com, per exemple, la temperatura de l'aigua. A la regió àrtica i subàrtica la temperatura de la superfície de l'aigua (SST) pot variar en el rang d'uns 5-25ºC al llarg de l'any, depenent de la temporada i l'àrea geogràfica. Per tant, les propietats del vessament de petroli, l'eficiència dispersant i la toxicitat del petroli poden ser molt diferents segons la SST i el tipus de petroli, influint en l'impacte i la resposta a aquests vessaments. Per tant, la investigació actual va tenir com a objectiu determinar com la temperatura modifica la toxicitat de diferents petrolis d'interès en mars glaçats (un petroli lleuger (Naphthenic North Atlantic crude oil; NNA), un diesel marí (Marine Gas Oil; MGO) i un fueloil pesat (Intermediate Fuel Oil 180; IFO) i com aquesta toxicitat es veu influenciada per l'aplicació d'un dispersant comunament utilitzat (Finasol OSR52®). Per a aquest propòsit es va aplicar una aproximació utilitzant multi-índex entre els que es van incloure la longitud larvària, la malformació larvària, l'alteració del desenvolupament i la genotoxicitat. Els perfils d'hidrocarburs policíclics aromàtics (PAH) van ser diferents segons el petroli i la temperatura de la producció de LEWAF (Low-Energy Water Accommodated Fraction). No obstant, la suma de PAHs només va mostrar mínimes variacions entre els LEWAF dels tres petrolis produïts a diferents temperatures. Després de l'aplicació de dispersant, la suma de PAHs en els LEWAFs va ser més baixa a altes temperatures de producció (20-25ºC) en els casos de NNA i MGO, i en canvi, en el cas d'IFO no estava relacionada amb la temperatura de producció. A més, en general, els valors eren molt més alts en el cas dels LEWAFs dels petrolis barrejats amb dispersant que els corresponents LEWAFs sense dispersant. El nivell de dany d'ADN causat per l'exposició al LEWAF del petroli, amb i sense dispersant, va variar en funció de la temperatura de producció dels LEWAFs de manera diferent per a cadascun dels petrolis. Després de l'aplicació dispersant el grau de genotoxicitat va augmentar. De la mateixa manera, el LEWAF dels tres petrolis va provocar una reducció de la longitud larvària, anormalitats i una deficiència en el desenvolupaments dels embrions d'eriçó de mar *Paracentrotus lividus* independentment de la temperatura de producció de LEWAF, tot i que la gravetat dels efectes va variar amb el tipus de petroli, l'aplicació de dispersant i la temperatura de producció del LEWAF. La toxicitat dels LEWAF dels tres petrolis, sols o en combinació amb el dispersant, només es va poder atribuir parcialment als PAHs individuals o a la mescla; per tant, una gran part de la toxicitat va ser deguda a les concentracions d'UCM i als compostos polars presents en aquests LEWAFs. No obstant, l'ús de concentracions nominals (% LEWAF) sembla ser una bona pràctica útil per a l'avaluació de toxicitat de fraccions aquoses de petroli produïdes a diferents temperatures en el rang de 5-25ºC.

Paraules clau: petroli, temperatura, dispersió, embrió d'eriçó de mar, toxicitat, mescles.

LABURPENA

Globalizazioaren eta klima-aldaketak eragindako urtzaldiaren ondorioz, itsas merkataritza bide berriak ireki dira. Bide horien erabilerak ordea petrolio isurien mehatxua dakar, artikoko eta azpiartikoko eremuetan, itsasontzi eta petrolio-ontzien istripuengatik, batik bat. Petrolio isuri baten inpaktuaren maila eta petrolio isuriari aurre egiteko neurrien eraginkortasuna ingurumen-baldintzen araberakoa da, hala nola uraren tenperaturaren araberakoa. Eskualde artikoan eta azpiartikoan, itsas-azaleko tenperaturak (SST) 5ºC eta 25ºC arteko balioak har ditzake urtean zehar, urtaroaren eta eremu geografikoaren arabera. Horrela, petrolio isuriaren propietateak, sakabanatzailearen eraginkortasuna eta petrolioaren toxikotasuna oso desberdinak izan daitezke, SSTaren eta olio motaren arabera, olio isuriaren eragina eta ematen zaion erantzuna baldintzatuz. Horregatik, ikerketa honen helburua SST tenperaturaren eragina aztertzea izango da. Batetik, itsaso izoztuetan olioen toxikotasunean temperatura horrek duen eragina aztertuko da, bereziki olio gordin eta bunker olio (Naphthenic North Atlantic petrolio gordina, itsas gasezko olioa eta tarteko erregai olio bat IFO 180) isurietan. Bestetik ohikoa den sakabanatzailearen (Finasol OSR52®) funtzionamenduan duen eragina ere aztertuko da. Helburu horiek lortzeko, itsas-trikuen larba-tamainaren gehikuntza, larba-malformazioa, garapen-nahastea eta genotoxikotasuna moduko adierazleak kontuan hartzen dituen indize multimodal bat erabili da. PAH (polycyclic aromatic hydrocarbon) profilak olioaren eta LEWAFaren (Low-Energy Water Accommodated Fraction) tenperaturaren arabera desberdinak direla ikusi da. Hala ere, PAHen (polycyclic aromatic hydrocarbons) batuketak aldaketa txikiak dituela antzeman da tenperatura ezberdinetan sorturiko hiru olioen LEWAFetan. Sakabanatzailea aplikatu ondoren, PAHen kopurua LEWAFetan baxuagoa izan da produkzio-tenperatura altuetan (20º-25ºC) NNA eta MGren kasuetan, eta ez zegoen produkzio-tenperaturarekin erlazionaturik IFOan. Gainera, PAH balioak askoz handiagoak izan dira sakabanatutako LEWAFetan sakabanatu gabekoetan baino. Petrolioa eta LEWAFen olioak eragindako ADN kalteen maila LEWAFen produkzio-tenperaturaren arabera aldatu da, modu ezberdin batean petrolio mota bakoitzarentzat. Gainera, genotoxikotasun maila handitu egin zen sakabantzailea erabili ondoren. Era berean, frogatutako hiru olioen LEWAFek *Paracentrotus lividus* itsas-trikuen larbaren luzera gutxitzea, anormaltasunak eta garapen urritasuna eragin ditu. LEWAFen produkzio tenperaturak eraginik ez duela ikusi ahal izan da, baina ondorioen larritasuna aldakorra da petroleo motaren, sakabanatzailea aplikatzearen eta LEWAFen produkzio tenperaturaren arabera. Aztertutako hiru olioen LEWAFen toxikotasuna, bakarrik edo sakabanatzailearekin konbinatuta, PAH indibidualaren edo nahasketaren eragina bakarrik dela ondorioztatu ahal izan da; horrela, toxikotasunaren zati handi bat LEWAFen dauden nahasketa konplexu (UCM) eta polar konposatuen ondorio dela pentsatzen da. Hala eta guztiz ere, proportzio nominalak (% LEWAF) erabiltzea praktika on bat dela dirudi, 5ºC eta 25ºC-ko tartean sortutako petrolio akuoso zatien toxikotasuna aztertzeko.

Hitz gakoak: olioa, tenperatura, sakabanatzailea, itsas-triku enbrioia, toxikotasuna, nahasteak.

1. INTRODUCTION

Oil spills after tanker accidents account for 10–15% marine oil pollution world-wide (Tornero and Hanke, 2016). The number of incidents resulting in oil pollution has declined in the last decades (ITOPF 2016), but it is still very likely that they will occur once and again. Particularly, enhanced by globalization and aided by climate change driven ice retreat, new maritime trade routes constitute an emerging threat in the Arctic and Subarctic region (Arctic Council, 2009; Pirotta et al., 2019), where meteorological and environmental conditions can be extreme and accessibility very limited due to remoteness, thus jeopardising oil spill response.

The severity of the impact of an oil spill and the efficiency of the oil spill response depend on environmental conditions, such as water temperature (USEPA, 1999). The annual average sea surface temperature (SST) in the hottest areas of the Arctic (Norwegian Sea, Greenland Sea and Barents Sea) shows values between -1 and 7°C, with seasonal maximum SST in summer-autumn around 4°C (Carvalho and Wang, 2020). Annual mean SST is around 5ºC in spring and 10ºC in late summer in the Baltic Sea and around 15ºC in temperate summer in the North Sea, whilst maximum SST in summer in the Gulf of Bothnia can be as high as 25ºC (Stigebrandt and Gustafsson, 2003; Siegel and Gerth, 2018). Thus, in the Arctic and Subarctic region the SST temperature may vary in the range of around 5-25ºC along the year, depending on the season and the geographical area.

The oil spill properties, dispersant efficiency and oil toxicity can be very different depending on SST and the type of oil. Oil is less likely to spread in very cold waters than in warmer waters because surface tension drops; and it depends on the oil pour point, the lower it is (e.g. -39ºC in naphthenic crude oils vs. -6ºC in IFO 180) the easier the oil will spread on the water surface (Faskness et al., 2008). Dispersants can work in cold water (Sørstrøm et al., 2010), albeit they seem to be less efficient than in warm water (Fingas et al., 1991; Chandrasekar et al., 2005). The degree of toxicity exerted by PAHs and other oil components is known to be influenced by seawater temperature (Vieira and

Guilhermino, 2012; Perrichon et al., 2018; Serafin et al., 2019), particularly upon dispersant addition (Ramachandran et al., 2004, 2006).

Moreover, the chemical profile of the water accommodated fraction (WAF) of oils, commonly used for oil toxicity assessment, also varies with the temperature in an oil type specific fashion (Faskness et al., 2008). This chemical profile is very unlike that of the parent oil due to different water solubility of the various oil components, the relatively highly soluble components (semi-volatiles such as naphthalenes and phenols) being generally dominant (Faskness et al., 2008). Generation of a saturated WAF takes longer time in colder seawater (2ºC) than at 13ºC, more remarkably in the case of oils with higher pour point (wax rich oils) than in oils with lower pour points (naphthenic oils) (Faskness et al., 2008).

However, studies about the influence of temperature in the toxicity of crude oils to marine organisms, alone or after dispersant application, are scarce and their experimental designs are disparate. Several deal with responses at different exposure temperatures but the test aqueous oil fractions were produced only at one temperature (Korn et al., 1979; Lyons et al., 2011; Camus et al., 2015; Perrichon et al., 2018; Serafin et al., 2019). Other studies use aqueous fractions produced at various temperatures. Saeed et al. (1998) used the Microtox test to compare the toxicity exerted by the water-soluble fraction (WSF) of Kuwait crude oil produced at different temperatures in the 15-35ºC range. Katsumiti et al. (2019) performed *in vitro* toxicity studies using hemocytes of marine mussel, *Mytilus galloprovincialis*, to compare the responses elicited by the WAF of a naphthenic North Sea crude oil produced at 10, 15 and 20ºC without and with dispersant. Li et al. (2021) investigated the toxicity to sea cucumber, *Apostichopus japonicus*, of Oman crude oil WAF produced at 2 temperatures (16 and 26ºC) after exposure to the WAF at the corresponding production temperatures.

Carried out within the framework of the UE-funded project GRACE (Jørgensen et al., 2019), the present study, was conceived to investigate the influence of temperature on the toxicity to sea urchin embryos of the low-energy WAF (LEWAF) of crude and bunker oils and how this is influenced by the application of dispersant. Standard conditions for

production of oil aqueous fractions (Singer et al., 2000) were modified in order to reflect regionally relevant temperatures in the Arctic and Subartic seas. The *Paracentrotus lividus* sea urchin embryo toxicity test (Beiras et al., 2012) was selected to interpret the toxicity of the tested chemicals against existing data on oil toxicity (Bellas et al., 2008; 2013; Saco-Álvarez et al., 2008; Beiras et al., 2012; Chapter 1) before the use of regionally relevant autochthonous test species is feasible. As a consequence, toxicity tests were conducted at one exposure temperature (20ºC; optimal for the test species employed) irrespective of the temperature of LEWAF production. Moreover, sea urchins are a well-known experimental model for DNA damage and repair studies (Le Bouffant et al., 2007, Le Bouffant et al., 2008; El-Bibany et al., 2014; Reinardy and Bodnar, 2015; et al., 2016).

2. MATERIALS AND METHODS

2.1. LEWAF production and chemical analyses

Three petroleum compounds and one dispersant were selected as relevant regarding potential oil spills in Arctic regions (Appendix I):

- *Naphthenic North Atlantic crude oil (NNA),* a very light crude oil of low viscosity, rich in branched and cyclic saturated hydrocarbons.
- *Marine gas oil (MGO),* a distillate marine gas oil, supplemented with the dye Dyeguard Green MC25 (John Hogg Technical Solutions; UK).
- *Intermediate Fuel Oil IFO 180 (IFO),* a heavy bunker oil of high viscosity with low amounts of volatile hydrocarbons (Polaroil, Greenland).
- *Finasol OSR52® dispersant (D),* a third-generation dispersant containing >30% non-ionic and 15–30% anionic surfactants (Total Special Fluids, France; SDS no. 30034 2015).

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 and IFO+D LEWAF) was produced in the darkness at 5, 10, 15, 20 and 25ºC according to Katsumiti et al. (2019), modified after Singer et al. (2000). Briefly, oils (1:200; w oil/v FSW), dispersant (1:2000; w D/v FSW) and their mixtures (1:10 w D/w oil+D 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 hr. Successive dilutions in FSW (8, 21, 34 and 55%) of LEWAF alone or mixed with dispersant were prepared. The dilutions were selected following a Fibonacci dose escalation between 0 and 100% LEWAF, after excluding some of the lower doses from the dilution series in order to optimise the experimental set up, as described in Chapter 1.

The specific PAH composition of LEWAFs was determined by gas chromatography-mass spectrometry (GC-MS) after Prieto et al. (2007). A mix standard solution of 18 PAHs[1] (CRM47543; Supelco, Bellefonte, USA) was used for calibration in the GC-MS analysis. A mixture of 5 deuterated compounds[2] (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 Appendix II.

[1] Naphthalene (Naph), 1-methylnaphthalene (1-MN), 2-methylnaphthalene (2-MN), acenaphthylene (Acy), acenaphthene (Ace), fluorene (Flu), anthracene (Ant), phenanthrene (Phe), pyrene (Pyr), fluoranthene (Fluo), benz[a]anthracene (B[a]A), chrysene (Chr), benzo[a]pyrene (B[a]P), benzo[b]fluoranthene (B[b]F), benzo[k]fluoranthene (B[k]F), benzo[g,h,i]perylene (B[g,h,i]P), dibenz[a,h]anthracene (D[a,h]A), indeno[1,2,3-cd]pyrene (I[1,2,3-cd]P).

[2] Norwegian Standard (S-4124-200-T): naphthalene-d^8, byphenyl-d^{10}, phenanthrene-d^{10}, pyrene-d^{10}, benzo[a]anthracene-d^{12}, benzo[a]pyrene-d^{10}, benzo[ghi]perylene-d^{12}.

2.2. Sea urchin embryo toxicity test (SET)

The sea urchin 48 hr 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 (43º26′01.1″N 2º53′56.1″W; Bay of Biscay) in spring (March-May) 2018. Spawning was induced by injecting 1 mL 0.5M 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 capped containing 10 mL of the test solutions (50 embryos/mL) to conduct toxicity assays (in completely darkness at 20ºC).

After 48 hr exposure, 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 exposure replicates) and the egg size at t_0 (L_0 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; Appendix III). Images were taken with NIS-Elements Imaging Software v4.30 (Nikon Instruments BV). Size increase ($\Delta L=L-L_0$) and its EC_{50} were calculated (Beiras et al., 2012).

2.3. Toxicity Index (TI) in pluteus larvae

Specific abnormalities of the pluteus larvae were recorded (n=100 larvae per vial × 3 exposure replicates per experimental group) and integrated in the Toxicity Index (TI, in a 0-100 range; after Carballeira et al., 2012; Appendix IV). 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).

2.4. Sea urchin embryo developmental disruption (SEDD) assay

Sublethal toxicity was evaluated as the capacity of sea urchin to undergo its developmental program (Appendix V). This was measured in terms of inhibition of pluteus larvae formation index (IPLFI), and potential mechanisms of toxic action on developmental processes were identified by examining main stages of developmental progression: cleavage disruption index (CDI) and gastrulation disruption index (GDI) during embryo development; and metamorphosis disruption index (MDI) during larval development.

Briefly, the longest dimension of each larvae (L_i in µm; n=35 larvae per vial × 3 exposure replicates) was measured as detailed 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 exposure replicates) to calculate the indices described in Chapter 1:

$$IPLFI = \frac{1}{r}\sum_{i=1}^{r}\frac{(L_{max} - L_i) \times 100}{(0.5 \times L_{max})};$$

$$CDI = \frac{100}{2 \log (C+G+N+P1)} \times \log \frac{(C+G+N+P1) \times C}{G+N+P1};$$

$$GDI = \frac{100}{2 \log (G+N+P1)} \times \log \frac{(G+N+P1) \times G}{N+P1}; \text{ and}$$

$$MDI = \frac{L_{max} - Li}{0.5 \times L_{max}} \times \frac{100}{2 \log (C+G+N+P1)} \times \log \frac{(C+G+N+P1) \times (C+G)}{(N+P1)};$$

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

2.5. Genotoxicity assay

Sublethal exposure concentrations were selected according to present ΔL results and preceding data (Chapter 1): 55% oil LEWAF and 34% oil+D LEWAF. After 48 hr exposure, sea urchin larvae were centrifuged (1800×g at 4ºC for 10 min) to obtain pellets made of 500 larvae that were directly frozen in 500 µL of RNAlater® (Life Technologies, Carlsbad, CA, USA) and stored at -80ºC until the genotoxicity assay was performed.

The amount of intact double-stranded DNA (dsDNA) was determined by the Fast Micromethod® DNA Single-Strand-Break Assay (Scröder et al., 2006), adapted to sea urchin larvae (Reinardy and Bodnar, 2015). Samples were assayed in quadruplicate by loading 20 µL (15 larvae) to each replicate well on a black-walled 96-well microplate (USA Scientific, INC., FL, USA), place on ice. Ca/Mg-free phosphate buffer solution (PBS) was used as blank. Lysis solution (20 µL, 9 M urea 0.1 % SDS, 0.2 M EDTA) containing 1:49 Picogreen (P7581, Life Technologies, NY, USA) was added and samples were left to lyse on ice in the dark for 40 min. Then, DNA unwinding solution (20 mM EDTA, 1 M NaOH) was added (200 µL) to initiate alkaline unwinding (pH 12.65 ± 0.02). Fluorescence (intact dsDNA) was recorded at an excitation wavelength of 480 nm and an emission wavelength of 520 nm (POLARstar® Omega Plate Reader, BMG LABTECH, Aylesbury, UK) as relative fluorescent units (RFU) at t_0 in the experimental

control group and after 20 min in both control and exposure groups. Blank values were subtracted from all the RFU values before calculations. Then, the strand scission factor (SSF) was calculated according to Scröder et al. (2006):

$$SSF = -\log \frac{\%\ dsDNA_i}{\%\ dsDNA_c};$$

where $\%dsDNA_i$ is the percentage of dsDNA in each exposure group and $\%dsDNA_c$ is the percentage of dsDNA in the experimental control group. The %dsDNA values were calculated as RFU for a given sample divided by the RFU recorded in the experimental control group at t_0 (Appendix VIII).

2.6. Toxic units

The concentrations in a mixture of individual pollutants expressed as fractions of the EC50 of each pollutant (toxic units –TUs–; Sprague, 1970), the relative contribution of each individual PAH to the TUs of LEWAFs (RT_i) and the relative concentration of each PAH in the mixtures (RC_i) were calculated according to Chapter 1. For this purpose, EC50 values for various individual PAHs (Naph, Fluo, 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, braquiopods, decapods, mollusc larvae, echinoderm larvae and fish juveniles) were used as consensus EC50 to calculate the TUs (Appendix VI; Ward et al., 1981; Holcombe et al., 1983; Trucco et al., 1983; Spehar, 1999; Lyons et al., 2002; Pillai et al., 2003; Calbet et al., 2007; Bellas et al., 2008; Frantzen et al., 2012; Renegar et al., 2017; Knap et al., 2017). Then, the relative contribution of each individual PAH to the TUs of the mixture ($\sum TU_{\sum PAHs}$) was determined as $RT_i = TU_{PAHi}/\sum TU_{\sum PAHs}$; where TU_{PAHi} means the TU estimated for this individual PAH. In parallel, the relative concentration of each PAH in the mixture was calculated as $RC_i = C_{PAHi}/\sum PAHs$; where C_{PAHi} stands for the individual concentration of each PAH. Thus, the ratio RT_i/RC_i 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).

2.7. Data treatment and 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 Dunett if the variances were homogenous and T3 Dunnett if they were not). For non-normal data sets, the non-parametric Kruskal-Wallis' test was used. Linear regressions were compared using the ANCOVA test. Differences in SSF were tested by one-way ANOVA on arcsine-transformed data, with *post hoc* Fisher's least significant difference (LSD) test for differences between each treatment and control ($p<0.05$). 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. Temperature-dependent LEWAF chemical composition

The concentration of Naph, 1-MN and 2-MN in NNA, and most remarkably in NNA+D LEWAF was higher than in the other oil LEWAFs with and without dispersant, but in all the cases the differences between temperatures were low to moderate (Tables 1-3). The values of ∑PAHs without naphthalenes were slightly variable among temperatures and amongst LEWAFs for the three tested oils (3.5-9.0 µg PAH/L) but varied largely depending on the temperature for NNA+D (4.5-27.0 µg PAH/L) and IFO+D (13.5-37.5 µg PAH/L) LEWAFs, with highest values at 10ºC in the former and at 5-15ºC in the latter (Tables 1-3). The composition profiles of PAHs other than naphthalenes were found to be comparable in NNA and MGO LEWAFs, with and without dispersant, with some slight deviations depending on the temperature of LEWAF production. The amplitude of variability in the profile was larger at 10-15ºC than at 5, 20 and 25ºC in NNA and NNA+D LEWAFs (Fig. 1). In MGO, $_{LMW}$PAHs gained relevance in LEWAF produced at 15-20ºC, and both $_{LMW}$PAHs and $_{HMW}$PAHs in LEWAF produced at 25ºC (Fig. 1). Alike, the PAH profiles for IFO LEWAFs were similar

irrespective of the temperature of WAF production but varied in the case of IFO+D LEWAFs (Fig. 1).

Thus, the concentrations of Ace, Flu and Phe were relatively high in oil LEWAFs produced at any temperature but the concentration of Acy was higher at 15ºC in all oil LEWAFs (Tables 1-3). Upon dispersant addition, the concentrations of Ace, Flu, Phe were higher in NNA+D LEWAF than in NNA LEWAF produced in the 5-10ºC range (Table 1). In NNA+D LEWAF, Ace, Pyr, Fluo and B[a]A+Chr concentration increased remarkably; however, at 20 and 25ºC, Ace and Flu dropped and Phe remained high (Table 1). In the case of MGO+D, Ace, Flu and Phe concentrations were low in LEWAF produced at 5ºC but Flu increased at 10-15ºC and Phe increased at 10-20ºC to finally drop at 25ºC, when the concentration of Fluo and Pyr rose (Table 2). The concentration of individual PAHs was higher in IFO+D LEWAF than in IFO LEWAF at any temperature of LEWAF production but most markedly below 20ºC (Table 3).

Overall, the values of ∑PAHs in NNA LEWAF were similar for LEWAF production temperatures in the range of 10-25ºC but they were reduced to a half when LEWAF production temperature was 5ºC (Table 1). Upon dispersant addition, ∑PAHs in LEWAF was higher than for NNA LEWAF, which was particularly remarkable at a LEWAF production temperature of 10ºC (Table 1). For MGO LEWAF, ∑PAHs slightly rose with temperature, which was less marked after dispersant addition (Table 2). The $\sum_{HMW}$PAHs and $\sum_{LMW}$PAHs were higher in NNA+D LEWAF produced at 10ºC (Table 1), meanwhile, the $\sum_{HMW}$PAHs in MGO+D LEWAF and $\sum_{LMW}$PAHs in MGO LEWAF were higher at 25ºC (Table 2). The values of ∑PAHs did not vary with the LEWAF production temperature in the case of IFO LEWAF but augmented upon dispersant addition following a reverse temperature gradient, which was observed regarding both $\sum_{LMW}$PAHs and $\sum_{HMW}$PAHs values (Table 3).

Table 1. GC-MS analysis of PAHs (ng/L) in NNA LEWAF and NNA+D LEWAF samples produced at 5ºC, 10ºC (taken from Chapter 1 (Table 3)), 15ºC, 20ªC and 25ºC. Asterisks indicate significant differences in each oil LEWAF type (Z-score). (UDL: under detection limits; $_{LMW}$PAHs: Low molecular weight polycyclic aromatic hydrocarbons; $_{HMW}$PAHs: High molecular weight polycyclic aromatic hydrocarbons; #: Total of PAHs without Naphthalene).

	5ºC		**10ºC**		**15ºC**		**20ºC**		**25ºC**	
	NNA	**NNA+D**	**NNA**	**NNA+D**	**NNA**	**NNA+D**	**NNA**	**NNA+D**	**NNA**	**NNA+D**
Naph	196117	313985	351221	439059	261523	525929	313874	437366	302362	486302
1-MN	40275	50945	72842	**173919***	45703	68255	65273	71741	68547	65019
2-MN	154973	153022	306563	365838	400476	**461692***	259801	215493	303218	270289
Acy (1)	38	13	98	46	**109***	27	71	16	74	19
Ace (1)	456	546	996	**2649***	828	861	953	652	994	664
Flu (1)	1684	1529	3158	**9111***	2238	1753	3022	1768	3199	1926
Ant (1)	UDL	UDL	UDL	UDL	UDL	UDL	UDL	UDL	UDL	UDL
Phe (1)	1113	2218	2269	**13569***	1665	2298	2260	2117	2389	2539
Pyr (2)	79	126	139	**575***	101	99	95	84	129	92
Fluo (2)	17	59	42	**294***	26	44	36	27	34	44
B[a]A + Chr (2)	15	132	41	**681***	21	141	31	33	20	63
B[a]P (2)	14	UDL	16	UDL	UDL	UDL	UDL	UDL	UDL	UDL
B[b]F+B[k]F (2)	14	UDL	53	UDL	13	UDL	13	UDL	UDL	UDL
B[g,h,i]P (2)	UDL	UDL	UDL	UDL	UDL	UDL	UDL	UDL	UDL	UDL
D[ah]A(2)	UDL	UDL	UDL	UDL	UDL	UDL	UDL	UDL	UDL	UDL
I[1,2,3-cd]P (2)	UDL	UDL	UDL	UDL	UDL	UDL	UDL	UDL	UDL	UDL
∑PAHs	**394795***	522574	737439	1005741	712706	**1061099***	645430	729299	680966	826958
$\sum_{LMW}$PAHs Σ(1)	3290	4305	6521	**25376***	4841	4939	6306	4554	6656	5149
$\sum_{HMW}$PAHs Σ(2)	139	317	292	**1549***	161	285	175	145	183	200
$\sum_{Naph}$PAHs#	**391366***	517952	730626	978816	707703	**1055876***	638949	724600	674127	821610
∑PAHs#	3430	4622	6813	**26925***	5003	5223	6481	4699	6838	5348

Table 2. GC-MS analysis of PAHs (ng/L) in MGO LEWAF and MGO+D LEWAF samples produced at 5ºC, 10ºC (taken from Chapter 1 (Table 3)), 15ºC, 20ªC and 25ºC. Asterisks indicate significant differences in each oil LEWAF type (Z-score). (UDL: under detection limits; $_{LMW}$PAHs: Low molecular weight polycyclic aromatic hydrocarbons; $_{HMW}$PAHs: High molecular weight polycyclic aromatic hydrocarbons; #: Total of PAHs without Naphthalene).

	5ºC		**10ºC**		**15ºC**		**20ºC**		**25ºC**	
	MGO	**MGO+D**	**MGO**	**MGO+D**	**MGO**	**MGO+D**	**MGO**	**MGO+D**	**MGO**	**MGO+D**
Naph	152117	69309	112311	71814	146229	115207	133438	97619	127367	**265637***
1-MN	38005	21921	32904	27011	44944	39061	36360	36693	**58138***	29674
2-MN	56534	27211	42043	26763	53913	46531	52468	42403	**95929***	87443
Acy (1)	119	81	142	120	**217***	203	77	102	96	170
Ace (1)	997	**470***	1144	585	1259	1072	1156	893	1388	852
Flu (1)	2230	**1613***	2043	2436	2439	2680	2697	2772	**3538***	2129
Ant (1)	UDL	UDL	UDL	UDL	UDL	UDL	UDL	UDL	UDL	UDL
Phe (1)	2089	1938	1992	2737	2632	3376	2784	3167	**4207***	3939
Pyr (2)	32	15	30	21	50	30	44	22	**82***	**78***
Fluo (2)	21	64	23	65	36	88	32	69	47	**117***
B[a]A + Chr (2)	UDL	18	7	22	UDL	22	7	20	13	7
B[a]P (2)	UDL	UDL	UDL	UDL	UDL	UDL	UDL	UDL	14	UDL
B[b]F+B[k]F (2)	UDL	UDL	UDL	UDL	UDL	UDL	UDL	UDL	12	UDL
B[g,h,i]P (2)	UDL	UDL	UDL	UDL	UDL	UDL	UDL	UDL	UDL	UDL
D[ah]A(2)	UDL	UDL	UDL	UDL	UDL	UDL	UDL	UDL	UDL	UDL
I[1,2,3-cd]P (2)	UDL	UDL	UDL	UDL	UDL	UDL	UDL	UDL	UDL	UDL
∑PAHs	252144	122640	192638	131576	251719	208271	229064	183759	290832	**390047***
$\sum_{LMW}$PAHs Σ(1)	5435	**4102***	5321	5879	6547	7332	6714	6933	**9230***	7091
$\sum_{HMW}$PAHs Σ(2)	53	97	60	108	86	141	84	111	168	**203***
$\sum_{Naph}$PAHs#	246656	118440	187258	125589	245086	200799	222266	176715	281434	**382754***
∑PAHs#	5488	**4199***	5380	5987	6633	7473	6798	7044	**9398***	7293

Table 3. GC-MS analysis of PAHs (ng/L) in IFO LEWAF and IFO+D LEWAF samples produced at 5ºC, 10ºC (taken from Chapter 1 (Table 3)), 15ºC, 20ªC and 25ºC. Asterisks indicate significant differences in each oil LEWAF type (Z-score). (UDL: under detection limits; $_{LMW}$PAHs: Low molecular weight polycyclic aromatic hydrocarbons; $_{HMW}$PAHs: High molecular weight polycyclic aromatic hydrocarbons; #: Total of PAHs without Naphthalene).

	5ºC		**10ºC**		**15ºC**		**20ºC**		**25ºC**	
	IFO	**IFO+D**	**IFO**	**IFO+D**	**IFO**	**IFO+D**	**IFO**	**IFO+D**	**IFO**	**IFO+D**
Naph	99501	91233	92285	73614	95471	131424	97489	61640	72488	**155908***
1-MN	67908	120292	77566	92047	75043	**150538***	81158	61928	75146	56985
2-MN	101968	142433	91365	107758	97381	**179222***	98066	84466	88721	172526
Acy (1)	329	733	419	1009	450	**1321***	374	316	134	212
Ace (1)	2296	**7788***	2607	5999	3084	4862	2672	1643	2769	3276
Flu (1)	1507	**5012***	1673	**5066***	1794	3942	2032	2760	1897	1947
Ant (1)	111	**1402***	188	1088	243	799	232	484	185	446
Phe (1)	2036	**16016***	2337	14774	2675	12940	2948	6353	3122	7334
Pyr (2)	40	2754	40	3056	68	2846	61	699	93	559
Fluo (2)	20	**671***	18	588	25	477	32	246	34	353
B[a]A + Chr (2)	11	**2557***	7	2176	17	1702	14	844	14	33
B[a]P (2)	UDL	144	UDL	115	UDL	99	UDL	41	UDL	27
B[b]F+B[k]F (2)	UDL	137	UDL	115	UDL	103	UDL	37	UDL	23
B[g,h,i]P (2)	UDL	122	UDL	89	UDL	72	UDL	32	UDL	23
D[ah]A(2)	UDL	98	UDL	84	UDL	106	UDL	26	UDL	UDL
I[1,2,3-cd]P (2)	UDL	27	UDL	13	UDL	29	UDL	6	UDL	UDL
∑PAHs	275728	391419	268504	307590	276252	**490482***	285078	221519	244603	399653
$\sum_{LMW}$PAHs Σ(1)	6280	**30952***	7223	27936	8246	23864	8259	11556	8107	13216
$\sum_{HMW}$PAHs Σ(2)	71	6510	65	6235	111	5434	106	1930	141	1019
$\sum_{Naph}$PAHs#	269377	353958	261216	273419	267895	**461184***	276713	208033	236355	385419
∑PAHs#	6351	**37461***	7288	34171	8357	29298	8365	13486	8248	14234

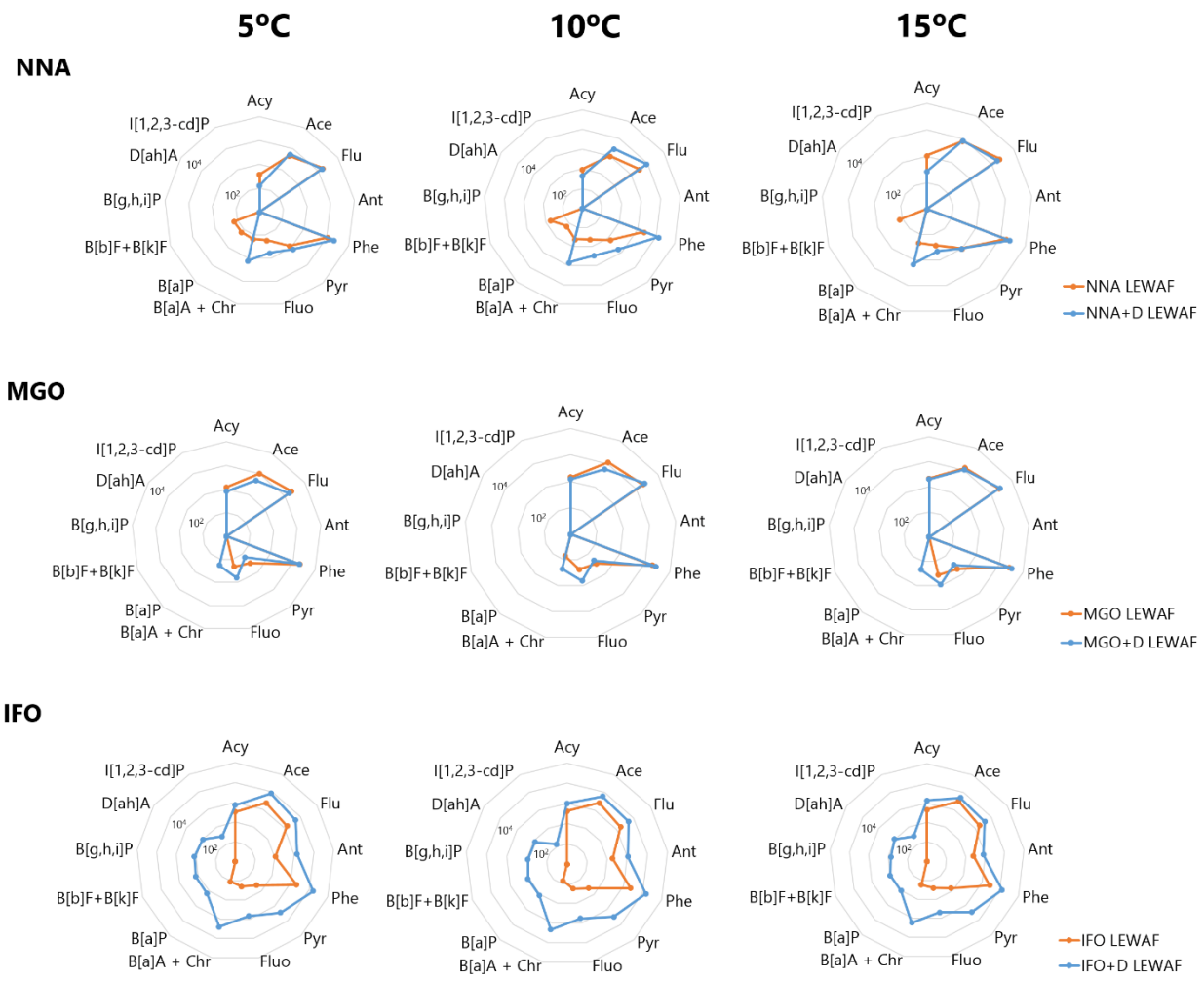

Figure 1. PAH profile of PAHs, represented in logarithmic scale, in oil and oil+D LEWAF of NNA, MGO and IFO produced at 5, 10, 15, 20 and 25ºC.

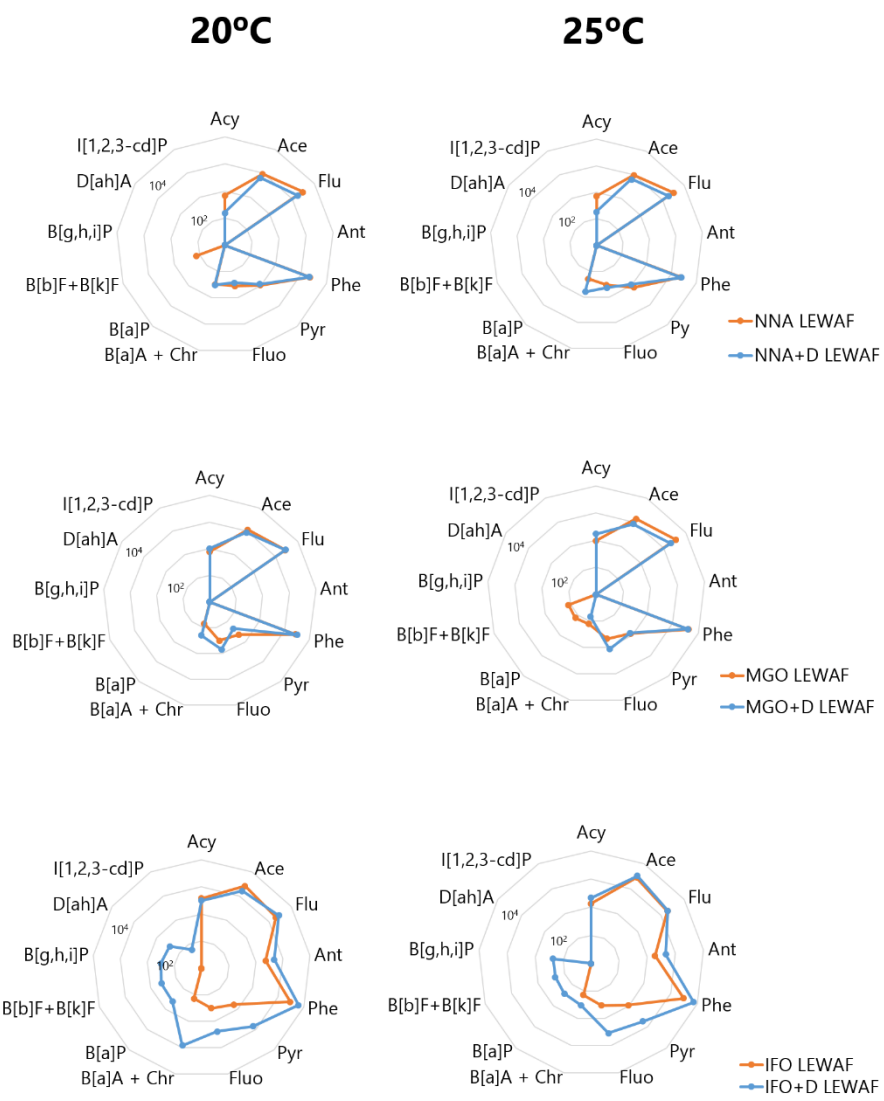

Figure 1. Continuation.

3.2. Temperature dependent toxicity of oils alone and combined with dispersant

3.2.1. Dispersant

The $EC50_{(D)}$ for the size increase (ΔL) in sea urchin larvae was 10% LEWAF (50±3 mg Finasol OSR52/L FSW) at 5ºC, 13% LEWAF (63±3 mg Finasol OSR52/L FSW) at 10ºC, 9% LEWAF (45±3 mg Finasol OSR52/L FSW) at 15ºC, 10% LEWAF (50±5 mg Finasol OSR52/L FSW) at 20ºC, and 21% LEWAF (105±5 mg Finasol OSR52/L FSW) at 25ºC.

3.2.2. NNA and NNA+D

NNA+D LEWAF exposure resulted to be genotoxic in comparison with the experimental control group, with significantly higher SSF values (Fig. 2B). The same trend was envisaged after NNA LEWAF exposure but the differences were not statistically significant (Fig. 2A). Thus, higher SSF values were recorded on NNA+D LEWAF exposure compared to NNA LEWAF exposure, irrespective of the LEWAF production temperature (Figs. 2A-2B).

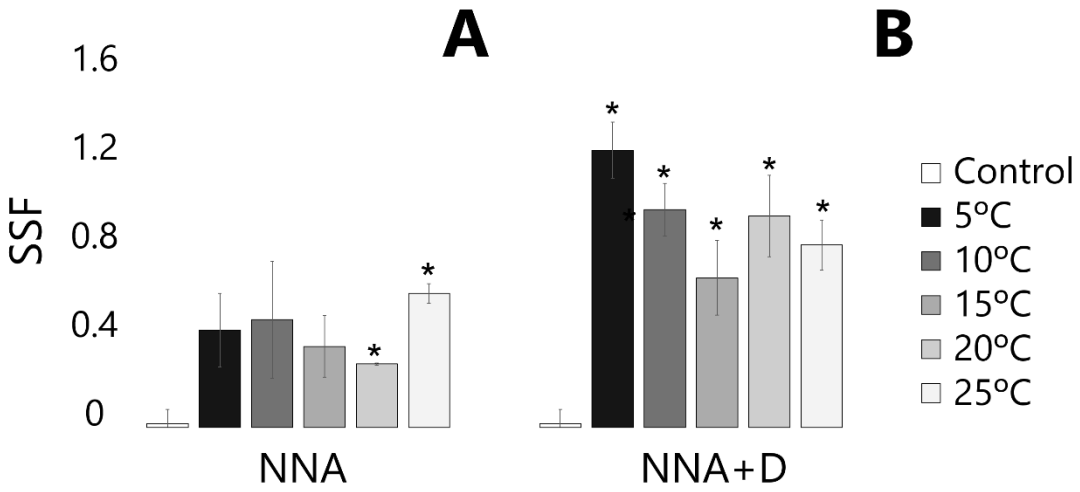

Figure 2. DNA damage measured in Strand Scission Factor (SSF ± SD) of sea urchin larvae exposed to **A)** NNA LEWAF and **B)** NNA+D LEWAF produced at different temperatures (5, 10, 15, 20 and 25 ºC). Asterisks indicate significant differences between NNA LEWAF and NNA+D LEWAF at each temperature. In the Duncan Matrix differences among temperatures in each condition (NNA LEWAF or NNA+D LEWAF) are shown (p<0.05).

There was an apparent progressive reduction in ΔL at increasing concentrations of both NNA LEWAF and NNA+D LEWAF, more markedly in the latter, but the NOEC values were always lower than 8%, except for NNA at 5ºC (Figs. 3A-3E; Table 4). Thus, ΔL decreased linearly at increasing concentrations of both NNA LEWAF produced at 10-20ºC and NNA+D LEWAF produced at any temperature in the 5-25ºC range (Figs. 3A-3E). The slope of ΔL against exposure concentration in NNA+D LEWAF was significantly steeper than in NNA LEWAF at all temperatures tested (ANCOVA; $p<0.05$; Figs. 3A-3E). In the case of NNA, moderately low EC50 values were recorded on exposure to LEWAF produced at 15-20ºC (EC50=46-55% LEWAF), whilst in the case of NNA+D the EC50 values were much lower (EC50=16-30% LEWAF) except for the case in which LEWAF was produced at 25ºC (Table 4).

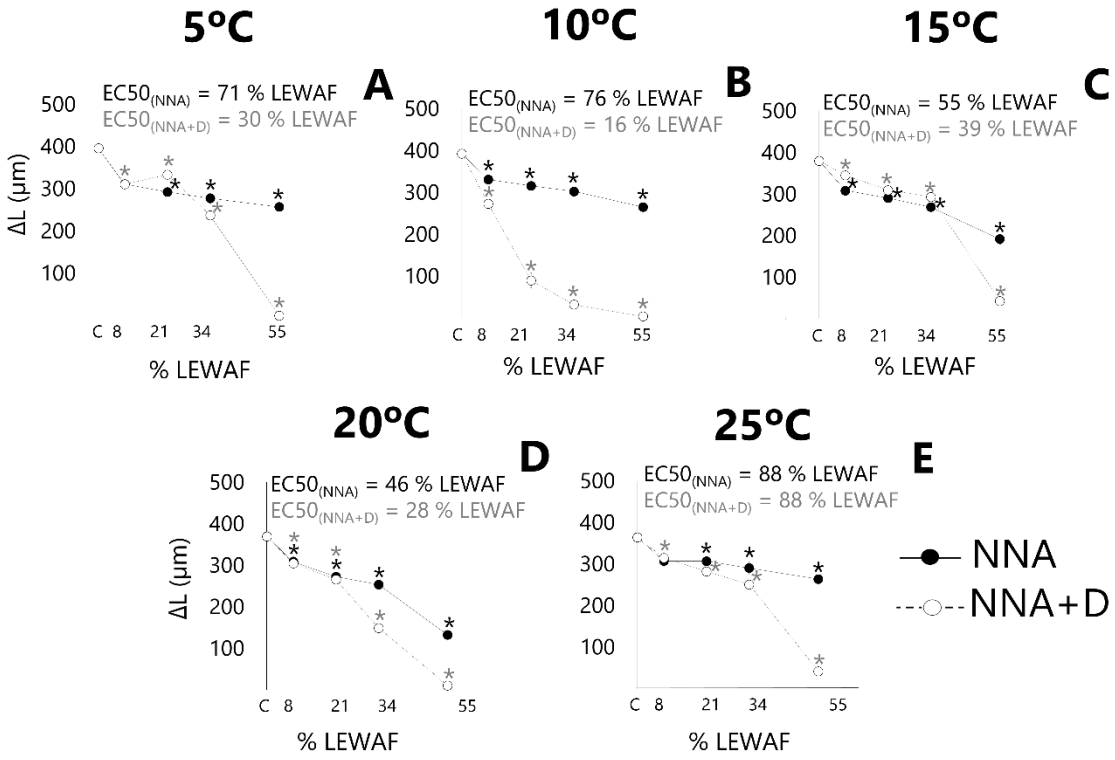

Figure 3. Size increase (ΔL in µm) of sea urchin larvae exposed to NNA LEWAF and NNA+D LEWAF produced at **A)** 5ºC, **B)** 10ºC, **C)** 15ºC, **D)** 20ºC and **E)** 25ºC. Values are given in µm (mean ± SD). Asterisks indicate significant differences between each exposure concentration and its respective control group (ANOVA; $p<0.05$). Median effective concentrations (EC50) were calculated after probit analysis.

Table 4. Summary of toxicity critical values (EC50 (95% confidence intervals)/NOEC, % LEWAFs) recorded in sea urchin embryos for CDI, GDI, MDI, IPLFI, TI and ∆L on exposure to NNA, NNA+D, MGO, MGO+D, IFO and IFO+D LEWAFs produced at 5, 10, 15, 20 and 25ºC.

	Index	NNA LEWAF	NNA+D LEWAF	MGO LEWAF	MGO+D LEWAF	IFO LEWAF	IFO+D LEWAF
5ºC	∆L	71(53-100)/21	30(26-36)/<8	31(28-34)/<8	18(16-20)/<8	29(25-34)/<8	11(10-12)/<8
	TI	>100/8	31(24-42)/21	33(26-45)/<8	14(10-19)/<8	33(26-45)/21	8(7-9)/<8
	IPLFI	77(60-100)/21	30(25-39)/<8	11(10-12)/<8	10(0-11)/<8	10(9-11)/<8	4(2-6)/<8
	CDI	>100/55	42(39-49)/34	32(26-43)/21	33(26-43)/21	33(25-45)/21	31(22-48)/21
	GDI	77(72-81)/55	42(41-44)/21	37(29-51)/21	26(20-35)/21	32(26-43)/21	9(8-11)/8
	MDI	>100/55	29(22-37)/21	28(25-31)/21	25(21-31)/21	29(25-33)/21	8(7-12)/<8
10ºC	∆L	76(61-100)/<8	16(13-27)/<8	53(46-64)/<8	34(30-38)/<8	39(33-48)/8	10(9-11)/<8
	TI	>100/21	12(10-14)/8	>100/21	40(33-47)/21	37(32-44)/21	5(4-7)/<8
	IPLFI	>100/21	11(10-12)/<8	25(21-31)/<8	12(10-13)/<8	14(12-16)/8	4(2-9)/<8
	CDI	>100/21	>100/8	>100/55	42(35-49)/34	42(23-54)/34	9(8-22)/<8
	GDI	>100/55	12(11-14)/8	>100/34	43(38-47)/34	29(24-36)/21	9(8-22)/8
	MDI	>100/55	31(24-42)/8	>100/21	41(40-47)/21	29(24-37)/21	8(7-13)/<8
15ºC	∆L	55(47-69)/<8	39(37-42)/<8	51(41-72)/<8	16(14-19)/<8	24(21-29)/<8	10(9-10)/8
	TI	>100/8	38(31-49)/8	32(26-42)/21	7(6-8)/<8	27(22-36)/21	3(2-4)/<8
	IPLFI	71(61-86)/21	37(32-43)/21	25(21-32)/<8	5(4-6)/<8	10(9-11)/<8	4(2-6)/<8
	CDI	>100/55	>100/55	>100/55	12(11-13)/8	42(39-47)/34	16(14-23)/8
	GDI	70(68-73)/34	44(40-47)/34	45(38-56)/21	8(7-9)/<8	27(22-36)/21	5(4-8)/<8
	MDI	>100/55	51(47-54)/21	41(34-53)/21	11(9-12)/8	25(20-31)/21	5(4-12)/<8
20ºC	∆L	45(41-52)/<8	26(25-29)/<8	59(50-75)/<8	57(51-66)/<8	43(36-54)/<8	25(23-28)/<8
	TI	70(47-100)/34	35(26-45)/21	>100/21	>100/8	42(37-48)/34	29(24-37)/21
	IPLFI	41(34-51)/21	27(25-30)/21	25(21-31)/<8	25(21-31)/21	26(21-33)/21	7(9-11)/<8
	CDI	82(60-100)/34	91(64-100)/55	>100/21	>100/55	>100/21	30(24-40)/21
	GDI	62(57-70)/21	43(35-54)/21	>100/34	62(60-66)/34	37(28-52)/21	28(23-36)/21
	MDI	77(70-86)/21	58(47-66)/21	80(69-100)/21	65(59-73)/21	37(35-41)/21	29(25-34)/21
25ºC	∆L	88(68-100)/<8	88(68-100)/<8	57(47-77)/<8	36(32-43)/8	64(54-79)/<8	29(26-33)/8
	TI	>100/21	37(30-46)/21	58(45-87)/21	36(29-46)/8	18(11-25)/8	31(25-39)/8
	IPLFI	93(72-100)/21	31(25-40)/21	25(20-31)/21	12(7-23)/8	25(20-32)/21	10(9-11)/8
	CDI	>100/55	>100/55	>100/55	40(31-52)/34	30(21-43)/8	30(25-37)/8
	GDI	>100/55	43(42-45)/34	65(48-100)/21	41(23-56)/21	45(36-61)/8	29(25-35)/21
	MDI	>100/55	50(47-55)/21	61(53-62)/21	38(35-43)/21	32(29-41)/21	28(23-38)/21

TI remained around 0-20% on exposure to NNA LEWAF produced at any temperature in the studied 5-25ºC range (Figs. 4A-4E). In contrast, in the case of NNA+D, TI abruptly rose to 100% on exposure to 55% LEWAF irrespective of the production temperature (Figs. 4A-4E). As a result, $EC50_{(NNA)}$ was as high as in the range of 70-100% LEWAF whilst $EC50_{(NNA+D)}$ was much lower (12-38% LEWAF); nonetheless, as a general rule NOEC values were relatively low (NOEC=8-34% LEWAF) both for NNA and NNA+D (Table 4).

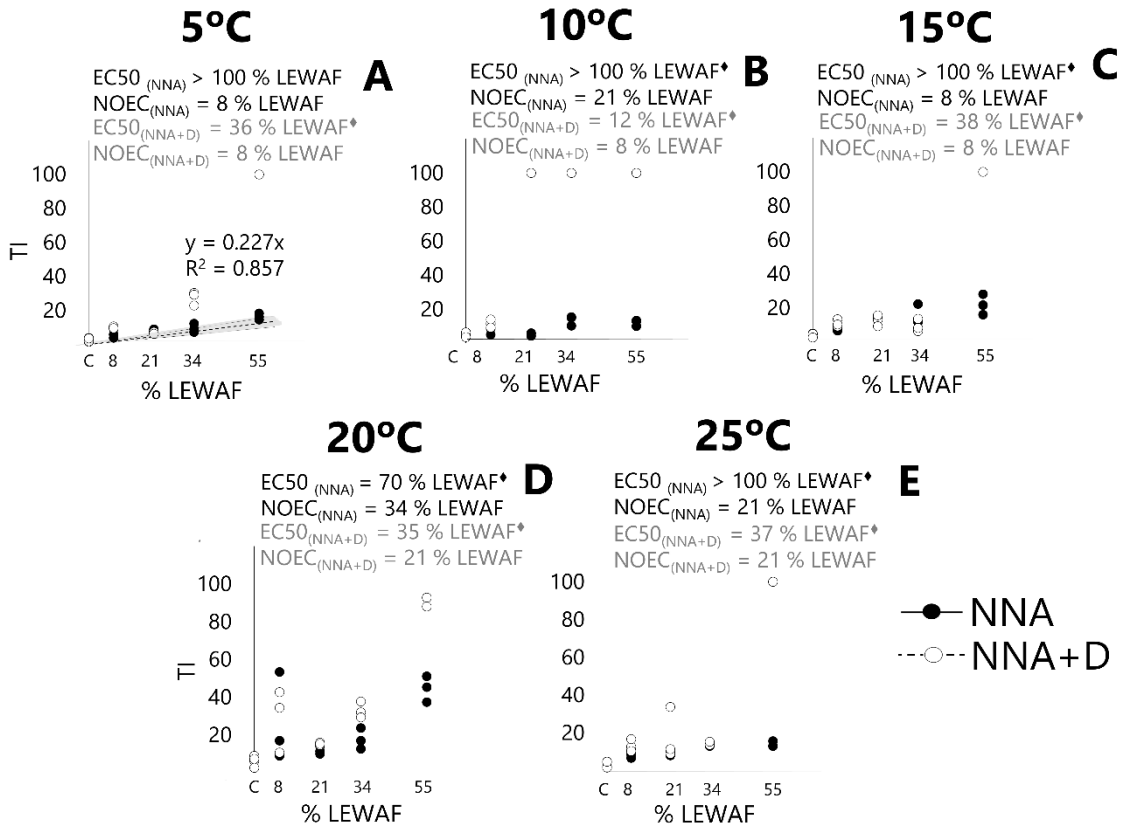

Figure 4. Toxicity Index (TI) recorded in sea urchin embryos exposed to NNA LEWAF and NNA+D LEWAFs produced at **A)** 5ºC, **B)** 10ºC, **C)** 15ºC, **D)** 20ºC and **E)** 25ºC. Median effective concentrations calculated upon linear regression models (EC50) or after probit analysis (EC50♦), and non-observed effect concentration (NOEC) values are shown for each case. No significant differences were found between linear regression coefficients of NNA and NNA+D LEWAF for each tested oil (ANCOVA; $p>0.05$).

For CDI, $EC50_{(NNA)}$ and $EC50_{(NNA+D)}$ were around 82-100% LEWAF along the whole range of temperatures of LEWAF production, except for NNA+D LEWAF produced at 5ºC in which $EC50_{(NNA+D)}$ was as low as 42% LEWAF (Table 4). $EC50_{(NNA)}$ values for GDI were in the 70-100% range whilst $EC50_{(NNA+D)}$ values were lower, in the 12-43% LEWAF range (Table 4). Similarly, $EC50_{(NNA)}$ values for MDI were in the 77-100% range whilst $EC50_{(NNA+D)}$ values were lower, especially at the lowest temperatures of LEWAF production (Table 4).

IPLFI increased linearly at increasing concentrations of NNA LEWAF at 10-20ºC (ANCOVA; $p>0.05$; Figs. 5B-5D), whereas it remained around 20% LEWAF when the production temperatures were 5 and 25ºC (Figs. 5A and 5E). Exposure to NNA+D caused an abrupt rise in IPLFI values at relatively low LEWAF concentrations (EC50=21-55% LEWAF) when LEWAF had been produced at low temperatures in the 5-15ºC range, and a concentration dependent linear increase when LEWAF had been produced at temperatures of 20-25ºC (ANCOVA; $p>0.05$; Figs. 5A-5E). Thus, $EC50_{(NNA)}$ was high (EC50=70-100% LEWAF) except when LEWAF had been produced at 20ºC (EC50=41% LEWAF), but $NOEC_{(NNA)}$ values were always low (21% LEWAF) irrespective of the LEWAF production temperature (Table 4). $EC50_{(NNA+D)}$ was at least 2-3 times lower than the corresponding $EC50_{(NNA)}$, with values in the 11-37% LEWAF range (Table 4). In agreement, $NOEC_{(NNA+D)}$ was also lower than $NOEC_{(NNA)}$, especially at the lowest temperatures of LEWAF production (5-10ºC) (Table 4).

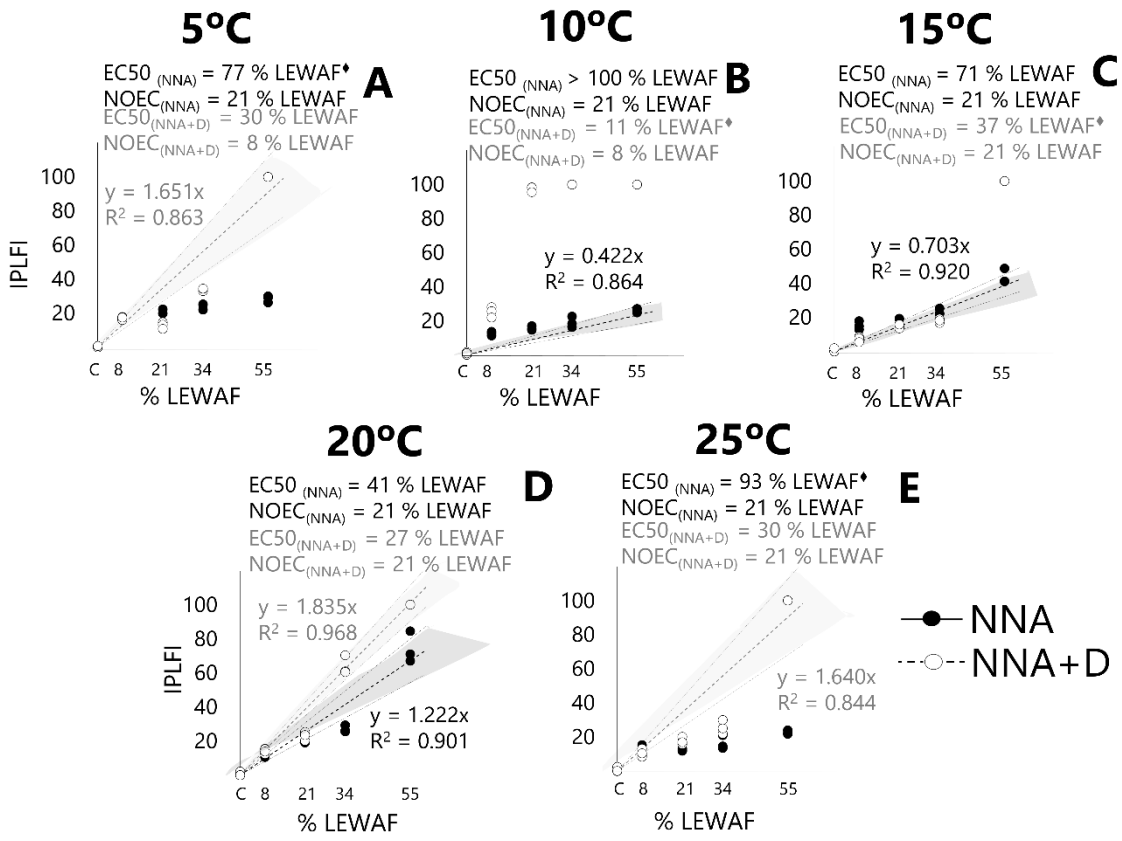

Figure 5. Inhibition pluteus larvae formation index (IPLFI) recorded in sea urchin embryos exposed to NNA and NNA+D LEWAFs produced at **A)** 5ºC, **B)** 10ºC, **C)** 15ºC, **D)** 20ºC and **E)** 25ºC. Median effective concentrations calculated upon linear regression models (EC50) or after probit analysis (EC50♦), and non-observed effect concentration (NOEC) values are shown for each case. No significant differences were found between linear regression coefficients of NNA and NNA+D LEWAF for each tested oil (ANCOVA; p>0.05).

The sum of TUs was higher in NNA+D LEWAFs than in the case of NNA LEWAFs but still below "1" for all the toxicity endpoints investigated irrespective of the LEWAF production temperature, except for CDI after exposure to NNA+D LEWAF produced at 10, 20 and 25ºC and for ΔL after exposure to NNA+D LEWAF produced at 25ºC (Table 5). Conversely, the sum of TUs exceed the value of "1" for all the toxicity endpoints on exposure to NNA+D LEWAF produced at 15ºC (Table 5). RT_i values greater than "1" revealed that one or more individual PAHs exhibited more toxicity than predicted for the mixture toxicity. Accordingly, RT_i/RC_i values were greater than "1" for several individual PAHs (Table 5) including 2-MN, Pyr, Fluo, B[a]A+Chr and B[a]P in NNA LEWAF and NNA+D LEWAF produced at any temperature in the 5-25ºC range, and Phe in NNA+D LEWAF produced at 5, 20 and 25ºC.

Table 5. Summary of the TU analysis of the toxicity of NNA and NNA+D LEWAFs produced at different temperatures based on the mixture of identified PAHs. The sum of TUs (∑TU) for each toxicity endpoint (ΔL, TI, IPLFI, CDI, GDI and MDI) is "1" if there is additive toxicity, ">1" if there is synergistic effect and "<1" if the toxicity is not caused by the mixture assuming the CA joint action. The sum of the TUs of individual PAHs vs. the TUs of the sum of PAHs ($\sum TU_{PAHi}/TU_{\sum PAHs}$) is "1" if all the PAHs in the mixture exert the same toxicity, ">1" if there are one or more individual PAHs with more toxicity than expected from its contribution to the mixture according to the CA model; and "<1" otherwise. The balance between the relative contribution of an individual PAH to the toxicity of the mixture and its relative contribution to the chemical composition of the mixture (RT_i/RC_i) is "1" if the individual toxicity of this PAH is the one expected due to its proportion in the mixture (CA model); "<1" if it is not a contributor to the mixture toxicity; and ">1" if there this PAH exerts toxicity beyond the one expected as a part of the mixture.

	5ºC		10ºC		15ºC		20ºC		25ºC	
	NNA	**NNA+D**	**NNA**	**NNA+D**	**NNA**	**NNA+D**	**NNA**	**NNA+D**	**NNA**	**NNA+D**
$\sum TU_{\Delta L}$	0.09	0.32	0.18	0.42	0.22	1.15	0.15	0.42	0.18	1.59
$\sum TU_{TI}$	0.08	0.33	0.16	0.32	0.20	1.12	0.14	0.52	0.16	0.67
$\sum TU_{IPLFI}$	0.04	0.32	0.08	0.29	0.10	1.09	0.07	0.40	0.08	0.56
$\sum TU_{CDI}$	0.32	0.45	0.62	2.64	0.77	2.96	0.53	1.35	0.61	1.81
$\sum TU_{GDI}$	0.09	0.45	0.18	0.32	0.22	1.30	0.15	0.64	0.18	0.78
$\sum TU_{MDI}$	0.08	0.31	0.16	0.82	0.20	1.51	0.14	0.86	0.16	0.91
$\sum TU_{PAHi}/TU_{\sum PAHs}$	2.20	1.73	2.31	2.22	2.96	2.36	2.25	1.73	2.45	1.86
RT/RC_{Naph}	0.08	0.10	0.08	0.08	0.06	0.08	0.08	0.10	0.07	0.10
$RT/RC_{1\text{-}MN}$	0.62	0.79	0.59	0.61	0.46	0.58	0.61	0.79	0.56	0.74
$RT/RC_{2\text{-}MN}$	2.27	2.88	2.16	2.24	1.68	2.11	2.22	2.89	2.03	2.69
RT/RC_{Acy}	0.45	0.57	0.43	0.44	0.33	0.42	0.44	0.57	0.40	0.53
RT/RC_{Ace}	0.50	0.63	0.48	0.50	0.37	0.47	0.49	0.64	0.45	0.59
RT/RC_{Flu}	0.19	0.25	0.19	0.19	0.14	0.18	0.19	0.25	0.17	0.23
RT/RC_{Ant}	-	-	-	-	-	-	-	-	-	-
RT/RC_{Phe}	0.90	1.14	0.86	0.89	0.67	0.84	0.88	1.15	0.81	1.07
RT/RC_{Pyr}	2.99	3.79	2.85	2.96	2.22	2.78	2.92	3.81	2.68	3.54
RT/RC_{Fluo}	1.52	1.93	1.45	1.51	1.13	1.42	1.49	1.94	1.37	1.80
$RT/RC_{B[a]A + Chr}$	38.55	48.88	36.72	38.13	28.58	35.87	37.72	49.09	34.58	45.65
$RT/RC_{B[a]P}$	2.57	-	2.45	-	-	-	-	-	-	-

3.2.3. MGO and MGO+D

Exposure to MGO LEWAF and MGO+D LEWAF resulted to be genotoxic in comparison with the experimental control group, with significantly higher SSF values (Figs. 6A-6E). Moreover, SSF values were higher after exposure to MGO+D LEWAF than after exposure to MGO LEWAF, irrespective of the LEWAF production temperature (Figs. 6A-6E).

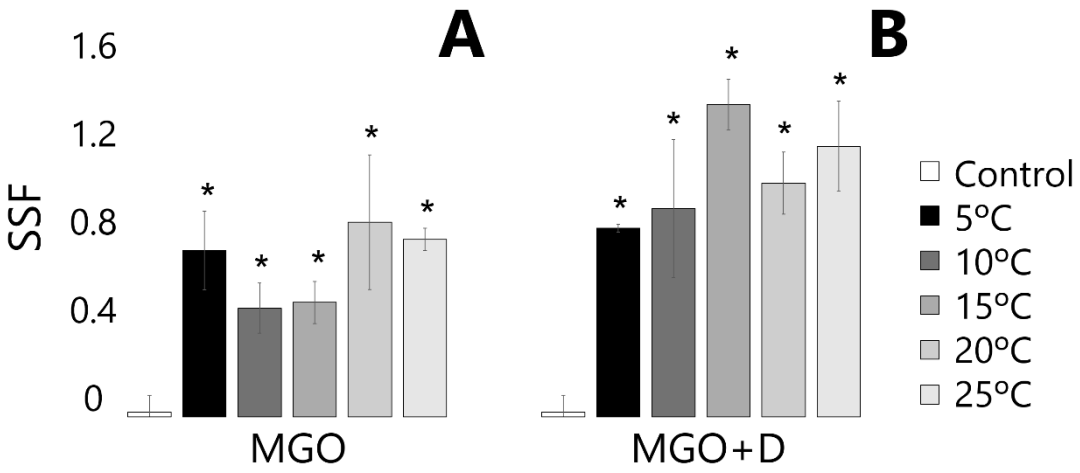

Figure 6. DNA damage measured in Strand Scission Factor (SSF ± SD) of sea urchin larvae exposed to **A)** MGO LEWAF and **B)** MGO+D LEWAF produced at different temperatures (5, 10, 15, 20 and 25 ºC). Asterisks indicate significant differences between MGO LEWAF and MGO+D LEWAF at each temperature. In the Duncan Matrix differences among temperatures in each condition (MGO LEWAF or MGO+D LEWAF) are shown (p<0.05).

There was an evident progressive reduction in ΔL at increasing concentrations of MGO and MGO+D LEWAF; this was more marked in the latter but the NOEC values were always lower than 8% in both cases (Figs. 7A-7E; Table 4). Thus, ΔL decreased linearly at increasing concentrations of both MGO LEWAF produced at 5-20ºC (not when produced at 25ºC) and MGO+D LEWAF produced at any temperature in the 5-25ºC range (Figs. 7A-7E). The slope of ΔL against exposure concentration in MGO+D LEWAF was significantly lower than in MGO LEWAF only at 10, 15 and 25ºC (ANCOVA; p<0.05; Figs. 7B, 7C and 7E). In the case of MGO, the lowest EC50 value was recorded on exposure to LEWAF produced at 5ºC (EC50 = 31% LEWAF), whilst in the case of MGO+D EC50 values were always low (EC50= 16-57% LEWAF) (Table 4).

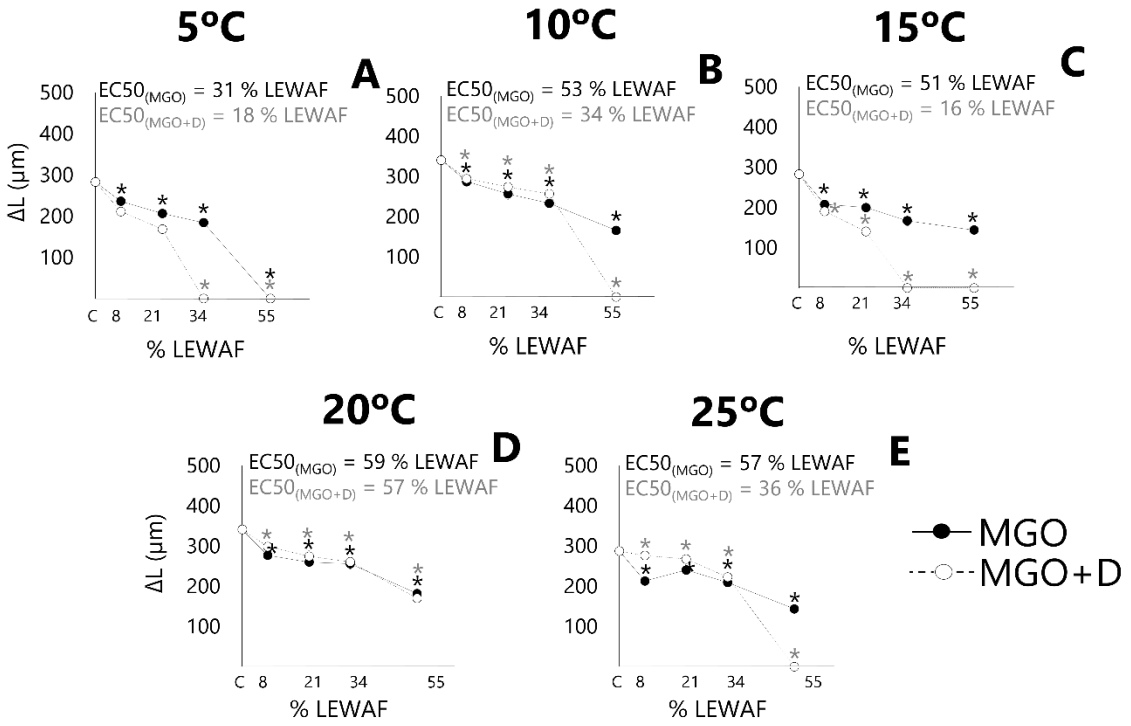

Figure 7. Size increase (ΔL in µm) of sea urchin larvae exposed to MGO LEWAF and MGO+D LEWAF produced at **A)** 5ºC, **B)** 10ºC, **C)** 15ºC, **D)** 20ºC and **E)** 25ºC. Values are given in µm (mean ± SD). Asterisks indicate significant differences between each exposure concentration and its respective control group (ANOVA; $p<0.05$). Median effective concentrations (EC50) were calculated after probit analysis.

TI increased linearly at increasing concentrations of MGO LEWAF at 5 and 15ºC (ANCOVA; $p>0.05$; Figs. 8A and 8C), whereas it remained around 0-30% on exposure to MGO LEWAF produced at other temperatures (Figs. 8B-8E). In the case of MGO+D LEWAF, TI abruptly rose to 100% on exposure to 21-55% LEWAF except for LEWAF produced at 20ºC, in which a linear increase in TI was found at increasing LEWAF exposure concentrations (ANCOVA; $p>0.05$; Figs. 8A-E). Overall, $EC50_{(MGO)}$ values were higher than the corresponding $EC50_{(MGO+D)}$ values and a high variability was found amongst LEWAFs produced at different temperatures, without any clear trend, for both MGO and MGO+D (Table 4). NOEC values for MGO, without and with dispersant, were always low (from <8% to 21% LEWAF) irrespective of the temperature of LEWAF production (Table 4).

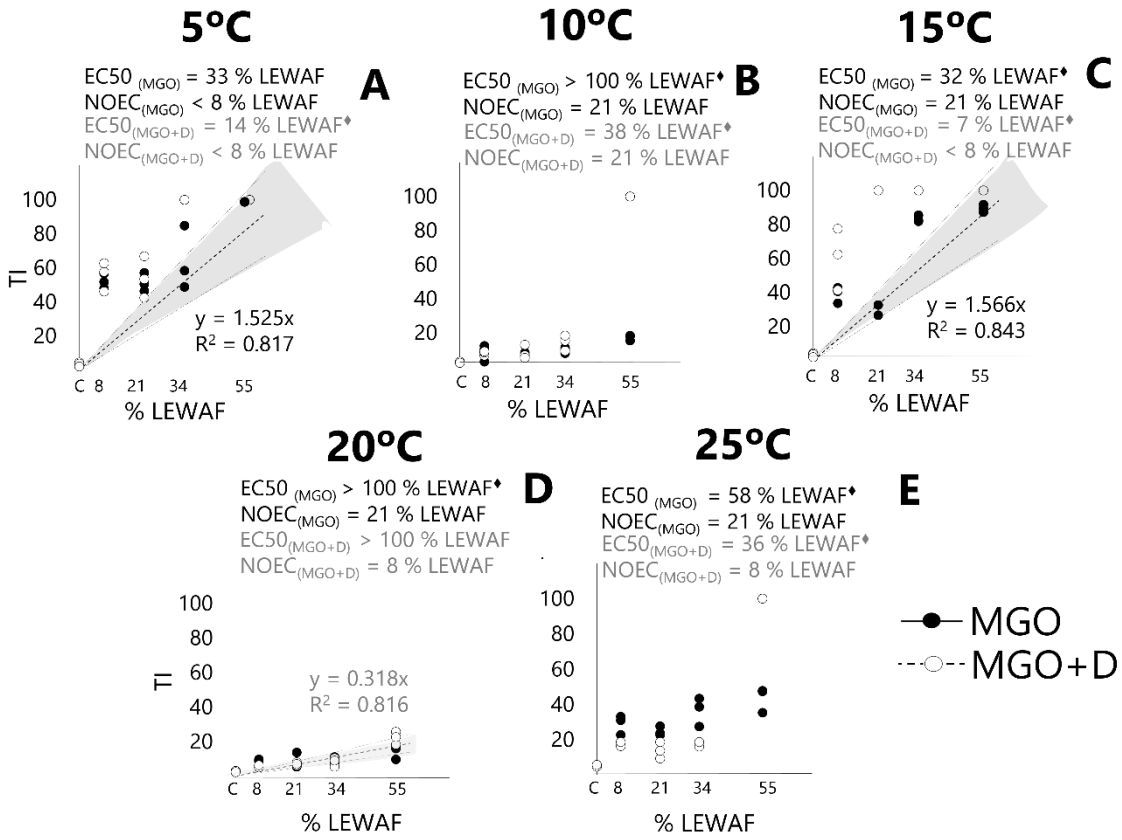

Figure 8. Toxicity Index (TI) recorded in sea urchin embryos exposed to MGO LEWAF and MGO+D LEWAFs produced at **A)** 5ºC, **B)** 10ºC, **C)** 15ºC, **D)** 20ºC and **E)** 25ºC. Median effective concentrations calculated upon linear regression models (EC50) or after probit analysis (EC50♦), and non-observed effect concentration (NOEC) values are shown for each case. No significant differences were found between linear regression coefficients of MGO and MGO+D LEWAF for each tested oil (ANCOVA; $p>0.05$).

For CDI, $EC50_{(MGO)}$ was higher than 100% LEWAF for a wide range of LEWAF production temperatures but it was severely dropped (32% LEWAF) when the LEWAF had been produced at 5ºC (Table 4). Upon dispersant addition to MGO, $EC50_{(MGO+D)}$ was highly variable (12-100% LEWAF) without a clear pattern in relation to the LEWAF production temperatures (Table 4). For GDI, values of $EC50_{(MGO)}$ (40-100% LEWAF) and $EC50_{(MGO+D)}$ (5-43% LEWAF) were also highly variable and seemingly not related with the LEWAF production temperatures, but they were markedly lower in presence of the dispersant than in MGO alone (Table 4). Similarly, $EC50_{(MGO)}$ and $EC50_{(MGO+D)}$ calculated for MDI were also highly variable (28->100% LEWAF) but the values were lower for $EC50_{(MGO+D)}$ than for $EC50_{(MGO)}$ for every LEWAF production temperature used herein (Table 4).

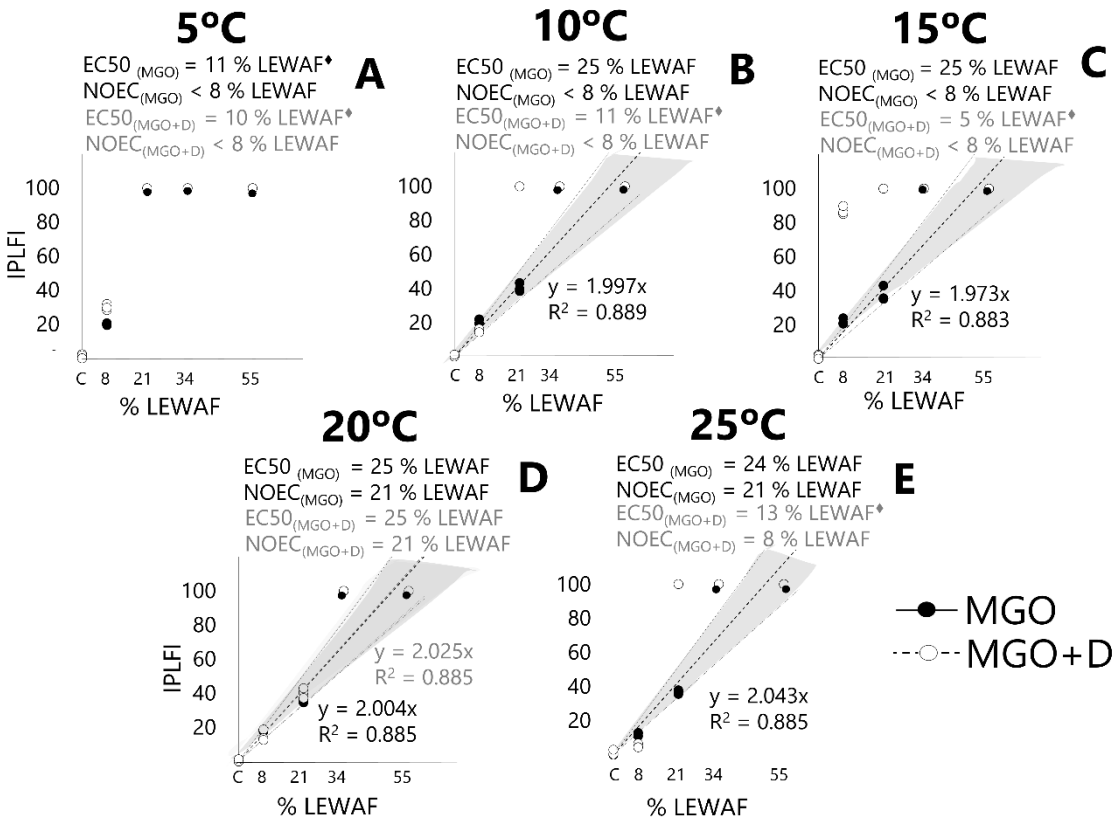

Figure 9. Inhibition pluteus larvae formation index (IPLFI) recorded in sea urchin embryos exposed to MGO and MGO+D LEWAFs produced at **A)** 5ºC, **B)** 10ºC, **C)** 15ºC, **D)** 20ºC and **E)** 25ºC. Median effective concentrations calculated upon linear regression models (EC50) or after probit analysis (EC50♦), and non-observed effect concentration (NOEC) values are shown for each case. No significant differences were found between linear regression coefficients of MGO and MGO+D LEWAF for each tested oil (ANCOVA; p>0.05).

IPLFI increased linearly at increasing concentrations of MGO LEWAF at 10-25ºC (ANCOVA; p>0.05; Figs. 9B-9E), whereas it abruptly rose to values of 100% at an exposure concentration of 20% LEWAF when the production temperature was 5ºC (Fig. 9A). Exposure to MGO+D caused an abrupt rise in IPLFI values at low LEWAF concentrations (8-21% LEWAF) with a concentration dependent linear increase only significant when LEWAF had been produced at 20ºC (ANCOVA; p>0.05; Figs. 9B-9E). Thus, $EC50_{(MGO)}$ was always low (EC50=11-25% LEWAF), most remarkably when LEWAF had been produced at the lowest temperature; and even lower upon dispersant application (EC50=5-25% LEWAF; Table 4). $NOEC_{(MGO)}$ was lower than 8% LEWAF at production temperatures of 5-20ºC and 21% LEWAF at 25ºC whilst $NOEC_{(MGO+D)}$ was 8% LEWAF or lower than 8% LEWAF except when LEWAF had been produced at 20ºC where $NOEC_{(MGO+D)}$ was 21% LEWAF (Table 4).

The sum of TUs was below "1" for all the toxicity endpoints investigated after exposure to MGO LEWAF and MGO+D LEWAF produced at different temperatures in the 5-25ºC range (Table 6). RT_i values were greater than "1" for all the endpoints investigated. Both without and with dispersant application, regardless of the temperature of LEWAF production in the 5-25ºC range, RT_i/RC_i values were greater than "1" for several individual PAHs (Table 6): 2-MN, Pyr, Phe, Fluo, B[a]A+Chr and B[a]P.

Table 6. Summary of the TU analysis of the toxicity of MGO and MGO+D LEWAFs produced at different temperatures based on the mixture of identified PAHs. The sum of TUs (∑TU) for each toxicity endpoint (ΔL, TI, IPLFI, CDI, GDI and MDI) is "1" if there is additive toxicity, ">1" if there is synergistic effect and "<1" if the toxicity is not caused by the mixture assuming the CA joint action. The sum of the TUs of individual PAHs vs. the TUs of the sum of PAHs ($\sum TU_{PAHi}/TU_{\sum PAHs}$) is "1" if all the PAHs in the mixture exert the same toxicity, ">1" if there are one or more individual PAHs with more toxicity than expected from its contribution to the mixture according to the CA model; and "<1" otherwise. The balance between the relative contribution of an individual PAH to the toxicity of the mixture and its relative contribution to the chemical composition of the mixture (RT_i/RC_i) is "1" if the individual toxicity of this PAH is the one expected due to its proportion in the mixture (CA model); "<1" if it is not a contributor to the mixture toxicity; and ">1" if there this PAH exerts toxicity beyond the one expected as a part of the mixture.

	5ºC		**10ºC**		**15ºC**		**20ºC**		**25ºC**	
	MGO	**MGO+D**	**MGO**	**MGO+D**	**MGO**	**MGO+D**	**MGO**	**MGO+D**	**MGO**	**MGO+D**
$\sum TU_{\Delta L}$	0.13	0.04	0.18	0.08	0.22	0.06	0.24	0.19	0.40	0.23
$\sum TU_{TI}$	0.14	0.03	0.33	0.09	0.14	0.03	0.41	0.34	0.41	0.23
$\sum TU_{IPLFI}$	0.05	0.02	0.08	0.03	0.11	0.02	0.10	0.09	0.18	0.08
$\sum TU_{CDI}$	0.14	0.07	0.33	0.10	0.43	0.04	0.41	0.34	0.70	0.25
$\sum TU_{GDI}$	0.16	0.06	0.33	0.10	0.19	0.03	0.41	0.21	0.46	0.26
$\sum TU_{MDI}$	0.12	0.05	0.33	0.09	0.18	0.04	0.32	0.22	0.43	0.24
$\sum TU_{PAHi}/TU_{\sum PAHs}$	1.46	1.51	1.46	1.46	1.45	1.52	1.50	1.58	2.04	1.37
RT/RC_{Naph}	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.11	0.09	0.13
$RT/RC_{1\text{-}MN}$	0.94	0.91	0.94	0.93	0.94	0.90	0.91	0.87	0.67	1.00
$RT/RC_{2\text{-}MN}$	3.42	3.31	3.41	3.41	3.44	3.27	3.32	3.16	2.44	3.64
RT/RC_{Acy}	0.68	0.65	0.67	0.67	0.68	0.65	0.66	0.63	0.48	0.72
RT/RC_{Ace}	0.76	0.73	0.75	0.75	0.76	0.72	0.73	0.70	0.54	0.80
RT/RC_{Flu}	0.29	0.28	0.29	0.29	0.30	0.28	0.29	0.27	0.21	0.31
RT/RC_{Ant}	-	-	-	-	-	-	-	-	-	-
RT/RC_{Phe}	1.36	1.31	1.35	1.35	1.37	1.30	1.32	1.26	0.97	1.44
RT/RC_{Pyr}	4.51	4.36	4.49	4.49	4.54	4.31	4.38	4.17	3.22	4.79
RT/RC_{Fluo}	2.30	2.22	2.29	2.29	2.31	2.20	2.23	2.13	1.64	2.44
$RT/RC_{B[a]A\ +\ Chr}$	-	56.22	57.98	57.92	-	55.62	56.44	53.80	41.52	61.80
$RT/RC_{B[a]P}$	-	-	-	-	-	-	-	-	2.77	-

3.2.4. IFO and IFO+D

Exposure to IFO LEWAF and IFO+D LEWAF resulted to be genotoxic in comparison with the experimental control group, with significantly higher SSF values (Figs. 10A-10C). Moreover, SSF values were higher after exposure to LEWAF produced at low temperatures in the 5-15ºC range than after exposure to LEWAF produced at 20-25ºC (Fig. 10A). In contrast, no differences in SSF were observed amongst LEWAFs produced at different temperatures regarding exposure to IFO+D LEWAF (Fig. 10B). As a result, SSF values were higher in IFO LEWAF than in IFO+D LEWAF within the range of 5-15ºC of LEWAF production temperature (Figs. 10A-10B).

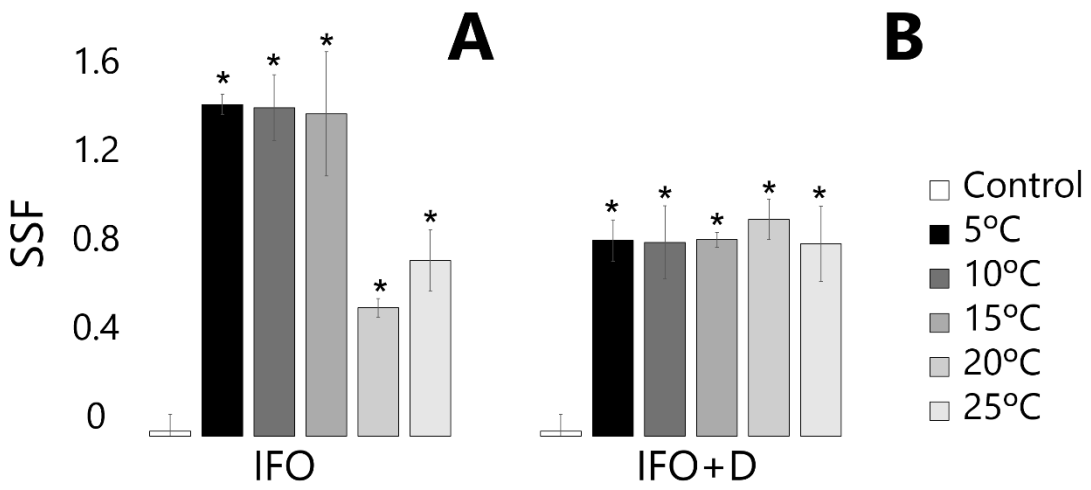

Figure 10. DNA damage measured in Strand Scission Factor (SSF ± SD) of sea urchin larvae exposed to **A)** IFO LEWAF and **B)** IFO+D LEWAF produced at different temperatures (5, 10, 15, 20 and 25 ºC). Asterisks indicate significant differences between IFO LEWAF and IFO+D LEWAF at each temperature. In the Duncan Matrix differences among temperatures in each condition (IFO LEWAF or IFO+D LEWAF) are shown (p<0.05).

There was a progressive reduction in ΔL at increasing concentrations of IFO and IFO+D LEWAF, more markedly in the latter, but the NOEC values were lower than 8% LEWAF at all temperatures tested (Figs. 11A-11E; Table 4). ΔL decreased linearly at increasing concentrations of both IFO LEWAF (at all temperatures tested) and IFO+D LEWAF (at 20 and 25ºC) (Figs. 11A-11E). The slope of ΔL against exposure concentration in IFO+D LEWAF was significantly lower than in IFO LEWAF at all temperatures tested (ANCOVA; p<0.05; Figs. 11A-11E). In the case of IFO, the lowest EC50 values were recorded at the lowest LEWAF production temperatures (5-20ºC;

EC50=24-43% LEWAF), whilst in the case of IFO+D, EC50 was low at 20-25ºC (EC50=25-29% LEWAF) and extremely low at 5-15ºC (EC50=10-11% LEWAF) (Table 4).

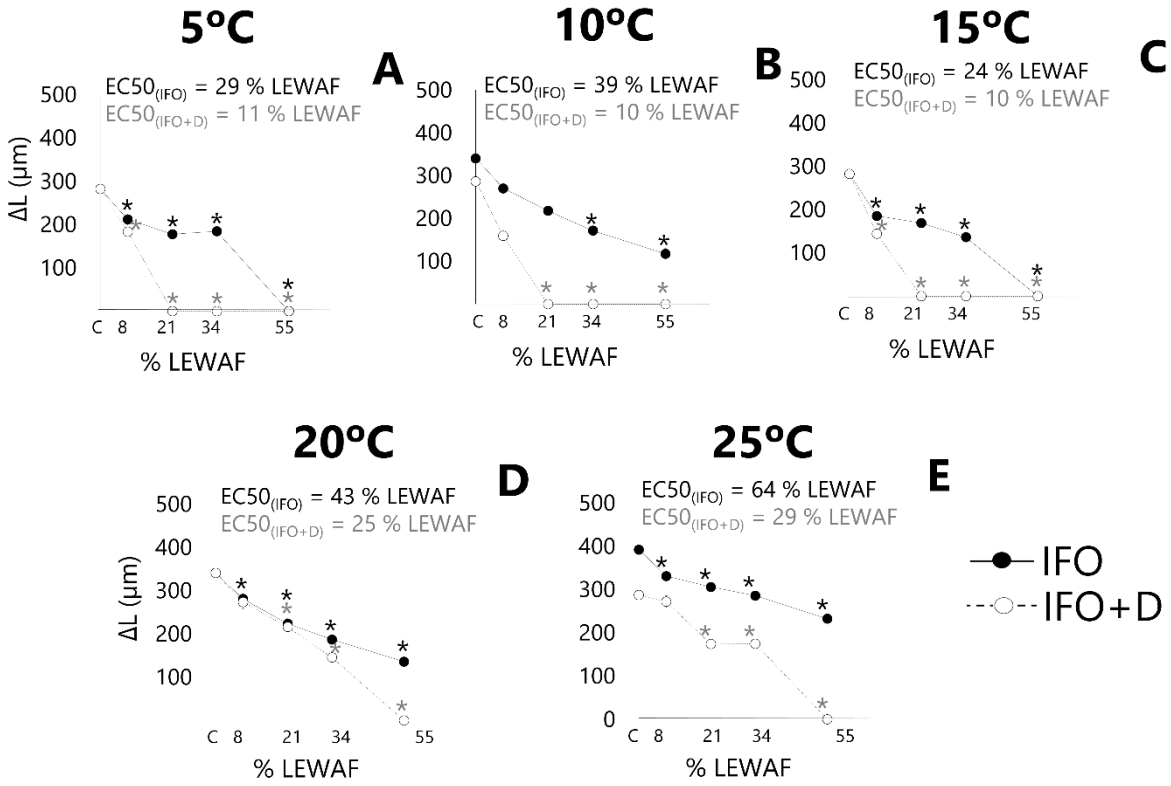

Figure 11. Size increase (ΔL in µm) of sea urchin larvae exposed to IFO LEWAF and IFO+D LEWAF produced at **A)** 5ºC, **B)** 10ºC, **C)** 15ºC, **D)** 20ºC and **E)** 25ºC. Values are given in µm (mean ± SD). Asterisks indicate significant differences between each exposure concentration and its respective control group (ANOVA; $p<0.05$). Median effective concentrations (EC50) were calculated after probit analysis.

TI sharply rose to 80-100% on exposure to 34-55% IFO LEWAF produced at the various temperatures used herein, with a concentration-dependent linear trend only significant in the cases of LEWAFs produced at 5 and 15ºC (ANCOVA; $p>0.05$; Figs. 12A-12E). In the case of IFO+D, the effect was more severe and TI values reached 100% on exposure to 8-21% LEWAF irrespective of the LEWAF production temperature, whilst a concentration-dependent linear trend was only significant when LEWAF had been produced at 20-25ºC (ANCOVA; $p>0.05$; Figs. 12A-12E). Thus, $EC50_{(IFO)}$ was lower for LEWAF produced at 25ºC (EC50=18% LEWAF) than for LEWAF produced at lower temperatures in the 5-20ºC range (EC50=27-42% LEWAF) (Table 4). In presence of dispersant, EC50 values were lower in IFO+D than for IFO oil alone;

and $EC50_{(IFO+D)}$ was extremely low (EC50=4-8% LEWAF) for IFO+D LEWAF produced at 5-15ºC and low (EC50=39-31% LEWAF) for IFO+D LEWAF produced at 20-25ºC (Table 4). In parallel, $NOEC_{(IFO)}$ was lower (NOEC=8% LEWAF) for the highest LEWAF production temperature (25ºC) than for LEWAF produced at 5-20ºC (NOEC=21-34% LEWAF), and $NOEC_{(IFO+D)}$ values were always 8% LEWAF or lower except for LEWAF produced at 20ºC where it was 21% LEWAF (Table 4).

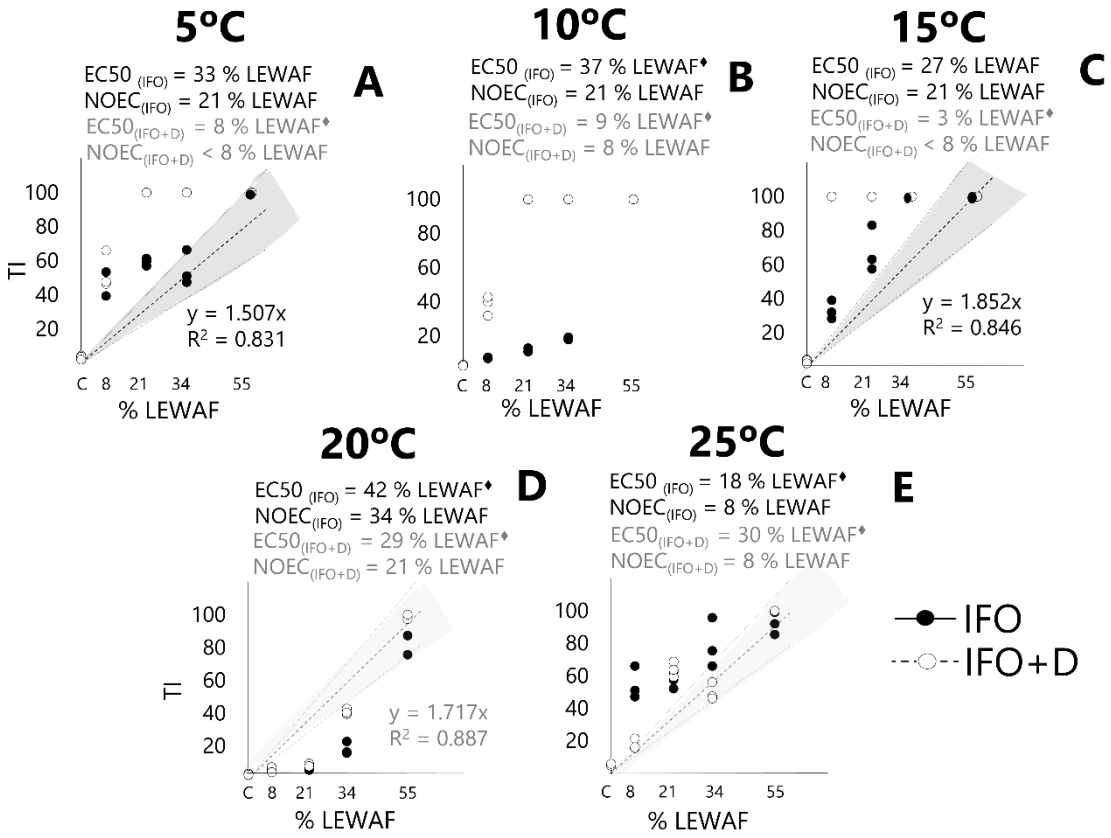

Figure 12. Toxicity Index (TI) recorded in sea urchin embryos exposed to IFO LEWAF and IFO+D LEWAFs produced at **A)** 5ºC, **B)** 10ºC, **C)** 15ºC, **D)** 20ºC and **E)** 25ºC. Median effective concentrations calculated upon linear regression models (EC50) or after probit analysis (EC50♦), and non-observed effect concentration (NOEC) values are shown for each case. No significant differences were found between linear regression coefficients of IFO and IFO+D LEWAF for each tested oil (ANCOVA*; p*>0.05).

For CDI, $EC50_{(IFO)}$ was relatively low (EC50=30-42% LEWAF) irrespective of the LEWAF production temperature, except for LEWAF produced at 20ºC; and dispersant addition resulted in even lower $EC50_{(IFO+D)}$ values in the range of 9-30% LEWAF (Table 4). For GDI, $EC50_{(IFO)}$ was around 27-45% LEWAF and the addition of dispersant, once again, resulted in much lower EC50 values, especially at low LEWAF production temperatures: $EC50_{(IFO+D)}$ was 5-9% LEWAF at 5-15ºC and 28-29% LEWAF at 20-25ºC

(Table 4). Similarly, $EC50_{(IFO)}$ for MDI was around 25-37% LEWAF whilst $EC50_{(IFO+D)}$ was between 5-8% LEWAF at 5-15ºC, and 28-29% at 20-25ºC (Table 4).

IPLFI sharply rose to 100% on exposure to 20% IFO LEWAF produced at the various temperatures used herein, with a concentration-dependent linear trend only significant in the cases of LEWAFs produced at 20-25ºC (ANCOVA; $p<0.05$; Figs. 13A-13E). Likewise, IPLFI also reached values of 100% on exposure to 8% IFO+D LEWAF produced at 5-15ºC and on exposure to 21% IFO+D LEWAF produced at 20-25ºC (Figs. 13A-13E). Thus, $EC50_{(IFO)}$ was extremely low at 5-15ºC (EC50=10-14% LEWAF) and low at 20-25ºC (EC50=25-26% LEWAF). $EC50_{(IFO+D)}$ was even lower, with values of 4% LEWAF at 5-15ºC and 10% LEWAF at 20-25ºC (Table 4). $NOEC_{(IFO)}$ and $NOEC_{(IFO+D)}$ were 8% LEWAF or lower except when IFO LEWAF had been produced at 20ºC, where it was 21% LEWAF (Table 4).

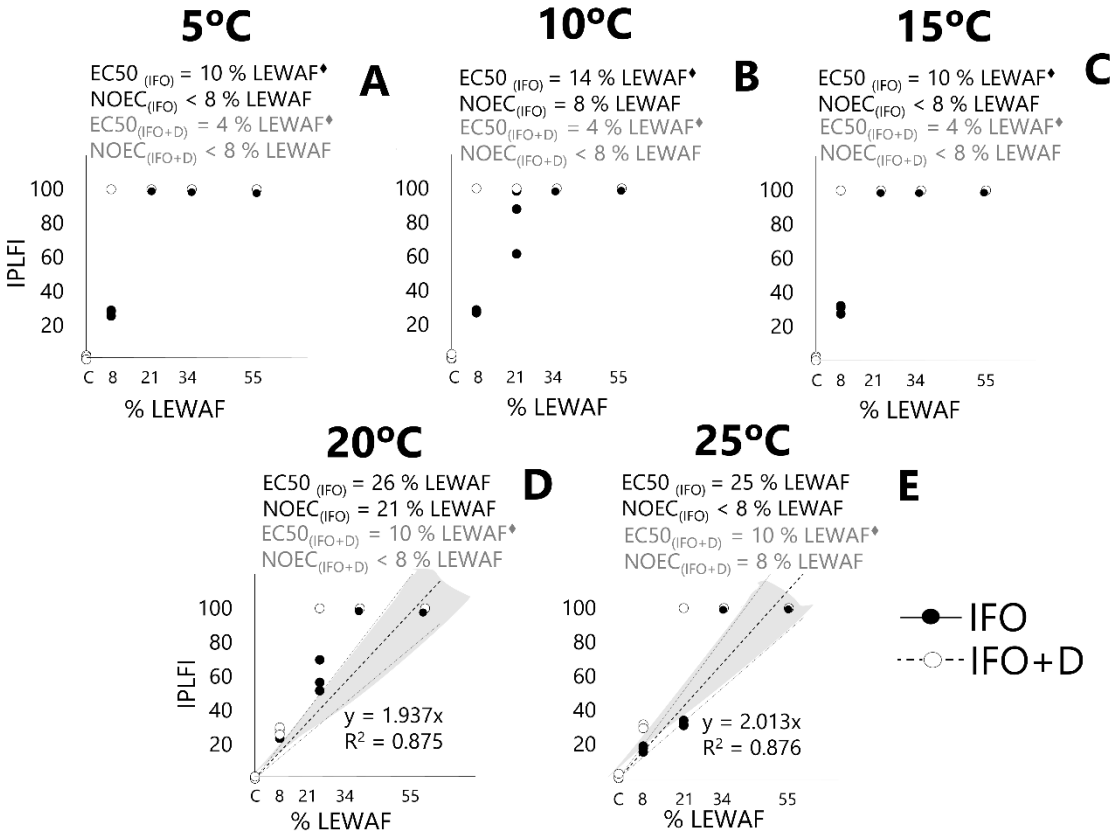

Figure 13. Inhibition pluteus larvae formation index (IPLFI) recorded in sea urchin embryos exposed to IFO and IFO+D LEWAFs produced at **A)** 5ºC, **B)** 10ºC, **C)** 15ºC, **D)** 20ºC and **E)** 25ºC. Median effective concentrations calculated upon linear regression models (EC50) or after probit analysis (EC50♦), and non-observed effect concentration (NOEC) values are shown for each case. No significant differences were found between linear regression coefficients of IFO and IFO+D LEWAF for each tested oil (ANCOVA; $p>0.05$).

The sum of TUs was below "1" for all the embryo toxicity endpoints investigated after exposure to IFO LEWAF and IFO+D LEWAFs irrespective of the production temperature in the 5-25ºC range (Table 7). RT_i values were greater than "1" suggesting that one or more individual PAHs exhibited more toxicity than predicted for the mixture toxicity. Particularly, RT_I/RC_I values greater than "1" were recorded for several individual PAHs after exposure to IFO without and with dispersant application and irrespective of the LEWAF production temperature (Table 7): 2-MN, Pyr, Ant, Fluo, B[a]A+Chr and B[a]P.

Table 7. Summary of the TU analysis of the toxicity of IFO and IFO+D LEWAFs produced at different temperatures based on the mixture of identified PAHs. The sum of TUs (∑TU) for each toxicity endpoint (ΔL, TI, IPLFI, CDI, GDI and MDI) is "1" if there is additive toxicity, ">1" if there is synergistic effect and "<1" if the toxicity is not caused by the mixture assuming the CA joint action. The sum of the TUs of individual PAHs vs. the TUs of the sum of PAHs ($\sum TU_{PAHi}/TU_{\sum PAHs}$) is "1" if all the PAHs in the mixture exert the same toxicity, ">1" if there are one or more individual PAHs with more toxicity than expected from its contribution to the mixture according to the CA model; and "<1" otherwise. The balance between the relative contribution of an individual PAH to the toxicity of the mixture and its relative contribution to the chemical composition of the mixture (RT_i/RC_i) is "1" if the individual toxicity of this PAH is the one expected due to its proportion in the mixture (CA model); "<1" if it is not a contributor to the mixture toxicity; and ">1" if there this PAH exerts toxicity beyond the one expected as a part of the mixture.

	5ºC		10ºC		15ºC		20ºC		25ºC	
	IFO	**IFO+D**	**IFO**	**IFO+D**	**IFO**	**IFO+D**	**IFO**	**IFO+D**	**IFO**	**IFO+D**
$\sum TU_{\Delta L}$	0.22	0.16	0.27	0.11	0.18	0.16	0.32	0.19	0.44	0.35
$\sum TU_{TI}$	0.25	0.12	0.26	0.10	0.20	0.05	0.32	0.22	0.12	0.37
$\sum TU_{IPLFI}$	0.07	0.06	0.10	0.05	0.07	0.06	0.20	0.07	0.17	0.12
$\sum TU_{CDI}$	0.25	0.45	0.30	0.10	0.31	0.26	0.75	0.22	0.20	0.36
$\sum TU_{GDI}$	0.24	0.13	0.20	0.10	0.20	0.08	0.28	0.21	0.31	0.35
$\sum TU_{MDI}$	0.22	0.12	0.20	0.09	0.18	0.08	0.28	0.22	0.22	0.33
$\sum TU_{PAHi}/TU_{\sum PAHs}$	2.29	3.15	2.22	3.15	2.27	2.77	2.24	2.85	2.36	2.53
RT/RC_{Naph}	0.08	0.06	0.08	0.06	0.08	0.06	0.08	0.06	0.08	0.07
$RT/RC_{1\text{-}MN}$	0.60	0.43	0.62	0.43	0.60	0.49	0.61	0.48	0.58	0.54
$RT/RC_{2\text{-}MN}$	2.17	1.58	2.25	1.58	2.20	1.80	2.23	1.75	2.11	1.97
RT/RC_{Acy}	0.43	0.31	0.44	0.31	0.43	0.36	0.44	0.35	0.42	0.39
RT/RC_{Ace}	0.48	0.35	0.50	0.35	0.48	0.40	0.49	0.39	0.47	0.43
RT/RC_{Flu}	0.19	0.14	0.19	0.14	0.19	0.15	0.19	0.15	0.18	0.17
RT/RC_{Ant}	18.48	13.46	19.11	13.45	18.67	15.32	18.92	14.89	17.94	16.74
RT/RC_{Phe}	0.86	0.63	0.89	0.63	0.87	0.72	0.88	0.70	0.84	0.78
RT/RC_{Pyr}	2.86	2.09	2.96	2.08	2.89	2.38	2.93	2.31	2.78	2.60
RT/RC_{Fluo}	1.46	1.06	1.51	1.06	1.48	1.21	1.50	1.18	1.42	1.32
$RT/RC_{B[a]A + Chr}$	36.96	26.92	38.22	26.89	37.33	30.65	37.83	29.78	35.88	33.48
$RT/RC_{B[a]P}$	-	1.79	-	1.79	-	2.04	-	1.99	-	2.23

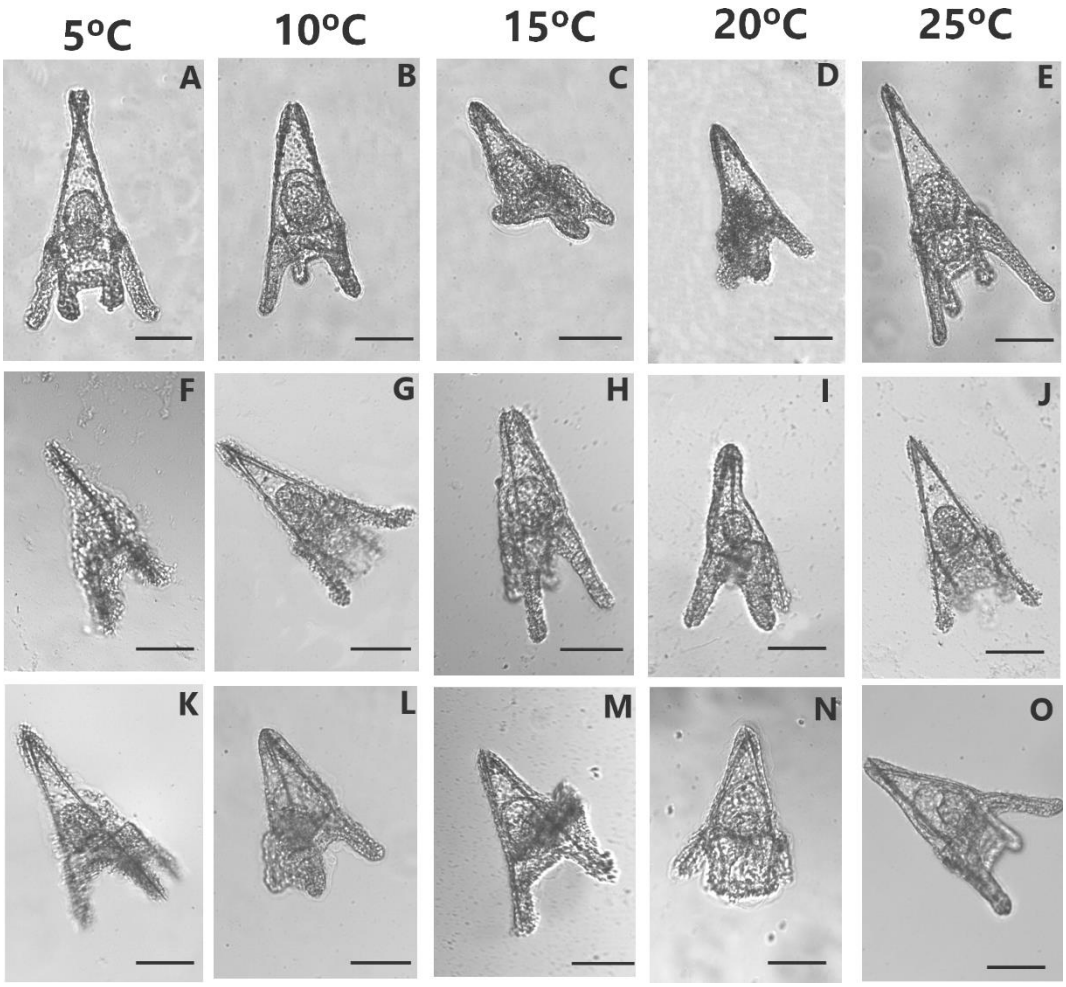

Figure 14. Photographs of sea urchin *P. lividus* larvae observed after 48 hr of exposure to oil NNA (A-E), MGO (F-J) and IFO LEWAF (K-O) produced at 5ºC (A,F,K), 10ºC (B,G,L), 15ºC (C,H,M), 20ºC (D,I,N) and 25ºC (E,J,O). Pictures corresponding to EC50 values in size increase (ΔL) or near to them. **A)** 55% NNA LEWAF; **B)** 55% NNA LEWAF; **C)** 55% NNA LEWAF; **D)** 55% NNA LEWAF; **E)** 55% NNA LEWAF; **F)** 34% MGO LEWAF; **G)** 55% MGO LEWAF; **H)** 55% MGO LEWAF; **I)** 55% MGO LEWAF; **J)** 55% MGO LEWAF; **K)** 21% IFO LEWAF; **L)** 34% IFO LEWAF; **M)** 21% IFO LEWAF; **N)** 55% IFO LEWAF; **O)** 55% IFO LEWAF. Scale bars 100 µm.

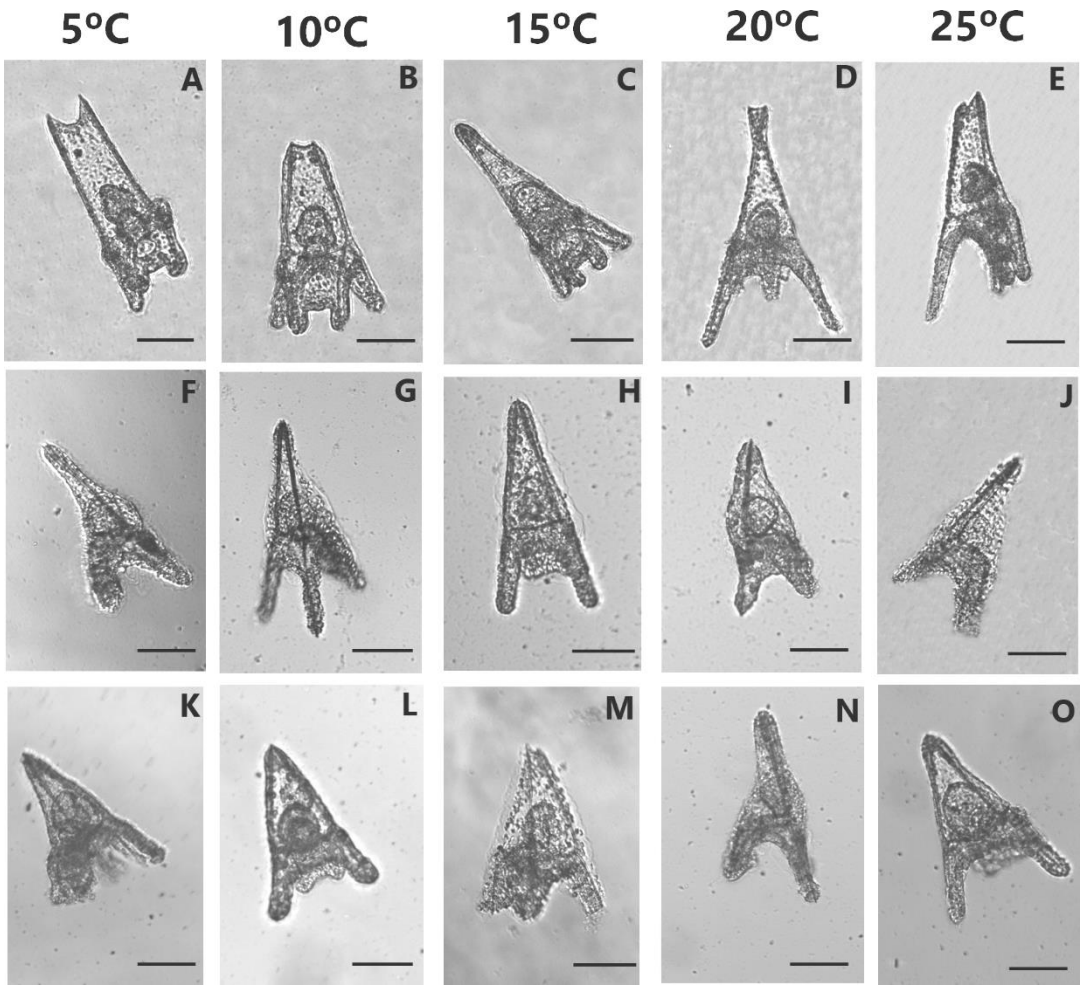

Figure 15. Photographs of sea urchin *P. lividus* larvae observed after 48 hr of exposure to NNA+D (A-E), MGO+D (F-J) and IFO+D LEWAF (K-O) produced at 5ºC (A,F,K), 10ºC (B,G,L), 15ºC (C,H,M), 20ºC (D,I,N) and 25ºC (E,J,O). Pictures corresponding to EC50 values in size increase (ΔL) or near to them. **A)** 34% NNA+D LEWAF; **B)** 21% NNA+D LEWAF; **C)** 34% NNA+D LEWAF; **D)** 21% NNA+D LEWAF; **E)** 55% NNA+D LEWAF; **F)** 21% MGO+D LEWAF; **G)** 34% MGO+D LEWAF; **H)** 21% MGO+D LEWAF; **I)** 55% MGO+D LEWAF; **J)** 34% MGO+D LEWAF; **K)** 8% IFO+D LEWAF; **L)** 8% IFO+D LEWAF; **M)** 8% IFO+D LEWAF; **N)** 21% IFO+D LEWAF; **O)** 21% IFO+D LEWAF. Scale bars 100 µm.

4. DISCUSSION

4.1. Influence of production temperature on oil LEWAF chemistry

The PAH profiles of oil LEWAFs were dominated by conspicuous concentrations of Naph, 1-MN, 2-MN, Ace, Flu and Phe when the LEWAFs were produced at 10ºC following standard procedures (Singer et al., 2000; Chapter 1) as well as when the LEWAFs were produced at lower (5ºC) and higher (15-25ºC) temperatures. However, the concentration of these PAHs was lower in NNA LEWAF produced at 5ºC and the concentrations of Ace, Flu and Phe were lower in NNA LEWAF produced at temperatures higher than 10ºC. Alike, the concentrations of Flu and Phe were higher when MGO LEWAF was produced at 25ºC than when it was produced at lower temperatures. Meanwhile, the concentration of these PAHs did not change with the LEWAF production temperature in the case of IFO. Thus, in agreement with previous studies (Faksness et al., 2008), the PAH profiles were found to be different depending on the oil and the temperature of LEWAF production. Nevertheless, the sum of PAHs (without naphthalenes) only showed minor variations amongst the LEWAFs of the three oils produced at different temperatures. The fraction of hydrocarbons that comprises the aqueous fraction of oils depends on the temperature (Perkins et al., 2003; 2005; Camus et al., 2015; Brown et al., 2016). The concentrations of PAHs and their methylated derivatives increased (more or less uniformly) significantly with increasing production temperature (from 15 to 25ºC) in the water-soluble fraction (WSF) of a light crude oil (Kuwait Oil) but decreased when temperature was taken to 35ºC; the concentration of aliphatics, however, decreased at increasing production temperatures in the 15-35ºC range (Saeed et al., 1998). MGO LEWAF produced at 0ºC presented low <n-C_9, equal n-C_9-C_{18} and high n-C_{19}-C_{28} PAHs (Brown et al., 2016). IFO LEWAF presented lowered levels of n-C_9-C_{18} at 0ºC but a slight increase in n-C_9-C_{18} at 5ºC (Brown et al., 2016). These temperature dependent differences in the PAH composition of the aqueous fraction of different oils might be explained because temperature plays an important role in PAH solubility. Small decreases in temperature may cause significant decreases in solubility of Phe, Ant and B[a]P (Whitehouse, 1984). In addition, the aqueous fractions are saturated differently depending on the pour point of the oil (Faskness et al., 2008).

Like in the case of oil LEWAFs, Naph, 1-MN, 2-MN, Ace, Flu and Phe were also the dominant individual PAHs in the profile of LEWAFs of the three oils upon addition of dispersant. However, other individual PAHs were also relevant in oil+D LEWAFs to a different degree depending on the oil and the temperature of LEWAF production. Thus, the concentrations of 1-MN and 2-MN in NNA+D LEWAFs at 10-15ºC were higher than at lower and higher production temperatures. Meanwhile, the concentrations of Naph and 2-MN in MGO+D LEWAF were higher when LEWAF was produced at 25ºC than when it was produced at lower temperatures; and no clear pattern regarding the effect of LEWAF production temperature was observed in the case of IFO+D LEWAFs. Likewise, in NNA+D LEWAF produced at 10ºC the concentrations of Pyr, Fluo and B[a]A+Chr were higher than in any other case, followed by the LEWAF produced at 5ºC. Meanwhile, the concentrations of Pyr and Fluo in MGO+D LEWAF were the highest at the highest LEWAF production temperature (25ºC) and the lowest at the lowest LEWAF production temperature (5ºC). In IFO+D LEWAFs, the concentrations of Ace, Flu, Phe, Acy, Ant, Pyr, Fluo and B[a]A+Chr were higher at the lowest temperatures of LEWAF production (5-15ºC), most remarkably at 5ºC. As a result, the sum of PAHs in LEWAFs upon dispersant application was lower at high production temperatures in the cases of NNA and MGO (20-25ºC in NNA+ LEWAF and 25ºC in MGO+D LEWAFs) and unrelated to production temperature in IFO; yet, the values were overall much higher than in the case of the corresponding oil LEWAFs without dispersant. Accordingly, total PAH concentrations are found to be higher in chemically dispersed mixtures (Saeed et al., 1998; Lyons et al. 2011; Li et al., 2021). Moreover, the $\sum_{HMW}$PAHs and $\sum_{LMW}$PAHs in oil+D LEWAFs varied depending on the production temperatures and the oil type. The light crude oil (NNA) presented the highest values of $\sum_{LMW}$PAHs and $\sum_{HMW}$PAHs upon dispersant application at 10ºC. In the case of the light bunker oil, whereas in MGO LEWAF the highest $\sum_{LMW}$PAHs was recorded at 25ºC, in MGO+D LEWAF highest $\sum_{HMW}$PAHs was found at 25ºC. However, at the lowest temperature (5ºC), the $\sum_{HMW}$PAHs was the highest in IFO LEWAF upon dispersant application. Increasing temperature of the water causes higher molecular weight aromatic compounds to dissolve more replacing lower molecular weight aromatic and non-aromatic compounds (Saeed et

al., 1998). Thus, elevated production temperature increases the proportion of $\sum_{HMW}$PAHs, and a decrease in $\sum_{LMW}$PAHs in oil aqueous fractions (Li et al., 2021).

Temperature and dispersant addition modified differently the PAH profile and levels in the LEWAFs of the three tested oils, which can be explained because MGO has lower viscosity than NNA and IFO thus rendering it more easily dispersible (EMSA, 2010). In addition to viscosity, the chemical composition of the oils also influences dispersant effectiveness, which is higher in presence of high saturate content and lower when the levels of asphaltene, and aromatic and polar components are high (Fingas et al., 1991). Temperature has an important role in determining dispersant effectiveness (Chandrasekar et al., 2005; Moles et al., 2001). For instance, dispersion increased in light refined oil (N.2 Fuel Oil), light crude oil (South Louisiana Crude Oil) and medium crude oil (Prudhoe Bay Crude Oil) when temperature increased from 5 to 22ºC, which was attributed to a decrease in oil viscosity (Chandrasekar et al., 2005). Likewise, the effectiveness of dispersants Corexit 9500 and SPC 1000 was found to be dependent on the temperature (Moles et al., 2001; Li et al., 2010).

As result of the aforementioned differences in PAH profiles and levels amongst oils depending on the LEWAF production temperature and the addition of dispersant, the toxicity level and toxicological profile of the LEWAFs can be expected to be different depending on the temperature of LEWAF production in a distinctive manner for each oil.

4.2. Influence of production temperature on oil LEWAF genotoxicity

In the present study, exposure to oil LEWAF produced DNA damage in sea urchin larvae, in agreement with previous results (Chapter 4). Two of the dominant PAHs identified in the LEWAFs, Ant and B[a]P, are known to cause DNA strand-breaks and cause genotoxicity to juvenile fish *(Trachinotus carolinus),* and to oyster *(Crassostrea gigas)* and mussel embryos *(Mytilus galloprovincialis)* (Wessel et al., 2007; Banni et al., 2010; Hasue et al., 2013; Ewa and Danuta 2017). Genotoxicity did not change with LEWAF production temperature in the cases of NNA and MGO LEWAF, whilst IFO LEWAFs produced at lower temperatures (5-10ºC) were more genotoxic than IFO

LEWAFs produced at high temperatures (20-25ºC). Yet, in both cases (MGO and IFO) the higher levels of DNA damage do not appear to be related to higher concentrations of the measured PAHs in the corresponding LEWAFs. In shrimp, *Pandalus borealis*, genotoxicity of the aqueous fraction of North Sea oil was lowered at a low production/exposure temperature of 5ºC compared to 10ºC, but in this case it was interpreted as the consequence of reduced sensitivity of the shrimp at low exposure temperature (Bechmann et al., 2010). This does not apply to the present study because exposure of sea urchin embryos to the various LEWAFs was carried out at one standard temperature of 20ºC. Further on, genotoxicity was enhanced upon dispersant application in the three oils. The level of DNA damage caused by exposure to oil and oil+D LEWAFs varied depending on the LEWAF production temperature in a different way for each oil. NNA+D LEWAFs produced at low temperatures (5-10ºC) were more genotoxic than the ones produced at higher temperatures (20-25ºC), which might be related to the higher levels of carcinogenic PAHs ($\sum_{HMW}$PAHs) and higher concentrations of certain individual PAHs (e.g., Ace, Flu, Phe, Pyr, Fluo and B[a]A+Chr) presently found in NNA+D LEWAFs produced at 5-10ºC. In contrast, genotoxicity did not vary with LEWAFs production temperature neither in MGO+D LEWAFs nor in IFO+D LEWAF, even though the concentrations of individual PAHs in MGO+D LEWAFs produced at 25ºC and in IFO+D LEWAFs produced at 5-15ºC were much higher than in LEWAFs produced at other temperatures. The 5-ring PAHs identified in IFO+D LEWAF (e.g., B[a]P+Chr) are known to cause genotoxicity through direct interaction with the DNA (Baird et al., 2005). However, it seems that the measured PAHs are not necessarily the cause of the genotoxic effects recorded in the LEWAFs of the three studied oils, in agreement with previous results about the genotoxicity of these oils weathered under ice (Chapter 4).

4.3. Influence of production temperature on oil LEWAF embryo toxicity

ΔL decreased and TI and IPLFI increased on exposure to the LEWAF of the three tested oils, without and with dispersant, irrespective of the temperature of LEWAF production, although the severity of the effects varied with the oil type, dispersant application and LEWAF production temperature. Similarly, a decrease in ΔL was also

observed in larvae of various sea urchin species. In *Hemicentrotus pulcherrimus* exposed to LEWAFs of N.0 diesel oil prepared at 24ºC (Lv and Xiong, 2009), *Echinometra lucunter* exposed to WSF of diesel oil (Pereira et al., 2018), *Strongylocentrotus droebachiensis* exposed to North Sea crude oil at 6.7ºC (Arnberg et al., 2018), and *P. lividus* exposed to WAFs of Angolan crude oil and heavy fuel oil prepared at 20ºC (Rial et al., 2013), and heavy fuel oil WAF (Prestige fuel oil) and intermediate fuel oil LEWAF (IFO 380) produced at 20ºC (Saco-Álvarez et al., 2008). A progressive decrease in ΔL was recorded at increasing LEWAF concentrations after exposure to NNA LEWAF produced at any temperature in the 5-25ºC range. Similarly, the same trend was observed in MGO except at the highest LEWAF production temperature tested (25ºC). In the case of IFO, an all-or-nothing response was observed on exposure to LEWAF produced at 5-15ºC whilst at higher temperatures (20 and 25ºC) a progressive decrease was recorded at increasing LEWAF concentrations. As a general rule, NNA and MGO LEWAFs were equally toxic in the range of 10-25ºC of LEWAF production temperature (EC50>50% LEWAF) but MGO LEWAF was more toxic than NNA LEWAF when was produced at 5ºC, as indicated by the lowered EC50 value. Meanwhile, IFO LEWAF was more toxic than NNA and MGO LEWAFs only when production temperature was below 25ºC. In all the oils, the toxic effects were exacerbated upon dispersant application, as revealed by steeper linear regression of ΔL against % LEWAF. Thus, EC50 values indicated that the LEWAFs of the three oils become much more toxic upon dispersant application for the whole range of LEWAF production temperatures studied herein (Fig. 8A). Nevertheless, in all the cases, irrespective of the oil, dispersant application and production temperature, the LEWAFs caused significant effects on $NOEC_{(\Delta L)}$ at the lowest exposure concentration tested herein (8% LEWAF) and therefore, potential long-term toxic effects cannot be disregarded in any case, even though in some cases EC50 values were as high as >100% LEWAF. Other studies with *P. lividus* exposed to oil LEWAF also found high EC50 values and low toxicity thresholds (NOEC, LOEC or EC10) (Saco-Álvarez et al., 2008; Rial et al., 2013).

TI remained around 0-20% on exposure to NNA LEWAF produced at any temperature in the studied 5-25ºC range but it increased linearly after exposure to MGO LEWAF produced at 5 and 15ºC, and abruptly to values of 80-100% on exposure to IFO LEWAF produced at any temperature. Upon dispersant addition to the three studied oils, TI rapidly reached 100% on exposure to different threshold concentrations of LEWAF, depending on the oil, at any temperature in the cases of NNA and IFO, and most especially at the lowest LEWAF production temperatures (5-15ºC) in the case of MGO. As a result, NNA was the least toxic (EC50>50% LEWAF) and IFO the most toxic (EC50<40% LEWAF), with MGO in between, for the whole range of LEWAF production temperatures (5-25ºC). The toxic effects were always more severe upon dispersant application, as revealed by lowered EC50 values, which was more marked in the case of IFO at low LEWAF production temperatures of 5-15ºC (Fig. 16B). Like in the case of ΔL, in all the cases, irrespective of the oil, dispersant application and production temperature, the LEWAFs caused significant effects on TI at low exposure concentrations (NOEC=<8-21% LEWAF).

IPLFI remained around 0-20% on exposure to NNA LEWAF produced at any temperature (5-25ºC). In contrast, it increased linearly at increasing concentrations of MGO LEWAF produced at 10-25ºC and IFO LEWAF produced at 20-25ºC, and abruptly to values of 100% upon exposure to a concentration of 20% MGO LEWAF produced at 5ºC, and to 20% IFO LEWAF produced at any of the temperatures used herein. In the three oils, dispersant addition caused an abrupt rise in IPLFI values at relatively low LEWAF concentrations, which was more marked in LEWAFs produced at low temperatures in the 5-15ºC range. As a result, NNA was less toxic (EC50>70% LEWAF) than MGO and IFO (EC50<30% LEWAF) for the whole range of LEWAF production temperatures, but much more remarkably at the lowest temperatures of LEWAF production (5ºC for MGO and 5-15ºC for IFO; Fig. 16C). Toxicity was enhanced by dispersant addition for the three oils, which was particularly critical for the case of NNA, with a $EC50_{(NNA+D)}$ 2-3 times lower than the $EC50_{(NNA)}$. Following the same scheme than ΔL and TI, irrespective of the oil, dispersant application and production temperature, the LEWAFs caused significant effects on IPLFI at low exposure concentrations (NOEC=<8-21% LEWAF).

The responses recorded in IPLFI are the result of the combination of alterations in particular phases of the embryo development process, indicated by changes in cleavage (CDI), gastrulation (GDI) and metamorphosis (MDI) (Chapter 1). According to the CDI values, NNA and MGO, without and with dispersant application, did not show a clear toxicity pattern except when oil+D LEWAF was produced at 5ºC, with EC50 values lower than 50% LEWAF. In contrast, CDI indicated that IFO was toxic (EC50<50% LEWAF) irrespective of the LEWAF production temperature, except for LEWAF produced at 20ºC; and dispersant addition resulted in extreme toxicity with EC50 values of 9-30% LEWAF (Fig. 17A). Regarding GDI, NNA exerted low toxicity (EC50>70%) irrespective of the LEWAF production temperature; however, dispersant addition enhanced NNA LEWAF toxicity (EC50<50% LEWAF) with an anomalous extremely low EC50 value recorded when LEWAF was produced at 10ºC (Fig. 17B). In contrast, IFO was shown to be more toxic, especially upon dispersant addition and at LEWAF production temperatures of 5-15ºC. The temperature-dependent toxicity pattern for MGO was unclear both without and with dispersant addition (Fig. 17B). Very similar integration of results can be done concerning MDI (Fig. 17C). In general, for all the three oils and regardless of the LEWAF production temperature, CDI, GDI and MDI toxicity critical values were lower upon dispersant application, as previously reported for LEWAF produced at 10ºC under standard conditions, when CDI was also less sensitive than the other two endpoints (Chapter 1).

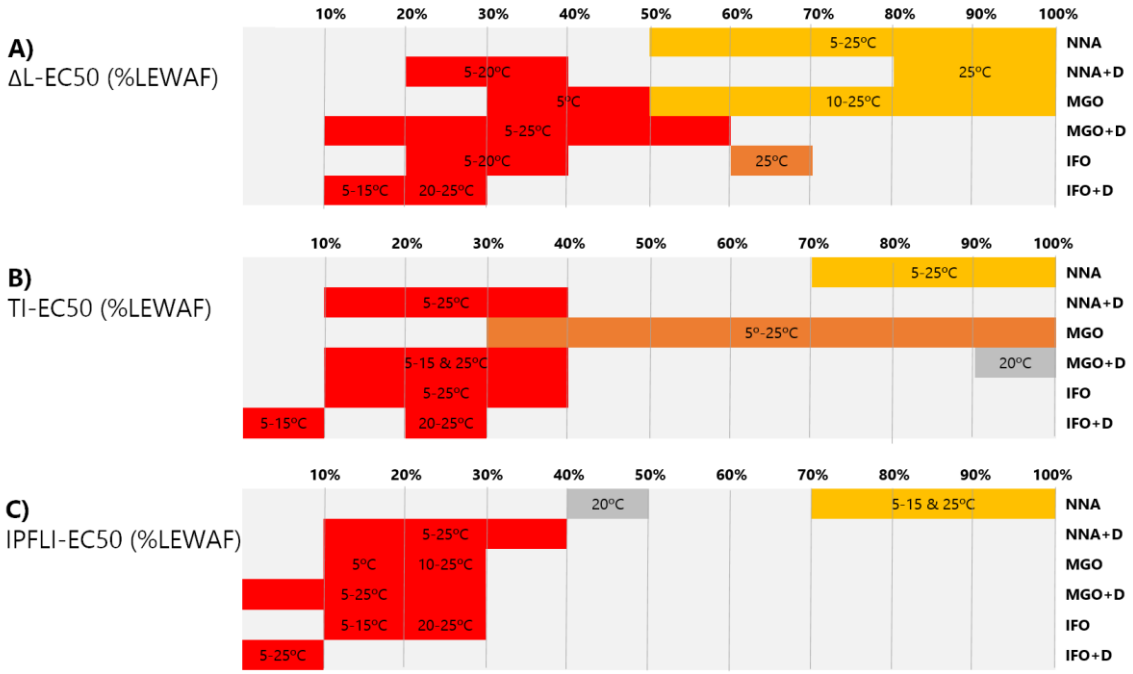

Figure 16. Range of variation along the LEWAF production temperatures (5-25ºC) in EC50 values represented for **A)** ΔL; **B)** TI; and **C)** IPFLI for each oil alone (NNA, MGO, IFO) and combined with dispersant (NNA+D,MGO+D, IFO+D). **Light orange**, low toxicity range (EC50<60% LEWAF); **dark orange**, mid or highly varying toxicity range; **red**, high toxicity range (EC50>50% LEWAF); **grey**, anomalous result.

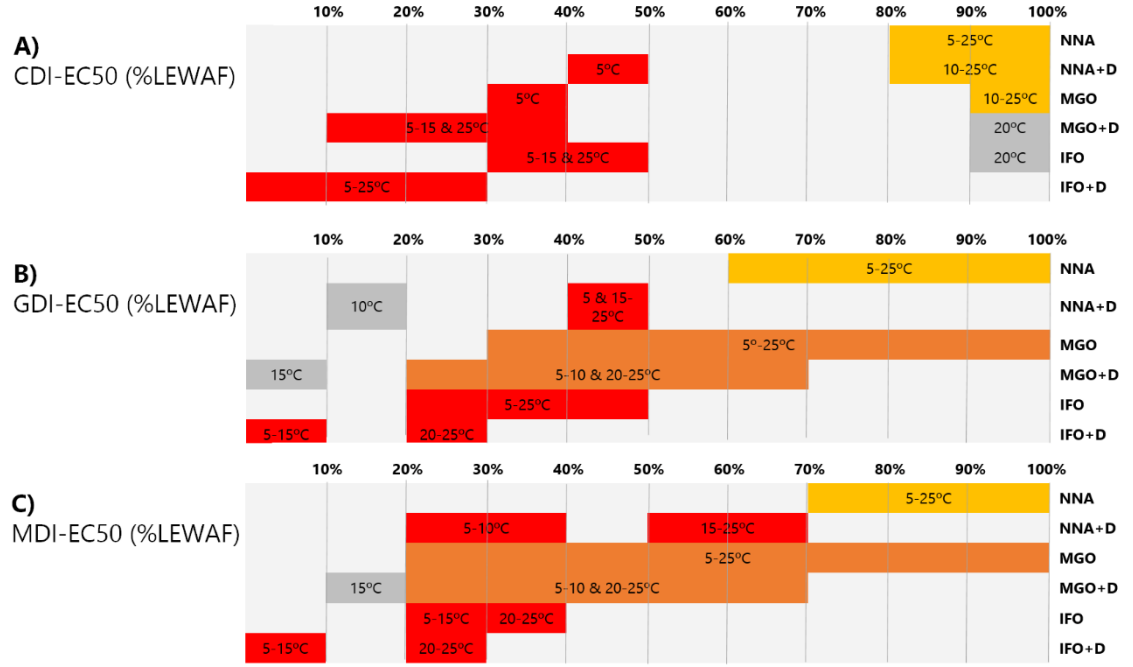

Figure 17. Range of variation along the LEWAF production temperatures (5-25ºC) in EC50 values represented for **A)** CDI; **B)** GDI; and **C)** MDI for each oil alone (NNA, MGO, IFO) and combined with dispersant (NNA+D,MGO+D, IFO+D). **Light orange**, low toxicity range (EC50<60% LEWAF); **dark orange**, mid or highly varying toxicity range; **red**, high toxicity range (EC50>50% LEWAF); **grey**, anomalous result.

Synergistic effects are reported between oil exposure and temperature in larvae of sea urchin *Strongylocentrotus droebachiensisk* and *Arbacia lixula* (Gianguzza et al., 2014; Arnberg et al., 2018), shrimp larvae *Pandalus borealis* (Arnberg et al., 2018), juvenile cod fish *Gadus morhua* (Lyons et al., 2011), mahi-mahi fish *Coryphaena hippurus* (Pasparakis et al., 2016; Perrichon et al., 2018), and Gulf killifish *Fundulus grandis* (Serafin et al., 2019). However, this explanation does not apply to the the present study because the temperature of exposure was only one (20ºC) irrespective of the LEWAF preparation temperature, which is the optimal one for sea urchin development (Shpigel et al., 2004). Thus, lower EC50 values at lower temperatures can be explained due to an increased persistence of toxic LEWAF compounds, and not to changes in the sensitivity of sea urchin embryos at low temperarures. Moreover, there is a greater loss of toxicants at higher temperaratures, as before reported (Korn et al., 1979), which could account for lowered toxicity when LEWAF was produced at higher temperatures.

The toxicity of mixtures of oil and dispersant could be attributed to the narcosis caused by the 2- and 3-ring PAHs and to toxicity caused by 4- to 6-ring PAHs (Irwin et al., 1997; Incardona et al., 2004, 2005; Hodson et al., 2007; Lee et al 2011; Le Bihanic et al., 2014). For *P. lividus* embryos, 4-ring PAHs (Pyr and Fluo) are more toxic than 3-ring PAHs (Flu and Phe) and these are more toxic than 2-ring PAHs (Naph) (Bellas et al., 2008). The concentration of these compounds in the LEWAFs varied depending on the production temperature; however, the concentrations of individual PAHs such as 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). Therefore, the identified individual PAHs seem not to be the main cause for toxicity of the oil LEWAFs. In agreement, TUs values were below "1" for all the embryo toxicity endpoints investigated after exposure to the three oils, alone or in combination with dispersant, regardless of the LEWAF production temperature, with a few exceptions, say:

- ΔL after exposure to NNA+D LEWAF produced at 25ºC;
- CDI after exposure to NNA+D LEWAF produced at 10, 20 and 25ºC;

- All the toxicity endpoints on exposure to NNA+D LEWAF produced at 15ºC.

However, RT_i values higher than "1" indicated that one or more individual PAHs exhibited more toxicity than predicted for the mixture toxicity of all the LEWAFs tested in this study. Accordingly, 2-MN, Pyr, Fluo, B[a]A+Chr and B[a]P in all the LEWAFs produced at any temperature in the 5-25ºC range, Phe in NNA+D LEWAF produced at 5 and 20-25ºC and in MGO LEWAF produced at any temperature, and Ant in all the IFO LEWAFs could be responsible for a part of the toxicity ($RT_i/RC_i>1$).

The TU approach limits the characterization of the mixture toxicity only in terms of the sum of identified PAHs. However, individual PAHs are not necessarily the major determinant of toxicity because the oil aqueous fractions are a cocktail of PAHs (many not identified) combined with other chemicals (Neff et al., 2000, Barron et al., 1999; Meador and Nahrgang, 2019; Wheeler et al., 2020). In most crude oils, most of the compounds are unidentified and commonly known as the unresolved complex mixture or UCM (Sammarco et al., 2013; Farrington and Quinn, 2015), which are likely important contributors to the oil toxicity (Meador and Nahrgang, 2019). In a preceding study (Chapter 1), the toxicity of the NNA, MGO and IFO LEWAFs, alone or in combination with Finasol OSR52®, could be only partially attributed to individual PAHs (only the USEPA 16 list PAHs) or the CA action of the mixture (Pelletier et al., 1997), and a large part of the toxicity was suggested to be due to the UCM and polar compounds (Chapter 1). The same conclusion seems to be reasonable in the present study as well. Nevertheless, the use of nominal proportion loading (% LEWAF) values seems to be a useful best available practice for toxicity assessment of oil aqueous fractions produced at different temperatures in the 5-25ºC range.

5. CONCLUDING REMARKS

The toxicity level and toxicological profile of the LEWAFs of the oils alone or combined with dispersant were different depending on the temperature of LEWAF production in a distinctive manner for each oil. The PAH profiles were found to be different depending on the oil and the temperature of LEWAF production. However, the sum of PAHs only showed minor variations amongst the LEWAFs of the three oils produced at different temperatures. Upon dispersant application, the sum of PAHs in LEWAFs was lower at high production temperatures (20-25ºC) in the cases of NNA and MGO, and unrelated to production temperature in IFO. Moreover, the values were overall much higher than in the case of the corresponding oil LEWAFs without dispersant. The level of DNA damage caused by exposure to oil and oil+D LEWAFs varied depending on the LEWAF production temperature in a different way for each oil. Moreover, the degree of genotoxicity was augmented after dispersant application. Likewise, oil LEWAF of the three tested oils caused length reduction, abnormalities and development impairment in pluteus larvae of *P. lividus,* irrespective of the temperature of LEWAF production, although the severity of the effects varied with the oil type, dispersant application and LEWAF production temperature. The toxicity of the LEWAFs of the three studied oils, alone or in combination with dispersant, could be only partially attributed to individual PAHs or to the mixture.

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CHAPTER 3

Heavy bunker oil burn residues toxicity to sea urchin embryos

ABSTRACT

In the past decades, climate change has promoted the retrieve of ice in Arctic regions boosting oil transportation and, therefore, increasing the risk of oil spill accidents in these areas. Conventionally countermeasures against oil spills are often unfeasible under the Arctic extreme conditions. Thus, *in-situ* burning (ISB), which offers a logistically simple and highly efficient means of eliminating large quantities of oil quickly and cheaply, is described as more suitable response tool. ISB is known to reduce the amount of oil spill residues remaining in water but these can persist on the sea surface or sink because their density increases rising the risk to affect marine organisms. In the present study, the sea urchin *(Paracentrotus lividus)* embryo toxicity assay (SET), toxicity index (TI), and sea urchin embryo development assay (SEDD) were applied to assess the toxicity of IFO burn residues (IFO-BR) obtained in a large-scale field experimental oil spill (project GRACE). The concentration of PAHs in the LEWAF (Low-Energy Water Accommodated Fraction) of ISB residues was lower than in the LEWAF of untreated oil. Particularly, $_{LMW}$PAH (low molecular weight PAH) concentration decreased and $_{HMW}$PAH (high molecular weight PAH) concentration increased but burn residues did not seem to be more toxic than the parental oil. Indeed, TU values were always very low and PAHs were not the main contributors to the observed toxicity, although some $_{HMW}$PAHs exhibited augmented their contribution to the toxicity of the mixture (LEWAF) after burning. Thus, toxicity might be attributed to other chemicals not identified in this study. The long-term effect and the composition of burned oil residues is not known and, in addition, burning produce smoke, volatiles, soot particles, additives and unburnt oil that may have some hazard potential to nearby wildlife, as well as, heavier PAHs which have a higher potential for bioaccumulation and may include mutagens and carcinogens that are persistent. Thus, further research in burn residues toxicity is needed using a battery of marine organisms and other type of oils, including changes in the oil weathering to have a multifactorial scenario that will provide a better understanding and knowledge in oil spill response decision-making.

Keywords: oil, *in-situ* burning, iced seas, sea urchin embryo, toxicity, mixtures.

RESUMEN

En las últimas décadas el cambio climático ha favorecido una reducción del hielo en las regiones árticas aumentando el tráfico petrolífero y, por lo tanto, incrementando el riesgo de accidentes causando vertidos de petróleo en estas áreas. Normalmente las medidas habituales que se utilizan para hacer frente a los vertidos de petróleo son inviables en ambientes con condiciones extremas como es el caso de las regiones árticas. Por esto, la quema *in-situ*, que sigue siendo una estrategia logísticamente simple y altamente eficiente para eliminar grandes cantidades de petróleo de forma rápida y barata, está descrita como uno de los procedimientos más adecuados en estos casos. Se sabe que esta estrategia de quema *in-situ* es una técnica que reduce la cantidad de residuos de petróleo que quedan en el agua tras un vertido. No obstante, estos residuos pueden persistir en la superficie del mar o hundirse, dado que su densidad incrementa tras la quema, y, por lo tanto, aumenta el riesgo para los organismos marinos. En el presente estudio se evaluó la toxicidad de los residuos de quema del petróleo IFO (IFO-BR) obtenido tras un experimento de campo a gran escala (proyecto GRACE) utilizando el test con embriones de erizo de mar *Paracentrotus lividus* (SET), la determinación del índice de toxicidad (TI) y el ensayo de la alteración del desarrollo de dicho organismo (SEED). La concentración de hidrocarburos policíclicos aromáticos (PAH) en el LEWAF (Low-Energy Water Accommodated Fraction) de los residuos de quema fue más baja que en el LEWAF del petróleo sin quemar. De hecho, la concentración de PAH de bajo peso molecular disminuyó y la concentración de PAH de alto peso molecular aumentó. No obstante, los residuos de quema *in-situ* no fueron más tóxicos que el petróleo sin quemar. Además, los valores de unidades tóxicas (TU) fueron siempre muy bajos y los PAH no fueron los contribuyentes principales a la toxicidad observada. Sin embargo, algunos PAH de alto peso molecular aumentaron su contribución a la toxicidad de la mezcla (LEWAF) tras la quema. Por ello, la toxicidad observada puede ser atribuida a la presencia de otros químicos no identificados en este estudio. El efecto de la quema *in-situ* a largo plazo y la composición de dichos residuos no se conoce. Además, la quema produce gases y partículas, y contiene aditivos y restos de petróleo no quemados que pueden tener un riesgo potencial para la fauna salvaje de las proximidades. De la misma forma, los PAH de alto peso molecular tienen una gran capacidad de bioacumulación y entre ellos se incluyen algunos mutagénicos y carcinogénicos que son persistentes. Por ello, es necesaria más investigación sobre la toxicidad de estos residuos de quema utilizando tanto una batería de distintos organismos marinos, así como de distintos tipos de petróleo, incluyendo cambios en el envejecimiento de este petróleo, para obtener un escenario multifactorial que nos ayude a obtener datos para mejorar el entendimiento y el conocimiento para la gestión y manejo de vertidos de petróleo.

Palabras clave: petróleo, quema *in-situ*, mares helados, embrión de erizo de mar, toxicidad, mezclas.

RESUM

En les dècades passades el canvi climàtic ha promogut la desaparició del gel en les regions àrtiques afavorint el transport de petroli i, per tant, incrementant el risc d'accidents que causen vessaments de petroli en aquestes àrees. Normalment, les mesures habituals que s'utilitzen per combatre els vessaments de petroli son inviables en ambients de condicions extremes com és el cas de les regions àrtiques. Per això, la crema *in-situ*, que és una estratègia logísticament simple i altament eficient per eliminar grans quantitats de petroli de forma ràpida i barata, està descrita com un dels procediments més adequats en aquests casos. Se sap que aquesta estratègia de crema *in-situ* es una tècnica que redueix la quantitat de residus de petroli que queden a l'aigua després d'un vessament. No obstant, aquests residus poden persistir en la superfície del mar o enfonsar-se, perquè la seva densitat augmenta després de la crema, augmentant el risc pels organismes marins. En el present estudi es va avaluar la toxicitat dels residus de crema del petroli IFO (IFO-BR) obtingut en un experiment de camp a gran escala (projecte GRACE) utilitzant el test amb embrions d'eriçó de mar *Paracentrotus lividus* (SET), la determinació de l'índex de toxicitat (TI) i l'assaig d'alteració del desenvolupament d'aquest organisme (SEED). La concentració d'hidrocarburs policíclics aromàtics (PAH) en el LEWAF (Low-Energy Water Accommodated Fraction) dels residus de crema va ser més baixa que en el LEWAF del petroli sense cremar. De fet, la concentració de PAHs de baix pes molecular va disminuir i la concentració de PAHs d'alt pes molecular va augmentar. No obstant, els LEWAFs dels residus de la crema *in-situ* no van ser més tòxics que els LEWAFs del petroli sense cremar. A més, els valors d'unitats tòxiques (TU) van ser sempre molt baixos i els PAHs no van ser els contribuents principals a la toxicitat observada. Malgrat això, alguns PAHs d'alt pes molecular van augmentar la seva contribució a la toxicitat de la mescla (LEWAF) després de la crema. Per això, la toxicitat observada pot ser atribuïda a la presència d'altres químics no identificats en aquest estudi. L'efecte de la crema *in-situ* a llarg termini i la composició dels seus residus es desconeix. A més, la crema produeix gasos i partícules, conté additius i restes de petroli sense cremar, que poden tenir un risc potencial per la fauna salvatge de les proximitats. De la mateixa manera, els PAHs d'alt pes molecular tenen una gran capacitat de bioacumular-se, i entre ells s'inclouen alguns mutagènics i carcinogènics que són persistents. Per això, és necessària més investigació sobre la toxicitat d'aquests residus de crema utilitzant tant una àmplia bateria d'organismes marins, així com de diferents tipus de petroli, incloent canvis en l'envelliment d'aquest petroli, per obtenir un escenari multifactorial que ens ajudi a obtenir dades per millorar la comprensió i el coneixement de cara a la gestió i maneig dels vessaments de petroli.

Paraules clau: petroli, crema *in-situ*, mars gelats, embrió d'eriçó de mar, toxicitat, mescles.

LABURPENA

Azken hamarkadetan, klima-aldaketaren eraginez, eskualde Artikoetan izotza urtzen ari da. Horren ondorioz, petrolioaren garraioa gune hauetan handitu egin da, baita horrek dakarren petrolio isurien arriskua ere. Petrolio isurien aurkako neurriak askotan ez dira bideragarriak Artikoko muturreko baldintzetan. Petrolioa bertan erretzea (ISB edo *in-situ* burning deritzon teknika) da logistikoki aukera errazena eta efizienteena. Petrolio kantitate handiak azkar eta era merkean kentzeko aukera ematen du eta hobekien egokitzen den sistema dela dirudi. Jakina da ISB teknikak uretan petrolio isurien ondorioz agertzen diren hondakinak gutxitzen dituela, baina hauek itsas azalean ere geratzen dira edo dentsitatea handitzen zaie eta hondoa jo dezakete itsasoko organismoak kaltetuz. Hemen aurkezten den ikerketan, itsas-trikuen *(Paracentrotus lividus)* toxikotasun testa (SET), toxikotasun-indizea (TI) eta enbrioien garapen proba (SEDD) aplikatu dira, IFO bat erretzearen ondorioz sortutako hondakinen toxikotasuna aztertzeko. Hain zuzen ere, GRACE proiektuan eskala handian lortutako hondakinak aztertu dira. ISB teknika aplikatu ondoren lortutako hondakinen LEWAFetan (Low-Energy Water Accommodated Fraction), PAH kontzentrazioak txikiagoak direla ikusi da tratatu gabeko petrolioen LEWAFetan baino. Batez ere, $_{LMW}$PAH (pisu molekular baxuko PAH) kontzentrazioa murrizten da eta $_{HMW}$PAH (pisu molekular handiko PAH) kontzentrazioa handitu egiten da, baina errekuntza ondoren lortutako hondakinek ez dute jatorrizko petrolioa baino toxikotasun handiagorik. Izan ere, TU balioak oso baxuak izan dira beti eta PAHak ez dira toxikotasunaren jatorri nagusia, nahiz eta $_{HMW}$PAH batzuek beren ekarpena gehitzen dioten nahasketari erre ondoren. Horrela, toxikotasuna azterketa honetan identifikatuta ez dauden beste produktu kimiko batzuei lepora dakieke. Epe luzerako eragina eta erretako petrolio hondakinen konposizioa ez da ezagutzen, eta, horrez gain, erretzeak kea, lurrunak, kedar-partikulak, eta gehigarriak uzten ditu, baita erre gabeko petrolioa ere. Hau inguruko faunarentzat arriskutsua izan daiteke, baita PAH astunagoak ere, biometatzeko potentzial handiagoa dutenak eta mutageno eta kartzinogeno iraunkorrak barne har ditzaketenak. Horrela, erretako hondakinetan toxikotasun ikerketa gehiago behar da itsas organismoen eta beste olio mota batzuen sorta zabalago bat erabiliz, baita petrolioaren higadura aldaketak kontuan hartuz aniztasun handiko agertoki bat izateko, olio isurien aurkako neurriak hobeto ulertzeko eta erabakiak hartzeko ezagutza handitzeko.

Hitz gakoak: olioa, ISB erretze teknika, itsaso izoztuak, itsas-trkiu enbrioia, toxikotasuna, nahasketak.

1. INTRODUCTION

In the past decades, climate change has promoted the retrieve of ice in Arctic regions (AMAP, 2019) boosting new spaces to be exploited as oil and gas resources and the possibility of new maritime routes. Those new traffic lines imply more oil transportation and, therefore, the risk of oil spill accidents in these areas is gaining concern. Low temperature, reduced visibility in winter-season and the presence of ice imply a challenge in oil spill response in iced seas, due to logistic difficulties and remoteness (Sørstrøm et al., 2010; EPPR, 2015). Mechanical recovery and the application of chemical dispersants, conventionally the first choices of countermeasure against oil spills (Ventikos et al., 2004), are often unfeasible under the Arctic extreme conditions; alternatively, *in-situ* burning (ISB) is described as more suitable response tool (Sørstrøm et al., 2010; API, 2015). ISB offers a logistically simple and highly efficient means of eliminating large quantities of oil quickly and cheaply, which can be conducted when other techniques are less effective or unavailable (Allen and Ferek, 1993). Thus, burning conditions and efficiency have been extensively investigated (Fritt-Rasmussen, 2010; Fritt-Rasmussen et al, 2012; Van Gelderen, 2017; Faksness and Altin, 2019). ISB is known to reduce the amount of oil spill residues remaining in water (Fritt-Rasmussen et al., 2015) but these can persist on the sea surface or sink because their density increases (Buist et al., 1977). Moreover, the burning process is different for different oils so that the residues also present a different chemical composition (Fritt-Rasmussen et al., 2015; Van Gelderen, 2017).

Although the investigations on the toxicity of ISB residues are scarce, overall it seems that burn residues exhibit a low toxicity to aquatic organisms. Thus, based on acute toxicity tests using three-spine stickleback *(Gasterosteus aculeatus)* embryos and White Sea urchin *(Lytechinus pictus)* gametes, the water-accommodated fraction (WAF) of the burn residue of a light crude oil (Alberta Sweet Mixed Blend; ASMB) was shown not to be more toxic than the WAF of the weathered crude oil (Blenkinsopp et al., 1996). Likewise, the WAF of burned ASMB residues was not toxic to eccentric sand dollar gametes and embryos, oyster embryos and inland silverside juveniles (Daykin et al., 1994). The WAF of burned Bass Strait (BS) stabilised crude oil was found to exert

very low lethal toxicity to amphipods, *Allorchestes compressa*, and low sublethal toxicity to marine sand snails, *Polinices conicus* (Gulec and Holdway, 1999); and to have no-effect on behavioural responses in asteroids (Georgiades et al., 2003). For Australian bass and Australian 11-armed asteroid exposed to BS crude oil, dispersed crude oil, and WAF of burnt crude oil WAFs in the laboratory, it was concluded that the dispersed oil was more acutely toxic than the crude oil and that the burn residue was least toxic overall (Cohen and Nugegoda 2000; Georgiades et al. 2003). The WAF of the ISB residues of IFO180 intermediate fuel oil did not increase the toxicity to zebrafish early life stages compared with the WAF of the fresh oil (Johann et al., 2020). Finally, the water soluble fraction (WSF) of the burn residues of Naphthenic Norwegian crude oil (Troll B) was found not to be more toxic than the WSF of the crude oil, as derived from toxicity testing using the Microtox bioassay and the marine copepod *Calanus finmarchicus* (Faksness et al., 2012).

In this context, within the framework of the UE-funded project GRACE (Jørgensen et al., 2019), ISB was applied after a large-scale field experimental oil spill carried out in Greenland coast (Wegeberg et al., 2018b). Approximately 1000 L of Intermediate Fuel Oil 180 (IFO; Appendix I) were released on the water surface into a fire-resistant boom in a fjord close to Kangerluarsoruseq (Greenland; July 2017) and ignited for 40 min. Burn residues were collected on absorbent oil-wetting cloth and stored in plastic bags at -20ºC (Wegeberg et al., 2018a) for further chemical and toxicological analyses. The toxicity of IFO burn residues to zebrafish early life stages (Johann et al., 2020) and copepods (Marigómez et al., 2019) was investigated. The present study sea urchin *(Paracentrotus lividus)* embryo toxicity assay (SET; Beiras et al., 2012), toxicity index (TI; Carballeira et al., 2012), and sea urchin embryo development assay (SEDD; Chapter 1) were applied to contribute to the toxicological characterisation of the IFO burn residues obtained in the Greenland field experiment. Sea urchin embryo toxicity tests are commonly used to assess the toxicity of metals, petroleum compounds, pharmaceuticals, biocides and complex mixtures of chemicals (Fernández et al., 2006; Saco-Álvarez et al., 2008; Bellas et al., 2013; Magesky et al., 2016; Morroni et al., 2016; Mijangos et al., 2020; Chapters 1, 2 and 4).

2. MATERIALS AND METHODS

2.1. LEWAF production and chemical analyses

The Low-Energy Water Accommodated Fraction (LEWAF) of the burn residue of IFO (IFO-BR LEWAF) and untreated IFO oil (IFO LEWAF) in filtered sea water (FSW) was produced in the darkness at 10ºC according to Katsumiti et al. (2019), modified after Singer et al. (2000). Briefly, oils (1:200; w oil /v FSW) were poured into filtered seawater (FSW) in 200 ml glass bottles and stirred at 200±20 rpm (no vortex; low energy) for 40 h.

The specific PAH composition of IFO-BR LEWAF was determined by gas chromatography-mass spectrometry (GC-MS) after Prieto et al. (2007). A mix standard solution of 18 PAHs[1] (CRM47543; Supelco, Bellefonte, USA) was used for calibration in the GC-MS analysis. A mixture of 5 deuterated compounds[2] (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 Appendix II.

The specific PAH composition of the IFO LEWAF was characterised following the same procedure (Prieto et al., 2007), as detailed in Chapter 1.

[1] Naphthalene (Naph), 1-methylnaphthalene (1-MN), 2-methylnaphthalene (2-MN), acenaphthylene (Acy), acenaphthene (Ace), fluorene (Flu), anthracene (Ant), phenanthrene (Phe), pyrene (Pyr), fluoranthene (Fluo), benz[a]anthracene (B[a]A), chrysene (Chr), benzo[a]pyrene (B[a]P), benzo[b]fluoranthene (B[b]F), benzo[k]fluoranthene (B[k]F), benzo[g,h,i]perylene (B[g,h,i]P), dibenz[a,h]anthracene (D[a,h]A), indeno[1,2,3-cd]pyrene (I[1,2,3-cd]P).

[2] Norwegian Standard (S-4124-200-T): naphthalene-d^8, byphenyl-d^{10}, phenanthrene-d^{10}, pyrene-d^{10}, benzo[a]anthracene-d^{12}, benzo[a]pyrene-d^{10}, benzo[ghi]perylene-d^{12}

2.2. Sea urchin embryo toxicity test (SET)

The sea urchin 48 hr 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 (43º26'01.1"N 2º53'56.1"W; Bay of Biscay) in spring (May-June) 2020. Spawning was induced by injecting 1 mL 0.5M 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 capped containing 10 mL of the test solutions (50 embryos/mL) to conduct toxicity assays (in completely darkness at 20ºC).

Successive dilutions of either IFO-BR or IFO LEWAF in FSW (0, 8, 21, 34, 55, and 89%) were prepared following a Fibonacci dose escalation after excluding some of the lower doses from the dilution series in order to optimise the experimental set up as described in Chapter 1.

After 48 hr exposure, 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 exposure replicates) and the egg size at t_0 (L_0 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; Appendix III). Images were taken with NIS-Elements Imaging Software v4.30 (Nikon Instruments BV). Size increase ($\Delta L=L-L_0$) and its EC_{50} were calculated (Beiras et al., 2012).

2.3. Toxicity Index (TI) in pluteus larvae

Specific abnormalities of the pluteus larvae were recorded (n=100 larvae per vial × 3 exposure replicates) and integrated in the Toxicity Index (TI, in a 0-100 range; after Carballeira et al., 2012; Appendix IV). 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).

2.4. Sea urchin embryo developmental disruption (SEDD) assay

Sublethal toxicity was evaluated as the capacity of sea urchin to undergo its developmental program (Appendix V); this was measured in terms of inhibition of pluteus larvae formation index (IPLFI), and potential mechanisms of toxic action on developmental processes were identified by examining main stages of developmental progression: cleavage disruption index (CDI) and gastrulation disruption index (GDI) during embryo development; and metamorphosis disruption index (MDI) during larval development.

Briefly, the longest dimension of each larvae (L_i in µm; n=35 larvae per vial × 3 exposure replicates) was measured as detailed 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 exposure replicates) to calculate the indices described in Chapter 1:

$$IPLFI = \frac{1}{r}\sum_{i=1}^{r} \frac{(L_{max} - L_i) \times 100}{(0.5 \times L_{max})};$$

$$CDI = \frac{100}{2 \log (C+G+N+P1)} \times \log \frac{(C+G+N+P1) \times C}{G+N+P1};$$

$$GDI = \frac{100}{2 \log (G+N+P1)} \times \log \frac{(G+N+P1) \times G}{N+P1}; \text{ and}$$

$$MDI = \frac{L_{max} - Li}{0.5 \times L_{max}} \times \frac{100}{2 \log (C+G+N+P1)} \times \log \frac{(C+G+N+P1) \times (C+G)}{(N+P1)};$$

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

2.5. Toxic units

The concentrations in a mixture of individual pollutants expressed as fractions of the EC50 of each pollutant (toxic units –TUs–; Sprague, 1970), the relative contribution of each individual PAH to the TUs of LEWAFs (RT_i) and the relative concentration of each PAH in the mixtures (RC_i) were calculated according to Chapter 1. For this purpose, EC50 values for various individual PAHs (Naph, Fluo, 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, braquiopods, decapods, mollusc larvae, echinoderm larvae and fish juveniles) were used as consensus EC50 to calculate the TUs (Appendix VI; Ward et al., 1981; Holcombe et al., 1983; Trucco et al., 1983; Spehar, 1999; Lyons et al., 2002; Pillai et al., 2003; Calbet et al., 2007; Bellas et al., 2008; Frantzen et al., 2012; Renegar et al., 2017; Knap et al., 2017). Then, the relative contribution of each individual PAH to the TUs of the mixture ($\sum TU_{\sum PAHs}$) was determined as $RT_i = TU_{PAHi} / \sum TU_{\sum PAHs}$; where TU_{PAHi} means the TU estimated for this individual PAH. In parallel, the relative concentration of each PAH in the mixture was calculated as $RC_i = C_{PAHi} / \sum PAHs$; where C_{PAHi} stands for the individual concentration of each PAH. Thus, the ratio RT_i/RC_i 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).

2.6. Data treatment and 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 sets, the non-parametric Kruskal-Wallis test was used. Differences between each exposure group among treatments were tested using the parametric Student's t-test for normal data sets and U Mann-Whitney for non-normal data sets. Linear regressions were compared using the ANCOVA test. Level of significance for all analyses was $p<0.05$.

3. RESULTS & DISCUSSION

The ∑PAHs (Naph included) was reduced to a 50% in IFO-BR LEWAF (103.2 µg/L) in comparison with IFO LEWAF (268.5 µg/L), whilst ∑PAHs (without Naph) was similar in both LEWAFs (Table 1). Overall, the concentration of PAHs in the LEWAF of ISB residues seems to be lower than in the LEWAF of untreated oil, as previously reported (Blenkinsopp et al. 1996; Bender et al. 2018; Johan et al., 2020). Thus, using the same IFO-BR than in the present study, Johann et al. (2020) found that ∑PAHs (Naph included) was reduced to a 30% in IFO-BR LEWAF compared with IFO LEWAF (52.2 vs. 184.8 µg/L; LEWAF produced in fish medium at 1:50 w/v), whilst ∑PAHs (without Naph) was in a comparable range (7-15 µg/L) in both LEWAFs. In agreement, in the NOBE field experiment, the burn residue was depleted in PAHs and only 25-30% remained, compared with the parent oil. In parallel, whilst $\sum_{LMW}$PAHs (without Naph) was similar in both LEWAFs, $\sum_{LMW}$PAHs (Naph included) was reduced in IFO-BR LEWAF and $\sum_{HMW}$PAHs was higher in IFO-BR than in IFO LEWAF (Table 1). Accordingly, the ∑PAHs (16 USEPA list) was decreased and pyrogenic PAH species of highest boiling-point were enriched in burn residues of Statfjord crude oil (Garrett et al. 2000). Also, the ISB residues of Louisiana crude oil were depleted in light PAHs (3-rings or less), the main decrease corresponding to semi-volatile PAHs such as Naph (Li et al., 1992). Seemingly, the more volatile toxic compounds (e.g., Naph) are removed during the burning process (Buist et al., 1999; Buist, 2000). In parallel, the release of total petroleum

hydrocarbons (TPHs) and PAHs into the underlying water is enhanced during burning (Cohen et al., 2001; 2005). ∑PAHs (Naph included) in post-burnt water samples was found to be twice higher than in pre-ignition water samples for the ASMB light crude oil (4 *vs.* 2 µg/L; Daykin et al., 1994), and 3 times for BS stabilised crude oil (6 *vs.* 2 µg/l; Gulec and Holdway, 1999). This loss of PAHs could also contribute to the lower levels PAHs presently recorded in IFO-BR LEWAF in comparison with IFO LEWAF.

Table 1. GC-MS analysis of PAHs (ng/L) in IFO-BR LEWAF and IFO LEWAF (*Characterised in Chapter 1). (UDL: under detection limits; $_{LMW}$PAHs: Low molecular weight polycyclic aromatic hydrocarbons; $_{HMW}$PAHs: High molecular weight polycyclic aromatic hydrocarbons; #: Total of PAHs without Naphthalene).

	IFO-BR LEWAF	**IFO LEWAF**
Naph	42129	92285
1-MN	41887	77566
2-MN	13021	91365
Acy (1)	2751	419
Ace (1)	336	2607
Flu (1)	1487	1673
Ant (1)	132	188
Phe (1)	1361	2337
Pyr (2)	58	40
Fluo (2)	48	18
B[a]A + Chr (2)	17	7
B[a]P (2)	UDL	UDL
B[b]F + B[k]F (2)	11	UDL
B[g,h,i]P (2)	UDL	UDL
D[a,h]A (2)	UDL	UDL
I[1,2,3-cd]P (2)	UDL	UDL
∑PAHs	103239	268504
∑PAHs #	6202	7288
∑$_{LMW}$PAHs	103105	268439
∑$_{LMW}$PAHs Σ(1)	6068	7223
∑$_{HMW}$PAHs Σ(2)	134	65

Moreover, the PAH profile of the water accommodated and water soluble fractions of oils seems to be altered after burning (Fig 1). Burned residues can be enriched in $_{HMW}$PAHs, pyrogenic PAHs, and metals (Buist, 2004; Shigenaka et al., 2015; Fingas, 2017), and include an altered unresolved complex mixtures (UCM; Bender et al., 2018).

Thus, it was reported that $_{LMW}$PAHs decrease and $_{HMW}$PAHs increase, both waterborne and in burn residues (Georgiades et al., 2003; Faksness et al., 2012; Fritt-Rasmussen et al., 2015), as the result of the loss of volatile PAHs and the formation of pyrogenic PAHs during burning (Wang et al 1999; Faksness et al., 2012; Fritt-Rasmussen et al, 2013). Due to their lower boiling point, the lighter compounds are more efficiently removed by burning and, though the heavy compounds are also removed, the heaviest ones concentrate in the oil residues (Fritt-Rasmussen et al., 2015). Thus, Alaska North Slope burn residues lack light compounds and contain a 90% heavy compounds (50% of asphaltenes and resins), including very high molecular weight hydrocarbons (Buist and Trudel, 1995). These latter are most likely originated by incorporation of salt from evaporated water during the vigorous burning phase or from ash or soot from the combustion (Buist and Trudel, 1995). In agreement, Faksness et al. (2012) reported a reduction of the light oil components and a small increase of 4-6 ring PAHs in burn residues of Troll B crude oil, even in absence of evaporation. Conversely, Buist and Bjerkelund (1986) and Benner et al. (1990) did not find differences in composition and concentration of PAHs in burn residues compared to fresh crude oil. It is conceivable that the final chemical composition of the burn residues may depend on the initial oil type and the efficiency of the burning, as previously suggested (Buist et al. 1999; Fritt-Rasmussen et al. 2013). Yet, in the present study the PAH profile of the LEWAFs is clearly altered after ISB of IFO oil.

Thus, Naph, 1-MN, 2-MN, Phe and Ace concentrations were lower in IFO-BR LEWAF than in IFO LEWAF, especially 2-MN (Table 1). Similarly, Naph, Fluo and Phe, the dominant PAHs in IFO-BR and IFO LEWAF produced in fish medium (1:50 w/v), were reduced by 60-70% in IFO-BR LEWAF in comparison with IFO LEWAF (Johann et al., 2020). In contrast, the concentration of Pyr, Fluo, B[a]A+Chr and B[b]F + B[k]F, and more markedly Acy, was higher in IFO-BR LEWAF than in IFO LEWAF (Fig. 1; Table 1). In fresh and weathered Troll B crude oil Naph was greatly depleted after ISB but Ace, Flu, and Phe were only partially reduced in the residue and Ant, Fluo and Pyr were enriched (Fritt-Rasmussen et al., 2015). The chemical profile of burnt diesel oil also was shown to change after burning (Wang et al., 1999). These changes included:

- depletion of alkylbenzenes;
- decrease of Naph and its alkylated homologues;
- increase of Chr and its alkylated homologues; and
- increase of >4 ring unsubstituted PAHs such as I[1,2,3-c,d]P, D[a,h]A and B[g,h,i]P.

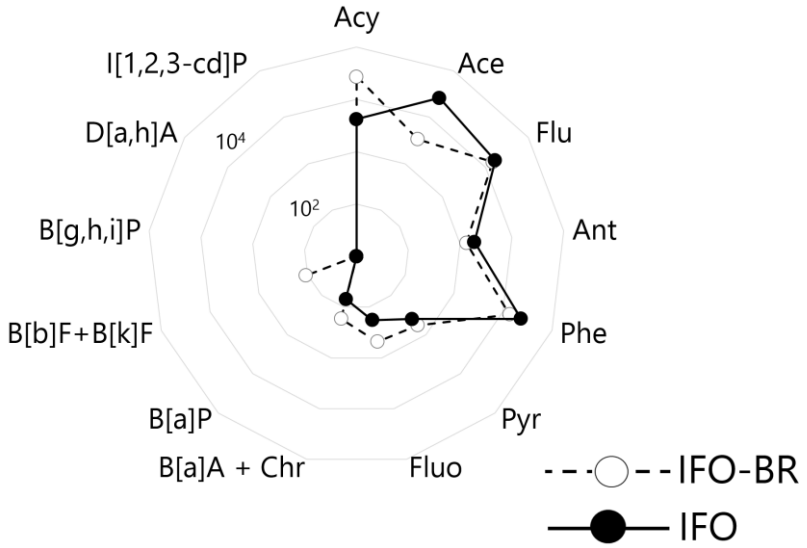

Figure 1. Profile of PAHs, represented in logarithmic scale, in IFO-BR LEWAF and IFO LEWAF.

ΔL was progressively reduced at increasing concentrations of IFO-BR LEWAF and IFO LEWAF, slightly less markedly in the former (ANOVA; p<0.05; Fig. 2 and 3A), with NOEC values of <8% LEWAF in both cases and not disparate EC50 values ($EC50_{IFO-BR}$=74% LEWAF; $EC50_{IFO}$=59% LEWAF). Likewise, a concentration dependent linear increase was observed for both IFO-BR LEWAF and IFO LEWAF in TI, GDI, MDI and IPLFI (ANCOVA; p>0.05; Figs. 3B and 3D-3F). In contrast, no consistent changes were recorded in CDI after exposure to either IFO-BR or IFO LEWAF (Fig. 3C). Thus, the degree of developmental completion (ΔL; Beiras et al., 2012), frequency of abnormalities (TI, Carballeira et al., 2012), developmental progression (gastrulation –GDI– or metamorphosis –MDI– arrest; Chapter 1), and potential ecological fitness (IPLFI; Chapter 1) were compromised in sea urchin embryos by both IFO LEWAF and IFO-BR LEWAF. Yet, the levels of toxicity were always moderate to low. Comparable toxicity results were found for zebrafish embryos exposed to the LEWAF of the same burn residues, which were determined to be less toxic than the IFO LEWAF ($EC50_{IFO-BR}$=41% LEWAF vs. $EC50_{IFO}$=23.7% LEWAF; Johann et al. 2020). The EC50 (Microtox) was 47% WSF prior to burning and 39% WSF after burning, and the 96-hr LC50 (copepod,

Calanus finmarchicus) was above 50% WSF indicating that the WSF did not become more toxic after burning (Faksness et al., 2012). The 96-hr LC50 (amphipod, *Allorchestes compressa)* and the 24-hr LC50 (snail, *Polinices conicus)* were, respectively, 80% LEWAF and 60% LEWAF of the burn residues of BS crude oil (Gulec and Holdway, 1999). As a rule, burn residues seem not to be more toxic than the parental oil (Blenkinsopp et al., 1996; Cohen and Nugegoda, 2000; Cohen et al., 2006; Faksness et al., 2012; Georgiades et al., 2003; Gulec and Holdway, 1999; Johann et al., 2020).

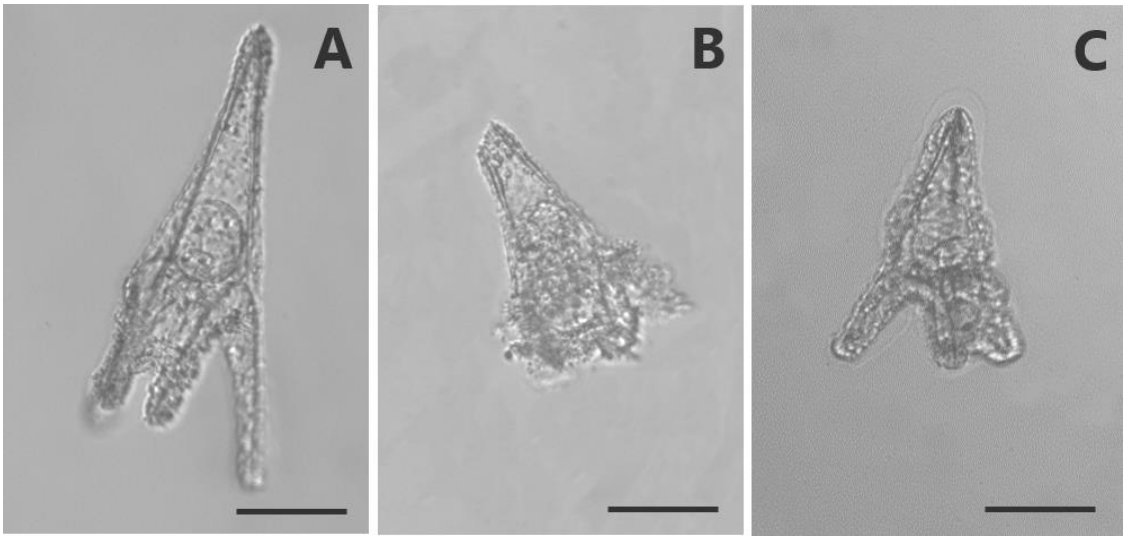

Figure 2. Photographs of sea urchin *P. lividus* larvae observed after 48 hr of exposure to **A)** Control, **B)** 55% IFO-BR LEWAF and **C)** 55% IFO LEWAF. Pictures corresponding to near EC50 values in size increase (ΔL). Scale bars 100 µm.

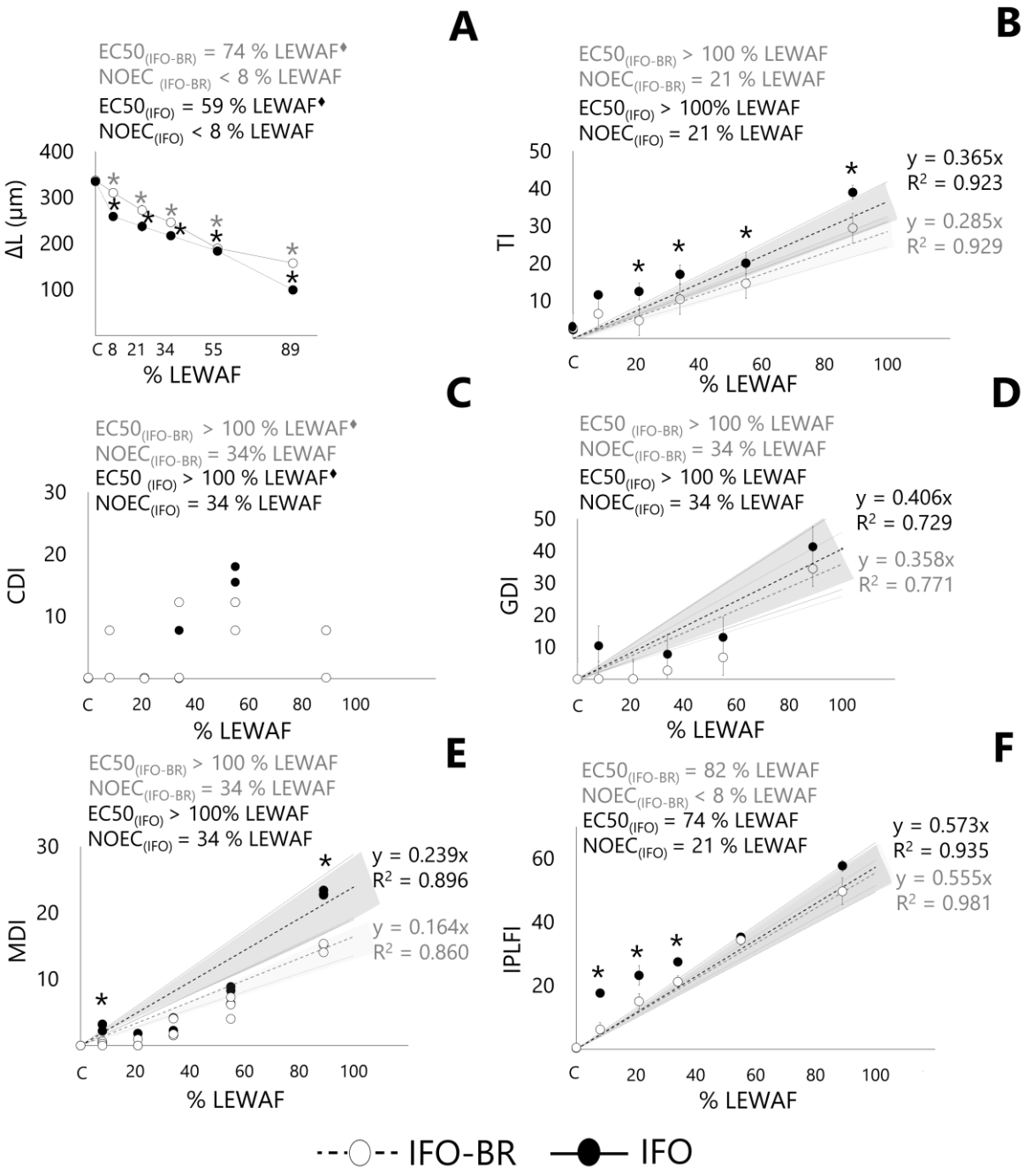

Figure 3. Endpoints recorded in sea urchin embryo after exposure to IFO-BR LEWAF and IFO LEWAF. **A)** Size increase (ΔL in µm), **B)** TI (Toxicity Index), **C)** CDI (Cleavage Disruption Index), **D)** GDI (Gastrulation Disruption Index), **E)** MDI (Metamorphosis Disruption) and **F)** IPLFI (Inhibition Pluteus Larvae Formation Index). Median effective concentration (EC50) calculated upon linear regression models or after probit analysis (EC50♦) and non-observed effect concentration (NOEC) values are shown for each case. Only significant regression models are represented (p<0.05). No significant differences were found between linear regression coefficients of IFO-BR and IFO LEWAF for each tested oil (ANCOVA; p>0.05). Asterisks indicate significant differences between IFO-BR and IFO LEWAF for each concentration (Student's t test or U Mann-Whitney; p<0.05).

On the other hand, TU values were always very low (TU<0.1) after IFO-BR LEWAF exposure and, although still below 1, they were 10× higher on exposure to IFO LEWAF (TU<0.7; Table 2). Thus, it seems that PAHs in any case, not even the higher levels of $_{HMW}$PAHs in the case of IFO-BR LEWAF, were the main contributors to the observed toxicity. The augmented relative presence of $_{HMW}$PAHs and their hydroxyl and alkyl derivatives, know to be toxic (Heintz et al., 1999; Saco-Álvarez et al., 2008). Overall, the most toxic fraction of WAF would include the unresolved complex mixture and polar compounds (Neff et al., 2000; Booth et al., 2007), which could be enriched in the water column after burning (Fritt-Rasmussen et al. 2015; Bender et al., 2018). Unfortunately, these chemicals were not quantified in the present study despite their potential contribution to adverse effects. RT_i values were greater than "1" on exposure to both LEWAFs, more remarkably on exposure to IFO (Table 2), revealing that some individual PAHs deviated from the Concentration Addition joint action (Altenburger et al., 2003). RT_i/RC_i values greater than "1" (Table 2) were found for Phe on exposure to IFO-BR LEWAF, and for 2-MN, Pyr and Fluo, and most remarkably for Ant and for B[a]A+Chr on exposure to both LEWAFs. Moreover, RT_i/RC_i values were always higher in IFO-BR than in IFO LEWAF, especially for Ant and B[a]A+Chr (RT/RC_{Ant}: 30 *vs.* 19; $RT/RC_{B[a]A+Chr}$: 61 *vs.* 38). The $_{HMW}$PAHs cause genotoxicity, carcinogenicity and endocrine disruption (Banni et al., 2010; Booc et al., 2014; Alharty et al., 2017).

Nevertheless, we must be cautious regarding the lower (or at least not higher) toxicity of burn residues LEWAF in comparison with parental oil LEWAF. Indeed, burning might change the chemical profile of a spilled oil and hence lead to modified toxicity (Wang et al., 1999; Georgiades et al., 2003; Faksness et al., 2012; Fritt-Rasmussen et al. 2015; Bender et al., 2018). For instance, the levels of $_{HMW}$PAHs in seawater increases after burning (as discussed above) and these compounds are known to cause sublethal toxicity (Fritt-Rasmussen et al. 2015). Together with changes in the chemical profile, burning may also alter the physical characteristics of the oil (viscosity, stickiness, droplet size), which may modify the exposure route and exposure time, thus enhancing toxicity (Bender et al., 2018).

Table 2. Summary of the TU analysis of the toxicity of IFO-BR LEWAF and IFO LEWAF based on the mixture of identified PAHs. The sum of TUs (∑TU) for each toxicity endpoint (ΔL, TI, IPLFI, CDI, GDI and MDI) is "1" if there is additive toxicity, ">1" if there is synergistic effect and "<1" if the toxicity is not caused by the mixture assuming the CA joint action. The sum of the TUs of individual PAHs *vs.* the TUs of the sum of PAHs ($\sum TU_{PAHi}/TU_{\sum PAHs}$) is "1" if all the PAHs in the mixture exert the same toxicity, ">1" if there are one or more individual PAHs with more toxicity than expected from its contribution to the mixture according to the CA model; and "<1" otherwise. The balance between the relative contribution of an individual PAH to the toxicity of the mixture and its relative contribution to the chemical composition of the mixture (RT_i/RC_i) is "1" if the individual toxicity of this PAH is the one expected due to its proportion in the mixture (CA model); "<1" if it is not a contributor to the mixture toxicity; and ">1" if there this PAH exerts toxicity beyond the one expected as a part of the mixture.

	IFO-BR LEWAF	**IFO LEWAF**
$\sum TU_{\Delta L}$	0.02	0.41
$\sum TU_{TI}$	0.01	0.70
$\sum TU_{IPLFI}$	0.01	0.52
$\sum TU_{CDI}$	0.05	0.70
$\sum TU_{GDI}$	0.02	0.70
$\sum TU_{MDI}$	0.01	0.70
$\sum TU_{PAHi}/TU_{\sum PAHs}$	1.39	2.22
RT/RC_{Naph}	0.13	0.08
$RT/RC_{1\text{-}MN}$	0.98	0.62
$RT/RC_{2\text{-}MN}$	3.58	2.25
RT/RC_{Acy}	0.71	0.44
RT/RC_{Ace}	0.79	0.50
RT/RC_{Flu}	0.31	0.19
RT/RC_{Ant}	30.46	19.11
RT/RC_{Phe}	1.42	0.89
RT/RC_{Pyr}	4.72	2.96
RT/RC_{Fluo}	2.41	1.51
$RT/RC_{B[a]A + Chr}$	60.91	38.22

Interestingly, thought high EC50 values (from 74% to 100% LEWAF) indicated that the toxicity of IFO-BR LEWAF was low, as a general rule recorded NOEC values were quite low (e.g., <8% LEWAF for ΔL and IPLFI and 21% for TI; which are the most environmentally relevant endpoints; Fig. 3; Table 3). In agreement low NOEC values and high EC50 values were also recorded on exposure to burnt ASMB oil (Daykin et al., 2004). Noteworthy, whilst survival and growth were not affected in the short-term in polar cod, *Boreogadus saida*, exposed to burn residues, long-term effects on reproduction occurred (Bender et al., 2018). Similarly, higher toxicity was reported for

Menidia beryllina and *Palaemonetes pugio* after 16-d subacute exposure to partially combusted Kuwait crude oil compared to the pure crude oil (Gundersen et al., 1996). Therefore, enhanced long-term effects of ISB residues cannot be fully disregarded.

Table 3. Schematic representation of toxicity critical values recorded in sea urchin embryos for CDI, GDI, MDI, IPLFI, TI and ΔL on exposure to IFO, IFO-BR and IFO+D LEWAFs. Green: NOEC or lower concentration; yellow: concentration between NOEC and EC50; orange: EC50 or higher concentration; red: 100% effect (EC100). Data of IFO+D LEWAF obtained from Chapter 1).

TOXICITY ENDPOINT	TESTED OIL	% LEWAF						
		0	8	21	34	55	89	100
CDI	IFO							
	IFO-BR							
GDI	IFO							
	IFO-BR							
MDI	IFO							
	IFO-BR							
IPLFI	IFO							
	IFO-BR							
TI	IFO							
	IFO-BR							
ΔL	IFO							
	IFO-BR							

4. CONCLUDING REMARKS

In summary, the concentration of PAHs in the LEWAF of ISB residues is lower than in the LEWAF of untreated oil. Particularly, $_{LMW}$PAH concentration decreases and $_{HMW}$PAH concentration increases but burn residues do not seem to be more toxic than the parental oil. Indeed, TU values were always very low and PAHs were not the main contributors to the observed toxicity, although some $_{HMW}$PAHs exhibited augmented their contribution to the toxicity of the mixture (LEWAF) after burning. Thus, toxicity might be attributed to other chemicals not identified in this study.

Overall, it seems that ISB would reduce a large mass of the oil without increasing the toxicity of the remaining burn residues. However, the environmental impacts from residues may be more severe than the ones derived from the low toxicity anticipated

from the toxicity tests, as in the present study. The long-term effect and the composition of burned oil residues is not known and, in addition, burning produce smoke, volatiles, soot particles, additives and unburnt oil that may have some hazard potential to nearby wildlife (Potter et al., 2012; Fritt-Rasmussen et al., 2015). Moreover, heavier PAHs have a higher potential for bioaccumulation and, in addition, may include mutagens and carcinogens that are persistent (USEPA-IRIS, 2021).

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CHAPTER 4

Toxicity to sea urchin embryos of crude and bunker oils of interest in iced seas weathered under ice alone and mixed with dispersant

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De Miguel L, Reinardy H, Lekube X, Izagirre U, Marigómez I. Toxicity to sea urchin embryos of three types of WAFs weathered under ice, alone and mixed with dispersant.

ABSTRACT

Arctic regions are highly vulnerable to oil spills due to their peculiar environmental conditions (e.g. presence of ice-cover) and remoteness, which can influence oil spill impact and response differently compared to temperate regions. Spill consequences and countermeasures may also vary depending on whether the oil has been spilled on or under the ice. Therefore, the present investigation aims at determining how under ice weathering modifies the toxicity of crude and bunker oils of interest in iced seas (Naphthenic North Atlantic crude oil, marine gas oil, and an intermediate fuel oil IFO 180) and how this toxicity is influenced by the application of a commonly used dispersant (Finasol OSR52®). For these purposes, a multi-index approach including larval size increase, larval malformation, developmental disruption and genotoxicity as endpoints, was applied. The long-term weathering under ice contributed to enhance WAF toxicity irrespective of the use dispersant. The PAH levels measured in UIWAFs (Under Ice Water Accommodated Fraction) were lower than in the corresponding LEWAF (Low-Energy Water Accommodated Fraction), and they were similar amongst the UIWAFs of different oils tested as the direct consequence of long-term weathering under ice. Moreover, the addition of dispersant produced minimal and less consistent changes in the PAH levels, composition and toxicity in the UIWAFs in comparison with the LEWAFs. Overall, oil UIWAF and LEWAF of the three tested oils caused length reduction, abnormalities, development impairment and DNA damage in pluteus larvae of *Paracentrotus lividus*. However, the individual PAH levels found in UIWAF were 1-3 times lower than the effective concentrations reported for those PAHs. Hence, it is quite unlikely that these individual PAHs constitute a realistic concern regarding UIWAF toxicity.

Keywords: oil, under ice weathering, dispersant, sea urchin embryo, toxicity, mixtures.

RESUMEN

Las regiones árticas son altamente vulnerables a los vertidos de petróleo debido a sus peculiares condiciones ambientales (p. ej. la presencia de una capa de hielo) y por su inaccesibilidad, que puede influenciar de forma diferente el impacto que tenga el vertido de petróleo y de las maniobras de respuesta a este en comparación a las regiones temperadas. Las consecuencias de los vertidos de petróleo y las acciones en contra de estos pueden también variar dependiendo de si el petróleo ha sido vertido por encima o por debajo del hielo. Por eso, el objetivo de la presente investigación fue determinar cómo las condiciones de envejecimiento bajo hielo modifican la toxicidad de tres tipos de petróleo (el crudo ligero: Naphthenic North Atlantic (NNA)); el diésel: Marine Gas Oil (MGO); y el fueloil: Intermediate Fuel Oil 180 (IFO)), de interés en mares boreales mediante y cómo esta toxicidad se ve influenciada por la aplicación de un dispersante comúnmente utilizado (Finasol OSR52®). Para estos propósitos se aplicó una aproximación utilizando multi-índices entre los que se incluyeron los indicadores de longitud larvaria, malformación larvaria, alteración del desarrollo y genotoxicidad. El envejecimiento bajo hielo contribuyó a aumentar la toxicidad del WAF (Water Accommodated Fraction) independientemente del uso de dispersante. Los niveles de hidrocarburos policíclicos aromáticos (PAH) en los UIWAFs (Under Ice Water Accommodated Fraction) fueron más bajos que los medidos en los correspondientes LEWAFs (Low-Energy Water Accommodated Fraction), y fueron similares entre los UIWAFs de los diferentes petróleos como una consecuencia directa del envejecimiento del petróleo a largo plazo. Además, la adición de dispersante produjo unos cambios mínimos y menos consistentes en los niveles de PAHs, en la composición y en la toxicidad de los UIWAFs en comparación de los LEWAFs. En general, los UIWAFs y LEWAFs de los tres petróleos causaron una reducción de la longitud larvaria, anormalidades y una alteración en el desarrollo, así como daño en el DNA de las larvas de erizo *Paracentrotus lividus*. No obstante, los niveles de PAHs individuales encontrados en los UIWAFs fueron de 1-3 veces más bajos que sus concentraciones efectivas registradas para esos PAHs y, por lo tanto, es bastante improbable que estos PAHs individuales constituyan una preocupación realista en referencia a la toxicidad del UIWAF.

Palabras clave: petróleo, envejecimiento bajo hielo, dispersante, embrión de erizo de mar, toxicidad, mezclas.

RESUM

Les regions àrtiques són altament vulnerables al vessaments de petroli degut a les condicions ambientals peculiars que presenten (p. ex. la presència d'una capa de gel) i per la seva inaccessibilitat, que poden influenciar de forma diferent l'impacte que tingui el vessament de petroli i de les maniobres de resposta a aquest en comparació amb les regions temperades. Les conseqüències dels vessaments de petroli i les accions en contra d'aquests poden també variar depenent de si el petroli ha sigut vessat per sobre o per sota del gel. Per això, l'objectiu de la present investigació va ser determinar com les condicions d'envelliment sota el gel modifiquen la toxicitat del petroli de diferents tipus de petroli en mars glaçats (un petroli lleuger (Naphthenic North Atlantic crude oil; NNA), un diesel marí (Marine Gas Oil; MGO) i un fueloil pesat (Intermediate Fuel Oil 180; IFO) i com aquesta toxicitat es veu influenciada per l'aplicació d'un dispersant regularment utilitzat (Finasol OSR52®). Per aquest propòsit es va aplicar una aproximació utilitzant multi-índex entre els que es van incloure la longitud larvària, malformació larvària, alteració del desenvolupament i genotoxicitat. L'envelliment sota el gel va contribuir a augmentar la toxicitat del WAF independentment de l'ús de dispersant. Els valors dels nivells d'hidrocarburs policíclics aromàtics (PAH) en els UIWAFs (Under Ice Water Accommodated Fraction) van ser més baixos que els nivells mesurats en els LEWAFs (Low-Energy Water Accommodated Fraction) corresponents i van ser similars entre els UIWAFs dels diferents petrolis com a conseqüència directa de l'envelliment del petroli a llarg termini. A més, l'addició de dispersant va produir uns canvis mínims i menys consistents en els nivells de PAHs, en la composició i en la toxicitat dels UIWAFs en comparació amb els LEWAFs. En general, els UIWAFs i els LEWAFs dels tres petrolis van causar una reducció de la longitud larvària, anormalitats i una alteració en el desenvolupament així com dany a l'ADN de les larves d'eriçó de mar *Paracentrotus lividus*. No obstant, els nivells de PAHs individuals trobats en els UIWAFs van ser d'1 a 3 vegades més baixos que les concentracions efectives registrades per aquests PAHs i, per tant, és bastant improbable que aquests PAHs individuals constitueixin una preocupació realista en referència a la toxicitat del UIWAF.

Paraules clau: petroli, envelliment sota el gel, dispersant, embrió d'eriçó de mar, toxicitat, mescles.

LABURPENA

Artikoko eskualdeak oso ahulak dira petrolio isurien aurrean, ingurumen-egoera bereziak dituztelako (izotz-azalaren presentzia, adibidez) eta urrun daudelako. Horrek eragina izan dezake isuriaren inpaktuan eta horiei aurre egiteko neurrietan, eskualde epelekin alderatuz. Isurien ondorioak eta kontrako neurriak ere alda daitezke, olioa izotzaren gainean edo azpian egon baitaiteke. Horregatik, ikerketa honen helburua da izoztutako itsaso batean, izotzaren azpian dagoen petrolioaren (Naphthenic North Atlantic olio gordina, itsas gasezko olioa eta tarteko erregai olio bat IFO 180) toxikotasunean zahartze baldintzek eta ohikoa den sakabanatzaile batek (Finasol OSR52®)) duten eragina aztertzea. Ikerketarako, indize anitzeko teknika bat aplikatu da, non larba-tamainaren handitzea, larba-malformazioa, garapen-nahastea eta genotoxikotasuna kontuan hartu diren. Izotzaren azpian luzaroan egoteak WAFaren (Water Accommodated Fraction) toxikotasuna handitzen lagundu du eta sakabanatzaileak ez du eraginik izan. UIWAFen (Under Ice Water Accommodated Fraction) neurtutako PAH (polycyclic aromatic hydrocarbons) mailak dagokion LEWAFen (Low-Energy water Accommodated Fraction) baino baxuagoak izan ziren, eta antzekoak izan ziren olio ezberdinen UIWAFen artean, izotzaren eraginpean epe luzeko ehunaren ondorio zuzena izanik. Gainera, sakabanatzaileak gehitzeak aldaketa minimoak eta ez hain koherenteak eragin zituen PAH mailetan, konposizioan eta toxikotasunean UIWAFetan, LEWAFen aldean. Gainera, UIWAFek eta hiru petrolioen LEWAFek larben luzera murriztea eragin zuten, anormaltasunak, garapen-nahasteak eta DNAren kalteak *Paracentrotus lividus* itsas-trikuetan. Hala ere, UIWAFen aurkitu ziren PAH mailak 1 eta 3 aldiz baxuagoak izan ziren PAH-entzat jakinarazitako kontzentrazio efektiboak baino. Beraz, oso litekeena da banakako PAHs horiek ez izatea benetako kezka UIWAF toxikotasunari dagokionean.

Hitz gakoak: olioa, izotz ehunaren azpian, sakabanatzailea, itsas-triku enbrioia, toxikotasuna, nahasketak.

1. INTRODUCTION

Nowadays, interest in Arctic regions is blooming because they contain a noteworthy amount of undiscovered oil and gas reserves (Gautier et al., 2009). In parallel, sea ice retreat driven by climate change is enhancing petroleum exploration and maritime transport in Arctic seas, thus increasing the threat of oil spills (OGP, 2013; Yang et al., 2018). Regrettably, Arctic ecosystems are highly vulnerable to oil spills due to their peculiar environmental conditions and remoteness, which can influence oil spill impact and response differently compared to temperate regions.

The low temperature of seawater and, more remarkably, the presence of ice-cover cause deferred oil spreading, diminished oil droplet emulsification due to wind-waves flattening, and attenuated physico-chemical weathering and biodegradation (Brandvik and Faksness, 2009; Sørstrøm et al., 2010; Daling et al., 2012). Moreover, in the presence of ice-cover the oil spill impact and response may also vary depending on whether the oil has been spilled on or under the ice, as well as on characteristics of the ice-cover. Spreading and encapsulation of the spilled oil, and weathering and potential toxicity of spill products are crucial. These processes can be largely modified depending on the duration of the ice-cover season, ice type and concentration, thickness, growth rate, drift velocity, and physical and mechanical properties of the ice-cover (Faksness and Brandvik, 2008; Nordam et al., 2017; Wilkinson et al., 2017).

Remoteness and severe climatological conditions in the Arctic can be major factors hampering clean-up operations after oil spills and therefore the use of chemical dispersants and *in-situ* burning manoeuvres, together with natural attenuation, are considered suitable alternative response tools (Sørstrøm et al., 2010; Wilkinson et al., 2017). *In-situ* burning operations are not expected to generate additional residues but the remaining residues will persist on the sea surface or sink (Fritt-Rasmussen et al., 2015), and may produce and atmospheric impact due to the release of large quantities of particles derived from the combustion of oil (McGrattan et al., 1997; Sartz and Aggarwal, 2016). Dispersants reduce interfacial tensions thus enhancing fragmentation of the oil slick into small droplets and allowing hydrocarbon-degrading bacteria to

breakdown the oil more rapidly (Prince et al., 2016; Soloviev et al., 2016). On the other hand, these processes can modify the chemical composition of the water accommodated fraction (WAF) of the spill products and intensify the toxicity to marine biota (Lee et al., 2013; Adeyemo et al., 2015; DeLeo et al., 2016; Chapter 1). Oil-in-ice behaviour (encapsulation, spreading, migration and weathering) has been thoroughly investigated in order to improve oil spill response in iced seas (Fingas and Hollebone, 2003; Brandvik and Faksness, 2009; Afenyo et al., 2016; Boccadoro et al., 2018; Øksenvåg et al., 2019; Nordam et al., 2020; Singsaas et al., 2020). Conversely, toxicity of under ice weathered oil is, to our knowledge, lacking.

Therefore, the present investigation aims at determining how under ice weathering modifies the toxicity of crude and bunker oils of interest in iced seas (Naphthenic North Atlantic crude oil (NNA), marine gas oil (MGO), and an intermediate fuel oil IFO 180 (IFO)) and how this toxicity is influenced by the application of a commonly used dispersant (Finasol OSR52®). For these purposes, sea urchin embryo toxicity assay (SET; Beiras et al., 2012), toxicity index (TI; Carballeira et al., 2012), and sea urchin embryo development assay (SEDD; Chapter 1) were applied. Sea urchin embryos are sensitive to oil exposure and have been often used to assess the toxicity of the oil WAF (Fernández et al., 2006; Saco-Álvarez et al., 2008; Bellas et al., 2013; Chapter 1). In parallel, genotoxicity was also determined using the Fast Micromethod® DNA Single-Strand-Break Assay adapted to sea urchin larvae (Scröder et al., 2006; El-Bibany et al., 2014; Reinardy and Bodnar, 2015; Reinardy et al., 2016).

2. MATERIALS AND METHODS

2.1. LEWAF production and chemical analyses

Three petroleum compounds and one dispersant were selected as relevant regarding potential oil spills in Arctic regions (Appendix I):

- *Naphthenic North Atlantic crude oil (NNA),* a very light crude oil of low viscosity, rich in branched and cyclic saturated hydrocarbons.
- *Marine gas oil (MGO),* a distillate marine gas oil, supplemented with the dye Dyeguard Green MC25 (John Hogg Technical Solutions; UK).
- *Intermediate Fuel Oil IFO 180 (IFO),* a heavy bunker oil of high viscosity with low amounts of volatile hydrocarbons (Polaroil, Greenland).
- *Finasol OSR52® dispersant (D),* a third-generation dispersant containing >30% non-ionic and 15–30% anionic surfactants (Total Special Fluids, France; SDS no. 30034 2015).

The water accommodated fraction of oils weathered under ice (UIWAF), alone (NNA UIWAF, MGO UIWAF and IFO UIWAF) or mixed with dispersant (NNA+D UIWAF, MGO+D UIWAF and IFO+D UIWAF) was produced in a cold chamber at -4±2ºC in complete darkness for 2 months. For this purpose, glass tanks (35×25×25 cm) were filled with 15 L filtered seawater (FSW; 32 psu) and capped tightly with a thick ice-cover. The ice-cover was produced using a 25 L polypropylene tank mould (50×35×11 cm), which was filled with FSW and frozen at –80ºC for a minimum of 3 d. PVC tubes (25 mm Ø) were inserted in the moulding system to produce one ice-free borehole per ice-cover for contaminant delivery into seawater. Two ice-covers of 35×25×11 cm were obtained from each moulding tank. The ice-cover was carefully placed on top of the seawater in each glass tank and the borderline ice was left melting to tightly close the cap. The three oils (1:200; w oil/v FSW) and their mixture with dispersant (1:10 w D/w oil+D in 1:200; w oil/v FSW) were injected into their respective glass tanks through the delivery borehole, which was immediately sealed with melted fresh ice. After 2 months weathering, the UIWAFs of oil and oil+D (100% stock solution) were retrieved from the depth of each glass tank with the aid of a peristaltic pump (Watson-Marlow 323E),

using a serological pipette introduced through a newly made retrieval borehole (5 mm Ø). Photographs of the experiment are shown in Appendix VII.

The Low-Energy Water Accommodated Fraction (LEWAF) in FSW of the three oils, alone (NNA LEWAF, MGO LEWAF and IFO LEWAF) or mixed with the dispersant (NNA+D LEWAF, MGO+D LEWAF and IFO+D LEWAF), was produced in the darkness at 10ºC according to Katsumiti et al. (2019), modified after Singer et al. (2000). Briefly, oils (1:200; w oil/v FSW) and their mixtures (1:10 w D/w oil+D 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 hr.

The specific PAH composition of UIWAFs was determined by gas chromatography-mass spectrometry (GC-MS) after Prieto et al. (2007). A mix standard solution of 18 PAHs[1] (CRM47543; Supelco, Bellefonte, USA) was used for calibration in the GC-MS analysis. A mixture of 5 deuterated compounds[2] (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 Appendix II.

[1] Naphthalene (Naph), 1-methylnaphthalene (1-MN), 2-methylnaphthalene (2-MN), acenaphthylene (Acy), acenaphthene (Ace), fluorene (Flu), anthracene (Ant), phenanthrene (Phe), pyrene (Pyr), fluoranthene (Fluo), benz[a]anthracene (B[a]A), chrysene (Chr), benzo[a]pyrene (B[a]P), benzo[b]fluoranthene (B[b]F), benzo[k]fluoranthene (B[k]F), benzo[g,h,i]perylene (B[g,h,i]P), dibenz[a,h]anthracene (D[a,h]A), indeno[1,2,3-cd]pyrene (I[1,2,3-cd]P).

[2] Norwegian Standard (S-4124-200-T): naphthalene-d^8, byphenyl-d^{10}, phenanthrene-d^{10}, pyrene-d^{10}, benzo[a]anthracene-d^{12}, benzo[a]pyrene-d^{10}, benzo[ghi]perylene-d^{12}

The specific PAH composition of the IFO LEWAF (Table 1) was characterised following the same procedure (Prieto et al., 2007), as detailed in Chapter 1.

2.2. Sea urchin embryo toxicity test (SET)

The sea urchin 48 hr 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 (43º26'01.1"N 2º53'56.1"W; Bay of Biscay) in spring (March-May) 2019. Spawning was induced by injecting 1 mL 0.5M 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 capped containing 10 mL of the test solutions (50 embryos/mL) to conduct toxicity assays (in completely darkness at 20ºC).

Successive dilutions in FSW (0, 8, 21, 34, 55, 89 and 100%) of UIWAF and LEWAF alone or mixed with dispersant were prepared. The dilutions were selected following a Fibonacci dose escalation between 0 and 100% UIWAF or LEWAF, after excluding some of the lower doses from the dilution series in order to optimise the experimental set up, as described in Chapter 1.

After 48 hr exposure, 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 exposure replicates) and the egg size at t_0 (L_0 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; Appendix III). Images were taken with NIS-Elements Imaging Software v4.30 (Nikon Instruments BV). Size increase ($\Delta L=L-L_0$) and its EC_{50} were calculated (Beiras et al., 2012).

2.3. Toxicity Index (TI) in pluteus larvae

Specific abnormalities of the pluteus larvae were recorded (n=100 larvae per vial × 3 exposure replicates per experimental group) and integrated in the Toxicity Index (TI, in a 0-100 range; after Carballeira et al., 2012; Appendix IV). 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).

2.4. Sea urchin embryo developmental disruption (SEDD) assay

Sublethal toxicity was evaluated as the capacity of sea urchin to undergo its developmental program (Appendix V). This was measured in terms of inhibition of pluteus larvae formation index (IPLFI), and potential mechanisms of toxic action on developmental processes were identified by examining main stages of developmental progression: cleavage disruption index (CDI) and gastrulation disruption index (GDI) during embryo development; and metamorphosis disruption index (MDI) during larval development.

Briefly, the longest dimension of each larvae (L_i in µm; n=35 larvae per vial × 3 exposure replicates) was measured as detailed 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 exposure replicates) to calculate the indices described in Chapter 1:

$$IPLFI = \frac{1}{r}\sum_{i=1}^{r}\frac{(L_{max} - L_i) \times 100}{(0.5 \times L_{max})};$$

$$CDI = \frac{100}{2 \log (C+G+N+P1)} \times \log \frac{(C+G+N+P1) \times C}{G+N+P1};$$

$$GDI = \frac{100}{2 \log (G+N+P1)} \times \log \frac{(G+N+P1) \times G}{N+P1}; \text{ and}$$

$$MDI = \frac{L_{max} - L_i}{0.5 \times L_{max}} \times \frac{100}{2 \log (C+G+N+P1)} \times \log \frac{(C+G+N+P1) \times (C+G)}{(N+P1)};$$

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

2.5. Genotoxicity assay

Sublethal exposure concentrations were selected according to present ΔL results and preceding data (Chapter 1): 55% oil UIWAF or oil LEWAF, and 34% oil+D UIWAF or oil+D LEWAF. After 48 hr exposure, sea urchin larvae were centrifuged (1800×g at 4ºC for 10 min) to obtain pellets made of 500 larvae that were directly frozen in 500 µL of RNAlater® (Life Technologies, Carlsbad, CA, USA) and stored at -80ºC until the genotoxicity assay was performed.

The amount of intact double-stranded DNA (dsDNA) was determined by the Fast Micromethod® DNA Single-Strand-Break Assay (Scröder et al., 2006), adapted to sea urchin larvae (Reinardy and Bodnar, 2015; Appendix VIII). Samples were assayed in quadruplicate by loading 20 µL (15 larvae) to each replicate well on a black-walled 96-

well microplate (USA Scientific, INC., FL, USA), placed on ice. Ca/Mg-free phosphate buffer solution (PBS) was used as blank. Lysis solution (20 µL, 9 M urea 0.1 % SDS, 0.2 M EDTA) containing 1:49 Picogreen (P7581, Life Technologies, NY, USA) was added and samples were left to lyse on ice in the dark for 40 min. Then, DNA unwinding solution (20 mM EDTA, 1 M NaOH) was added (200 µL) to initiate alkaline unwinding (pH 12.65 ± 0.02). Fluorescence (intact dsDNA) was recorded at an excitation wavelength of 480 nm and an emission wavelength of 520 nm (POLARstar® Omega Plate Reader, BMG LABTECH, Aylesbury, UK) as relative fluorescent units (RFU) at t_0 in the experimental control group and after 20 min in both control and exposure groups. Blank values were subtracted from all the RFU values before calculations. Then, the strand scission factor (SSF) was calculated according to Scröder et al. (2006):

$$SSF = -\log \frac{\%\ dsDNA_i}{\%\ dsDNA_c};$$

where $\%dsDNA_i$ is the percentage of dsDNA in each exposure group and $\%dsDNA_c$ is the percentage of dsDNA in the experimental control group. The %dsDNA values were calculated as RFU for a given sample divided by the RFU recorded in the experimental control group at t_0 (Appendix VIII).

2.6. Toxic units

The concentrations in a mixture of individual pollutants expressed as fractions of the EC50 of each pollutant (toxic units –TUs–; Sprague, 1970), the relative contribution of each individual PAH to the TUs of UIWAFs and LEWAFs (RT_i) and the relative concentration of each PAH in the mixtures (RC_i) were calculated according to Chapter 1. For this purpose, EC50 values for various individual PAHs (Naph, Fluo, 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, braquiopods, decapods, mollusc larvae, echinoderm larvae and fish juveniles) were used as consensus EC50 to calculate the TUs (Appendix VI; Ward et al., 1981; Holcombe et al., 1983; Trucco et al., 1983; Spehar, 1999; Lyons et al., 2002; Pillai et al., 2003; Calbet et al., 2007; Bellas et al., 2008; Frantzen et al., 2012; Renegar et al., 2017; Knap et al., 2017). Then, the relative contribution of each individual PAH to the

TUs of the mixture ($\sum TU_{\sum PAHs}$) was determined as $RT_i=TU_{PAHi}/\sum TU_{\sum PAHs}$; where TU_{PAHi} means the TU estimated for this individual PAH. In parallel, the relative concentration of each PAH in the mixture was calculated as $RC_i=C_{PAHi}/\sum PAHs$; where C_{PAHi} stands for the individual concentration of each PAH. Thus, the ratio RT_i/RC_i 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).

2.7. Data treatment and 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 Dunett if the variances were homogenous and T3 Dunnett if they were not). For non-normal data sets, the non-parametric Kruskal-Wallis' test was used. Linear regressions were compared using the ANCOVA test. Differences in SSF were tested by one-way ANOVA on arcsine-transformed data, with *post hoc* Fisher's least significant difference (LSD) test for differences between each treatment and control ($p<0.05$). Significant differences in chemical data were tested with the Z-score test. Level of significance for all analyses was $p<0.05$.

3. RESULTS

The Naph concentration was similar in MGO UIWAF and IFO UIWAF, with values around 30 µg Naph/L, whilst for NNA UIWAF the values were in the range of 75 µg Naph/L (Table 2). The concentration of methylnaphthalenes (1-MN and 2-MN) was around 30 µg/L in NNA UIWAF and IFO UIWAF and 10 µg/L in MGO UIWAF (Table 2). The Naph concentration was 80-110 µg/L in NNA+D UIWAF and MGO+D UIWAF, and above 20 µg/L in IFO+D UIWAF (Table 2). The concentration of 1-MN was similar in all the oil+D UIWAFs (5-15 µg/L), whilst the concentration of 2-MN was always lower than

55 µg/L but diverse depending on the oil (Table 2). The concentration of Ace, Flu and Phe was high in all the UIWAFs (Fig. 1; Table 2). The addition of dispersant increased the concentrations of Flu and Phe in NNA+D UIWAF and MGO+D UIWAF, whilst in IFO+D UIWAF the concentrations of Ace, Flu and Phe decreased (Fig. 1; Table 2). Pyr, Fluo, B[a]A+Chr and B[a]P were detected at relatively low concentrations in the three oil UIWAFs (<100 ng/L) whilst Ant was only detected in IFO UIWAF (Table 2). After the addition of dispersant, the concentration of these five individual PAHs in UIWAF decreased in IFO, whilst the concentration of Pyr, Fluo and B[a]A+Chr increased in NNA and MGO (Table 2). Overall, the values of ∑PAHs (without Naph), $\sum_{LMW}$PAHs and $\sum_{HMW}$PAHs were low in all the UIWAFs (Table 2); yet, after dispersant addition, the values increased in NNA+D UIWAF and MGO+D UIWAF, and decreased in IFO+D UIWAF (Table 2).

Table 1. GC-MS analysis of PAHs (ng/L) in oil LEWAF and oil+D LEWAF samples of NNA, MGO and IFO after (Taken from Chapter 1 (Table 3)). Asterisks indicate significant differences in each oil LEWAF type (Z-score). (UDL: under detection limits; $_{LMW}$PAHs: Low molecular weight polycyclic aromatic hydrocarbons; $_{HMW}$PAHs: High molecular weight polycyclic aromatic hydrocarbons; #: Total of PAHs without Naphthalene).

	NNA LEWAF	**NNA+D LEWAF**	**MGO LEWAF**	**MGO+D LEWAF**	**IFO LEWAF**	**IFO+D LEWAF**
Naph	351221	439059	112311	71814	92285	73614
1-MN	72842	**173919***	32904	27011	77566	92047
2-MN	306563	365838	42043	26763	91365	107758
Acy (1)	98	46	142	120	419	**1009***
Ace (1)	996	2649	1144	585	2607	**5999***
Flu (1)	3158	**9111***	2043	2436	1673	5066
Ant (1)	UDL	UDL	UDL	UDL	188	1088
Phe (1)	2269	13569	1992	2737	2337	14774
Pyr (2)	139	575	30	21	40	**3056***
Fluo (2)	42	294	23	65	18	**588***
B[a]A + Chr (2)	41	681	7	22	7	**2176***
B[a]P (2)	16	UDL	UDL	UDL	UDL	115
B[b]F+B[k]F (2)	53	UDL	UDL	UDL	UDL	115
B[g,h,i]P (2)	UDL	UDL	UDL	UDL	UDL	89
D[ah]A(2)	UDL	UDL	UDL	UDL	UDL	84
I[1,2,3-cd]P (2)	UDL	UDL	UDL	UDL	UDL	13
∑PAHs	737439	1005741	192578	131576	268439	301355
$\sum_{LMW}$PAHs Σ(1)	6521	25376	5321	5879	7223	27936
$\sum_{HMW}$PAHs Σ(2)	292	1549	60	108	65	**6235***
$\sum_{Naph}$PAHs#	730626	978816	187258	125589	261216	273419
∑PAHs#	6813	26925	5380	5987	7288	34171
∑(1)/ ∑(2	22	16	89	54	111	4
Phe/Ant	-	-	-	-	12.43	14
Fluo/Pyr	0.30	0.51	0.79	**3.04***	0.46	0.19

Table 2. GC-MS analysis of PAHs (ng/L) in oil UIWAF and oil+D UIWAF samples of NNA, MGO and IFO. Asterisks indicate significant differences in each oil LEWAF type (Z-score). (UDL: under detection limits; $_{LMW}$PAHs: Low molecular weight polycyclic aromatic hydrocarbons; $_{HMW}$PAHs: High molecular weight polycyclic aromatic hydrocarbons; #: Total of PAHs without Naphthalene).

	NNA UIWAF	**NNA+D UIWAF**	**MGO UIWAF**	**MGO+D UIWAF**	**IFO UIWAF**	**IFO+D UIWAF**
Naph	74898	107030	22700	80908	31565	21025
1-MN	24180	15515	8708	10953	27530	5270
2-MN	33663	52508	8883	30440	28385	10095
Acy (1)	UDL	25	28	55	95	15
Ace (1)	215	320	350	415	655	120
Flu (1)	818	1148	525	1100	660	185
Ant (1)	UDL	UDL	UDL	UDL	98	15
Phe (1)	563	1698	655	1715	1130	340
Pyr (2)	50	**93***	40	70	70	30
Fluo (2)	33	150	20	70	28	25
B[a]A + Chr (2)	33	40	UDL	15	73	28
B[a]P (2)	33	UDL	38	UDL	35	UDL
B[b]F+B[k]F (2)	UDL	UDL	28	UDL	UDL	UDL
B[g,h,i]P (2)	UDL	UDL	53	UDL	45	UDL
D[ah]A(2)	UDL	UDL	UDL	UDL	UDL	UDL
I[1,2,3-cd]P (2)	UDL	UDL	UDL	UDL	UDL	UDL
∑PAHs	134483	178525	42025	125740	90368	37148
$\sum_{LMW}$PAHs Σ(1)	1595	3190	1558	3285	2638	675
$\sum_{HMW}$PAHs Σ(2)	148	283	178	155	250	83
$\sum_{Naph}$PAHs#	132740	175053	40290	122300	87480	36390
∑PAHs#	1743	3473	1735	3440	2888	758
∑(1)/ ∑(2	11	11	9	21	11	8
Phe/Ant	-	-	-	-	11.59	22.67
Fluo/Pyr	0.65	**1.62***	0.5	1	0.39	0.83

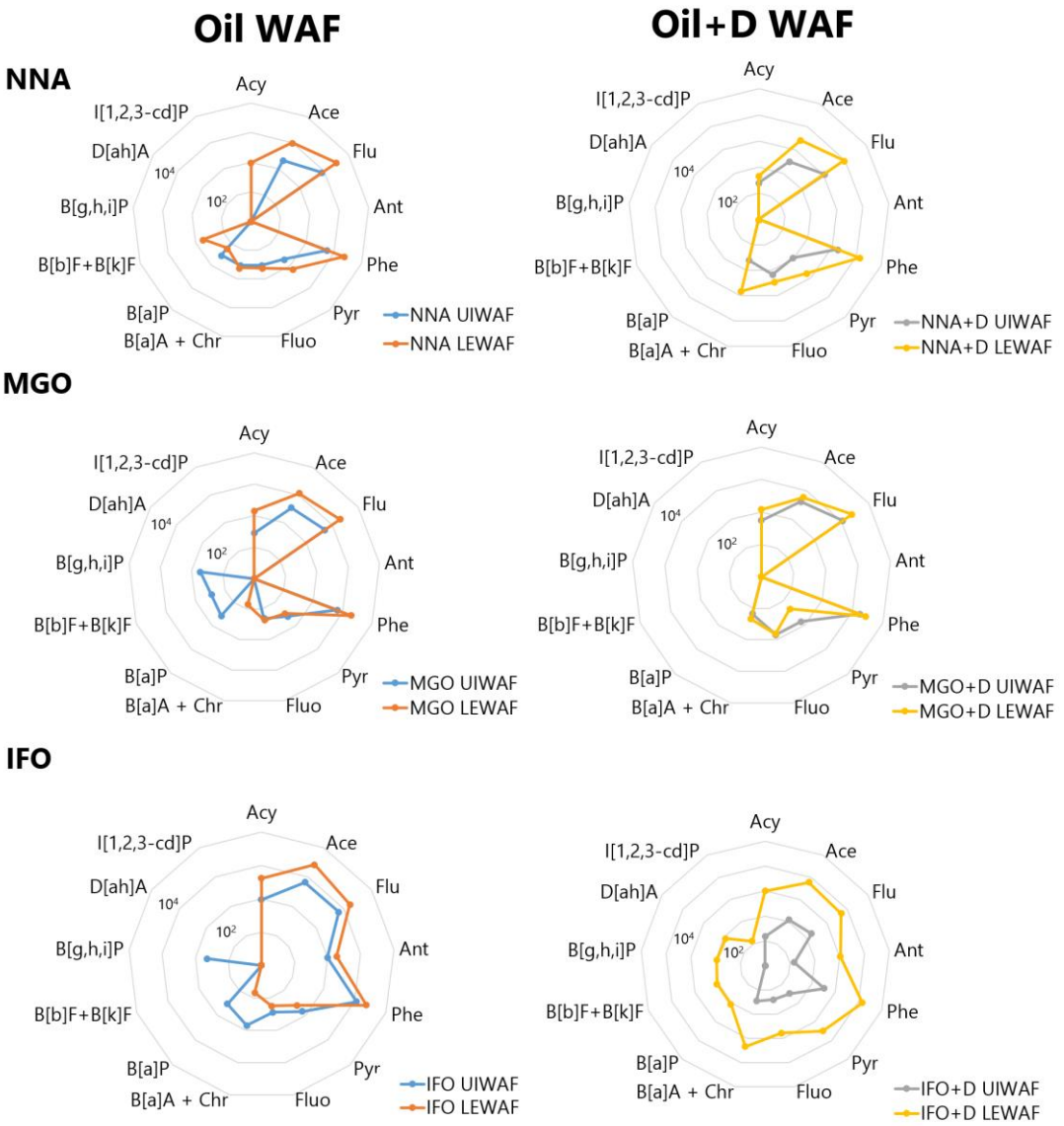

Figure 1. Profile of PAHs, represented in logarithmic scale, in oil UIWAF, oil LEWAF, oil+D UIWAF and oil+D LEWAF samples of NNA, MGO and IFO.

For the three tested oils, ΔL decreased at increasing concentrations of both oil UIWAF and oil LEWAF, more markedly for the former (Figs. 2A, 2C, 2E and 9A-F). In parallel, TI increased linearly at increasing concentrations of both oil UIWAF and oil LEWAF, with a higher slope for the case of UIWAF than for LEWAF (ANCOVA, $p<0.05$; Figs. 3A, 3C and 3E). CDI increased linearly at increasing concentrations of the UIWAF of the three oils and of MGO LEWAF, but remained unchanged with exposure to NNA and IFO LEWAF (Figs. 4A, 5A and 6A). Likewise, GDI, MDI and IPLFI increased linearly at increasing concentrations of both oil UIWAF and oil LEWAF, more markedly for the former (ANCOVA; $p<0.05$; Figs. 4C, 4E, 5C, 5E, 6C, 6E, 7A, 7C and 7E). Finally, on exposure to the three oils, SSF increased as a result of both oil UIWAF and oil LEWAF, more markedly in the latter, especially in the case of MGO LEWAF (Fig. 8).

After addition of dispersant, ΔL decreased in a comparable manner at increasing concentrations of both UIWAF and LEWAF of the three oils (Figs. 2B, 2D, 2F and 9G-L). Likewise, no differences between UIWAF and LEWAF were found for the other biological endpoints (ANCOVA, $p>0.05$; Figs. 3-7). Concretely, a concentration dependent linear increase was observed in TI, CDI, GDI, MDI and IPLFI for NNA+D and MGO+D UIWAF and LEWAF (Figs. 3B, 3D, 4B, 4D, 4F, 5B, 5D, 5F, 7B and 7D). In contrast, TI, CDI, GDI and IPLFI reached the maximum value of 100 on exposure to 34% IFO+D UIWAF and 21% IFO+D LEWAF (Figs. 3F, 6B, 6D and 7F). Meanwhile, on exposure to IFO+D, MDI increased linearly at increasing concentrations of UIWAF but reached the maximum value of 100 on exposure to 21% LEWAF (Fig. 6F). SSF increased upon exposure to UIWAF and LEWAF of the three oils, with and without dispersant, except for IFO+D UIWAF, DNA damage being always more marked in the case of LEWAF than in UIWAF (Fig. 8).

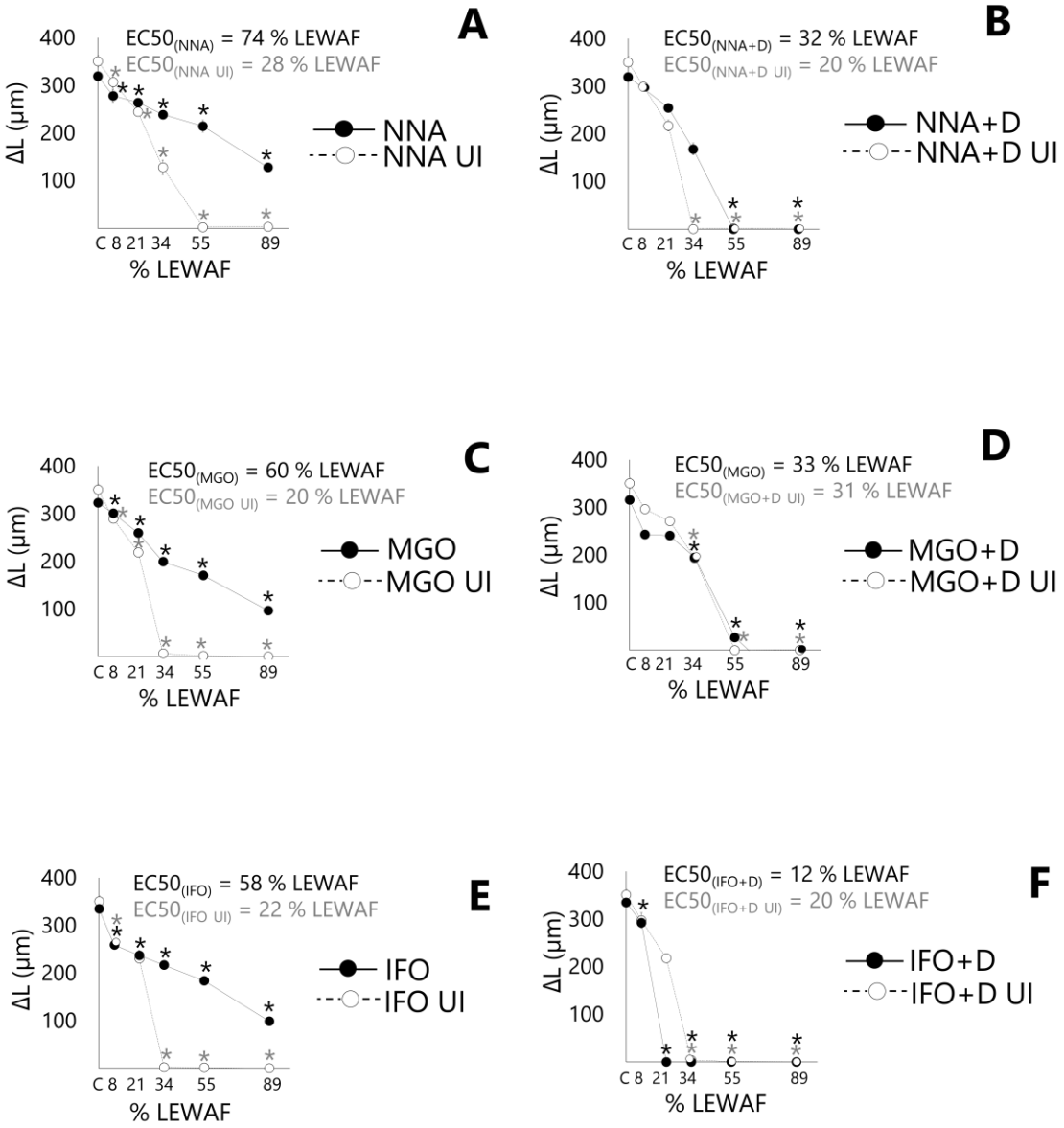

Figure 2. Size increase (ΔL in µm) of sea urchin larvae exposured to oil and oil+D WAF weathered under ice (UI) or produced at 10ºC (LEWAF). **A)** NNA UIWAF and NNA LEWAF. **B)** NNA+D UIWAF and NNA+D LEWAF. **C)** MGO UIWAF and MGO LEWAF. **D)** MGO+D UIWAF and MGO+D LEWAF. **E)** IFO UIWAF and IFO LEWAF. **F)** IFO+D UIWAF and IFO+D LEWAF. Values are given in µm (means ± SD). Asterisks indicate significant differences among concentrations of each treatment and control (p<0.05).

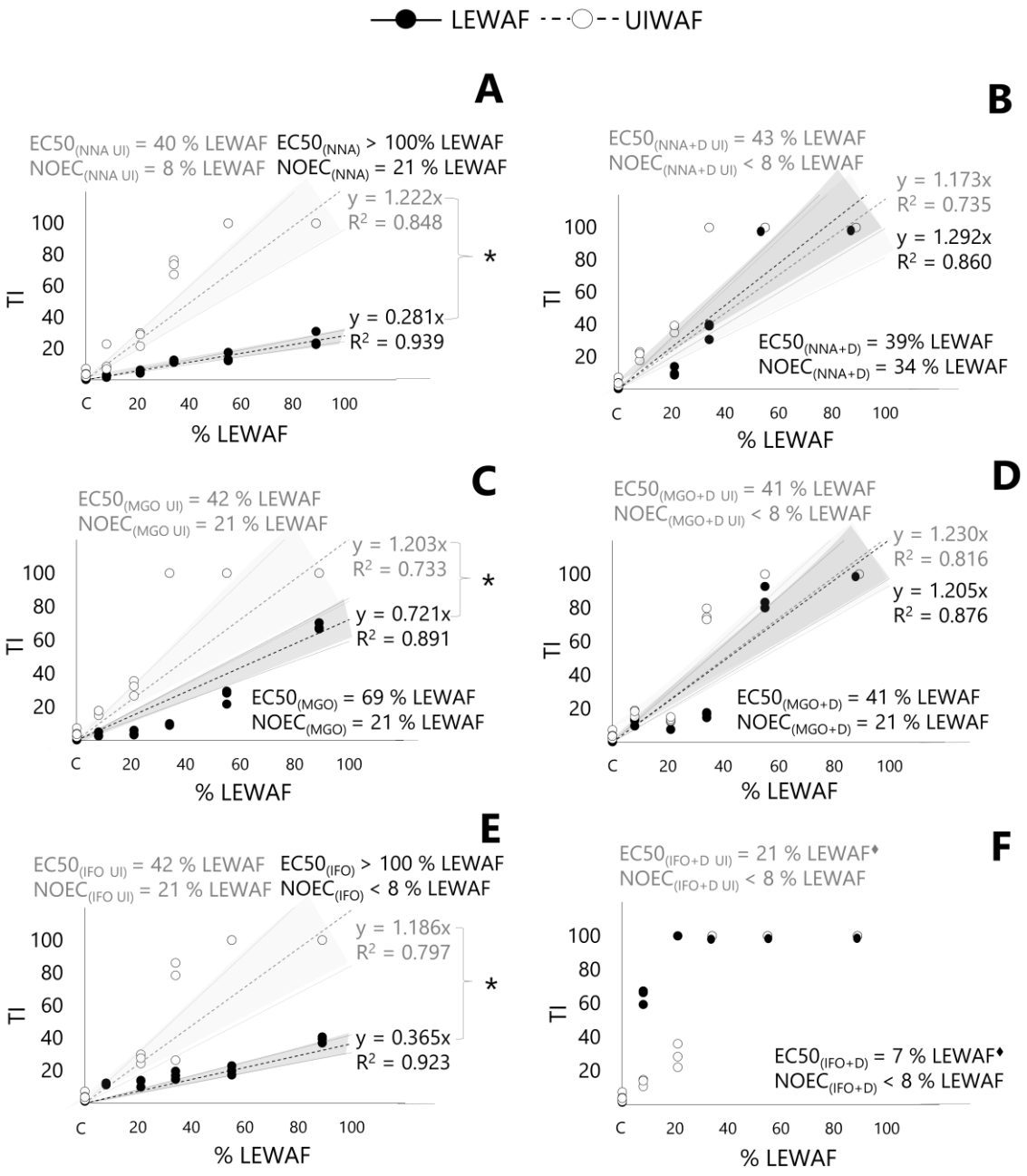

Figure 3. TI (Toxicity Index) on exposure to oil and oil+D WAF weathered under ice (UI) or produced at 10ºC (LEWAF). **A)** NNA UIWAF and NNA LEWAF. **B)** NNA+D UIWAF and NNA+D LEWAF. **C)** MGO UIWAF and MGO LEWAF. **D)** MGO+D UIWAF and MGO+D LEWAF. **E)** IFO UIWAF and IFO LEWAF. **F)** IFO+D UIWAF and IFO+D LEWAF. Half-effective concentration (EC50 from regression equations, EC50♦ based on Probit analyses) and NOEC values are shown. Asterisks indicate significant differences between linear regression coefficients (R^2, ANCOVA (p<0.05)).

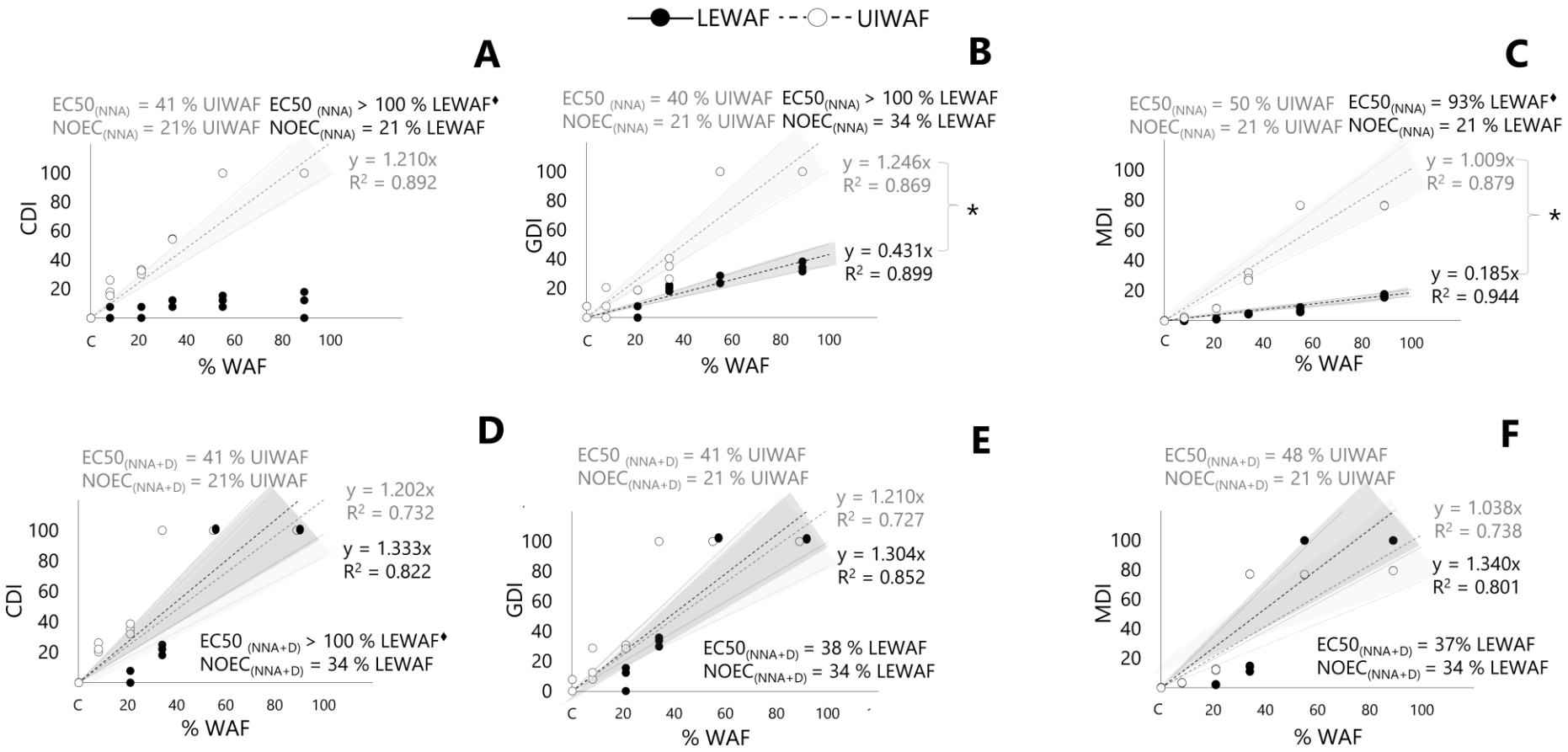

Figure 4. CDI (Cleavage Disruption Index), GDI (Gastrulation Disruption Index), MDI (Metamorphosis Disruption Index) on exposure to oil and oil+D WAF weathered under ice (UI) or produced at 10ºC (LEWAF). **A)** CDI for NNA UIWAF and NNA LEWAF. **B)** CDI for NNA+D UIWAF and NNA+D LEWAF. **C)** GDI for NNA UIWAF and NNA LEWAF. **D)** GDI for NNA+D UIWAF and NNA+D LEWAF. **E)** MDI for NNA UIWAF and NNA LEWAF. **F)** MDI for NNA+D UIWAF and NNA+D LEWAF. Half-effective concentration (EC50 from regression equations, EC50♦ based on Probit analyses) and NOEC values are shown. Asterisks indicate significant differences between linear regression coefficients (R^2, ANCOVA (p<0.05)).

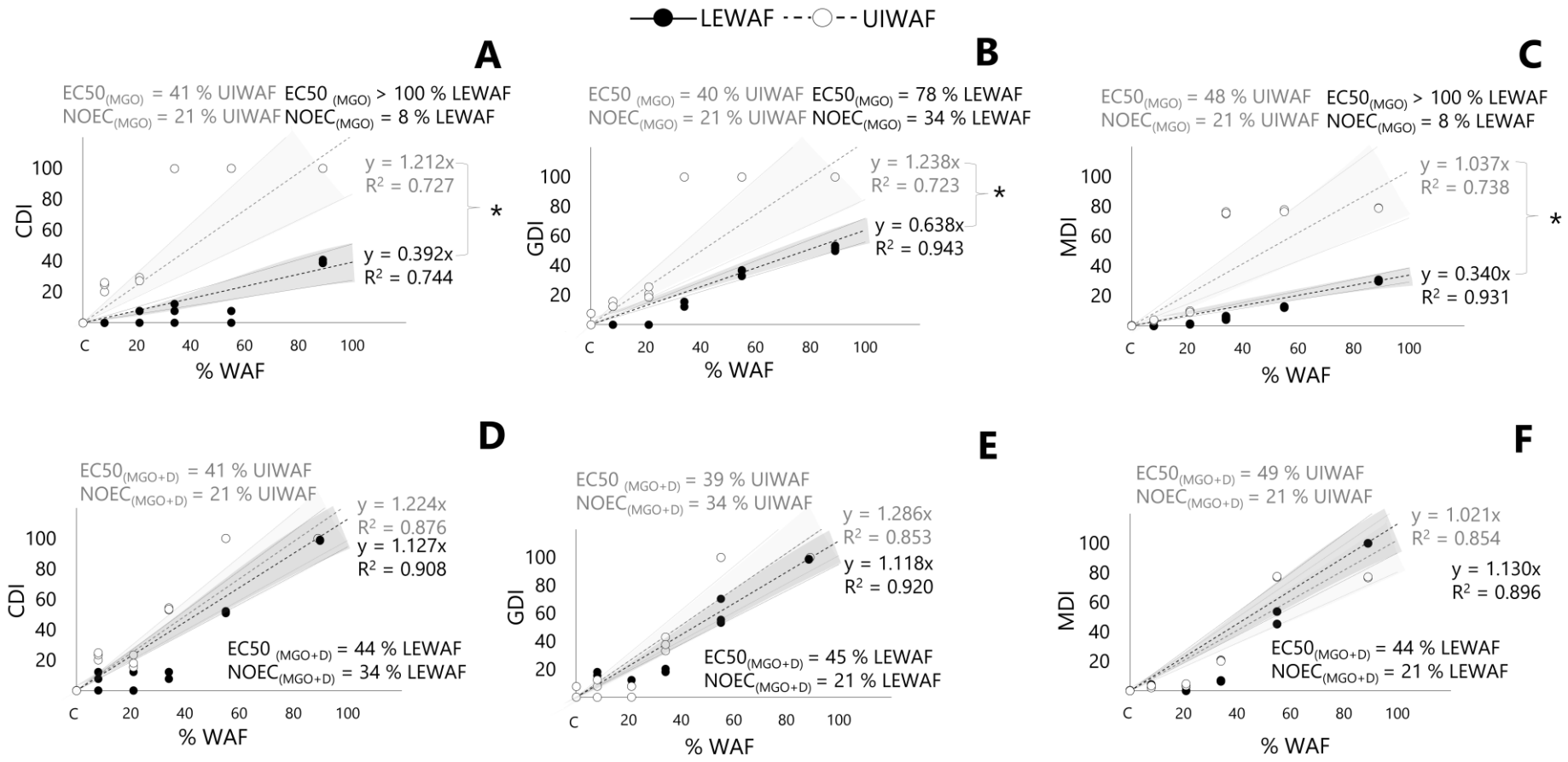

Figure 5. CDI (Cleavage Disruption Index), GDI (Gastrulation Disruption Index), MDI (Metamorphosis Disruption Index) on exposure to oil and oil+D WAF weathered under ice (UI) or produced at 10ºC (LEWAF). **A)** CDI for MGO UIWAF and MGO LEWAF. **B)** CDI for MGO+D UIWAF and MGO+D LEWAF. **C)** GDI for MGO UIWAF and MGO LEWAF. **D)** GDI for MGO+D UIWAF and MGO+D LEWAF. **E)** MDI for MGO UIWAF and MGO LEWAF. **F)** MDI for MGO+D UIWAF and MGO+D LEWAF. Half-effective concentration (EC50 from regression equations, EC50♦ based on Probit analyses) and NOEC values are shown. Asterisks indicate significant differences between linear regression coefficients (R^2, ANCOVA (p<0.05)).

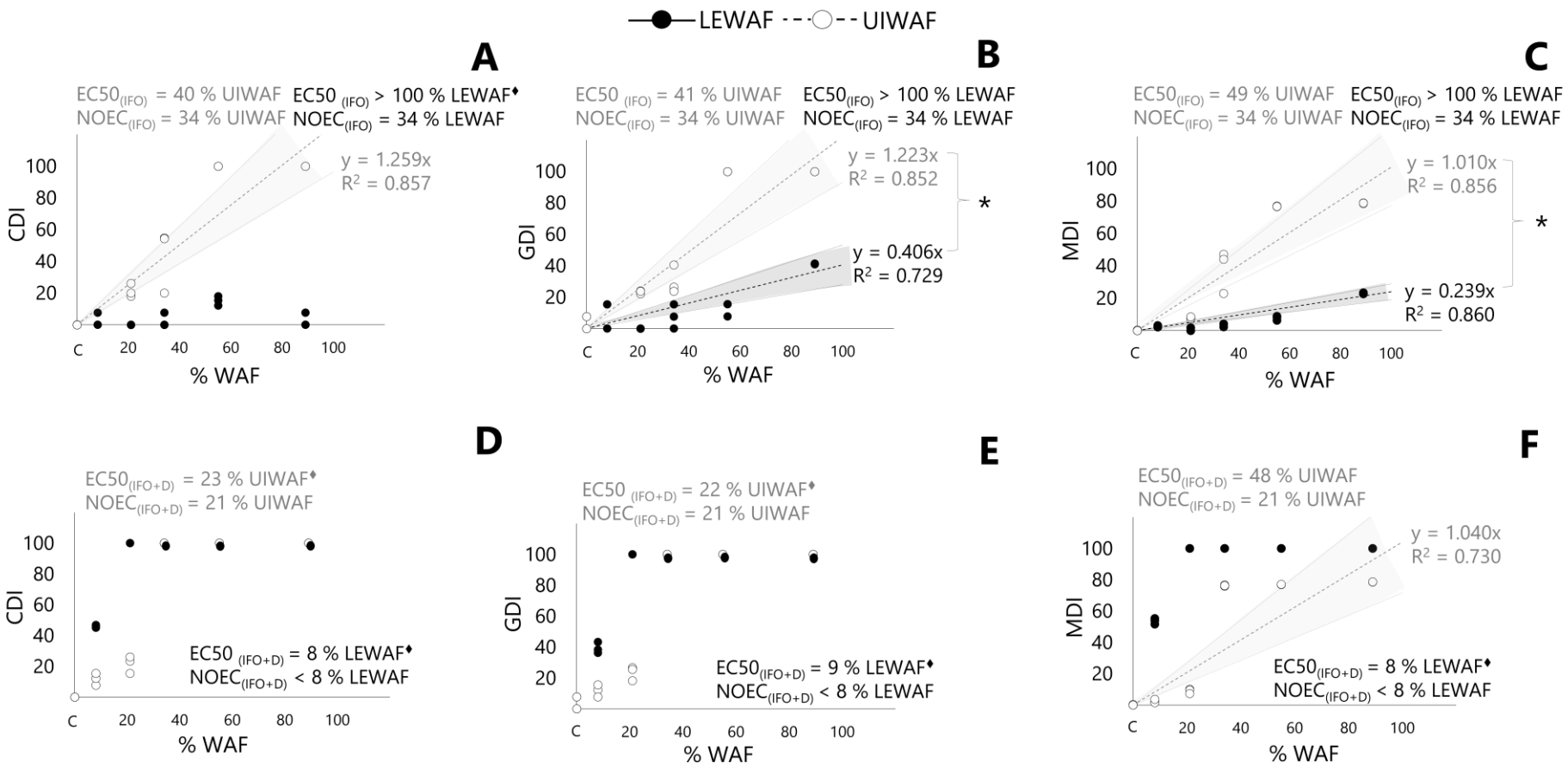

Figure 6. CDI (Cleavage Disruption Index), GDI (Gastrulation Disruption Index), MDI (Metamorphosis Disruption Index) on exposure to oil and oil+D WAF weathered under ice (UI) or produced at 10ºC (LEWAF). **A)** CDI for IFO UIWAF and IFO LEWAF. **B)** CDI for IFO+D UIWAF and IFO+D LEWAF. **C)** GDI for IFO UIWAF and IFO LEWAF. **D)** GDI for IFO+D UIWAF and IFO+D LEWAF. **E)** MDI for IFO UIWAF and IFO LEWAF. **F)** MDI for IFO+D UIWAF and IFO+D LEWAF. Half-effective concentration (EC50 from regression equations, EC50♦ based on Probit analyses) and NOEC values are shown. Asterisks indicate significant differences between linear regression coefficients (R^2, ANCOVA (p<0.05)).

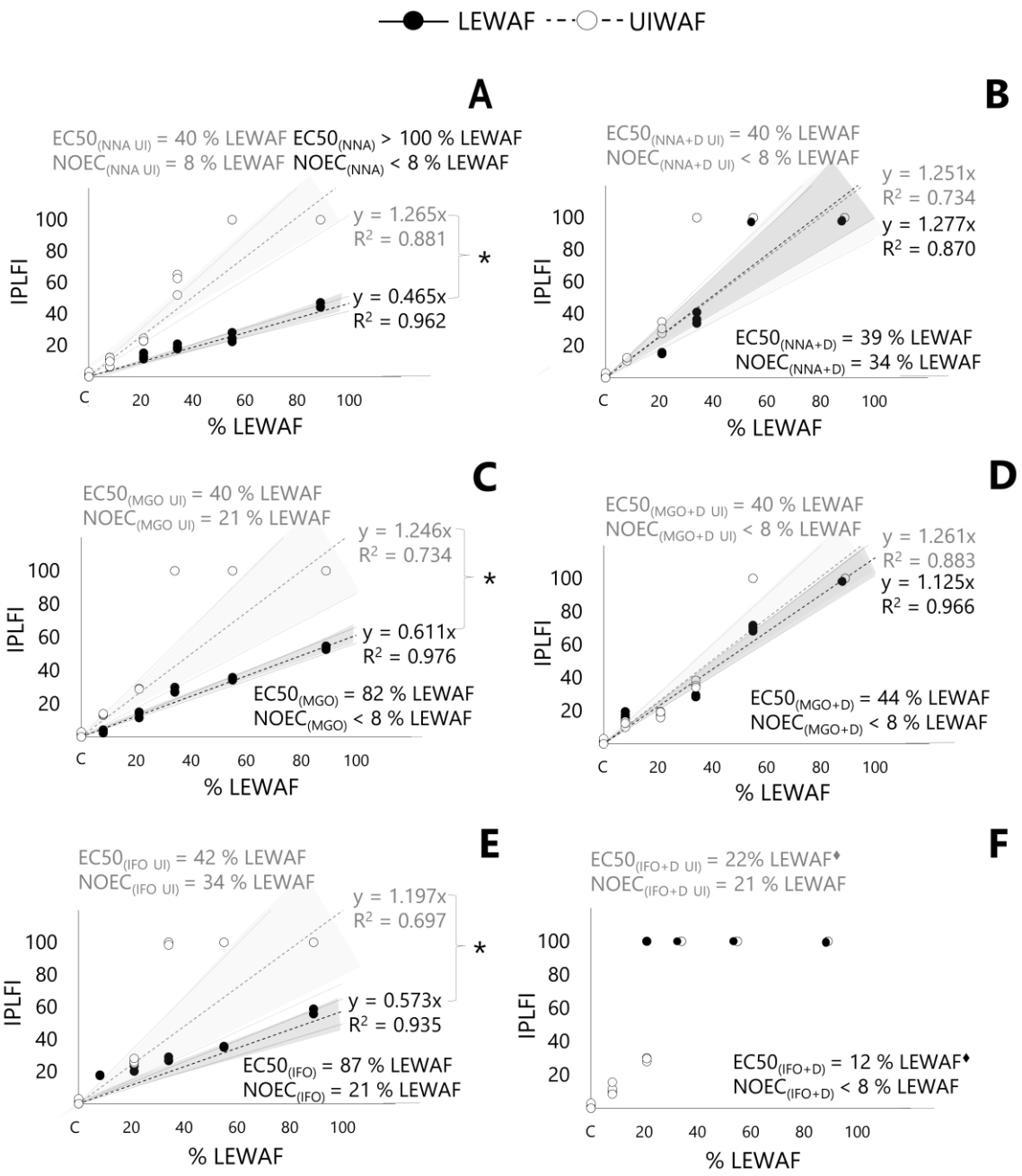

Figure 7. IPLFI (Inhibition Pluteus Larvae Formation Index) on exposure to oil and oil+D WAF weathered under ice (UI) or produced at 10ºC (LEWAF). **A)** NNA UIWAF and NNA LEWAF. **B)** NNA+D UIWAF and NNA+D LEWAF. **C)** MGO UIWAF and MGO LEWAF. **D)** MGO+D UIWAF and MGO+D LEWAF. **E)** IFO UIWAF and IFO LEWAF. **F)** IFO+D UIWAF and IFO+D LEWAF. Half-effective concentration (EC50 from regression equations, EC50♦ based on Probit analyses) and NOEC values are shown. Asterisks indicate significant differences between linear regression coefficients (R^2, ANCOVA (p<0.05)).

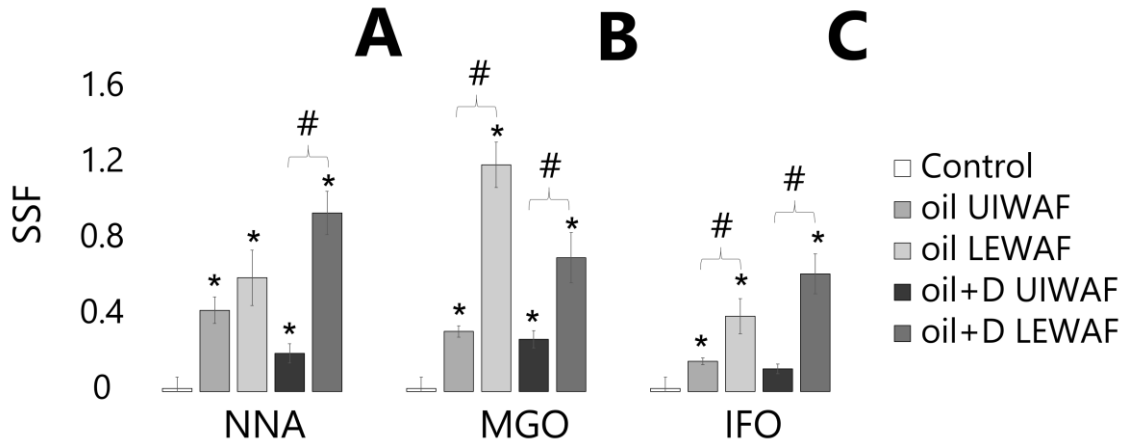

Figure 8. DNA damage measured in Strand Scission Factor (SSF± SD) of sea urchin larvae exposed to oil LEWAF (55%), oil UIWAF (55%), oil+D LEWAF (34%) and oil+D UIWAF (34%) of: **A)** NNA, **B)** MGO and **C)** IFO. Asterisks indicate significant differences between each treatment and control (ANOVA $p<0.05$). Pads indicate significant differences between each oil condition (LEWAF or UIWAF) (t-Student $p<0.05$).

TU values were always very low (<0.20) after UIWAF exposure (Table 3). In contrast, on exposure to LEWAF these values were higher, both with and without dispersant, most remarkably for NNA (TU>1; Table 4). RT_i was greater than "1" after exposure to the UIWAF and LEWAF of the three oils alone or in combination with the dispersant, especially on exposure to IFO (Tables 3 and 4). RT_i/RC_i values greater than "1" were found for various individual PAHs after UIWAF exposure (Table 3):

- 2-MN, Pyr and Fluo (all the oils alone or with dispersant);
- Ant (IFO and IFO+D);
- B[a]A+Chr (NNA and IFO but not MGO);
- B[a]P (all the oils in absence of dispersant).

Alike, RT_i/RC_i was also greater than "1" after LEWAF exposure for various individual PAHs (Table 4):

- 2-MN, Pyr, Fluo and B[a]A+Chr (all the oils alone or with dispersant);
- Ace, Acy and Phe (NNA);
- Phe (MGO and MGO+D);
- Ant (IFO and IFO+D);
- B[a]P (NNA and IFO+D).

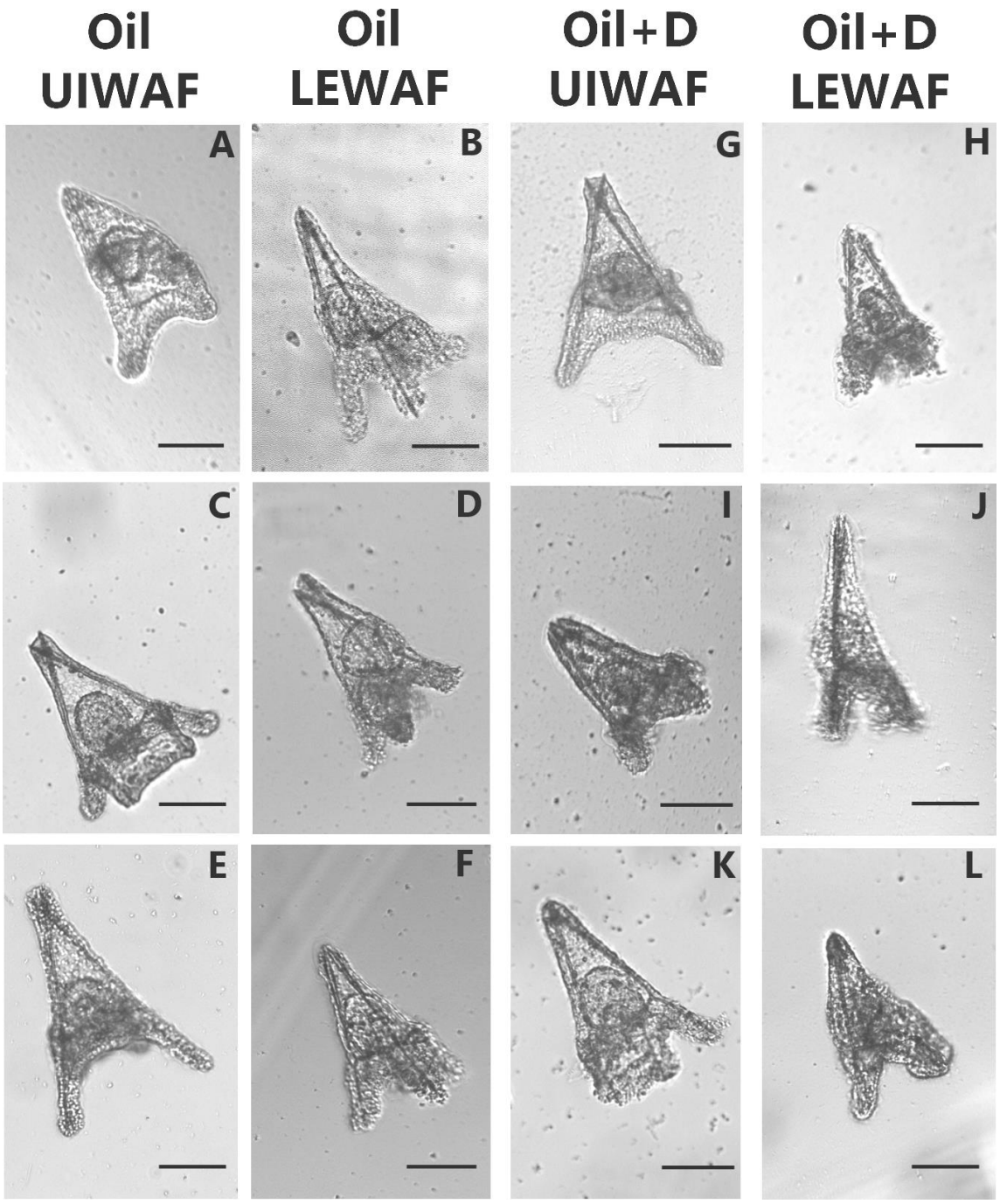

Figure 9. Photographs of sea urchin *P. lividus* larvae observed after 48 hr of exposure to oil (A-F) and oil+D (G-L) UIWAF and LEWAF. Pictures corresponding to EC50 values in size increase (ΔL) or near to them. **A)** 30% NNA UIWAF. **B)** 70% NNA LEWAF. **C)** 20% MGO UIWAF. **D)** 60% MGO LEWAF. **E)** 20% IFO UIWAF. **F)** 60% IFO LEWAF. **G)** 20% NNA+D UIWAF. **H)** 30% NNA+D LEWAF. **I)** 30% MGO+D UIWAF. **J)** 30% MGO+D LEWAF. **K)** 20% IFO+D UIWAF. **L)** 10% IFO+D LEWAF. Scale bars 100 µm.

Table 3. Summary of the TU analysis of the toxicity of under ice weathered WAF (UIWAF) based on the mixture of identified PAHs. The sum of TUs (∑TU) for each toxicity endpoint (ΔL, TI, IPLFI, CDI, GDI and MDI) is "1" if there is additive toxicity, ">1" if there is synergistic effects and "<1" if the toxicity is not caused by the mixture assuming the CA joint action. The sum of the TUs of individual PAHs vs. the TUs of the sum of PAHs ($\sum TU_{PAHi}/TU_{\sum PAHs}$) is "1" if all the PAHs in the mixture exert the same toxicity, ">1" if there are one or more individual PAHs with more toxicity than expected from its contribution to the mixture according to the CA model; and "<1" otherwise. The balance between the relative contribution of an individual PAH to the toxicity of the mixture and its relative contribution to the chemical composition of the mixture (RT_i/RC_i) is "1" if the individual toxicity of this PAH is the one expected due to its proportion in the mixture (CA model); "<1" if it is not a contributor to the mixture toxicity; and ">1" if there this PAH exerts toxicity beyond the one expected as a part of the mixture.

	NNA UIWAF	**NNA+D UIWAF**	**MGO UIWAF**	**MGO+D IUWAF**	**IFO UIWAF**	**IFO+D UIWAF**
$\sum TU_{\Delta L}$	0.05	0.07	0.01	0.07	0.05	0.02
$\sum TU_{TI}$	0.11	0.16	0.03	0.09	0.10	0.03
$\sum TU_{IPLFI}$	0.10	0.15	0.03	0.09	0.10	0.03
$\sum TU_{CDI}$	0.11	0.15	0.03	0.09	0.09	0.03
$\sum TU_{GDI}$	0.11	0.15	0.03	0.09	0.10	0.03
$\sum TU_{MDI}$	0.12	0.18	0.04	0.11	0.12	0.04
$\sum TU_{PAHi}/TU_{\sum PAHs}$	1.63	1.74	1.49	1.49	2.20	1.76
RT/RC_{Naph}	0.08	0.10	0.12	0.12	0.08	0.10
$RT/RC_{1\text{-}MN}$	0.59	0.79	0.91	0.92	0.62	0.78
$RT/RC_{2\text{-}MN}$	2.16	2.87	3.34	3.34	2.26	2.83
RT/RC_{Acy}	-	0.57	0.66	0.66	0.45	0.56
RT/RC_{Ace}	0.48	0.63	0.74	0.74	0.50	0.62
RT/RC_{Flu}	0.19	0.25	0.29	0.29	0.19	0.24
RT/RC_{Ant}	-	-	-	-	19.22	24.05
RT/RC_{Phe}	0.86	1.14	1.32	1.33	0.90	1.12
RT/RC_{Pyr}	2.84	3.78	4.40	4.41	2.98	3.73
RT/RC_{Fluo}	1.45	1.93	2.24	2.25	1.52	1.90
$RT/RC_{B[a]A + Chr}$	36.69	48.72	-	56.86	38.44	48.11
$RT/RC_{B[a]P}$	2.45	-	3.78	-	2.56	-

Table 4. Summary of the TU analysis of the toxicity of LEWAF based on the mixture of identified PAHs. The sum of TUs (∑TU) for each toxicity endpoint (ΔL, TI, IPLFI, CDI, GDI and MDI) is "1" if there is additive toxicity. ">1" if there is synergistic effects and "<1" if the toxicity is not caused by the mixture assuming the CA joint action. The sum of the TUs of individual PAHs vs. the TUs of the sum of PAHs ($\sum TU_{PAHi}/TU_{\sum PAHs}$) is "1" if all the PAHs in the mixture exert the same toxicity. ">1" if there are one or more individual PAHs with more toxicity than expected from its contribution to the mixture according to the CA model; and "<1" otherwise. The balance between the relative contribution of an individual PAH to the toxicity of the mixture and its relative contribution to the chemical composition of the mixture (RT_i/RC_i) is "1" if the individual toxicity of this PAH is the one expected due to its proportion in the mixture (CA model); "<1" if it is not a contributor to the mixture toxicity; and ">1" if there this PAH exerts toxicity beyond the one expected as a part of the mixture.

	NNA LEWAF	**NNA+D LEWAF**	**MGO LEWAF**	**MGO+D LEWAF**	**IFO LEWAF**	**IFO+D LEWAF**
$\sum TU_{\Delta L}$	1.43	0.84	0.20	0.07	0.41	0.14
$\sum TU_{TI}$	1.55	1.03	0.23	0.09	0.70	0.08
$\sum TU_{IPLFI}$	1.61	1.03	0.27	0.10	0.61	0.14
$\sum TU_{CDI}$	2.01	2.64	0.33	0.10	0.70	0.09
$\sum TU_{GDI}$	1.57	1.00	0.26	0.10	0.70	0.10
$\sum TU_{MDI}$	1.87	0.98	0.33	0.10	0.70	0.09
$\sum TU_{PAHi}/TU_{\sum PAHs}$	2.31	2.22	1.46	1.46	2.22	3.15
RT/RC_{Naph}	0.25	0.08	0.12	0.12	0.08	0.06
$RT/RC_{1\text{-}MN}$	1.92	0.61	0.94	0.93	0.62	0.43
$RT/RC_{2\text{-}MN}$	7.01	2.24	3.41	3.41	2.25	1.58
RT/RC_{Acy}	1.39	0.44	0.67	0.67	0.44	0.31
RT/RC_{Ace}	1.55	0.50	0.75	0.75	0.50	0.35
RT/RC_{Flu}	0.60	0.19	0.29	0.29	0.19	0.14
RT/RC_{Ant}	-	-	-	-	19.11	13.45
RT/RC_{Phe}	2.79	0.89	1.35	1.35	0.89	0.63
RT/RC_{Pyr}	9.24	2.96	4.49	4.49	2.96	2.08
RT/RC_{Fluo}	4.71	1.51	2.29	2.29	1.51	1.06
$RT/RC_{B[a]A\ +\ Chr}$	119.22	38.13	57.98	57.92	38.22	26.89
$RT/RC_{B[a]P}$	7.95	-	-	-	-	1.79

4. DISCUSSION

The ∑PAHs (with and without Naph) in UIWAF was always lower than in the corresponding LEWAF (Chapter 1). These lower PAH levels measured in UIWAF in comparison with LEWAF can be explained upon considering that the conditions used to produce LEWAF (short-term, low-energy stirring, closed system, 10ºC; Katsumiti et al., 2019, after Singer et al., 2000) were very different to those used to produce UIWAF (long-term –2 mo–, static, open ice-cover system, -4±2ºC). Although many of the same factors (e.g., evaporation, dissolution, droplet dispersion, and emulsification) are important, oil weathering in the presence of sea ice is significantly different from oil weathering in ice-free waters (Payne et al., 1991). Under ice weathering is a long-term process, which includes low evaporation, enhanced dissolution, and specific photoxidation and biodegradation of oil compounds (Payne et al., 1991; Faksness et al., 2008; 2011; Desmond et al., 2019; Saltymakova et al., 2020).

After long-term under ice weathering of a light-medium crude oil, the majority of the hydrocarbons (70.2%; with a low percentage of PAHs, alkylcyclohexanes, and alkylcyclopentanes) were found in the ice, whilst a large fraction of the hydrocarbons (19%) was evaporated despite of the presence of ice (Desmond et al., 2019). Evaporation seems to be relevant for aliphatic compounds (alkanes, alkylcyclohexanes, and alkylcyclopentanes), LMW alkylbenzenes and even for some of the most water soluble compounds (e.g., Naph), but less relevant for HMW compounds and PAHs (Desmond et al., 2019). Thus, only a 2.5% of the hydrocarbons (mainly PAHs, in particular, Naph and 1-MN, 2-MN and 3-MN-; and LMW alkylbenzenes and aryl-isoprenoids) are dissolved in the water column (Desmond et al., 2019). Indeed, ∑PAHs (Naph incl.) values presently recorded are in the range of 42 to 134 ng/mL, depending on the oil; which are comparable to the 48 ng PAH/mL reported after weathering under ice (Desmond et al., 2019). and 1-2 folds lower than the 100-1000 ng/mL reported when WAF was produced using sea water in closed systems (e.g., LEWAF[3], CEWAF[4],

[3] Low-Energy Water Accommodated Fraction.
[4] Chemically Enhanced Accommodated Fraction.

HEWAF[5]; Neff et al., 2000; Faksness et al., 2008; Forth et al., 2017; Bender et al., 2018; Johann et al., 2020b; Chapter 1). On the other hand, the high similarity found amongst the PAH levels measured in the UIWAFs of different oils, in contrast with the differences found amongst LEWAFs, could be the direct consequence of long-term weathering under ice. The presence of ice-cover is known to reduce the viscosity of the water-in-oil emulsion, the flash point and the pour point (Brandvik and Faksness, 2009), which are relevant parameters regarding oil behaviour and weathering, especially at low temperatures (Faksness et al., 2008). Thus, together with the above mentioned low levels of PAHs in water, altered oil behaviour could also contribute to reducing differences between the PAH levels in the WAFs of different oils in the long-term.

Certainly, the PAH concentrations recorded in UIWAFs were lower than in LEWAFs of their corresponding oil, yet UIWAFs and LEWAFs exhibited similar PAH profiles, which varied amongst oils. The "chemical profile" of a WAF is very unlike that of the parent oil; however, for a given oil load, if the mixing time and temperature allow reaching the equilibrium, each oil type WAF seems to have a characteristic PAH profile irrespective of how the WAF is produced (Faksness et al., 2008). Herein, as reported for the PAH composition of LEWAFs of the same oils (Chapter 1), naphthalenes were also at high concentrations in all the UIWAFs; however, the concentrations were overall much lower in UIWAF than in LEWAF. Naph ranged from 20 to 110 µg/L in UIWAFs and from 50 to 400 µg/L in LEWAFs. 1-MN ranged from 5 to 30 µg/L in UIWAF and from 50 to 200 µg/L in LEWAFs. 2-MN ranged from 10 to 50 µg/L in UIWAF and from 50 to 350 µg/L in LEWAFs. Accordingly, it has been reported that, contrary to the other PAHs, evaporation of Naph prevails during under ice weathering despite its high solubility because it quickly migrates up through the ice before dissolution is started (Desmond et al., 2019). Naph concentrations in the range of 100-300 µg/L have been reported in the LEWAF of a variety of oils (Neff et al., 2000; Gardiner et al., 2013; Faksness et al., 2008; Johann et al., 2020b). Likewise, as in the case of the LEWAFs (Chapter 1), the PAH profile excluding naphthalenes in UIWAFs was also oil specific and was modified by dispersant addition, except for the case of IFO. The concentration of Ace, Flu and Phe

[5] High-Energy Accommodated Fraction.

was high in all the UIWAFs. These three PAHs were also majoritary in the LEWAF of diverse crude and diesel oils with concentrations comparable to the ones presently recorded (Neff et al., 2000; Chapter 1): 200-3000 ng Ace/L, 600-3000 ng Flu/L, and 100-5000 ng Phe/L. After dispersant addition, Flu and Phe increased in NNA and MGO UIWAF, and Ace, Flu and Phe decreased in IFO UIWAF. In contrast, in the case of LEWAF (Chapter 1), dispersant addition caused a raise in the concentration of Ace, Flu and Phe in NNA and IFO but no change in the PAH profile of MGO. Pyr, Fluo, B[a]A+Chr and B[a]P in the three oil UIWAFs and Ant in IFO UIWAF were detected at low concentrations and after the addition of dispersant, they tended to decrease, unlike in the case of LEWAF in which IFO+D LEWAF presented much higher concentrations of these PAHs than IFO LEWAF (Chapter 1). Pyr and Ant were also detected at low concentrations in other oil LEWAFs (Neff et al., 2000; Chapter 1) although these were slightly higher than the ones presently recorded in UIWAF: 30-140 ng Pyr/L and 200 ng Ant/L.

It is known that exposure to WAF of diverse oils provokes toxic effects on 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; Chapter 1). However, no toxicity data to marine organisms exposed to the WAF of oil weathered under ice is available, to our knowledge. In the present study, exposure to the oil UIWAF and LEWAF of the three tested oils caused length reduction (ΔL), abnormalities (TI) and development impairment (CDI, GDI, MDI, IPLFI) in pluteus larvae of *P. lividus*. Overall, toxic effects were less marked after LEWAF exposure, especially for NNA and IFO in which CDI remained unchanged (Table 5). Long-term weathering under ice can contribute to enhance WAF toxicity irrespective of the measured PAH concentrations, thus explaining why toxicity is higher and earlier than LEWAF, as below discussed regarding the interpretation of TUs.

As previously reported, the responsiveness of the various developmental stages is different (Table 5). Comparable conclusions were obtained after exposing sea urchin embryos to the LEWAF of the three oils studied herein produced with a higher oil/FSW ratio (1:40 o/w v:v; Chapter 1). Certainly 1:40 LEWAFs were more toxic than the

presently used 1:200 LEWAFs but it was also concluded that LEWAFs caused a dose-dependent effect on gastrulation disruption (GDI) without evidence of preceding cleavage disruption (low CDI values; Chapter 1). Overall, early embryo stages seem to be less sensitive to WAF exposure than pluteus larvae. In the case of the Antarctic sea urchin, *Sterechinus neumayeri*, the unhatched blastula stage was the least sensitive stage upon fuel oil and metal exposure, whereas the 4-armed pluteus stage was the most sensitive one (King and Riddle, 2001; Alexander et al., 2017). Yet, it is also plausible that early embryos are a sensitive life stage but it takes some time for evidences to be observable. Indeed, Alexander et al. (2017) concluded that EC10 values suggested a potential for greater sensitivity of earlier life stages than anticipated from EC50 values. In agreement, in the present study, EC50 values of the three LEWAFs for CDI were >100% but the corresponding NOEC values were lower than 21% LEWAF (Table 5). Thus, the occurrence of early effects that are later evidenced cannot be disregarded.

On the other hand, according to Chapter 1, the toxicity of the 1:40 LEWAFs was only to a low degree attributable to the measured PAHs and it was seemingly caused by individual or combined toxic action of other non-identified compounds present in the LEWAFs. Presently, the PAH concentration in LEWAFs (1:200 o/w v:v) was lower than the ones reported in 1:40 LEWAFs of the same oils in Chapter 1. This difference could explain that LEWAFs produced at low oil/FSW ratios (1:200 LEWAFs, herein) are less toxic than the ones produced at high oil/FSW ratios (e.g., 1:40). Even if the PAHs are not the main toxic agents in the LEWAFs, the lower level of PAHs could be interpreted as indicative of the presence lower levels of other non-identified compounds in 1:200 LEWAFs in comparison with 1:40 LEWAFs.

Table 5. Schematic representation of toxicity critical values recorded in sea urchin embryos for CDI. GDI. MDI. IPLFI. TI and ΔL on exposure to NNA. NNA+D. MGO. MGO+D. IFO and IFO+D UIWAFs and LEWAFs. Green: NOEC or lower concentration; yellow: concentration between NOEC and EC50; orange: EC50 or higher concentration; red: 100% effect (EC100).

TOXICITY ENDPOINT	TESTED UI OIL(+D)	% UIWAF							% LEWAF						
		0	8	21	34	55	89	100	0	8	21	34	55	89	100
CDI	NNA														
	NNA+D														
	MGO														
	MGO+D														
	IFO														
	IFO+D														
GDI	NNA														
	NNA+D														
	MGO														
	MGO+D														
	IFO														
	IFO+D														
MDI	NNA														
	NNA+D														
	MGO														
	MGO+D														
	IFO														
	IFO+D														
IPLFI	NNA														
	NNA+D														
	MGO														
	MGO+D														
	IFO														
	IFO+D														
TI	NNA														
	NNA+D														
	MGO														
	MGO+D														
	IFO														
	IFO+D														
ΔL	NNA														
	NNA+D														
	MGO														
	MGO+D														
	IFO														
	IFO+D														

The toxic effects of the oil LEWAFs were much higher after the application of dispersant in the three oils, reaching toxicity levels comparable to those recorded after UIWAF exposure. In agreement, the 1:40 oil+D LEWAFs were more toxic than the 1:40 oil LEWAFs for the three oils studied herein (Chapter 1). 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. Dispersant enhanced toxicity was especially remarkable in the cases of IFO+D UIWAF and LEWAF, in which an all-or-nothing response was found instead of a linear dose-response. Overall, adding dispersant to crude oils enhances the toxicity of the WAF of the oils (Epstein et al., 2000; Lee et al., 2013; Rial et al., 2014; Dussauze et al., 2015; DeLeo et al., 2016; Katsumiti et al., 2019; Johann et al 2020b). The influence of dispersant on oil toxicity could be explained because dispersant addition may increase the amount of PAHs and alter the PAH profile in the LEWAFs (Yamada et al., 2003; DeLorenzo et al., 2017). However, although chemical dispersion is effective even in presence of 90% ice-coverage (Brandvik et al., 2010) and in nearly freezing water (Belore et al., 2009), the toxicity of the UIWAF of oils alone or in combination with dispersant was similar. It is likely that under long-term weathering under ice the dispersant effect is less evident because the WAF reaches its full stability in both cases and differences in the chemical profiles of the two stable conditions are minimal. Indeed, whilst the PAH levels and composition in the LEWAFs changed upon the addition of dispersant in comparison with the LEWAFs of oils without dispersant (Chapter 1), changes were minimal and less consistent in the case of UIWAFs. For instance, ∑PAHs was two times higher in oil+D UIWAF than in oil UIWAF for NNA and MGO and three times lower for IFO, in contrast with the four times increase reported for MGO and IFO LEWAF after dispersant application.

Conversely, although both UIWAF and LEWAF of the three oils were shown to be genotoxic (increased SSF), DNA damage was more marked in the case of LEWAF. Crude oil WAF and individual PAHs such as e.g. Pyr and B[a]P produce oxidative stress mediated genotoxicity in marine invertebrates (Wessel et al., 2007; Banni et al., 2010; Han et al., 2017; Xie et al., 2017). Accordingly, in the present study, sea urchin larvae exposed to oil and oil+D LEWAF, irrespective to weathering under ice, exhibited DNA

damage, but the capability to produce DNA damage varied from oil to oil depending on the application of dispersant and/or the weathering under ice. Overall, UIWAF was less genotoxic than LEWAF and the addition of dispersant did not modify genotoxicity consistently. In addition, IFO was seemingly less genotoxic than NNA and MGO, which is in agreement with the results obtained after applying the micronucleus test to zebrafish liver cell cultures (Johann et al., 2020a). NNA was the most genotoxic and IFO the least, which correlated positively with the concentrations of Naph in the LEWAFs, and the application of dispersant did not modify the genotoxicity of IFO (Johann et al., 2020a). Interestingly, in the present study, lower concentrations of Naph were recorded in UIWAF compared to LEWAF, which might explain the lower genotoxicity of the former to sea urchin embryos; even though, as below discussed, UIWAF is more toxic than LEWAF regardless of the toxicity of identified PAHS.

TU values were always very low (<0.20) after UIWAF exposure. In contrast, on exposure to LEWAF these values were higher, both with and without dispersant, most remarkably for NNA (TU>1). Oil toxicity is frequently attributed only to identified compounds (e.g., PAHs) known to be toxic; yet, other hydrocarbons and organic compounds are likely contributors to crude oil toxicity (Melbye et al., 2009). Presently, the higher toxicity of UIWAF in comparison with LEWAF is not related to the concentrations of measured PAHs. Therefore, PAHs do not seem to be the major determinants of oil WAF toxicity, in agreement with previous studies (Barron et al., 1999; Johann et al., 2020b). Unfortunately, in the present study 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). Yet, PAHs constitute less than 1% of the total petroleum hydrocarbons (TPHs) in crude oil, whilst the majority of the compounds are unidentified and commonly known as the unresolved complex mixture (UCM), described as the most complex mixture of organic molecules in the environment (>250000 compounds), resistant to weathering and likely to persist (Melbye et al., 2009). The UCM usually contains branched alkyl benzenes, indanes, and tetralines, and other bioaccumulative compounds with a $\log_{10}K_{ow}$ in the 4-6 range (i.e., aliphatic naphthalenes and monocyclic acids, monocyclic thiophenic carboxylic acids,

and monoaromatic hydrocarbons; Booth et al., 2007). Crude oil contains a wide variety of organic compounds, amongst which polar compounds including alkylated heterocyclic compounds (quinolones, carbazoles, thioxanthones, benzothiophenes, benzofurans and a vast majority that remains unidentified) are at high concentrations, as high as 98%, in the aqueous fraction after oil weathering (Lang et al., 2009; Melbye et al., 2009). Alkyl phenols, alkyl benzenes, and alkylated heterocyclic compounds have been identified in the WAFs of crude oils, in which they occurred at much higher concentrations than PAHs, in the range of 100−1000 µg/L (Barron et al., 1999). Overall, the PAH concentration in water can be more than two orders of magnitude lower than the TPH concentration (Sammarco et al., 2013). Thus, the most toxic fraction of WAF would include UCM and polar compounds, usually not quantified and not even identified (Neff et al., 2000; Booth et al., 2007).

In Chapter 1 was also found that the relative contribution of the measured PAHs (16 USEPA list) to the toxicity of LEWAFs of the three oils studied herein, alone or in combination with dispersant, was low. However, according to the present results, contribution of these PAHs to the toxicity of the UIWAFs seems to be even much lower. After weathering, $_{HMW}$PAHs and their hydroxyl and alkyl derivatives, which are at relatively low proportion of the WAF compounds (Carls et al., 1999), could become relevant contributors to the toxicity of the mixture (e.g. as shown for fish embryos; Heintz et al., 1999). Hydroxyl PAHs, pyrenol and phenanthrol formed during oil weathering are known to be more toxic than parental PAHs to larvae of sea urchin, *P. lividus* (Saco-Álvarez et al., 2008). Likewise, the WAF of weathered Prestige fuel oil had lower PAH concentrations but higher toxicity to sea urchin embryos than the WAF of fresh fuel oil (Saco-Álvarez et al., 2008); and long-term weathering (80 d) caused up to eightfold increase in toxicity to sea urchin and mussel embryos that was unrelated to the PAH concentrations (Bellas et al., 2013).

Nevertheless, although the toxicity of the mixture seems to be caused by WAF compounds other than the measured PAHs, the potential risk posed by the toxicity of individual PAHs should not be neglected a priori, especially in the long-term. For instance, 3-ring PAHs such as e.g. Pyr induce AhR dependent toxicity at early

developmental stages in fish embryos (i.e., capelin, herring and zebrafish); in contrast, weathered oil toxicity is mainly mediated through AhR independent toxicity, which is belatedly evident (Incardona et al., 2004, 2005; 2009; Hendon et al., 2008; Frantzen et al., 2012). Thus, some of the identified PAHs might be expected to exert some toxicity in the long-term. Presently, RT_i values were higher than "1" on exposure to the UIWAF and LEWAF of the three oils alone or in combination with the dispersant, which suggests that one or more individual PAHs could exhibit more toxicity than the one that could be predicted for the mixture toxicity (Chapter 1). Particularly, 2-MN, Ant, Pyr, Fluo and B[a]A+Chr appeared to contribute for a part of the toxicity of both UIWAFs and LEWAFs of the three oils with and without dispersant (RT_i/RC_i >1). Likewise the potential contribution of B[a]P to the mixture toxicity was relevant for all the oil UIWAFs and some LEWAFs, whilst Ace, Acy and Phe contributed to LEWAF toxicity. More persistent and less biodegradable than the parent compound Naph, 2-MN is toxic to diverse marine species, seemingly via non-polar narcosis (Falk-Petersen et al., 1982; De Hoop et al., 2011; Olsen et al., 2011). Pyr causes severe oxidative stress and DNA damage in marine organisms, as well as immunotoxicity, peroxisome proliferation, neurotoxicity, reproductive disruption and behavioural alterations (Frantzen et al., 2012). Fluo is acutely toxic to aquatic organisms (Rossi and Neff 1978; Horne and Oblad 1983; Gendusa 1990; Suedel et al. 1993). B[a]P is a recognised genotoxic, carcinogenic (via the AhR-CYP1 pathway) and endocrine disrupting compound (Banni et al., 2010; Booc et al., 2014; Alharty et al., 2017). Concretely, Pyr, Fluo and B[a]A are toxic for sea urchin larvae affecting spicule formation and larvae elongation (Bellas et al., 2008; Sekiguchi et al., 2018). Phe and Chr are carcinogenic and their metabolites exhibit endocrine disrupting effects (USEPA, 2000). Nevertheless, the effective concentrations reported for these individual PAHs in the aforementioned studies are 1-3 orders of magnitude higher than the ones corresponding to the EC50 values presently calculated for the various endpoints measured during sea urchin embryo development. Therefore it is quite unlikely that these individual PAHs showing RT_i/RC_i ratios higher than "1" might constitute a realistic concern regarding UIWAF toxicity.

5. CONCLUDING REMARKS

The long-term weathering under ice contributed to enhance WAF toxicity of crude and bunker oils of interest in boreal iced seas irrespective of the use of a third generation dispersant, as revealed by a battery of endpoints derived from the SET, TI and SEDD.

The PAH levels measured in UIWAFs were lower than in the corresponding LEWAF, and they were similar amongst the UIWAFs of different oils tested as the direct consequence of long-term weathering under ice. Moreover, the addition of dispersant produced minimal and less consistent changes in the PAH levels, composition and toxicity in the UIWAFs in comparison with the LEWAFs.

Overall, oil UIWAF and LEWAF of the three tested oils caused length reduction, abnormalities, development impairment and DNA damage in pluteus larvae of *P. lividus*. However, the individual PAH levels found in UIWAF were 1-3 times lower than the effective concentrations reported for those PAHs. Hence, it is quite unlikely that these individual PAHs constitute a realistic concern regarding UIWAF toxicity.

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V. CONCLUSIONS AND THESIS

CONCLUSIONS

1. The PAH profiles were found to be different depending on the oil and the temperature of LEWAF production. The PAH levels measured in UIWAFs were lower than in the corresponding LEWAF, and they were similar amongst the UIWAFs of different oils tested, irrespective of the use of dispersant, as the direct consequence of long-term weathering under ice.

2. Upon dispersant application, the sum of PAHs in LEWAFs was lower at high production temperatures (20-25ºC) in the cases of NNA and MGO, and unrelated to production temperature in IFO.

3. The toxicity of LEWAFs and UIWAFs of the three oils studied alone or in combination with dispersant, could be only partially attributed to individual PAHs or to the mixture.

4. The exposure to LEWAFs and UIWAFs from crude oil and bunker oils caused length reduction, abnormalities, development impairment and DNA damage in pluteus larvae of *Paracentrotus lividus*. The degree of effect varied depending on the oil type, the temperature of LEWAF production and the addition or not of dispersant.

5. Under standard LEWAF production conditions (10ºC, 1:200 w oil/v FSW), toxicity to sea urchin embryos was different among oil types. The heavy bunker oil IFO was more toxic than the light crude oil NNA with the light bunker oil MGO in between.

6. The application of a third-generation dispersant (Finasol OSR52®) influenced the toxicity of crude and bunker oils. Thus, the LEWAFs obtained after adding dispersant to the oils were more toxic than the LEWAFs obtained from the pure oils. Moreover, the degree of genotoxicity was augmented after dispersant application. Conversely, the addition of dispersant produced minimal and less

consistent changes in the PAH levels, composition and toxicity in the UIWAFs in comparison with the LEWAFs.

7. The toxicity of IFO LEWAF was not enhanced after *in-situ* burning. Thus, *in-situ* burning would reduce a large mass of the oil without increasing the toxicity of the remaining burn residues in marine waters. However, the long-term effect of the burned oil residues (which are richer in heavier PAHs) is still unknown.

8. The long-term weathering under ice contributed to enhance WAF toxicity of crude and bunker oils irrespective of the use of a third-generation dispersant (Finasol OSR52®), as revealed by a battery of endpoints derived from the SET, TI and SEDD.

9. Whereas no effect on early sea urchin embryo developmental stages was shown for any of the LEWAF of the three oils produced under standard LEWAF production conditions (10ºC, 1:200 w oil/v FSW), disruption in gastrulation and metamorphosis was observed confirming that the responsiveness of the various developmental stages to oil toxicity is different.

10. The sea urchin embryo toxicity assays that include various developmental stages in their analyses provide us with improved sensitivity to discriminate from slight to severe levels of toxicity, being particularly relevant for moderately toxic but environmentally realistic mixtures such as the LEWAFs of the studied oils alone and in combination with dispersant.

THESIS

Oil response strategies envisaged to respond against prospective oil spills in cold seas of the Arctic and Sub-Arctic regions (dispersant application, under ice weathering and oil burning) do not reduce and even often enhance oil toxicity, in a different manner depending on the oil type and the temperature in which the aqueous fraction is produced. Toxicity only depends partially and to a minor extent on the cocktail of 16 USEPA PAHs and, until suitable toxicity testing approaches using autochthonous species are available, it can be reliably assessed using toxicity testing with allochthonous sea urchin embryos, using both standard and novel biological endpoints as targets.

VI. APPENDIX

Appendix I. Physicochemical properties of petroleum products and the dispersant: Naphthenic North Atlantic crude oil (NNA), Marine Gas Oil (MGO), Intermediate Fuel Oil 180 (IFO) and Finasol OSR52® dispersant (D). Note that values given in Table 1 should be considered as indicative and not absolute properties of such oils as refined products (e.g. IFO) may have varying properties depending on the origin of the crude oil and refinery process. (API, American Petroleum Institute; SDS, sodium dioctyl sulfosuccinate).

	API gravity	Density (g/mL)	Pour Point (°C)	Viscosity	Wax (%)	Sulphur content (% wt)	Asphaltenes (%)	Information source	Reference
NNA[1]	35.9	0.845	-36	2.8 cSt at 50°C	0.9	0.139	0.04	Document: NNA201101	Statoil (2011)[2]
MGO[3]	34.2	0.856	< -6	3.66 cSt at 40°C	-	0.049	-	Document Nº COA18014002	Esso Norge AS (2018)[4]
IFO[5]	17.3	0.951	-10	178 cSt at 50°C	2	3.01	-	[6]	Government of Canada[6]

	Surfactant SDS (%)	Density (g/mL)	Pour Point (°C)	Viscosity	Hydrocarbons (%)	Organic solvent	Other compounds (%)	Information source	Reference
D[7]	20-25	0.990 – 1.015	- 37	30.1 – 36.7 cSt at 40°C	15-20*	15 – 20%**	0-2%***	[8]	Total Special Fluids (2015)[8]

[1] kindly provided by Statoil (Norway)

[2] Statoil. 2011. Crude Summary Report of Crude NNA 2011 01. Reference: NNA201101

[3] Esso Norge AS (Norway)

[4] Esso Norge AS. 2018. Certificate of quality Gasoil_GO-12/MGO. Document Nº COA18014002

[5] Polaroid (Greenland)

[6] Government of Canada. IFO180 properties (http://etc-cte.ec.gc.ca/databases/OilProperties/pdf/WEB_Intermediate_Fuel_Oil_180.pdf)

[7] kindly provided by Total Spain

[8] Total Special Fluids. 2015. Finasol OSR 52® Safety Data Sheet # 30034, according to Regulation (EC) No 1970/2006

[*] C11-C14, n-alkanes, isoalkanes, cyclics and <2% aromatics

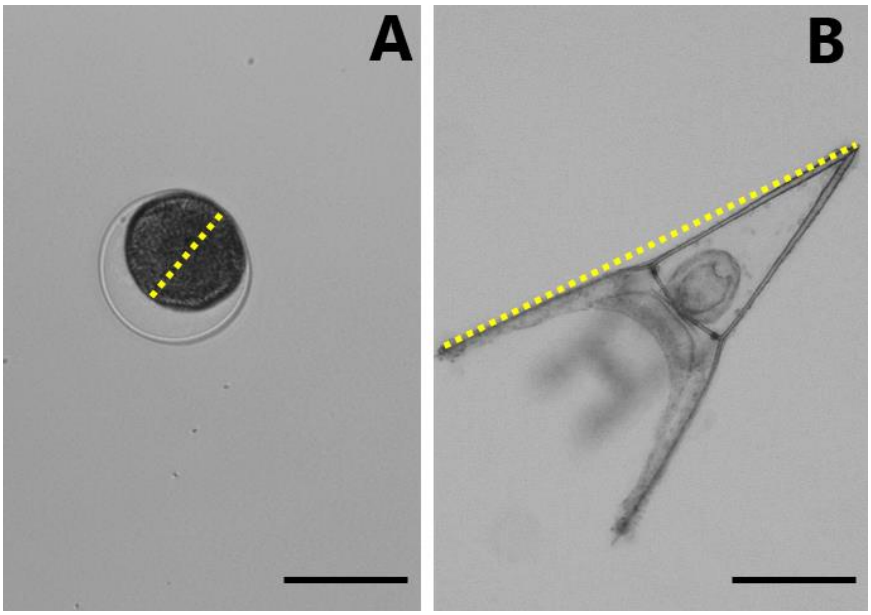
[**] 2-(methoxymethylethoxy)-propanol

[***] Different carboxylic acids and ethanolamine

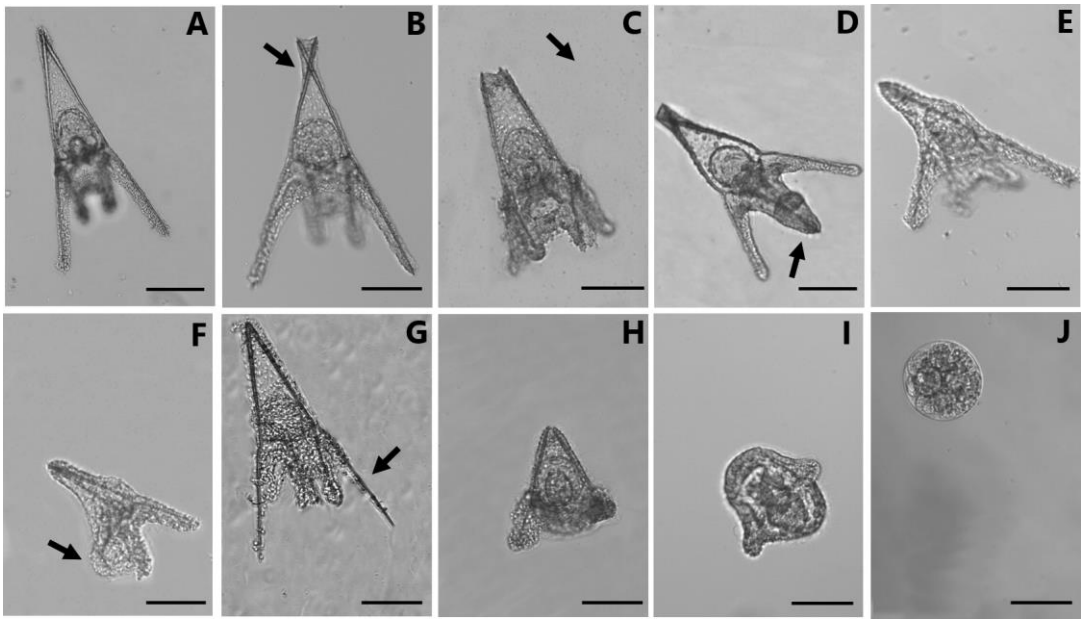
Appendix II. Detection limit values (DL, ng/L) for each PAH from GC-MS analysis.

PAH	PAH Abbreviation	DL
Naphthalene	Naph	3.3
1-Methylnaphthalene	1-MN	3.5
2-Methylnaphthalene	2-MN	4.6
Acenaphthylene	Acy	4.5
Acenaphthene	Ace	3.6
Fluorene	Flu	3.5
Anthracene	Ant	1.3
Phenanthrene	Phe	3.4
Pyrene	Pyr	4.3
Fluoranthene	Fluo	3.5
Benz[a]anthracene + Chrysene	B[a]A + Chr	6.1
Benzo[a]pyrene	B[a]P	7.6
Benzo[b]fluoranthene	B[b]F	10.8
Benzo[k]fluoranthene	B[k]F	9.5
Benzo[g,h,i]perylene	B[g,h,i]P	15
Dibenz[a,h]anthracene	D[a,h]A	28.7
Indeno[1,2,3-cd]pyrene	I[1,2,3-cd]P	35

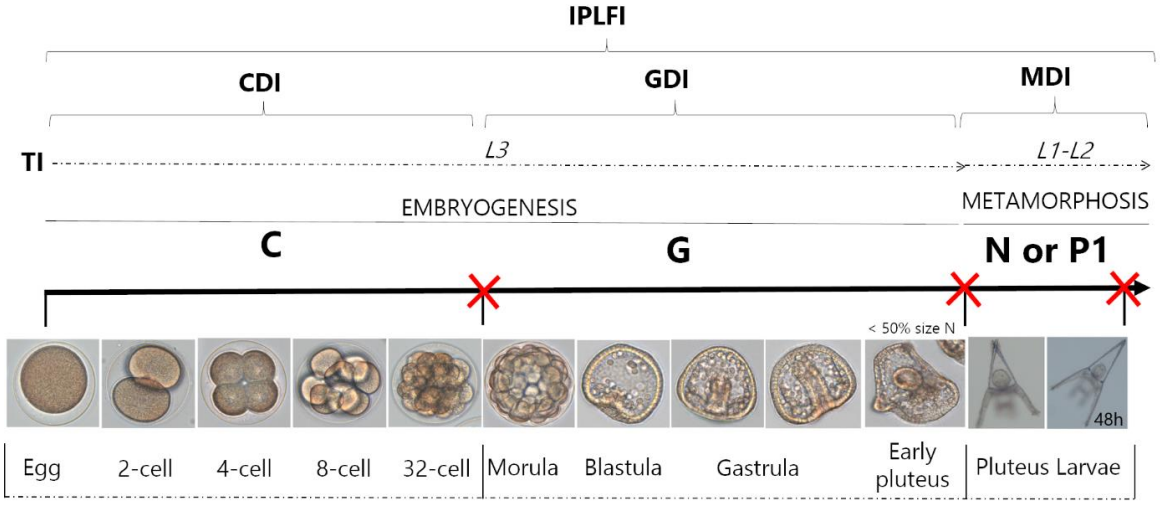
Appendix III. Photographs of the longest dimension measured in sea urchin *Paracentrotus lividus*, according to Beiras et al. (2012). **A)** Egg fertilized (L_0). **B)** Pluteus larvae after 48 hr post-fertilization. Scale bars 100 µm.



Appendix IV. Photographs of the type of developmental abnormalities (black arrows) of sea urchin *Paracentrotus lividus*, observed after 48 hr post-fertilization according to Carballeira et al. (2012) on exposure to oil LEWAF and oil+D LEWAF. **A)** Normal larva at pluteus stage. **B)** Crossed tip (L1). **C)** Separated tip (L1). **D)** Fused arms (L1). **E)** Incomplete or absent skeletal rods (L2). **F)** Folded tip (L2). **G)** Fractured ectoderm (L2). **H)** Pre-pluteus (L3). **I)** and **J)** Undeveloped embryo (L3). Scale bars 100 µm.



Appendix V. Developmental program of the sea urchin *Paracentrotus lividus*, which includes the progression throughout various main stages (egg, X-cell, morula, blastula, gastrula, early pluteus and pluteus larva). Those stages can be disturbed and classified in: C (undeveloped embryos), G (retarded or inhibited embryos), N (normal pluteus) and P1 (abnormal pluteus) to calculate a set of indices: Toxicity Index (TI) calculated scoring the frequencies of target abnormalities recorded at the pluteus larva stage (Carballeira et al., 2012); and the indices present in the SEDD assay: a high inhibition of pluteus larvae formation index (IPLFI) indicates the incompetence of pluteus larvae to successfully settle and progress to become a benthic adult; 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.



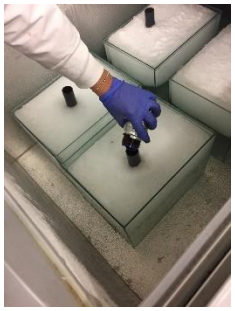
Appendix VI. EC50 values (mg PAH/L) for individual PAHs (Naph, 1-MN, 2-MN, Ace, Pyr, Fluo, Flu, Phe, Ant, Chr, B[a]A and B[a]P) found in literature.

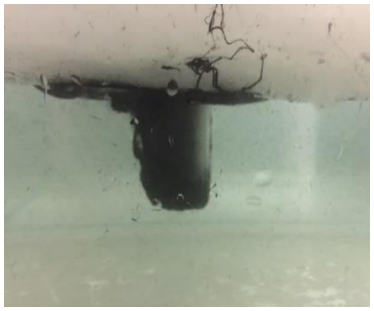
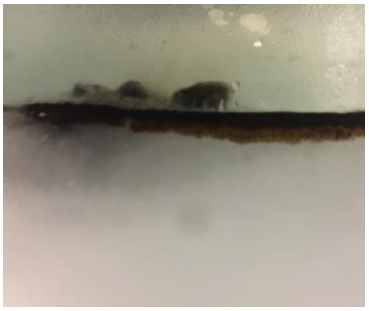
Individual PAH	Species (embryos)	EC50 (mg PAH/L)	Reference
Naph	Sea urchin *Paracentrotus lividus*	4.78	Bellas et al. (2008)
1-MN	Malacostraca *Americamysis bahia*	0.355	Knap et al. (2017)
2-MN	Coral *Porites divaricata*	0.171	Renegar et al. (2017)
Ace	Fish *Ciprinodon variegatus*	3.1	Ward et al. (1981)
Ace	Fish *Oncorhynchus mykiss*	0.67	Holcombe et al. (1983)
Ace	Fish *Salmo trutta*	0.58	Holcombe et al. (1983)
Pyr	Sea urchin *Paracentrotus lividus*	0.129	Bellas et al. (2008)
Pyr	Mollusc/Oyster *Crassostrea gigas*	0.1	Lyons et al. (2002)
Pyr	Fish *Mallotus villosus*	5.16	Frantzen et al. (2012)
Fluo	Sea urchin *P. lividus*	0.253	Bellas et al. (2008)
Fluo	Clam *Mulinea lateralis*	0.0028	Spehar et al. (1999)
Fluo	Sea urchin *Arbacia lixula*	0.13	Spehar et al. (1999)
Flu	Sea urchin *Lytechinus anemesis*	1.26	Pillai et al. (2003)
Flu	Sea urchin *P. lividus*	1.978	Bellas et al. (2008)
Phe	Sea urchin *P. lividus*	0.428	Bellas et al. (2008)
Ant	Clam *M. lateralis*	4.26	Pelletier et al. (1997)
Chr	Brachiopod *Artemia salina*	3	Pelletier et al. (1997)
B[a]A	Brachiopod *Daphnia pulex*	0.01	Trucco et al. (1983)
B[a]P	Brachiopod *D. pulex*	0.005	Trucco et al. (1983)
B[a]P	Mollusc/Oyster *C. gigas*	0.0025	Lyons et al. (2002)

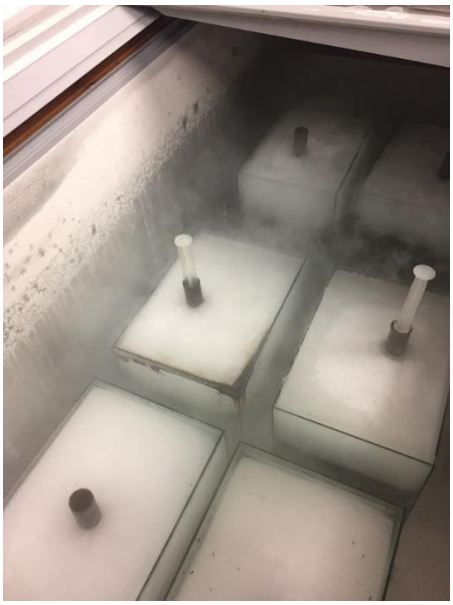
Appendix VII. Photographs of the experiment performed in Chapter 4.









Appendix VIII. Protocol of the Fast Micromethod (FMM) adapted from Walker Zoe and Reinardy Helena.

Objective: Establish a protocol for the use of Fast Micromethod (FMM) to determine the extent DNA damage in sea urchin *Paracentrotus lividus* larvae (pluteus stage).

Principle:

The FMM is an alternative to the comet assay, used for measuring the extent DNA damage in a sample of fresh cells or extracted DNA. It involves lysis alkaline denaturation of the sample(s) with pico-green dye which binds preferentially to dsDNA (double-strand). By taking 20-30 minutes of kinetics readings on the microplate the progress of dsDNA denaturing to ssDNA (simple-strand) is measured by decreasing fluorescence. This rate of reduction is proportionate to the amount of intact dsDNA at the beginning of the assay (>DNA damage = >rate of denaturation).

Equipment and reagents:

- Solutions:
    - Solution A: Pico Green dye stock solution
    - Solution B: Ca/Mg-free PBS *can be autoclaved or filter sterilised*

      For 500 mL:

137 mM NaCl	4.003 g NaCl (58.44)
2.7 mM KCl	0.1006 g KCl (74.55)
4.3 mM Na_2HPO_4	0.3052 g Na_2HPO_4 (141.96)
1.5 mM KH_2PO_4	0.102 g KH_2PO_4 (136.09)

    - Solution C: Lysing solution

      For 500 mL:

9.0 M urea	270.27 g urea (60.06) *cannot be autoclaved, filter to sterilise*
0.1% SDS	0.5 g SDS (288.4)
0.2 M EDTA	7.224 g EDTA (372.24)

    - Solution E: 20 mM EDTA (pH around 8-9, adjusted adding mL of NaOH stock solution(F)

20 mM	1.8612 g in 250 mL
0.5 M	46.53 g in 250 mL *dilute 1:25 to make 20 mM*

    - Solution F: NaOH stock solution

      For 250 mL:

1 M NaOH	10 g NaOH (40)
20 mM EDTA (Solution E)	1.8612 g EDTA (372.24)

- Ice
- Aluminum foil (to cover the plate)
- Polystyrene box with lid
- Multichannel pipette and 200-300 µL tips
- Micropipette set and tips
- Greiner 96-black-flat-bottom microplate + lid
- Fluorescence plate-reader (Polar OMEGAstar)

- Pico Green Dye (frozen at -20ºC, defreeze gently)
- pH meter

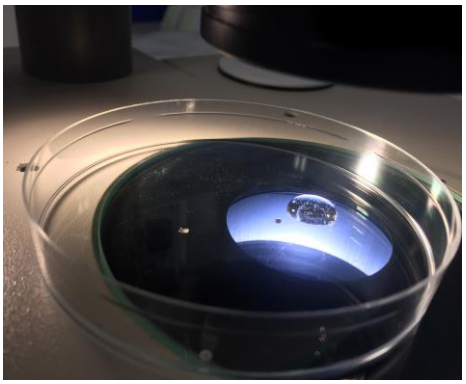
Protocol:

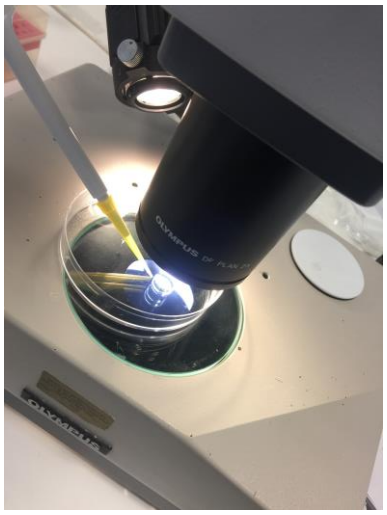
1. Prepare the above solutions prior to beginning the assay.
2. Solutions to prepare fresh (on day of the assay) depending on your nº of samples.
   2.1. Lysis solution (Solution D): `
   Prepare from 980 µL of lysis solution (Solution C) and 20 µL of Pico Green (Solution A). We need 20 µL of lysis solution per each sample.
   2.2. Unwinding solution (Solution G):
   We need 200 µL of unwinding solution per each sample/well, and need to be enough volume for the cuvette to charge the multichannel pipette (e.g. prepare double of volume in a falcon):

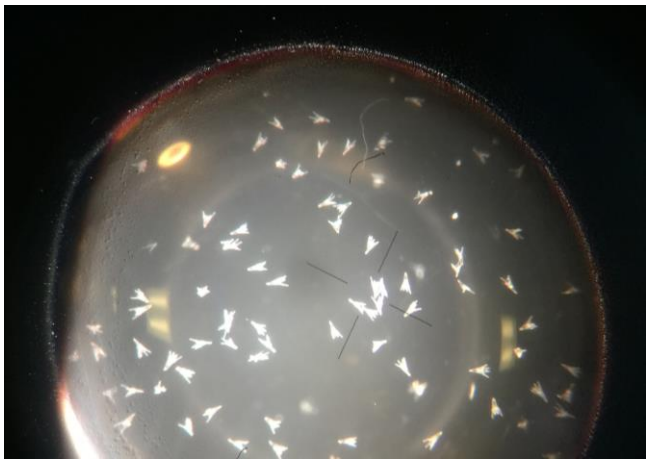
   You need:
   - 1 mL tips and pipette
   - Solution F (bottle of 1M NaOH + 20 mM EDTA; alkalinity, to increase pH)
   - Solution E (bottle of 20 mM EDTA: to decrease pH)
   - Lysis solution in falcon
   - Ca Mg Free PBS in falcon
   - pH check tube
   - Unwinding solution (G) tube

   Prepare freshly before use (in the 40 minutes lysing period) by mixing 2 mL of NaOH stock solution (Solution F) with 18 mL of EDTA (Solution E). Check the pH of the solution by adding 2.5 mL of Solution G to 0.25 mL of PBS (Solution B) and 0.25 mL of lysing solution (Solution C) and check that the pH is 12.65 ± 0.02 for sea urchin larvae. If the pH is too high, add some of Solution E, if it is too low add some of Solution F and recheck as before.

3. Prepare samples:
   - To perform FMM directly in fresh sea urchin larvae:
     - Defreeze your larvae pellet samples stored in RNAlater at -80ºC on ice, gently.
     - Place one plastic petri dish under the microscope and create some drops with 150 µL of ELGA H_2O.
     - Pipette 100 µL of the stock larvae tube inside one drop.
     - Pipette 10 µL counting how many larvae are you taking (up to 15 larvae/well). Do it twice for a final volume of 20 µL in each well.







4. Run assay:

   4.1. Make up lysis solution fresh (and warm up a little in lysis solution falcon)
   - Add 20 µL of Pico Green (Solution A) to 980 µL lysis solution (Solution C) (1:50 dilution).
   - Keep in the dark until use. The same tube can be used for future lysis solution and new solution can be mixed with old.

   4.2. Place a new black-walled 96-well microplate on ice to cool. Place the lid over the plate to help prevent condensation in the wells.

   4.3. Pipette 20 µL of blank (for extracted DNA is PBS) into duplicate wells.

   4.4. Pipette volume (µL) of ELGA H_2O that need each sample.

   4.5. Pipette volume (µL) of sample into triplicate/quadruplicate wells (ELGA H_2O + sample = 20 µL total volume).

   4.6. Once all samples/blanks are in, add 20 µL lysis solution (with Pico Green).

   4.7. Cover the plate with aluminium foil to prevent exposure to light and allow for a lysing period of 40 minutes.

4.8. During the 40 lysing period, prepare and pH check Solution G – unwinding solution (as described in step 2.2, pH 12.65 ± 0.02).

4.9. Also during the lysing period, turn on fluorescent plate-reader and adjust the settings for the readings.
- OMEGAstar plate reader with 1 spacer only.
- Gain 1500 for fresh larvae and Gain 2000 for DNA-extracted.
- Excitation wavelength of 480 nm and an emission wavelength of 520 nm.

4.10. After the lysis period, place plate on the plate reader.

4.11. Using the multichannel pipette, set to 200 µL and using 200/300 µL tips, carefully but quickly add 200 µL unwinding solution (Solution G) to all wells with sample.

4.12. Set plate to read immediately after adding the unwinding solution (Solution G).

4.13. After the reading is completed, save the plate file with the date or experiment title/number. Also, export and save the data file so that the data can be processed and analysed on excel.

5. Data analysis. The strand scission factor (SSF) is calculated according to Scröder et al. (2006):

$$SSF = -\log \frac{\%\ dsDNA_i}{\%\ dsDNA_c};$$

where $\%dsDNA_i$ is the percentage of dsDNA in each exposure group and $\%dsDNA_c$ is the percentage of dsDNA in the experimental control group. The %dsDNA values were calculated as RFU for a given sample divided by the RFU recorded in the experimental control group at t0.

Scröder HC, Batel R, Schwertner HR, Boreiko O, Müller WEG. 2006. Fast Micromethod DNA single-strand-break assay. In: Henderson DS (Ed.). *Methods in Molecular Biology: DNA Repair Protocols Mammalian System*. Humana Press Inc, 287-305.

VII. AGRAÏMENTS

Mirar atrás y ver cuánto te ha enseñado y enriquecido esta experiencia compensa el sacrificio invertido en ella.

Es cierto que nunca me han gustado las despedidas y que sigo sin acostumbrarme a ellas, pero quería dejar constancia que cada una de las personas que he tenido la oportunidad de conocer durante este viaje ha contribuido a hacer de éste, un camino difícil de olvidar. Como no siempre es fácil acordarse de todos con los que he podido compartir este trayecto, pido disculpas por aquellos que no aparezcan a continuación y deberían hacerlo.

Empezando por los inicios, en los que la Dra. Amparo Torreblanca, Dra. Inmaculada Varó y la Dra. Amparo González me introdujeron al mundo de la ciencia.

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"Si tienes un problema y no tiene solución, para que te preocupas, y si tiene solución, para que te preocupas". – Proverbio chino.

Following, my deepest gratitude to you Helena. Thank you for believing in me, for helping me at any time, for your guidance, suport, advices and kindness. Indeed, for understanding and sharing the same feeling for sea urchin power and love.

A Beñat, Pamela, Oihane y Urtzi, por acogerme como ayudante de profesorado, por vuestra buena disposición a enseñarme e impulsarme a ganar confianza en mí misma. Además de Javi, Beñat, Oihane y Eider, por vuestra ayuda, colaboración y conocimiento transferido. Esker anitz zure laguntzagatik.

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Duroudier, Dr. Nerea García-Velasco, Dr, Tifanie Briaudeau, Dr. Erik Urionabarrenetxea, Ander Urrutia, Denis B(u)enito, Tamer Hafez, Markel Sanz. I appreciate the pleasure of working with you, your help and fun moments. Eskerrik asko denoi. Y con les doctorandes más cercanes, por los momentos clave que han ido acompañados de apoyo mutuo con risas aseguradas y que serán difíciles de olvidar, tanto en congresos como en el despacho, con reflexiones diarias, viernes tardíos de musicota y teorías y/o debates (no) absurdos. Pero bueno, no me extenderé demasiado por si me cobran los caracteres del texto, katalanarra naiz eta, hori beti.

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A mis dos indomables favoritos: Unai & Nerea. Gracias por compartir momentos difíciles de olvidar, por nuestras locuras, vuestros ánimos y apoyo. Ya sabéis…estemos dónde estemos: "hasta que se seque el malecón".

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con el paso del tiempo y volver a recuperar buenísimos momentos durante las visitas express. A vosotros mutilak, Txomin, Xou, Asier, Ivan e Iraola, entre muchos otros, eskerrik asko zuen harrera onagatik, vacile, apoyo y ánimos que ha hecho mucho más llevadero todo este viaje. Ibanne eta Janire os habéis convertido en un pilar fundamental en esta última etapa del camino. Benetan, no lo hubiese disfrutado así de no ser por vosotras. Eskerrik anitz zure laguntzagatik, baita zure indarra eta babesa ere!

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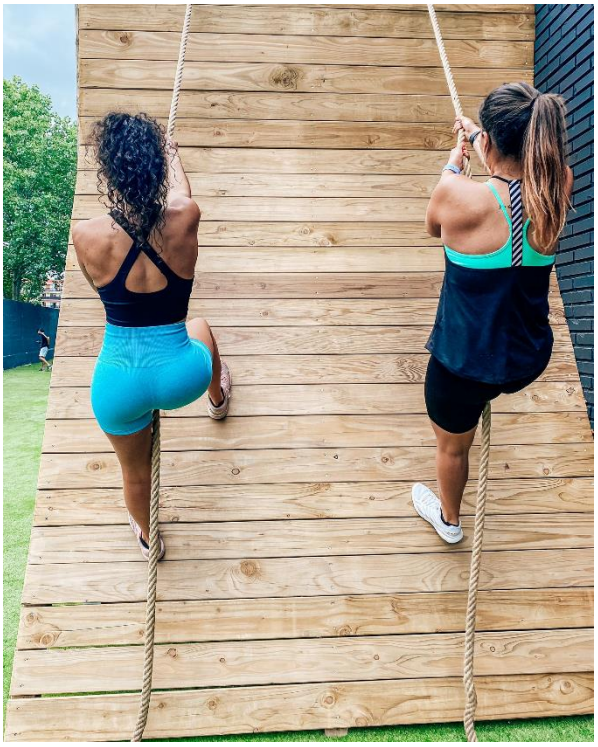
A ti tata por devolverme a la realidad de una bofetada y que, junto a vosotros, mama y papa, habéis creído en mí, habéis estado siempre a mi lado apoyándome en todo momento y, sobre todo, a lo largo de este camino en el que en numerosos momentos no veía el final. Gracias por toda la ayuda que me habéis brindado y sobre todo por recordarme qué es lo importante en esta vida. Us estimo moltíssim.

Txomin, zure aldera beti goian. Bihotzez eskertzen dizut. Gracias por seguir ahí, porqué fácil no te lo he puesto, por hacer que este camino haya ido mejorando con el paso del tiempo y por no dejar que nunca me rinda. Nire urduritasun aurrean beti irribarre batekin eta besarkada lasaigarri batekin erantzun dituzulako. Maite zaitut.

"Zu zinen protagonista, baina ni naiz gidoilari obra honetan. Antzezlana bukatu da eta orain libre naiz haizea bezala. Gorantz noa. Ikusten al dituzu nire hegalak urrunean?" – Lisboa, Anne Lukin.



"Tu ets el protagonista, però jo sóc la guionista d'aquesta obra. L'obra s'ha acabat i ara sóc lliure com el vent. Pujo. Pots veure les meves ales en la distància?" – Lisboa, Anne Lukin.