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

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ABSTRACT

This investigation deals with how temperature influences oil toxicity, alone or combined with dispersant (D). Larval lengthening, abnormalities, developmental disruption, and genotoxicity were determined in sea urchin embryos for assessing toxicity of low-energy water accommodated fractions (LEWAF) of three oils (NNA crude oil, marine gas oil –MGO-, and IFO 180 fuel oil) produced at 5–25 °C. PAH levels were similar amongst LEWAFs but PAH profiles varied with oil and production temperature. The sum of PAHs was higher in oil-dispersant LEWAFs than in oil LEWAFs, most remarkably at low production temperatures in the cases of NNA and MGO. Genotoxicity, enhanced after dispersant application, varied depending on the LEWAF production temperature in a different way for each oil. Impaired lengthening, abnormalities and developmental disruption were recorded, the severity of the effects varying with oil, dispersant application and LEWAF production temperature. Toxicity, only partially attributed to individual PAHs, was higher at lower LEWAF production temperatures.

1. Introduction

Enhanced by globalization and aided by climate change driven ice retreat, new maritime trade routes constitute an emerging threat in the Arctic and Subarctic regions, where meteorological and environmental conditions can be extreme and accessibility very limited due to remoteness, thus jeopardising the oil spill response (Arctic Council, 2009; Yang et al., 2018; Pirotta et al., 2019). In these regions, seawater surface temperature (SST) varies in the range of around 5–25 °C around the year, depending on the season and the geographical area. The annual average SST in the hottest areas of the Arctic (Norwegian Sea, Greenland Sea and Barents Sea) shows values between −1 and 7 °C (Carvalho and Wang, 2020). Annual mean SST is around 5 °C in spring and 10 °C in late summer in the Baltic Sea and around 15 °C in temperate summer in the North Sea, whilst maximum SST in summer in the Gulf of Bothnia can be as high as 25 °C (Stigebrandt and Gustafsson, 2003; Siegel and Gerth, 2018).

SST is a key environmental condition that may influence both the oil spill impact and the efficiency of the oil spill response (USEPA, 1999). Oil is less likely to spread in very cold waters than in warmer waters because surface tension drops; this depends on the oil pour point, the lower it is (e.g. -39 °C in naphthenic crude oils vs. -6 °C in IFO 180) the easier the oil will spread on the water surface (Faksness 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; Pasparakis et al., 2016; Perrichon et al., 2018; Serafin et al., 2019), particularly upon dispersant addition (Ramachandran et al., 2004, 2006). Moreover, the chemical profile of

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the water accommodated fraction (WAF) of oils, commonly used for oil toxicity assessment, also varies with the temperature in an oil type specific fashion (Faksness 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 (Faksness et al., 2008), and the solubility of PAHs depends on the temperature (Whitehouse, 1984). Generation of a saturated WAF takes longer time in colder seawater (2 °C) than at 13 °C, more remarkably in the case of oils with higher pour point (wax rich oils) than in oils with lower pour points (naphthenic oils) (Faksness et al., 2008).

Nevertheless, studies dealing with the influence of temperature in oil toxicity to marine organisms are scarce, and their experimental designs are disparate. Exceptionally, Li et al. (2021) investigated the toxicity to sea cucumber, *Apostichopus japonicus*, of Oman crude oil WAF produced at 2 temperatures (16 and 26 °C) after exposure to the WAF at the corresponding production temperatures. Yet, most frequently, experimental designs include exposure at various temperatures to test aqueous oil fractions produced only at one standard temperature (Korn et al., 1979; Lyons et al., 2011; Pasparakis et al., 2016; Camus et al., 2015; Perrichon et al., 2018; Serafin et al., 2019). These investigations provide valuable information about how temperature affects exposure conditions and organism responsiveness but do not consider that temperature also may change the composition and toxic potential of the oil aqueous fractions. Other studies use extracts or concentrates of aqueous fractions produced at various temperatures and conduct toxicity testing at one standard temperature. Saeed et al. (1998) used the Microtox test to compare the toxicity exerted by the water-soluble fraction of Kuwait crude oil produced at different temperatures in the 15–35 °C range. Katsumiti et al. (2019) performed in vitro toxicity studies using haemocytes 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 20 °C without and with dispersant. These investigations do not consider that temperature also affects exposure conditions and organism responsiveness but provide valuable information about how temperature modifies the composition and toxic potential of the oil aqueous fractions.

The present study, carried out within the framework of the EU-funded GRACE project (Jørgensen et al., 2019), was conceived to investigate the influence of temperature on the toxicity to sea urchin embryos of the low energy WAF 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 Sub-arctic seas. However, irrespective of the LEWAF production temperature, toxicity assessment was carried out using the *Paracentrotus lividus* sea urchin embryo toxicity test (Beiras et al., 2012) at only one exposure temperature (20 °C), optimal for the test species employed (Shpigel et al., 2004). Thus, we can compare the toxicity of aqueous fractions produced at various temperatures against existing data on oil toxicity (Bellas et al., 2008, 2013; Saco-Álvarez et al., 2008; Beiras et al., 2012; DeMiguel-Jiménez et al., 2021, 2022). The information provided by this relatively simple experimental design may be valuable to develop further research based on complex designs including combinations of various temperatures to produce oil aqueous fractions and various autochthonous test species of diverse temperature optima.

2. Materials and methods

2.1. Test chemicals

Three oils and one dispersant were selected as representative of prospective oil spill threats in Arctic and Sub-Arctic seas (Electronic Supplementary Material, ESM 1): (a) Naphthenic North Atlantic crude oil (NNA); (b) distillate marine gas oil (MGO); (c) intermediate fuel oil IFO 180 (IFO); and (d) the third-generation dispersant Finasol OSR52® (D). Test chemicals were directly obtained from the producer in sealed containers, and stored at a cold room (5 °C). Once the containers were opened, aliquots (90 g) for WAF production were retrieved all at the same time and stored until use in 100 mL Pyrex laboratory bottles with screw-cap sealed with aluminium foil at 5 °C in a dark chamber.

2.2. LEWAF production, and chemical analysis

The Low Energy Water Accommodated Fraction in filtered seawater (FSW) of the three oils, alone (NNA LEWAF, MGO LEWAF and IFO LEWAF) or mixed with the dispersant (NNA + D LEWAF, MGO + D LEWAF, IFO + D LEWAF), was produced in the darkness at 5, 10, 15, 20 and 25 °C, after Singer et al. (2000) and Katsumiti et al. (2019). Briefly, oils (1:200; w oil/v FSW) and their mixtures (1:10 w D/w oil+D in 1:200; w oil+D/v FSW) were poured into filtered seawater in 200 mL glass bottles and stirred at 200 ± 20 rpm (no vortex; low energy) for 40 h (Bilbao et al., 2022) in refrigerated boxes (Δ = ±2 °C).

The specific PAH composition of each LEWAF was determined by gas chromatography–mass spectrometry after Prieto et al. (2007). The full chemical names and abbreviations of the measured PAHs are listed in ESM 2. A standard solution of 18 PAHs (CRM47543; Supelco, USA) was used for calibration in the GC–MS analysis. A mixture of 5 deuterated compounds was used as surrogates (Norwegian Standard S-4124-200-T; Chiron, Trondheim, Norway). Stir-bars (10 mm length; 0.5 mm film thick; Gerstel GmbH & Co, Germany) were introduced in aqueous samples 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, desorption temperature, desorption flow (23 mL/min), cryo-focusing temperature and vent pressure. The chromatographic conditions were setup as described in Prieto et al. (2007), with recoveries estimated in the range of 80–120 %. Procedural detection limits are given in ESM 2.

2.3. Sea urchin embryo toxicity (SET) testing

The sea urchin 48-h embryo toxicity assay was carried out according to the International Council for the Exploration of the Sea methodology (ICES, Beiras et al., 2012). Gametes were obtained from sexually mature sea urchins (*Paracentrotus lividus*). These were collected from a relatively pristine rocky shore in Armintza (43°26′01.1″N 2°53′56.1″W; Bay of Biscay) in spring (March–May) 2018, transferred to the laboratory and maintained in recirculating-system tanks containing clean seawater at their natural conditions in the field (32 psu; 16 ± 1 °C). Spawning and fertilisation were carried out in filtered seawater (0.22 μm) at 32 psu and 20 °C, as described by DeMiguel-Jiménez et al. (2021). Within 30 min after fertilisation, the successfully fertilised eggs (50 embryos/mL) were transferred to glass vials containing 10 mL of the test solutions, capped with Teflon lids. Toxicity assays were conducted in complete darkness at 20 °C. Successive dilutions (0, 8 %, 21 %, 34 %, and 55 %) in FSW 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 doses from the dilution series in order to optimise the experimental set up, as in previous investigations (DeMiguel-Jiménez et al., 2021).

After 48 h exposure, larvae were fixed by adding two drops of 40 % formaldehyde. The longest dimension of larva (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) and NIS-Elements Imaging Software v4.30 (Nikon Instruments BV). Lengthening was calculated as $\Delta L = L\text{-}L_0$ (Beiras et al., 2012). Specific abnormalities of the pluteus larvae were recorded (n = 100 larvae per vial ×3 replicates per experimental group) and integrated into the Toxicity Index (TI, in a 0–100 range; after Carballeira et al., 2012), as detailed in DeMiguel-Jiménez et al. (2021).

Embryo development arrest indices (Cleavage Disruption Index: CDI; Gastrulation Disruption Index: GDI; Metamorphosis Disruption Index: MDI) and the Inhibition of Pluteus Larvae Formation Index (IPLFI) were determined according to DeMiguel-Jiménez et al. (2021).

2.4. Genotoxicity assay

The present results regarding the effect of the test oils on pluteus larvae lengthening (ΔL) were used, together with preceding data (DeMiguel-Jiménez et al., 2021), to select sublethal exposure concentrations: 55 % oil LEWAF and 34 % oil+D LEWAF. After 48 h exposure, sea urchin larvae were centrifuged (1800 ×*g* at 4 °C 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 −80 °C until the genotoxicity assay was performed. The amount of intact double-stranded DNA was determined by the Fast Micromethod® DNA Single-Strand-Break Assay (Scröder et al., 2006), adapted to sea urchin larvae (Reinardy and Bodnar, 2015), as detailed by DeMiguel-Jiménez et al. (2022). The strand scission factor (SSF) was calculated according to Scröder et al. (2006).

2.5. Toxic units

The relative contribution of each individual PAH to the toxic units (TUs; Sprague, 1970) of LEWAFs (RT_i) and the relative concentration of each PAH in the mixtures (RC_i) were calculated according to DeMiguel-Jiménez et al. (2021). For TU calculations, EC50 values of individual PAHs published for marine organisms were used as reference (ESM 3; Ott et al., 1978; Ward et al., 1981; Holcombe et al., 1983; Trucco et al., 1983; Spehar et al., 1999; Lyons et al., 2002; Pillai et al., 2003; Barata et al., 2005; Calbet et al., 2007; Bellas et al., 2008; Olsen et al., 2011; Frantzen et al., 2012; Renegar et al., 2017; Knap et al., 2017). RT_i was determined as $RT_i = TU_{PAHi}/\sum TU_{\sum PAHs}$; where TU_{PAHi} is the TU estimated for each individual PAH and $\sum TU_{\sum PAHs}$ is the TUs of the mixture. RC_i was determined as $RC_i = C_{PAHi}/\sum PAHs$; where C_{PAHi} stands for the individual concentration of each PAH. The ratio RT_i/RC_i was calculated as indicative of whether the toxicity of a given 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 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. 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 amongst temperatures and amongst oil LEWAFs for the three tested oils (3.4–9.4 μg PAH/L). In contrast, ∑PAHs varied largely depending on the temperature for NNA + D (4.6–26.9 μg PAH/L) and IFO + D (13.5–37.5 μg PAH/L) LEWAFs, with highest values at 10 °C in the former and at 5–15 °C in the latter (Tables 1-3). The PAH profiles (without napthalenes) were comparable in NNA and MGO LEWAFs, with and without dispersant, with some slight deviations depending on the LEWAF production temperature (Fig. 1). The profile was more variable at 10–15 °C than at 5, 20 and 25 °C in NNA and NNA + D LEWAFs. In MGO, low molecular weight PAHs ($_{LMW}$PAHs) were at higher levels in LEWAF produced at 15-25 °C than in LEWAF produced at lower temperatures; likewise, high molecular weight PAHs ($_{HMW}$PAHs) were at higher levels in LEWAF produced at 25 °C than in LEWAF produced at any lower temperature. The PAH profiles for IFO LEWAFs were also similar irrespective of the temperature of LEWAF production but varied in the case of IFO + D LEWAFs.

Table 1
GC–MS analysis of PAHs (ng/L) present in NNA LEWAF and NNA + D LEWAF samples produced at 5 °C, 10 °C (after DeMiguel-Jiménez et al., 2021), 15 °C, 20 °C and 25 °C. 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.

	5 °C		10 °C		15 °C		20 °C		25 °C	
	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$^{\sum(1)}$	3290	4305	6521	25376*	4841	4939	6306	4554	6656	5149
$\sum_{HMW}$PAHs$^{\sum(2)}$	139	317	292	1549*	161	285	175	145	183	200
∑PAHs[#]	3430	4622	6813	26925*	5003	5223	6481	4699	6838	5348

Table 2
GC–MS analysis of PAHs (ng/L) present in MGO LEWAF and MGO + D LEWAF samples produced at 5 °C, 10 °C (after DeMiguel-Jiménez et al., 2021), 15 °C, 20 °C and 25 °C. 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.

	5 °C		10 °C		15 °C		20 °C		25 °C	
	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$^{\sum(1)}$	5435	4102*	5321	5879	6547	7332	6714	6933	9230*	7091
$\sum_{HMW}$PAHs$^{\sum(2)}$	53	97	60	108	86	141	84	111	168	203*
∑PAHs[#]	5488	4199*	5380	5987	6633	7473	6798	7044	9398*	7293

Table 3
GC–MS analysis of PAHs (ng/L) present in IFO LEWAF and IFO + D LEWAF samples produced at 5 °C, 10 °C (after DeMiguel-Jiménez et al., 2021), 15 °C, 20 °C and 25 °C. 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.

	5 °C		10 °C		15 °C		20 °C		25 °C	
	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$^{\sum(1)}$	6280	30952*	7223	27936	8246	23864	8259	11556	8107	13216
$\sum_{HMW}$PAHs$^{\sum(2)}$	71	6510	65	6235	111	5434	106	1930	141	1019
∑PAHs[#]	6351	37461*	7288	34171	8357	29298	8365	13486	8248	14234

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 15 °C in all the 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 5–10 °C range (Table 1). In NNA + D LEWAF, Ant, Pyr, Fluo and B[*a*]A + Chr concentration increased remarkably; however, at 20 and 25 °C, 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 5 °C but Flu increased at 10–15 °C and Phe increased at 10–20 °C to finally drop at 25 °C, 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 20 °C (Table 3).

Overall, the values of ∑PAHs in NNA LEWAF were similar for LEWAF production temperatures in the range of 10–25 °C but half-reduced when LEWAF production temperature was 5 °C (Table 1). Upon dispersant addition, ∑PAHs in LEWAF was higher than for NNA LEWAF, which was particularly remarkable at a LEWAF production temperature of 10 °C (Table 1). For MGO LEWAF, ∑PAHs slightly rose with temperature, which was less marked after dispersant addition (Table 2). The $\sum_{LMW}$PAHs and $\sum_{HMW}$PAHs in NNA + D LEWAF were higher at 10 °C than at any other production temperature (Table 1). Meanwhile, the $\sum_{LMW}$PAHs in MGO LEWAF and $\sum_{HMW}$PAHs in MGO + D LEWAF were higher at 25 °C than at lower production temperatures (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, as observed regarding both $\sum_{LMW}$PAHs and $\sum_{HMW}$PAHs values (Table 3).

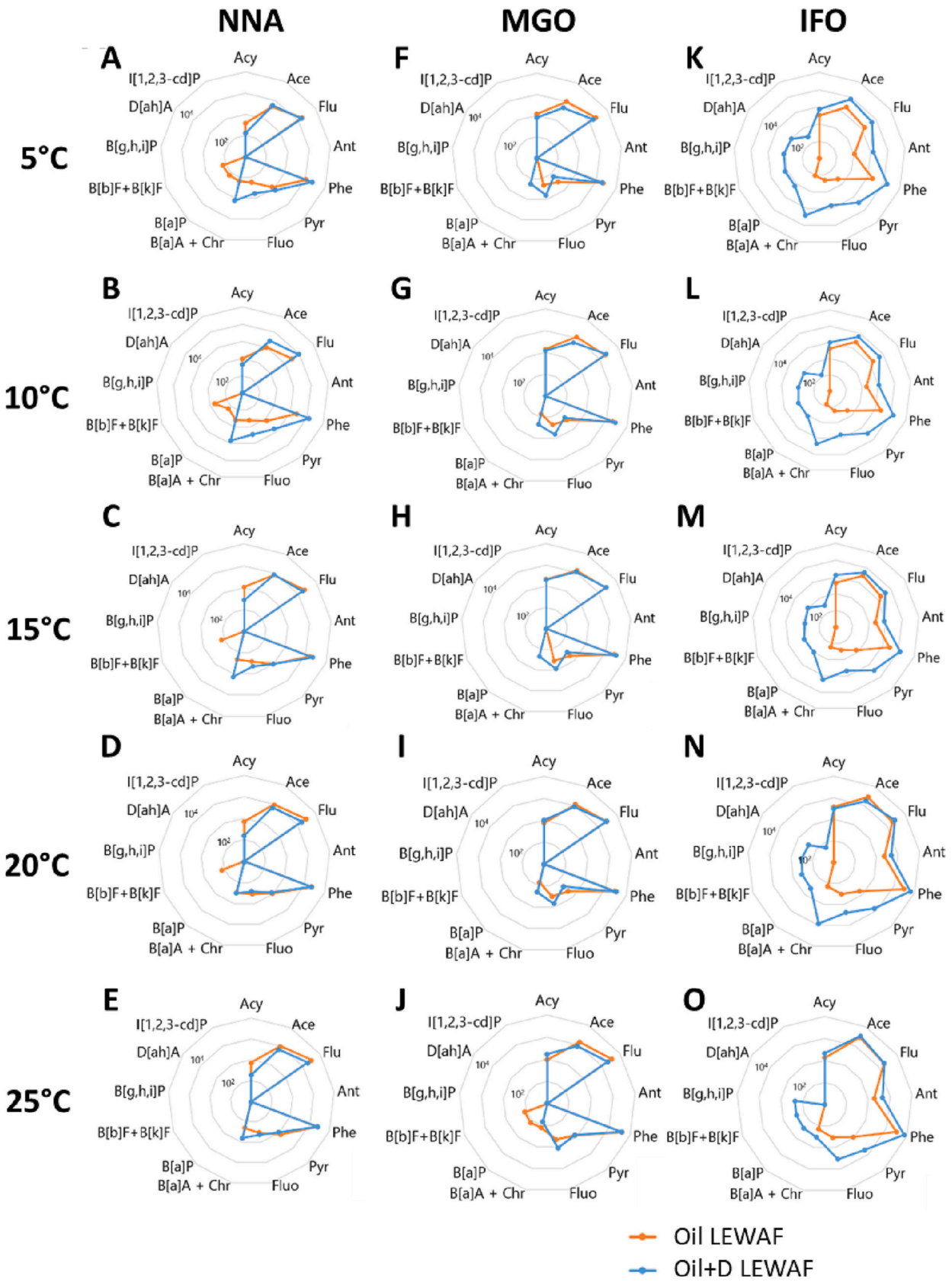


Fig. 1. PAH profiles in logarithmic scale (using concentrations in ng/L of individual PAHs other than Naph and MNs; Tables 1-3), present in oil and oil+D LEWAF of NNA, MGO and IFO produced at 5, 10, 15, 20 and 25 °C.

3.2. Temperature dependent toxicity of NNA and NNA + D

NNA + D LEWAF and, to a lesser extent, NNA LEWAF were more genotoxic in comparison with the experimental control group at all LEWAF production temperatures (Fig. 2A). ΔL decreased at increasing concentrations of both NNA LEWAF and NNA + D LEWAF produced at temperatures in the 5–25 °C range (Fig. 3A-E). NOEC values were lower than 8 % LEWAF except for NNA at 5 °C (Table 4). NNA + D LEWAF was seemingly more toxic than NNA LEWAF (Fig. 3A-E). Thus, moderately low EC50 values were recorded on exposure to NNA LEWAF produced at 15–20 °C, whilst in the case of NNA + D the EC50 values were lower except for LEWAF produced at 25 °C (Table 4; ESM 4). TI remained around 0–20 % on exposure to NNA LEWAF produced at any temperature in the studied 5–25 °C range (Fig. 4A-E). In contrast, in the case of NNA + D LEWAF, TI abruptly rose to 100 % on exposure to 55 % LEWAF irrespective of the production temperature (Fig. 4A-E). As a result, $EC50_{(NNA)}$ was in the range of 70–100 % LEWAF whilst EC50 was much lower (12–38 % LEWAF). Nonetheless, NOEC values were relatively low for both NNA and NNA + D (Table 4). For CDI, $EC50_{(NNA)}$ and EC50 were also high except for NNA + D LEWAF produced at 5 °C (Table 4; ESM 4). For GDI and MDI, $EC50_{(NNA)}$ values were in the 70–100 % range whilst EC50 values were much lower, especially at the lowest temperatures of LEWAF production in the case of MDI (Table 4; ESM 4). IPLFI increased at increasing concentrations of NNA LEWAF (Fig. 5B-D), less markedly when production temperatures were 5, 10 and 25 °C (Fig. 5A and E).

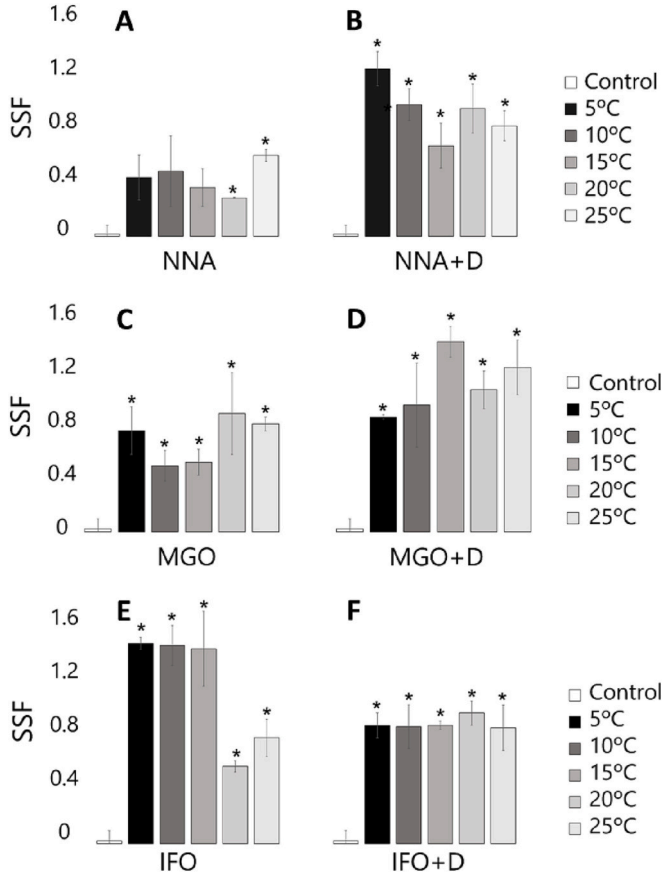


Fig. 2. DNA damage measured in Strand Scission Factor (SSF ± SD) of sea urchin larvae exposed to NNA LEWAF (A), NNA + D LEWAF (B), MGO LEWAF (C), MGO + D LEWAF (D), IFO LEWAF (E) and IFO + D LEWAF (F) produced at different temperatures (5, 10, 15, 20 and 25 °C). Asterisks indicate significant differences between each oil LEWAF and its corresponding oil+D LEWAF at each LEWAF production temperature. Differences amongst temperatures in each condition (oil LEWAF or oil+D LEWAF) according to the Duncan's post-hoc test are indicated by asterisk upper matrices.

Thus, $EC50_{(NNA)}$ was high except when LEWAF had been produced at 20 °C, but $NOEC_{(NNA)}$ values were always low irrespective of the LEWAF production temperature (Table 4; ESM 4). Exposure to NNA + D caused an abrupt rise in IPLFI values at relatively low concentrations of LEWAF produced at low temperatures, and a concentration dependent increase when LEWAF had been produced at 20–25 °C (Fig. 5A-E). EC50 was at least 2–3 times lower than the corresponding $EC50_{(NNA)}$ (Table 4; ESM 4). In agreement, NOEC was also lower than $NOEC_{(NNA)}$, especially at the lowest temperatures of LEWAF production (Table 4).

The sum of TUs was always higher in NNA + D LEWAFs than in NNA LEWAFs (Table 5). TU values higher than 1 were recorded for ΔL after exposure to NNA + D LEWAF produced at 25 °C, for CDI after exposure to NNA + D LEWAF produced at 10, 20 and 25 °C, and for all the toxicity endpoints on exposure to NNA + D LEWAF produced at 15 °C (Table 5). RTi values higher than 1 revealed that one or more individual PAHs exhibited higher toxicity than predicted for the mixture toxicity. Accordingly, RT/RC values were higher 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 5–25 °C range, and Phe in NNA + D LEWAF produced at 5, 20 and 25 °C.

3.3. Temperature dependent toxicity of MGO alone and combined with dispersant

MGO + D LEWAF, and to a lesser extent MGO LEWAF, was more genotoxic in comparison with the experimental control group, irrespective of the LEWAF production temperature (Fig. 2C-D). ΔL decreased at increasing concentrations of MGO LEWAF produced at 5–20 °C (not when produced at 25 °C) and MGO + D LEWAF produced at any temperature in the 5–25 °C range (Fig. 3F-J). When LEWAF was produced at 10, 15 and 25 °C, the decrease was more pronounced on exposure to MGO + D LEWAF than on exposure to MGO LEWAF (ANCOVA; $p < 0.05$; Fig. 3G, H and J). The lowest EC50 value for MGO LEWAF was recorded on exposure to LEWAF produced at 5 °C, whilst EC50 values were always low in the case of MGO + D LEWAF (Table 4; ESM 4). NOEC values were always lower than 8 % in for both MGO and MGO + D LEWAFs (Fig. 3F-J; Table 4). TI increased at increasing concentrations of MGO LEWAF produced at 5 and 15 °C and remained around 0–30 % for MGO LEWAF produced at other temperatures (Fig. 4F-J). Likewise, TI increased at increasing concentrations of MGO + D LEWAF produced at 20 °C and abruptly rose to 100 % on exposure to 21 %–55 % MGO + D LEWAF produced at any other temperature (Fig. 4F-J). Overall, $EC50_{(MGO)}$ values were higher than the corresponding EC50 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; ESM 4). NOEC values for MGO, without and with dispersant, were always low irrespective of the temperature of LEWAF production (Table 4). For CDI, $EC50_{(MGO)}$ was higher than 100 % LEWAF for a wide range of LEWAF production temperatures but it dropped sharply when the LEWAF had been produced at 5 °C (Table 4; ESM 4). EC50 was highly variable without a clear pattern in relation to the LEWAF production temperatures (Table 4; ESM 4). For GDI, values of $EC50_{(MGO)}$ and EC50 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, ESM 4). Similarly, $EC50_{(MGO)}$ and EC50 calculated for MDI were also highly variable but the values were lower for EC50 than for $EC50_{(MGO)}$ for all the LEWAF production temperatures (Table 4; ESM 4). IPLFI increased at increasing concentrations of MGO LEWAF at 10–25 °C (Fig. 5G-J), whereas it abruptly rose to values of 100 % on exposure to 20 % LEWAF produced at 5 °C (Fig. 5F). IPLFI increased at increasing concentrations of MGO + D LEWAF produced at 20 °C and abruptly rose to 100 % on exposure to 8–21 % MGO + D LEWAF produced at any other temperature (Fig. 5F-J). Thus, $EC50_{(MGO)}$ was always low, most remarkably when LEWAF had been produced at the lowest temperature; and EC50 was even lower (Table 4; ESM 4). $NOEC_{(MGO)}$ and NOEC were

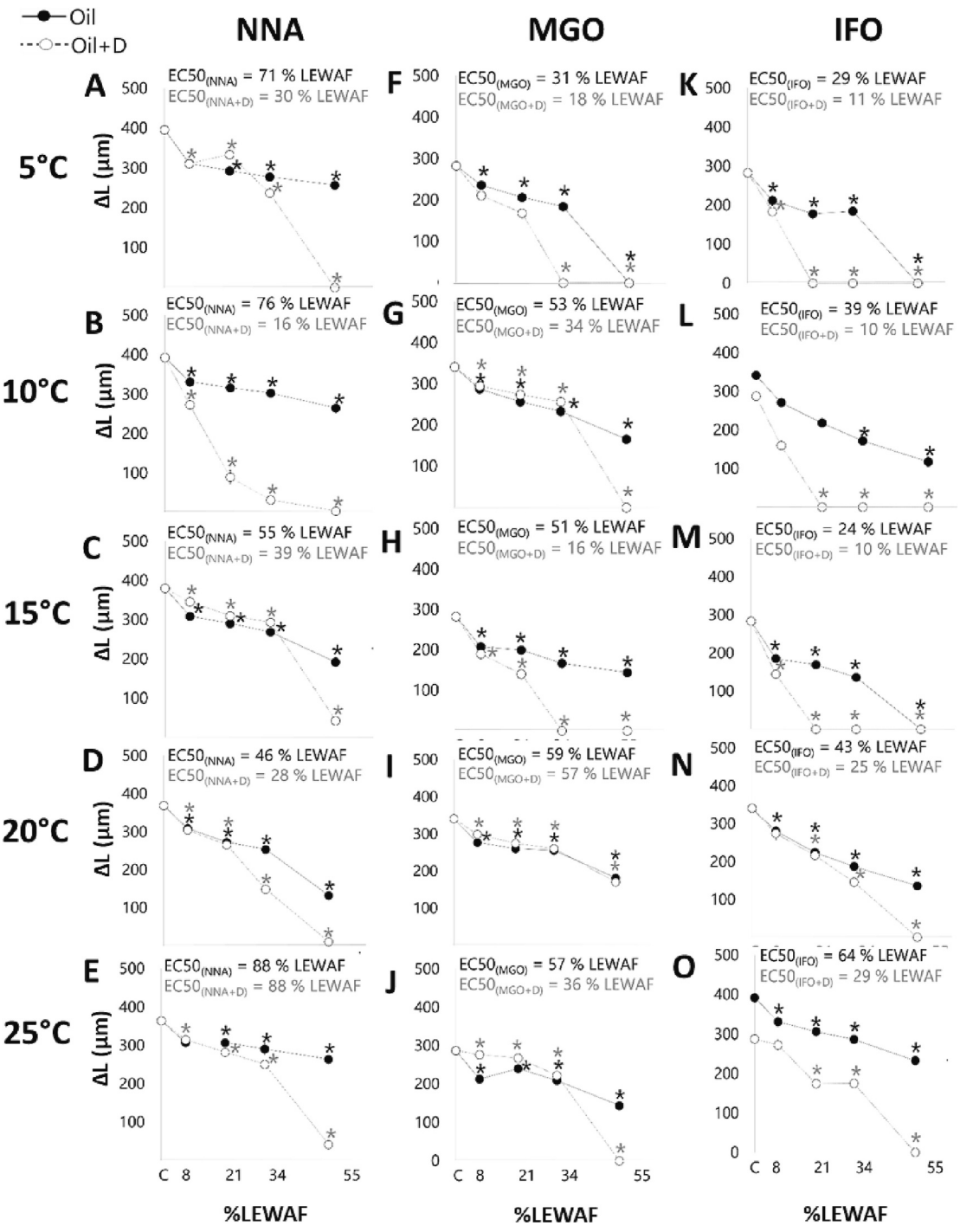


Fig. 3. Lengthening (ΔL in μm) of pluteus larvae exposed to oil LEWAF and oil+D LEWAF of NNA, MGO and IFO produced at 5, 10, 15, 20 and 25 °C. Values are given in μm (mean ± SD). Asterisks indicate significant differences between each exposure concentration and its respective control group (ANOVA; $p < 0.05$). Median effective concentrations (EC50) were calculated after probit analysis.

low at all the LEWAF production temperatures (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 5–25 °C range (Table 6). RTi values were higher than "1" for all the endpoints investigated. Both without and with dispersant application, regardless of the temperature of LEWAF production in the 5–25 °C range, RT/RC values were higher than "1" for several individual PAHs (Table 6): 2-MN, Pyr, Phe, Fluo, B[*a*]A + Chr and B[*a*]P.

3.4. Temperature dependent toxicity of IFO alone and combined with dispersant

IFO LEWAF and IFO + D LEWAF resulted to be genotoxic in comparison with the experimental control group (Fig. 2E-F). SSF values were higher after exposure to IFO LEWAF produced at low than at high (20–25 °C) temperatures (Fig. 2E) whilst no differences were observed amongst IFO + D LEWAFs produced at different temperatures (Fig. 2F). Thus, when LEWAF production temperature was low IFO LEWAF was more genotoxic than IFO + D LEWAF (Fig. 2E and F). ΔL decreased at

Table 4
Summary of toxicity critical values (EC50; NOEC), expressed as %LEWAF, recorded in sea urchin embryos for ΔL, TI, IPLFI, CDI, GDI, and MDI on exposure to NNA, NNA + D, MGO, MGO + D, IFO and IFO + D LEWAFs produced at 5, 10, 15, 20 and 25 °C.

	Index	NNA LEWAF		NNA + D LEWAF		MGO LEWAF		MGO + D LEWAF		IFO LEWAF		IFO + D LEWAF	
		EC50	NOEC	EC50	NOEC	EC50	NOEC	EC50	NOEC	EC50	NOEC	EC50	NOEC
5 °C	ΔL	71	21	30	<8	31	<8	18	<8	29	<8	11	<8
	TI	>100	8	31	21	33	<8	14	<8	33	21	8	<8
	IPLFI	77	21	30	<8	11	<8	10	<8	10	<8	4	<8
	CDI	>100	55	42	34	32	21	33	21	33	21	31	21
	GDI	77	55	42	21	37	21	26	21	32	21	9	8
	MDI	>100	55	29	21	28	21	25	21	29	21	8	<8
10 °C	ΔL	76	<8	16	<8	53	<8	34	<8	39	8	10	<8
	TI	>100	21	12	8	>100	21	40	21	37	21	5	<8
	IPLFI	>100	21	11	<8	25	<8	12	<8	14	8	4	<8
	CDI	>100	21	>100	8	>100	55	42	34	42	34	9	<8
	GDI	>100	55	12	8	>100	34	43	34	29	21	9	8
	MDI	>100	55	31	8	>100	21	41	21	29	21	8	<8
15 °C	ΔL	55	<8	39	<8	51	<8	16	<8	21	<8	10	8
	TI	>100	8	38	8	32	21	7	<8	27	21	3	<8
	IPLFI	71	21	37	21	25	<8	5	<8	10	<8	4	<8
	CDI	>100	55	>100	55	>100	55	12	8	42	34	16	8
	GDI	70	34	44	34	45	21	8	<8	27	21	5	<8
	MDI	>100	55	51	21	41	21	11	8	25	21	5	<8
20 °C	ΔL	45	<8	26	<8	59	<8	57	<8	43	<8	25	<8
	TI	70	34	35	21	>100	21	>100	8	42	34	29	21
	IPLFI	41	21	27	21	25	<8	25	21	26	21	7	<8
	CDI	82	34	91	55	>100	21	>100	55	>100	21	30	21
	GDI	62	21	43	21	>100	34	62	34	37	21	28	21
	MDI	77	21	58	21	80	21	65	21	37	21	29	21
25 °C	ΔL	88	<8	88	<8	57	<8	36	8	64	<8	29	8
	TI	>100	21	37	21	58	21	36	8	18	8	31	8
	IPLFI	93	21	31	21	25	21	12	8	25	21	10	8
	CDI	>100	55	>100	55	>100	55	40	34	30	8	30	8
	GDI	>100	55	43	34	65	21	41	21	45	8	29	21
	MDI	>100	55	50	21	61	21	38	21	32	21	28	21

increasing concentrations of IFO LEWAF at all temperatures tested) and IFO + D LEWAF at 20 and 25 °C (Fig. 3K-O). This decrease was always more pronounced on exposure to IFO + D LEWAF than on exposure to IFO LEWAF (ANCOVA; $p < 0.05$; Fig. 3K-O). Low EC50 values were recorded for IFO produced at 5–20 °C and IFO + D produced at 20–25 °C, and extremely low values for IFO + D LEWAF produced at 5–15 °C (Table 4). NOEC values were always lower than 8 % LEWAF for both IFO and IFO + D (Fig. 3K-O; Table 4). TI increased at increasing concentrations of IFO LEWAF produced at 5 and 15 °C and sharply rose to 80–100 % on exposure to 34–55 % IFO LEWAF produced at 10, 20 and 25 °C (Fig. 4K-O). Thus, $EC50_{(IFO)}$ and $NOEC_{(IFO)}$ were lower for LEWAF produced at 25 °C than for LEWAF produced at 5–20 °C (Table 4; ESM 4). In the case of IFO + D, TI values reached 100 % on exposure to 8–21 % LEWAF produced at 5–15 °C whilst increased depending on the LEWAF concentration when this was produced at 20–25 °C (Fig. 4K-O). EC50 values were lower for IFO + D than for IFO oil. Indeed, EC50 was extremely low for IFO + D LEWAF produced at 5–15 °C and low for IFO + D LEWAF produced at 20–25 °C (Table 4; ESM 4). Accordingly, NOEC values were extremely low except for LEWAF produced at 20 °C (Table 4). For CDI, $EC50_{(IFO)}$ was relatively low except for LEWAF produced at 20 °C, and dispersant addition resulted in even lower EC50 values (Table 4; ESM 4). For GDI and MDI, $EC50_{(IFO)}$ was moderately low, and the addition of dispersant resulted in much lower values, especially at low LEWAF production temperatures (Table 4; ESM 4). IPLFI increased at increasing concentrations of IFO LEWAF produced at 20–25 °C (Fig. 5N and O), whereas it abruptly rose to values of 100 % on exposure to 20 % LEWAF produced at 5–15 °C (Fig. 5K-M). Thus, $EC50_{(IFO)}$ was extremely low at 5–15 °C and low at 20–25 °C and $NOEC_{(IFO)}$ was very low except when IFO LEWAF had been produced at 20 °C (Table 4; ESM 4). IPLFI also reached values of 100 % on exposure to 8 % IFO + D LEWAF produced at 5–15 °C and on exposure to 21 % IFO + D LEWAF produced at 20–25 °C (Fig. 5K-O). EC50 and NOEC were extremely low, irrespective of the LEWAF production temperature (Table 4; ESM 4).

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 LEWAF production temperature (Table 7). RT_i values were higher than "1" suggesting that one or more individual PAHs exhibited more toxicity than predicted for the mixture toxicity. Particularly, RT/RC values higher than "1" were recorded for several individual PAHs (Table 7): 2-MN, Pyr, Ant, Fluo, B[*a*]A + Chr and B[*a*]P.

4. Discussion

4.1. Influence of production temperature on oil LEWAF chemistry

Like in oil LEWAF produced at 10 °C following standard procedures (Singer et al., 2000; DeMiguel-Jiménez et al., 2021), the PAH profiles of the three oil LEWAFs studied herein were dominated by Naph, 1-MN, 2-MN, Ace, Flu and Phe in the 5–25 °C range of production temperature. Yet, the concentrations of these PAHs varied depending on the production temperature in the cases of NNA LEWAF and MGO LEWAF, though not in the case of IFO LEWAF. Accordingly, the PAH profiles of the oil LEWAFs are known to be different depending on the oil and the temperature of LEWAF production (Perkins et al., 2005; Faksness et al., 2008; Camus et al., 2015; Brown et al., 2016). On the one hand, aqueous fractions of different oils saturate at different times depending on the oil pour point (different for the three tested oils; ESM 1) and the temperature (Faksness et al., 2008). On the other, the solubility of some PAHs such as Phe, Ant and B[*a*]P decreases as temperature decreases (Whitehouse, 1984) whilst high temperatures favour volatilisation. Thus, for instance, the concentration of PAHs and their methylated derivatives increased with increasing production temperature from 15 to 25 °C in the water-soluble fraction of a light crude oil but decreased when temperature was taken to 35 °C (Saeed et al., 1998).

Viscosity, chemical composition and temperature are known to play

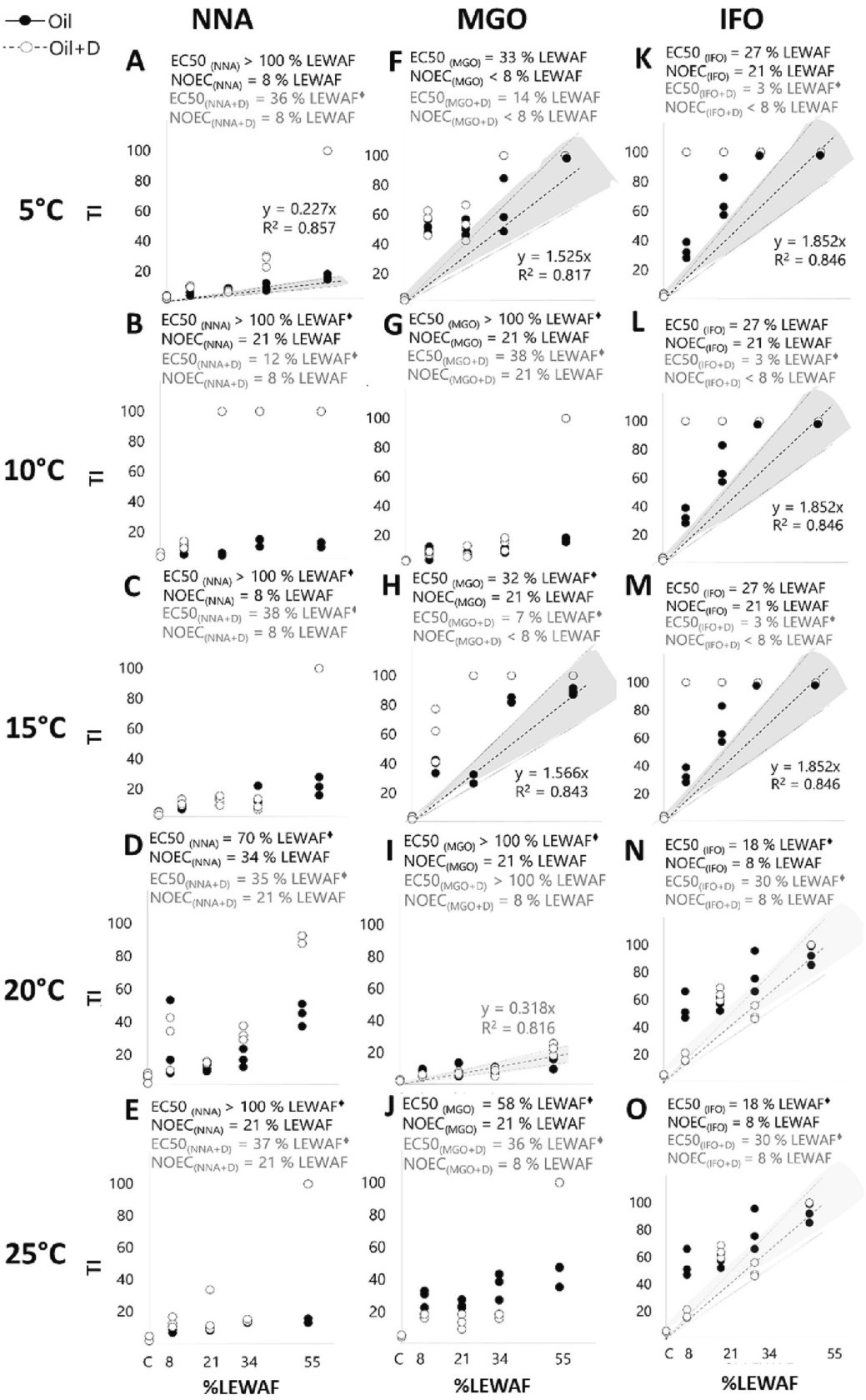


Fig. 4. Toxicity Index (TI) calculated for sea urchins exposed to oil LEWAF and oil+D LEWAF of p NNA, MGO and IFO produced at 5, 10, 15, 20 and 25 °C. Median effective concentrations calculated upon linear regression models (EC50) or after probit analysis, and non-observed effect concentration (NOEC) 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; $p > 0.05$).

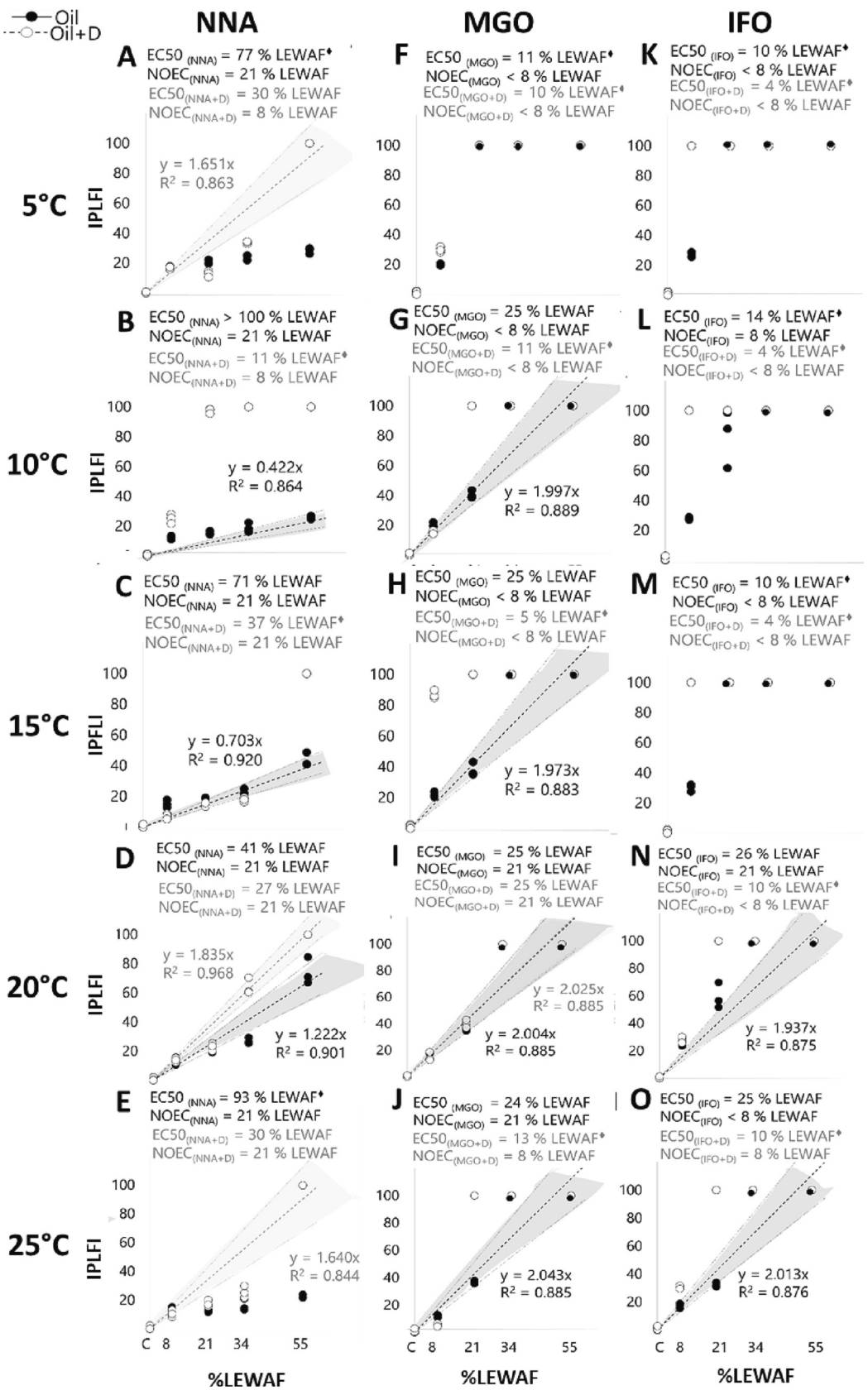


Fig. 5. Inhibition of Pluteus Larvae Formation Index (IPLFI) calculated for sea urchins exposed to oil LEWAF and oil+D LEWAF of p NNA, MGO and IFO produced at 5, 10, 15, 20 and 25 °C. Median effective concentrations calculated upon linear regression models (EC50) or after probit analysis, and non-observed 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; $p > 0.05$).

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. $\sum TU_{endpoint}$: sum of TUs for each toxicity endpoint (ΔL, TI, IPLFI, CDI, GDI and MDI); $\sum TU_{PAHi}/TU_{\sum PAHs}$: sum of the TUs of individual PAHs vs. the TUs of the sum of PAHs; RT_i/RC_i: ratio between the relative contribution of an individual PAH to the toxicity of the mixture (RT_i) and its relative contribution to the chemical composition of the mixture (RC_i).

	5 °C		10 °C		15 °C		20 °C		25 °C	
	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	–	–	–	–	–	–	–

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. $\sum TU_{endpoint}$: sum of TUs for each toxicity endpoint (ΔL, TI, IPLFI, CDI, GDI and MDI); $\sum TU_{PAHi}/TU_{\sum PAHs}$: sum of the TUs of individual PAHs vs. the TUs of the sum of PAHs; RT_i/RC_i: ratio between the relative contribution of an individual PAH to the toxicity of the mixture (RT_i) and its relative contribution to the chemical composition of the mixture (RC_i).

	5 °C		10 °C		15 °C		20 °C		25 °C	
	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	–

an important role in determining dispersant effectiveness (Fingas et al., 1991; Moles et al., 2001; Chandrasekar et al., 2005; Colcomb et al., 2005; EMSA, 2010; Li et al., 2010). Accordingly, temperature and dispersant addition modified differently the PAH profile and levels in the LEWAFs of the three tested oils, which differ in viscosity and chemical composition. Thus, together with the PAHs relevant for the oil LEWAF profiles (Naph, 1-MN, 2-MN, Ace, Flu and Phe), other individual PAHs were also relevant in the profile of the oil+D LEWAFs, to a different degree depending on the oil and the temperature of LEWAF production. The concentrations of individual PAHs in oil+D LEWAFs varied depending on the production temperature and the oil. The ∑PAHs in oil+D LEWAFs, much higher than in oil LEWAFs (as commonly reported in chemically dispersed oil aqueous fractions; Saeed et al., 1998; Lyons et al., 2011; Katsumiti et al., 2019; Li et al., 2021), was lower at higher production temperatures in the cases of NNA+ LEWAF and MGO + D LEWAF (25 °C), in agreement with previous studies (Katsumiti et al., 2019). The $\sum_{HMW}$PAHs and $\sum_{LMW}$PAHs in oil+D LEWAFs also varied depending on the production temperature and the oil type. Increasing water temperature causes HMW aromatic compounds to dissolve and replace LMW aromatic and non-aromatic compounds (Saeed et al., 1998). Thus, $\sum_{HMW}$PAHs increases and $\sum_{LMW}$PAHs decreases in oil aqueous fractions produced at elevated temperatures (Li et al., 2021). Further temperature increases can also cause volatilisation of HMW compounds (Saeed et al., 1998). The highest values of $\sum_{HMW}$PAHs were recorded at different temperatures in NNA + D LEWAF (10 °C), MGO + D LEWAF (25 °C) and IFO + D LEWAF (5 °C), which would be the consequence of differences in physical and chemical properties amongst the three oil+D mixtures.

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. $\sum TU_{endpoint}$: sum of TUs for each toxicity endpoint (ΔL, TI, IPLFI, CDI, GDI and MDI); $\sum TU_{PAHi}/TU_{\sum PAHs}$: sum of the TUs of individual PAHs vs. the TUs of the sum of PAHs; RT_i/RC_i: ratio between the relative contribution of an individual PAH to the toxicity of the mixture (RT_i) and its relative contribution to the chemical composition of the mixture (RC_i).

	5 °C		10 °C		15 °C		20 °C		25 °C	
	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

4.2. Influence of production temperature on oil LEWAF genotoxicity

Exposure to oil LEWAF produced DNA damage in sea urchin larvae, in agreement with previous results (DeMiguel-Jiménez et al., 2022). Two PAHs identified in oil LEWAFs, Ant and B[*a*]P, are known to cause genotoxicity to juvenile fish and mussel embryos (Wessel et al., 2007; Banni et al., 2010; Hasue et al., 2013; Ewa and Danuta, 2017). Genotoxicity did not change with LEWAF production temperature for NNA and MGO. Conversely, IFO LEWAF was more genotoxic when produced at low temperatures than at high ones (20–25 °C). Yet, elevated DNA damage did not appear to be related to high concentrations of the measured PAHs in the corresponding LEWAFs. Genotoxicity was enhanced upon dispersant application in the three oils, although the level of DNA damage varied depending on the oil+D LEWAF production temperature in a different way for each oil. NNA + D LEWAF was more genotoxic when produced at low temperatures than at higher ones, which might be linked to elevated levels of carcinogenic PAHs ($\sum_{HMW}$PAHs) and potentially genotoxic individual PAHs (e.g., Ace, Flu, Phe, Pyr, Fluo and B[*a*]A + Chr). In contrast, genotoxicity did not vary with LEWAF production temperature in MGO + D LEWAF and IFO + D LEWAF, even though individual PAHs in MGO + D LEWAF produced at 25 °C and in IFO + D LEWAF produced at 5–15 °C were at much higher concentrations than in LEWAFs produced at other temperatures. Thus, the measured PAHs are not necessarily the cause of LEWAF genotoxic effects, as reported for the under ice weathered aqueous fraction of these oils (DeMiguel-Jiménez et al., 2022).

4.3. Influence of production temperature on oil LEWAF embryo toxicity

LEWAF produced at different temperatures in the 5–25 °C range caused sea urchin embryo toxicity. Exposure to oil LEWAFs affected the normal lengthening of the pluteus larvae (low ΔL values), in agreement with previous studies in various sea urchin species (Saco-Álvarez et al., 2008; Lv and Xiong, 2009; Rial et al., 2013; Arnberg et al., 2018; Pereira et al., 2018; DeMiguel-Jiménez et al., 2021, 2022). This toxic effect was moderate and not clearly affected by the LEWAF production temperature for NNA LEWAF (Fig. 6A), but it was intensified when LEWAF was produced at 5 °C in the case of MGO (Fig. 6A) and at 5–20 °C in the case of IFO (Fig. 6A). Likewise, exposure to oil LEWAFs caused abnormalities (high TI values) and inhibition of pluteus larvae formation (higher IPLFI values), as previously reported (Saco-Álvarez et al., 2008; Carballeira et al., 2012; DeMiguel-Jiménez et al., 2021, 2022). Although the LEWAF production temperature did not influence TI following any recognisable pattern in the case of MGO (Fig. 6B), these toxic effects were more severe for IFO LEWAF produced at lower temperatures (Fig. 6C). In agreement with previous studies (DeMiguel-Jiménez et al., 2021, 2022), oil-LEWAF exposure affected early embryo development (CDI, GDI, MDI), especially in the case of IFO LEWAF, in which developmental toxicity was always high, irrespective of the LEWAF production temperature (EC50 < 50 % LEWAF; Fig. 6D-F).

Dispersant application always exacerbated the toxic effects. EC50 values lower than for oil LEWAFs were obtained for the whole range of oil+D LEWAF production temperatures in the case of ΔL (Fig. 6A) and more markedly for IFO + D LEWAF produced at 5–15 °C in the case of TI (Fig. 6B) and for NNA + D LEWAF in the case of IPLFI (Fig. 6C). TI was severely affected (EC50 < 50 % LEWAF) at any temperature in the case of NNA + D and IFO + D LEWAFs, and most extremely (EC50 < 10 % LEWAF) in the case of IFO + D LEWAF produced at low temperatures (5–15 °C). Likewise, dispersant addition enhanced toxicity regarding IPLFI (EC50 < 40 % LEWAF), especially when LEWAF was produced at low temperatures (Fig. 6C). Thus, whilst NNA LEWAF was not toxic, $EC50_{(IPLFI)}$ values were lower than 50 % NNA + D LEWAF irrespective of the LEWAF production temperature. Similarly, upon dispersant application, $EC50_{(CDI)}$, $EC50_{(GDI)}$, and $EC50_{(MDI)}$ values dropped beyond the values recorded on exposure to the corresponding oil LEWAFs alone (as previously reported for LEWAF produced at 10 °C under standard conditions; DeMiguel-Jiménez et al., 2021), more remarkably at the lowest LEWAF production temperatures for MGO + D and IFO + D (Fi. 6D-F).

The toxicity of oil aqueous fractions may vary with temperature, as shown after toxicity testing of oil aqueous fractions produced at one temperature in combination with various exposure temperatures (Lyons et al., 2011; Pasparakis et al., 2016; Perrichon et al., 2018; Serafin et al., 2019). In those studies, the combined effects of oil exposure and temperature were attributed to temperature-dependent sensitivity of the test organisms. Presently, the temperature optimum for sea urchin development (20 °C, Shpigel et al., 2004) was the only exposure temperature employed, irrespective of the LEWAF preparation temperature. Thus, rather than temperature-related differences in the sensitivity of sea urchin embryos, differences in the toxicity of the LEWAFs produced at different temperatures should be attributed to differences in the bioavailability of toxic components. For instance, a higher bioavailability of toxic compounds would explain why NNA + D LEWAF is more

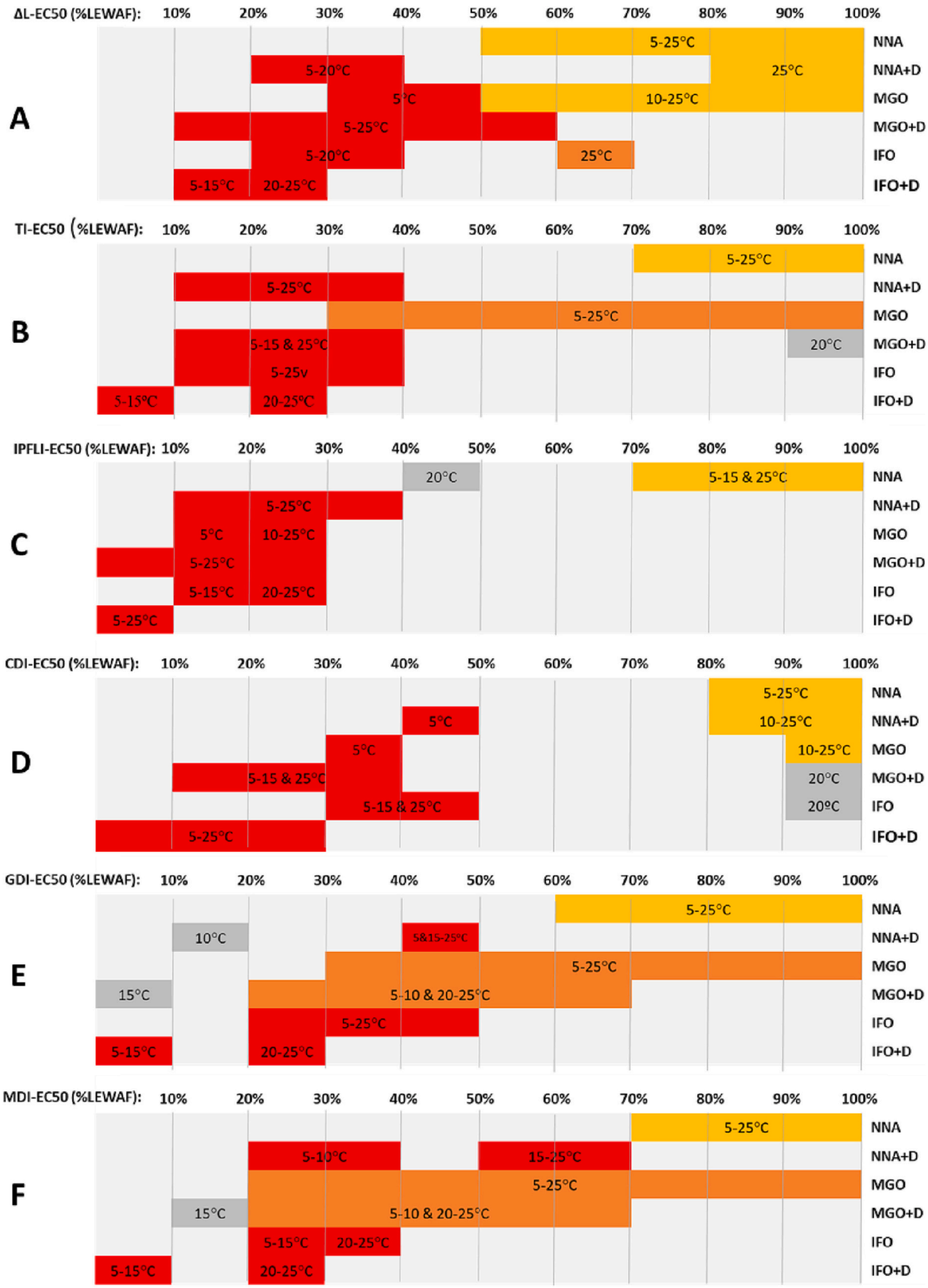


Fig. 6. Range of variation along the LEWAF production temperatures in EC50 values represented for ΔL (A), TI (B), IPFLI (C), CDI (D), GDI (E) and MDI (F) for each oil alone (NNA, MGO, IFO) and combined with dispersant (NNA + D,MGO + D, IFO + D). Light orange, low toxicity range (EC50 < 60 % LEWAF); dark orange, mid or highly varying toxicity range; red, high toxicity range (EC50 > 50 % LEWAF); grey, anomalous result. Anomalous results might represent either inconsistent data sets or critical temperature thresholds. This cannot be discerned from the present data; therefore, they are indicated in the scheme but not considered for discussion. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

toxic for mussel haemocytes when produced at 10 °C that when produced at 15 or 20 °C (Katsumiti et al., 2019). In parallel, a greater loss of toxic compounds may occur in oil aqueous fractions at elevated temperatures (Korn et al., 1979), thus accounting for a lowered toxicity of LEWAFs produced at high temperatures. Nevertheless, the results should be interpreted in terms of the operational procedure (i.e. various WAF production temperatures vs. one testing temperature). The TUs were estimated from the concentrations measured when the WAFs were prepared and thus the TU values can be considered as the worst scenario, since some PAHs might volatilize.

The toxicity of aqueous fractions of oil, alone or with dispersant, could be attributed to narcosis caused by 2- and 3-ring PAHs and 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; DeMiguel-Jiménez et al., 2021). 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. Actually, TU values were below "1" for the majority of the embryo toxicity endpoints investigated after exposure to the three oils, alone or in combination with dispersant, regardless of the LEWAF production temperature. TU values above "1" were recorded only in a few exceptional casess, say: (a) in ΔL after exposure to NNA + D LEWAF produced at 25 °C; (b) in CDI after exposure to NNA + D LEWAF produced at 10, 20 and 25 °C; and (c) in all the toxicity endpoints after exposure to NNA + D LEWAF produced at 15 °C. However, RTi 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 5–25 °C range, Phe in NNA + D LEWAF produced at 5 and 20–25 °C 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 preceding studies, the toxicity of the NNA, MGO and IFO LEWAFs, alone or in combination with Finasol OSR52, was only partially attributed to measured individual PAHs (USEPA 16 list) or the CA action of the mixture and a large part of the toxicity was suggested to be due to the UCM and polar compounds (DeMiguel-Jiménez et al., 2021, 2022). The same conclusion seems to be reasonable in the present study as well.

Finally, it is worth noting that LEWAFs caused significant effects at the lowest exposure concentration tested herein ($NOEC_{(\Delta L)}$: 8 % LEWAF; $NOEC_{(TI)}$: <8–21 % LEWAF; $NOEC_{(IPLFI)}$: <8–21 % LEWAF), irrespective of the oil, dispersant application and production temperature. Therefore, potential long-term toxic effects cannot be disregarded in any case, even though in some cases EC50 values were higher than 100 % LEWAF. In agreement, other studies with *P. lividus* exposed to oil LEWAF also found high EC50 values and low toxicity thresholds (NOEC, LOEC or EC10) for embryo toxicity endpoints such as ΔL (Saco-Álvarez et al., 2008; Rial et al., 2013).

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 (20–25 °C) in the cases of the crude oil and the light bunker oil, 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 augmented after dispersant application. Likewise, oil LEWAF of the three tested oils caused altered lengthening, 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, was only partially attributed to individual PAHs or to their mixture.

CRediT authorship contribution statement

Laura DeMiguel-Jiménez: Methodology, Investigation, Visualization, Writing original draft. **Dennis Bilbao:** Methodology. **Ailette Prieto:** Methodology. **Helena C. Reinardy:** Methodology, Formal analysis. **Xabier Lekube:** Methodology, Investigation. **Urtzi Izagirre:** Conceptualization, Investigation, Writing original draft, Supervision. **Ionan Marigómez:** Conceptualization, Funding acquisition, Formal analysis, Supervision, Writing original draft, Writing, review and editing.

Declaration of competing interest

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Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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