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Application of a biological multilevel response approach in the copepod *Acartia tonsa* for toxicity testing of three oil Water Accommodated Fractions

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ABSTRACT

Copepods play a critical role in the marine food webs, being a food source for marine organisms. In this study, we investigated the toxic effects of Water Accommodated Fractions (WAFs) from three types of oil: Naphthenic North Sea crude oil (NNS), Intermediate Fuel Oil (IFO 180) and a commercial Marine Gas Oil (MGO). The WAFs were prepared at 10 °C and 30 PSU (practical salinity unit), and tested on the marine copepod *Acartia tonsa* at different endpoints and at different levels of biological organization. We determined the median lethal concentrations after 96 h (LC50) and reproduction capabilities were calculated in adult females following seven days of exposure to sublethal WAF doses. The total lipid content was measured in reproductive females using Nile red lipophilic dye after 96 h of WAF exposure. We also measured the transcription levels of genes involved in antioxidant response and xenobiotic biotransformation after short exposure for 48 h. High doses (7% WAF) of MGO affected survival, percentage of fecund females, egg hatching success, and total lipid content. The IFO 180 WAF affected, at medium (20%) and high (40%) doses, the number of fecund females, mortality and produced significant effects on gene expression levels. In conclusion, toxicity assays showed that the WAFs prepared from refined oils were more toxic than crude oil WAF to *Acartia tonsa*.

1. Introduction

Due to the increasing oil demand and the melting of the polar ice caps, oil extraction and transport in the oceans have significantly increased during the last decade. Consequently, the risks of oil spill incidents are escalating rapidly. Between 2010 and 2018, 59 major oil spills occurred, resulting in the release of approximately 163,000 tons of oil into the marine environment (ITOPF, 2020). Water Accommodated Fractions (WAFs) are solutions of low molecular weight hydrocarbons, which with appropriate wave dynamics and weathering processes, are naturally released from petroleum hydrocarbon mixtures or spilled oils. WAFs contain harmful polycyclic aromatic hydrocarbons (PAHs), aliphatic compounds and alkylated chemicals known to be bioavailable and very toxic to several aquatic species (Abbriano et al., 2011; Barron and Káaihue, 2003). Several environmental factors, such as temperature and light, influence the degree of bioavailability and toxicity of PAHs in WAFs (Lyons et al., 2011; Lee, 2003). At low temperatures, PAHs show low solubility and reduced bioavailability, but at high temperatures both low and high molecular weight hydrocarbons can be introduced in the water column (Lyons et al., 2011). Photo-oxidation of PAHs results in the production of free radicals that react with oxygen to generate toxic reactive oxygen species (ROS) (Lee, 2003). Several members of the superfamily of cytochrome P450 genes participate in the metabolism and biotransformation of organic chemicals, including PAHs, and are considered biomarkers for WAF and oil exposure in aquatic animals (Snyder et al., 1998; Rhee et al., 2013). The role of the aryl-hydrocarbon

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receptor (AhR) dependent pathway and the activity of cytochrome P450 enzymes (CYPs) in the detoxification of PAHs is well characterized in vertebrates, but poorly described in invertebrates (Han et al., 2017; Nelson, 2018).

PAHs are known to have a deleterious impact on cellular metabolism and functions (Allan et al., 2012; Han et al., 2014). Inside the cell, the metabolism of PAHs generates ROS (Hannam et al., 2010) and the imbalance between generation of ROS and ROS neutralization via antioxidants or oxyradical scavenging systems. An imbalance of these processes in the cell triggers oxidative stress (Valavanidis et al., 2006) that can result in DNA damage, oxidation of biomolecules and cell death (Martins et al., 2013; Wilk et al., 2013). Consequently, the expression of several genes involved in antioxidant defenses represents a valuable tool for understanding the toxicity response mechanisms against oxidative stress. Antioxidant enzymes (e.g., catalase and glutathione-S-transferase, GST) (Kim et al., 2011), molecular chaperones (e.g., heat shock proteins) and iron storage proteins (e.g., ferritin) have been used as biomarkers of ROS induced responses in aquatic organisms (Gupta et al., 2015; Tarrant et al., 2019).

The calanoid copepod, *Acartia tonsa*, is a cosmopolitan species inhabiting temperate regions and is usually abundant in coastal and estuarine areas (Cervetto et al., 1995). Copepods are considered ecologically crucial to the marine food web, serving as a primary food source for secondary consumers, and exerting trophic effects on primary producers such as phytoplankton (Gorbi et al., 2012). The International Organization for Standardization (ISO) recommended the use of *Acartia tonsa* for the evaluation of lethality and toxicity of several contaminants (Gorbi et al., 2012), since it is sensitive to different types of contaminants and toxins (Andersen et al., 1999; Bielmyer et al., 2006; Bellas and Thor, 2007; Pinho et al., 2007; Sahlmann et al., 2019). Thus, *Acartia tonsa* is considered a valuable model organism for understanding pollutants' effects on the marine zooplankton communities.

Few studies have investigated the effects of oil WAFs on *Acartia tonsa* (Avila et al., 2010, Faksness et al., 2014), mainly focusing on lethality without concurrently considering different sublethal endpoints. The toxic effects of WAFs on several other copepod species with respect to mortality, development, egg production, and motility have been established (Cowles and Remillard, 1983; Bejarano et al., 2006; Jiang et al., 2012; Hansen et al., 2013; 2018; Lee et al., 2013). Exposure to dispersed oil alters the lipid sac volume in the cold-water copepod *Calanus finmarchicus* (Hansen et al., 2018), leading the authors to conclude that changes in lipid composition due to contaminant exposition might have adverse effects on the reproduction capabilities in copepods. It has been shown that fatty acid composition in *Acartia tonsa* influenced egg hatching success and egg production rates (Broglio et al., 2003; Cavallo and Peck, 2020; Kleppel et al., 1998), but to date, the effects of oil WAFs on the lipid composition in *Acartia tonsa* are poorly understood.

A potential role in PAH metabolism for genes belonging to the CYP family has been suggested in copepods. Several genes of CYP3 clan showed differential transcription profiles after exposure to individual PAHs or WAFs (Hansen et al., 2007, 2008; Han et al., 2014, 2015, 2017). A role for genes in the CYP3 clan in PAHs and oil metabolism has been suggested in marine copepods, such as *Tigriopus japonicus* and *Paracyclopina nana* (Han et al., 2014, 2015).

In order to better understand the effects of oil WAFs on *Acartia tonsa* copepods, we studied the effects of three oil WAFs (crude and refined) with different water solubility and bioaccumulative characteristics. WE selected a naphthenic crude oil from the North Sea (NNS) and two refined oils, Intermediate Fuel Oil 180 (IFO 180) and a commercial Marine gas oil (MGO). NNS and IFO 180 have low dispersion potential and a low concentration of water-soluble components (Hansen et al., 2013). In contrast, MGO, commonly used as fuel for different types of ships, has low viscosity and high volatility, which facilitate its dispersion in the marine environment (Hansen et al., 2013). We applied a biological multilevel response approach to integrate effects on lethality, fecundity (egg-laying), total lipid content and transcription levels of genes related to stress responses and PAH metabolism. In addition, potential transgenerational effects on egg hatching success were assessed.

2. Materials and methods

2.1. 1. Selected Oils and preparation of Water Accommodated Fractions (WAFs)

The three oils used for WAFs preparation were NNS, IFO 180, and commercial MGO. NNS oil is a naphthenic-based light crude oil characterized by a low aromatic content, a low wax content, a low viscosity index (299 cP at 2 °C) and an intermediate density of (0.900 g/mL). It is also rich in branched and cyclic saturated hydrocarbons (Katsumiti et al., 2019). IFO 180 is a blend of heavy fuel oil and gas oil composing approximately of 88% residual oil and 12% distillate oil. It is characterized by a very high viscosity (180 cSt at 50 °C), high density (>0.967 g/mL), and has a maximum sulfur content of up to 3.5% (Johann et al., 2021). IFO 180 is mainly used in the machinery and engines of large shipping vessels (Alexander et al., 2016). MGO is used in the engines of small ships and fast boats. It is a blend between gas oil and heavy fuel oils and is highly volatile, with low viscosity (2–4 cSt at 40 °C), low density (0.856 g/mL) containing up to 60% aromatic hydrocarbons (Hansen et al., 2013). Oils were provided by the Norwegian University of Science and Technology (NTNU; Trondheim, Norway), within the framework of the EU-funded project GRACE (Integrated oil spill response actions and environmental effects) (Jørgensen et al., 2019).

The WAFs were prepared according to the protocol by Singer et al. (2000) for low-energy WAF with slight modifications. Briefly, oil was added to 0.2 μm filtered seawater in 4 L glass bottles with bottom tap, at a final loading concentration of 5 g oil per 1 L of seawater. The water-oil mixture was continuously stirred at 800 rpm (low-energy) using a magnetic stirrer without forming any vortex for 40 h. The stirring was conducted at 10 °C under dark conditions to avoid photo oxidation and degradation of oil elements. Finally, 3.5 L of WAF were carefully extracted from the bottles using the tap, avoiding the presence of non diluted oil, and serial dilutions of each WAF were immediately prepared using clean seawater.

2.2. Copepod culture

Copepods, *Acartia tonsa* (Plentzia Marine Station, PiE-UPV/EHU), were maintained in a 10 L tank filled with 0.2 μm filtered seawater at 18 °C, a salinity of 30 practical salinity unit (PSU) and a constant photoperiod of 16/8 h light/dark cycle. Copepods were fed every two days using a 1:1 mixture of the brown microalgae *Isochrysis galbana* and green microalgae *Tetraselmis chuii.* To prevent potential cannibalism, recently hatched nauplii were transferred from the main tank into a smaller 5 L tank until adulthood.

2.3. Chemical analysis of the PAH content

The PAH composition and concentrations of the different types of WAFs were determined by GC/MS analysis. A standard solution containing a mixture of 16 PAHs was used [Norwegian Standard (NS 9815: S-4008-100-T): phenanthrene (Phe), anthracene (An), fluoranthene (Fluo), pyrene (Pyr), 11 H-benzo[a]fluorene (11H-B[a]F), 11 H-benzo[b]fluorene (11H-B[b]F), benz[a]anthracene (B[a]A), chrysene (Chry), benzo[b]fluoranthene (B[b]F), benzo[k]fluoranthene (B[k]F), benzo[a]pyrene (B[a]P), benzo[e]pyrene (B[e]P), indeno[1,2,3-cd]pyrene (Ind), dibenz[a,h]anthracene (D[a,h]A), benzo[g,h,i]perylene (B[g,h,i]P)] and dibenzo[a,e]pyrene (D[a,e]P). Passive samplers of poly dimethylsiloxane polymer coat (Gerstel GmbH, Germany), with 10 mm in length and 0.5 mm film thick, were introduced in aqueous samples (35 mL) and stirred during 315 min. Once the extraction step was over, samplers were cleaned with Milli-Q water in order to eliminate seawater and dried with a paper tissue. Passive samplers were desorbed using a

commercial thermal desorption TDS-2 unit (Gerstel GmbH) connected to a CIS-4 injector (Gerstel GmbH) 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). This desorption unit was coupled in an Agilent 6890 gas chromatograph also coupled to an Agilent 5975 mass spectrometer system (Agilent Technologies, USA). The Mass spectrometer (MS) was operated in selected ion monitoring (SIM) to quantify target compounds. An Agilent DB-5MS + DG column with the following dimensions 30 m × 0.25 mm and 0.25 μm was used. Analytes were separated using the following conditions: helium as carrier gas (1.3 mL/min); ion source transfer line and quadrupole analyzer temperatures maintained at 300, 230, and 150 °C, respectively. The following temperature program was used for target PAHs and lineal hydrocarbons: 170 °C for 5 min; ramp at 30 °C min^{-1} to 260 °C; ramp at 8 °C/min to 300 °C and hold 15 min. Three replicates per WAF were analyzed and concentrations calculated at the ng/L level.

2.4. Lethal toxicity assays 96 h

Lethality tests were conducted according to the international standard for water quality determination of acute lethal toxicity to marine copepods (ISO14669). Thirty adult copepods were evenly distributed among six replicates, 5 copepods in each container. During the assay duration, the copepods were not fed, and no sex preference was considered when selecting adult copepods. Copepods in each replicate were exposed to the contaminant for 96 h in 250 mL glass beakers containing 50 mL of 0% (control), 10%, 30%, 50%, 70% and 100% WAF diluted in seawater. Fifty percent WAF + seawater volume was replaced every 48 h. Each day of exposure, replicates were checked for mortality, and dead copepods were counted and removed. A copepod was considered dead if it did not move shortly after a gentle stimulation using a pipette.

The LC50 values obtained from the lethal toxicity assays determined the low, medium and high concentrations to be further used in the sublethal endpoints assays (Table 1).

2.5. Fecundity and egg hatching assays

Twenty adult gravid females were selected under a 10× magnification dissecting microscope. Additionally, five adult males were included in each treatment to maintain female fertility throughout the experiment (Holste et al., 2006). The fecundity assay was performed similarly to the exposure methods described by Olsen et al. (2013), with some modifications in exposure time and methods. The fecundity assay was divided into three consecutive stages: exposure, recovery and individual egg collection.

In the exposure stage, copepods were placed into 300 mL glass beakers filled with 150 mL of the corresponding WAF dilution. Copepods were exposed in a semi-static condition for a duration of seven days. Every two days, 50% of the WAF + seawater volume was renewed. Containers were cleaned every day to remove fecal pellets, dead algae and any dead copepods. Throughout the experiment, copepods were fed daily with the algae *Tetraselmis chuii* at a concentration of 3000 cells per mL. In the recovery stage, the surviving females were transferred to clean, WAF-free seawater, for four days. Water was changed every two days, and copepods were fed daily with *Tetraselmis chuii* as in the exposure stage. This experimental procedure allows the identification of delayed effects on exposed female reproductive capacity to mimic environmental realistic scenarios of point oil spills or discharges.

In the fecundity egg collection stage, each surviving female was individually transferred to an incubation chamber with clean seawater for three days. Incubation chambers were divided into two compartments, spawning chamber and brooding chamber, similar to the device proposed by Kleppel et al. (1998). The upper spawning chamber consisted of a plastic tube (50 mL, 10 cm in diameter), which housed an individual female for egg laying. A mesh of 200 μm was attached at the tube's base to prevent females from entering the bottom compartment (brooding chamber) where eggs were deposited. The copepods were fed daily as in the previous stages. Each day, eggs were counted, carefully collected by pipetting, and transferred to individual vials containing seawater to allow them to hatch. Only females producing two or more eggs during the three days of the fecundity test stage were considered reproductive females. After 48 h, the unhatched eggs were considered unviable.

2.6. Total lipid quantification using Nile red lipophilic dye

We used a slightly modified protocol from Tingaud-Sequeira et al. (2011) and Jordão et al. (2015) to quantify fecund females' total lipid content utilizing Nile red lipophilic dye. The working solution of Nile red dye (Sigma Aldrich, USA) was prepared by dissolving the dye in acetone at a concentration of 334 μg/L. Briefly, adult females ($n = 10$) were maintained and exposed to WAF for 96 h as described for the fecundity assay. Two doses for each WAF (medium and high, Table 1) were analyzed. After the exposure, copepods were rinsed with seawater for 5 min and then transferred to 20 mL of seawater mixed with 29 μL of Nile red dye working solution. The adult females were stained for 1 h at room temperature in darkness. For lipid level quantification in adult females, the stained copepods were washed with seawater for 5 min. Then, each copepod was placed into a vial with 200 mL isopropanol and homogenized using a tissue homogenizer (Precellys 24 homogenizer, Bertin Instruments, France) at 2500 vibrations per second for 45 s. The homogenate was centrifuged at 12000 rpm for 2 min. Then, 150 mL of the extract was pipetted in a black 96 well microplate for fluorescence determination, and the plate was read at a wavelength of 530/590 nm excitation/emission using a fluorimeter (Biotek Cytation 5 imaging reader, USA).

A histological and histochemical study of lipid distribution in copepod tissues was carried out in parallel to detect which tissue/organ showed the highest accumulation pattern for lipids. The procedure and results are presented in the Supplementary Materials section.

2.7. Gene transcription analysis

2.7.1. Exposure experiments

Adult copepods were exposed to 150 mL of WAF for 48 h. Exposure concentrations were the same as described in section 2.6. Copepods were divided in five replicates, 35 individuals each, per treatment ($n =$ 175). The copepods were fed daily with *Tetraselmis chuii*, and no gender

Table 1
Selected doses of water accommodated fractions (WAFs) of studied oils in the different analyzed biological endpoints.

	NNS WAF			IFO 180 WAF			MGO WAF		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
Reproductive assays	10%	30%	50%	10%	25%	40%	3%	5%	7%
Lipid levels	–	25%	50%	–	20%	40%	–	5%	7%
Gene transcription levels	–	25%	50%	–	20%	40%	–	5%	7%

NNS: Naphthenic crude oil from North Sea; IFO 180: Intermediate fuel oil 180; MGO: Marine gas oil.
(-): not tested.

preferences were taken during copepod collection for exposure. After 48 h of exposure, the copepods were transferred to cryovials filled with RNA later solution (AMBION, USA) and stored at −80 °C until extraction.

2.7.2. RNA extraction and cDNA synthesis

RNA was extracted using RNeasy Minikit (Qiagen) following the manufacturer protocol. Total RNA was treated with DNase-1 using the DNA-free kit (Invitrogen, USA) to remove genomic DNA. RNA integrity and concentration were measured using the Bioanalyzer 2100 (Agilent Technologies, USA). All RNA samples obtained a RIN score higher than 7, suitable for downstream qPCR analysis (Fleige and Pfaffl, 2006). RNA purity was determined by measuring sample absorbance ratios of 260/280 nm using a UV-spectrophotometer (Epoch, Biotek, USA). All samples had acceptable ratios between 1.8 and 2.3. cDNA was synthesized from purified RNA using the AffinityScript Multiple Temperature cDNA synthesis kit (Agilent Technologies, USA) with random primers and following the manufacturer's protocol.

2.7.3. Primer design

Catalase, *gst, hsp90, hsp70*, and ferritin genes were targeted as biomarkers for the ROS response. A CYP gene from clan 3 (*cyp3026b*) was targeted as a biomarker for PAH metabolism. Primers for catalase (*cat*), *cyp3026b* and *gst* were designed *de novo* from conserved regions in closely related crustacean species and scaffolds from the *Acartia tonsa* genome project (Jørgensen et al., 2019) (Accession number PRJEB20069). Clustal Omega (Madeira et al., 2019) was used for multiple sequence alignment. For *cyp3026b, Acartia tonsa* genome sequence LS054218.1 was aligned with the gene sequences from *Tigriopus japonicus* (KF639998.1) and *Eurytemora affinis* (XM 023493699.1). For catalase, the genome sequence LS054460.1 was aligned with sequences from *Calanus finmarchicus* (EL965956.1) and *Eurytemora affinis* (XM023472406.1). For *gst,* the genome sequence LS085988.1 was aligned with *Tortanus forcipatus* (KT755427.1) and *Acartia pacifica* (KT754520.1). Specific primers to amplify fragments of *hsp70*, *hsp90* and *ferritin* genes were obtained from Nilsson et al. (2014). *β-actin* gene was used for transcription normalization as previously proposed (Zhou et al., 2020), as it showed low coefficient of variability ($CV < 5\%$). The identity of targeted genes was confirmed by sequencing (General Genomics Service Sequencing and Genotyping Unit, SGIker, UPV/EHU). Primer sequences and the primers' amplicon regions' multi-sequence alignments are presented in Table 2 and Supplementary Materials Fig. S2, respectively. Amplification efficiency for each primer pairs was calculated by conducting a 1:2 serial dilution curve generated from pooled experimental samples. All primer pairs showed amplification efficiency between 90% and 100%.

Table 2
Primer sequences and melting temperature (Tm) for all the genes analyzed in this study.

Gene	Primer sequences 5′ to 3′	Tm (°C)	References
hsp70	*Fw*: TTCAATGATTCACAGAGACAAGC *Rv*: TCCTTGTGATGTTAAGACCAGCTAT	57.1 59.7	Nilsson et al. (2014)
hsp90	*Fw*:GTCACATCCCAGTATGGTTGG *Rv*:CCATGGTGGAGGTGTCACGG	59.8 63.5	Nilsson et al. (2014)
ferritin	*Fw*:ACGCTTGCACTGATAATCCA *Rv*:AGTTCTACCGTGACGCATCC	55.3 59.4	Nilsson et al. (2014)
β-actin	*Fw*:TTGGGTATGGAGTCCTGTGG *Rv*:CCTGGATACATAGTGGTGCC	59.4 59.4	Zhou et al. (2020)
cyp3026b	*Fw*:TGCCATTGGCATCCACCATGAC *Rv*:AGCCTCCAGCAAAGCAAACCTC	62.1 62.1	Current study
gst	*Fw*:TGCTTGATTCACTTCTACAAGAGA *Rv*:GTCACCATCAACAACAGTTGGA	57.6 58.4	Current study
cat	*Fw*:GGAGAGAATCCCAGAAAGAGTG *Rv*:ACAGTCCAATTTCCCCTCCC	60.3 59.4	Current study

2.7.4. RT-qPCR

Real-time quantitative PCR was conducted using FastStart SYBR Green fluorescence dye (ROCHE Diagnostics, Switzerland) with ROX passive dye included in the mix. The reaction medium (25 μL) consisted of 12.5 μL FastStart SYBR Green master mix, 0.3 μL forward primer (800 nM), 0.3 μL reverse primer (800 nM) and 3 μL diluted cDNA template (1:5 v/v). qPCR was performed in 384 optical well plates (Applied Biosystems, USA) using ViiA 7 Real-Time PCR system (Applied Biosystems, USA). The amplification conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and then 1 min of annealing at the corresponding Tm for each gene (Table 2). A dissociation curve was performed after the PCR cycles to determine the product specificity and check the absence of primer dimers. All qPCR assays were conducted in triplicates, and a non-template control (NTC) was included for each plate.

2.8. Integrated biological response (IBR)/n index

The selected endpoints reflect a hierarchy of biological organization from molecular to an individual level. The percentage of reproductive females, egg hatching success, total lipid levels, and *cyp3026b* and *hsp70* transcription levels were used to develop an integrated biomarker response index (IBR). The IBR constitutes a practical and robust tool to assess the responsiveness using multiple levels of biological responses (Broeg and Lehtonen, 2006). Standardized values of each biological response were calculated and plotted using star plots according to Beliaeff and Burgeot (2002). IBR index was calculated by summing up the triangular surfaces of the star plots ($IBR = \sum A_i$) according to the procedure discussed by Beliaeff and Burgeot (2002) with the modifications suggested by Devin et al. (2014), which avoids the biomarkers order bias in the IBR calculation. Since the IBR index is directly dependent on the number of biomarkers in the data set, the obtained IBR index value was divided by the number of biomarkers used (Broeg and Lehtonen, 2006, Marigómez et al., 2013). To facilitate data representation of the different concentrations of the selected endpoints for IBR/n index, only medium and high doses of each WAF type were used in the star plot visualization.

2.9. Statistical data analysis

Copepod mortality at different times (48, 72, 96 h) from lethal toxicity assays were analyzed using the PROBIT regression model (Vincent, 2008) to calculate the LC50 of the different WAF types. Significant differences among LC50 values were determined based on the absence of 95% confidence interval limits overlap. Data from egg production rate, egg-hatching success, Nile red fluorescence and gene expression ratios was initially checked for normal distribution using Kolmogorov-Smirnov test, while homogeneity of variances were tested using Levene's test. One-way ANOVA analyzed data showing normal distribution. Significant differences were further analyzed using Dunnett's post-hoc test to identify differences among treated and control groups. Data showing non-homogenous variance but normal distribution was analyzed using Welch one-way ANOVA, followed by the Games-Howell post-hoc test. Data showing non-normal distribution were analyzed using the non-parametric Kruskal-Wallis test, followed by the Dunn's post-hoc test for multiple comparisons. In all cases the Bonferroni correction for multiple comparisons was applied. All these analyses were performed with the aid of the statistical package IBM-SPSS v26. The relationship between the count data of the number of reproductive females and WAF doses was analyzed by a modified Fisher exact test (Freeman Hamilton test), due to the small sample size and the contingency table larger than 2×2 (VassarStats, Lowry, 1998–2019). The association between both variables was determined by a linear regression coefficient (IBM SPSS v26). Egg hatching success during individual fecundity tests was calculated as follows: (number of hatched egg per female/total number of eggs produced per female) x100. Finally,

relative gene transcription levels were determined using the Pfaffl method (Pfaffl, 2001). The transcript levels were log2 transformed and statistical differences between control and treatments were determined using one-way ANOVA. The Dunnet post-hoc test was further used to determine the statistical difference compared to control (IBM SPSS v26). *Z*-test was used to determine significant differences in the IBR/n index values among experimental groups.

3. Results

3.1. WAFs chemical composition

The three studied types of WAF contained mostly (99%) low-molecular-weight PAHs (<3 rings), with naphthalene being predominant (Table 3). Among the 16 quantified PAHs, high-molecular-weight PAHs (>4 rings) were below the detection limit for all three WAFs. NNS WAF showed the highest content of total PAHs, particularly naphthalene (Table 3). IFO 180 WAF had the lowest total PAH concentration.

3.2. WAFs Lethal toxicity

The lethal toxicity assays showed that WAF toxicity to adult *Acartia tonsa* was different according to the fuel oil. The MGO WAF showed to be the most toxic, while the NNS was the least toxic to copepods (Table 4). For MGO WAF, LC50 were as low as 34%, 10% and 8% after 48, 72 and 96 h, respectively. The IFO 180 WAF had the second most lethal effect to adult *Acartia tonsa* with LC50 of 74%, 52%, and 40% after 48, 72 and 96 h, respectively. The NNS WAF showed the least lethal effect with LC50 of 74% after 72 h and 52% after 96 h, and no lethal effects on the first 48 h. Noteworthy, the three types of WAF showed LC50 at 72 h and 96 h significantly lower than their respective LC50 at 48 h.

3.3. Effect of WAFs on fecundity

Exposure of female copepods to IFO 180 WAF and MGO WAF significantly reduced the proportion of individuals laying eggs (Fig. 1). The proportion of reproductive females was negatively associated with the doses of IFO 180 WAF (Fisher exact test $p < 0.0005$, $a = -23$) and MGO WAF (Fisher exact test $p < 0.05$, $a = -17.4$). The effect was very pronounced with the high IFO 180 WAF dose, with only two females laying eggs. Therefore, the high dose of IFO 180 WAF was removed from further statistical analysis. No significant difference was observed between the egg production rates of control fecund females and the fecund females exposed to NNS WAF, IFO 180 WAF or MGO WAF (Fig. 2). However, MGO WAF at high dose negatively affected the viability of produced eggs (Fig. 3) compared to controls, suggesting a disruption in the egg production process.

Table 3
Concentrations of the most prevalent PAHs in the three different types of 100% WAF prepared at 10 °C. All concentrations are presented at ng/L (mean values ± SD; $n = 3$). PAHs below detection limit for the three tested oils were: 11 H-benzo [a]fluorene (11H-B[a]F), 11 H-benzo[b]fluorene (11H-B[b]F), chrysene (Chry), benzo[b]fluoranthene (B[b]F), benzo[k]fluoranthene (B[k]F), benzo[a]pyrene (B[a]P), benzo[e]pyrene (B[e]P), indeno[1,2,3-cd]pyrene (Ind), dibenz[a,h] anthracene (D[a,h]A), benzo[g,h,i]perylene (B[g,h,i]P)] and dibenzo[a,e]pyrene (D[a,e]P).

PAH	# of rings	NNS	IFO 180	MGO
Naphthalene	2	19295 ± 759	8441 ± 660	11213 ± 1166
Acenaphthene	2	347 ± 25	965 ± 3	362 ± 37
Fluorene	2	1008 ± 3	909 ± 115	1161 ± 95
Phenanthrene	3	1115 ± 15	1968 ± 63	1953 ± 71
Anthracene	3	LDL	132 ± 18	LDL
Fluoranthene	3	LQL	23 ± 2.3	25 ± 3
Pyrene	4	141 ± 33	56 ± 2	37 ± 3
Benzo [a] anthracene	4	LQL	29 ± 3	LQL
∑ PAHs		21934 ± 7141	12524 ± 2861	14759 ± 4080

LDL: Low detection limit.
LQL: Lower than quantification limit.

Table 4
Lethal concentrations (LC50) for the adult *Acartia tonsa* exposed to the different WAFs after 48, 72, 96 h expressed as percentage of WAF diluted in seawater. The upper and lower limits of 95% confidence interval are indicated in parenthesis. Different superscript letters in each row denote significant differences among times, based on the absence of confidence interval overlap. Different superscript numbers in each column denote significant differences between oils, based on the absence of confidence interval overlap.

	48 h	72 h	96 h
NNS	>100%^{a1} (95–176)	74%^{b1} (61–94)	52%^{b1} (40–65)
IFO180	74%^{a2} (67–81)	52%^{b1} (45–61)	40%^{b1} (32–49)
MGO	34%^{a3} (29–42)	10%^{b2} (0.3–16)	8%^{b2} (0–13)

3.4. Effect of WAFs on total lipid

Ovaries were demonstrated as a vital accumulation tissue for lipids in female *Acartia tonsa* copepods (see Supplementary Material and Fig. S1). We quantified the lipid content using the Nile red lipophilic dye to give further insights into how exposure to the three types of WAFs affected the amount of accumulated lipids in females. Overall, the collected data was highly variable in all treatments and control (Fig. 4). Nevertheless, Nile red fluorescence values of the copepods exposed to the high MGO WAF dose were significantly lower than thaose observed in the control copepods (Fig. 4c), indicating a loss in total lipid levels.

3.5. Effect of WAFs on gene expression

Changes in transcription levels of the studied genes indicated toxic effects of tested WAFs in *Acartia tonsa* (Fig. 5). Down-regulation was observed for *hsp70* transcription levels in copepods exposed to WAFs, being statistically significant for copepods exposed to the medium dose of IFO 180 WAF. In contrast, *cyp3026b* up-regulation was observed in copepods exposed to the high dose of IFO 180 WAF condition. Statistical significant differences were absent for *cat*, *gst*, *hsp 70* and *ferritin*.

3.6. IBR/n index

Fig. 6(a–f) shows star plots for each experimental group. The surface value inside the dotted areas was calculated in order to obtain the IBR/n index value (Fig. 6g). The IBR/n index values were significantly higher for high IFO 180 WAF and MGO WAF doses than the corresponding control, indicating toxic effects of both WAFs for copepods. For the IFO 180 WAF, the low number of fecund females and up-regulation of *cyp3026b* contributed significantly to obtain a high IBR/n index value. Reduced egg hatchability and low total lipid amount explained the high IBR/n index value for the MGO WAF condition.

4. Discussion

In this study, a multilevel biological response approach was applied to assess the toxicity of three different oil WAFs in the copepod *Acartia tonsa*. Adult mortality, female fecundity, egg hatchability and individual total lipid levels were analyzed in copepods exposed to a light crude oil (NNS) and two refined oils (IFO 180 and MGO). Besides, transcriptional responses of genes involved in ROS metabolism (*cat* and *gst*), stress and iron homeostasis (*hsp70*, *hsp90,* and *ferritin*), and xenobiotic metabolism (*cyp3026b*) were investigated. As expected, throughout the different

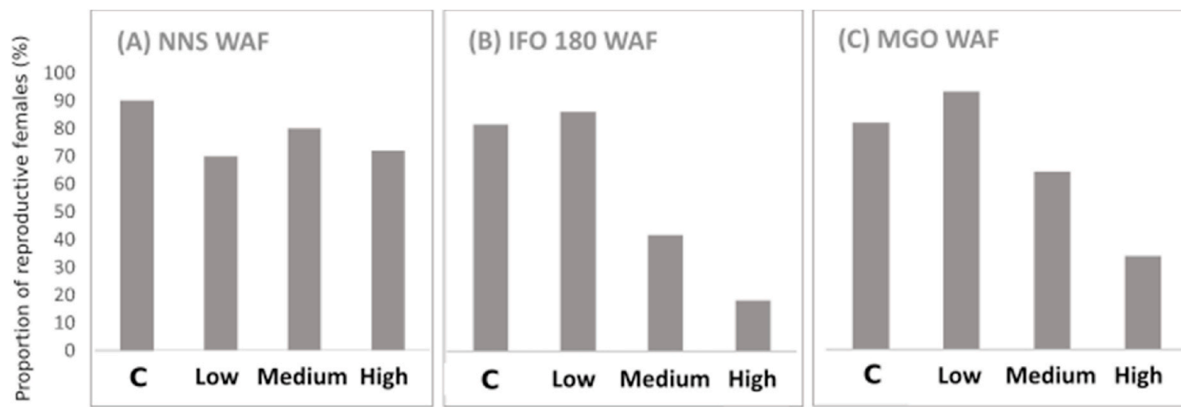


Fig. 1. Proportion of reproductive (egg producing) females after exposure to different doses of a) NNS WAF, b) IFO 180 WAF and c) MGO WAF. X-axis represents the selected sub-lethal WAF doses. A negative association between number of reproductive females and doses in IFO 180 WAF (Fisher exact test $p < 0.0005$, $a = -23$) and MGO WAF (Fisher exact test $p < 0.05$, $a = -17.4$) was detected. C: control. Only two females produced eggs in high IFO WAF treatment, therefore, high IFO 180 WAF dose was not included in the statistical analysis.

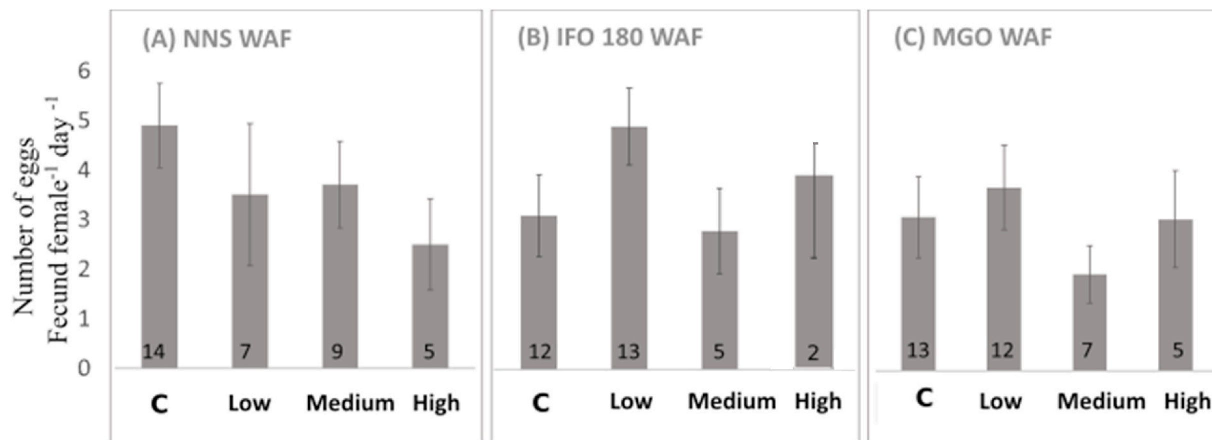


Fig. 2. Average egg production rate (number of eggs per fecund female^{-1} day^{-1}) over three days following exposure to a) NNS WAF, b) IFO 180 WAF and c) MGO WAF. Numbers in each bar indicate the total number of reproducing females in the experimental group. X-axis represents the selected sub-lethal WAF doses. C: control. Graphs show mean ± standard error.

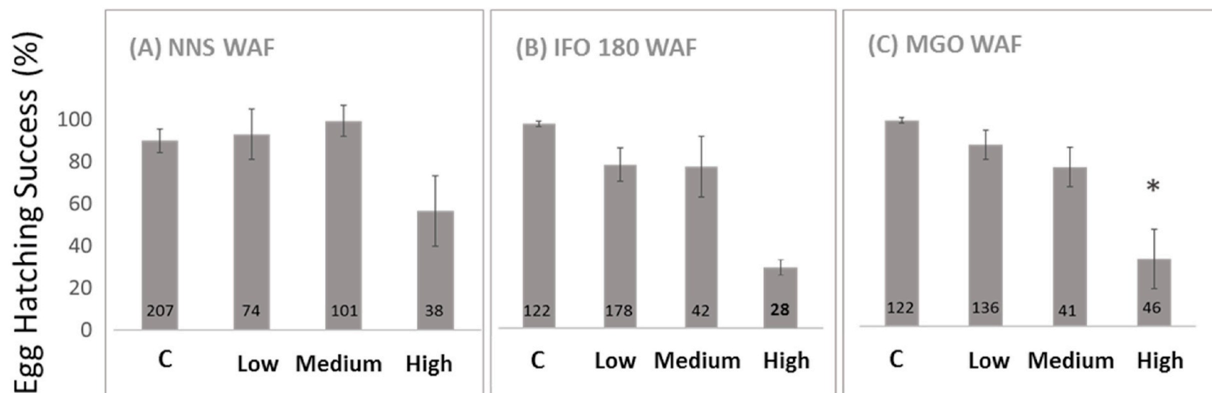


Fig. 3. Average percentage of egg hatching success of a) NNS WAF, b) IFO 180 WAF and c) MGO WAF. X-axis represents the selected sub-lethal WAF doses. Numbers in each bar indicate the total number of eggs analyzed in each treatment. Asterisks denote significant differences to control according to Kruskal-Wallis test, followed by Dunn's test (*p* < *0.05*). C: control. Graphs show mean ± standard error.

endpoints studied, MGO WAF caused the most detrimental effects on the copepods considering mortality, percentage of fecund females, egg hatching success, and total lipid content. Conversely, exposure to MGO WAF produced relatively mild effects on gene expression levels. IFO 180 WAF was also toxic to copepods, resulting in increased mortality, a decreased percentage of fecund females, and the most significant effects on gene expression. In contrast, NNS WAF was the least toxic considering mortality, reproductive, lipid endpoints and the gene expression

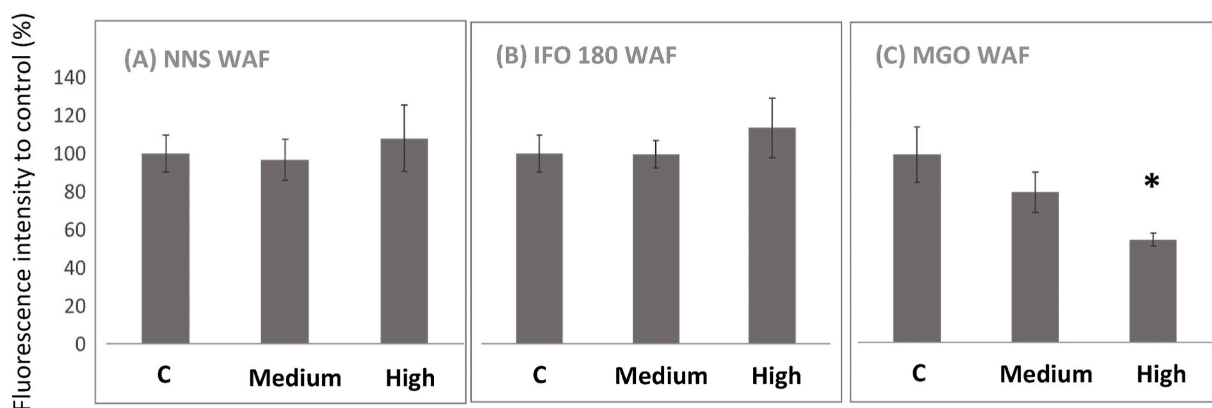


Fig. 4. Total lipid content corresponding to the percentage of Nile red fluorescence intensity compared to control (100%) after 96 h of exposure in a) NNS WAF, b) IFO 180 WAF and c) MGO WAF. X-axis represent the selected sub-lethal WAF doses. Asterisk denotes significant difference from control after one-way Welch ANOVA, followed by Games-Howell post hoc test ($p < 0.05$). C: control. Graphs show mean ± standard error.

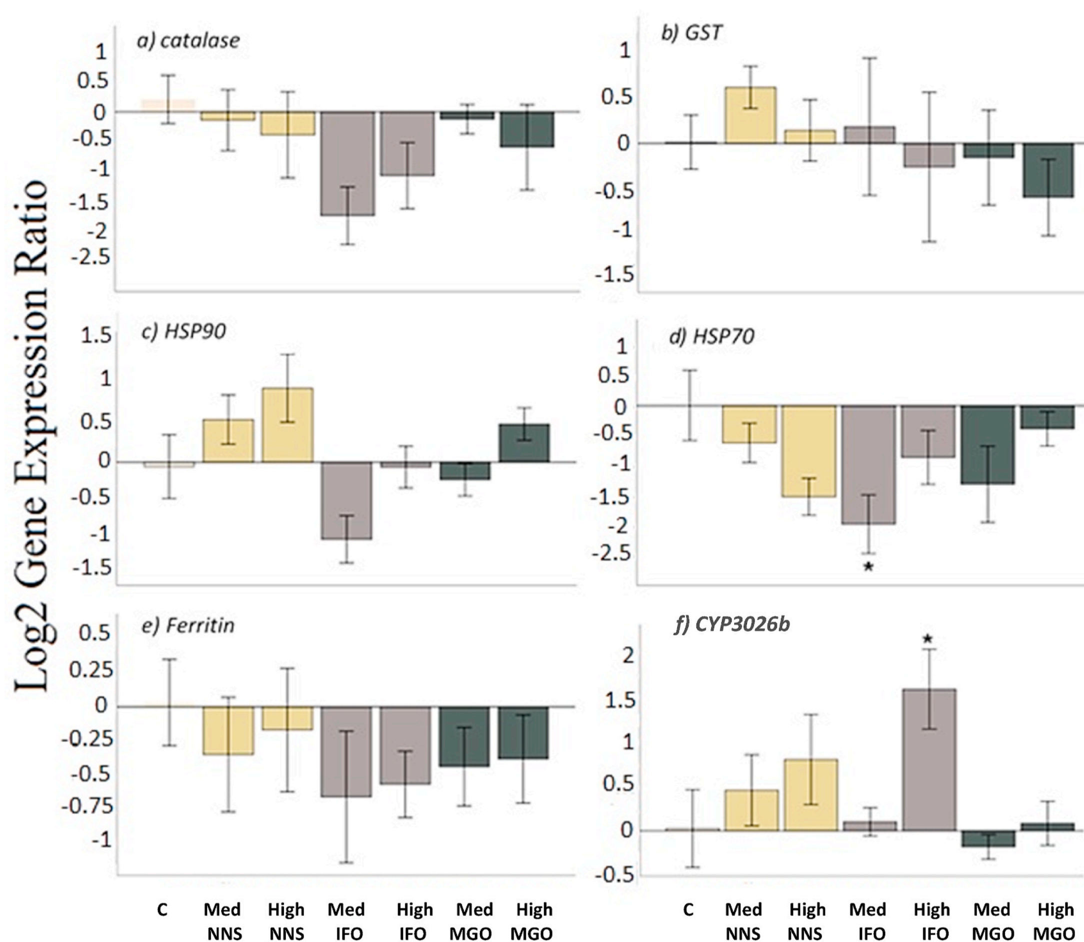
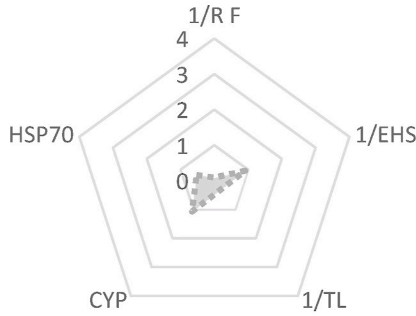
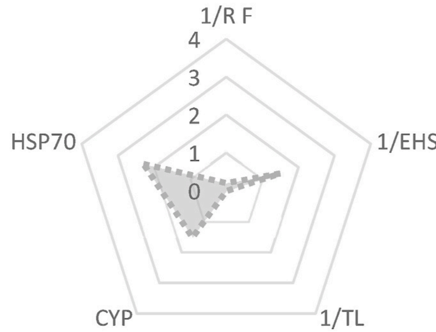


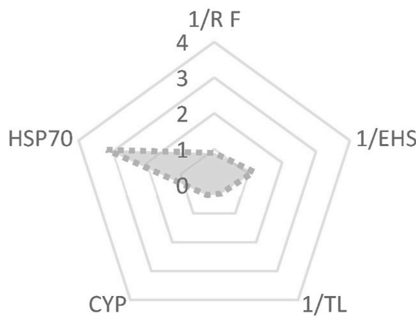
Fig. 5. Relative gene transcription levels (RQ) for a) Catalase (*cat*), b) Glutathione S-transferase (*gst*), c) Heat shock protein 90 (*hsp90*), d) Heat shock protein 70 (*hsp70*), e) Ferritin and f) Cytochrome P450 3026b (*cyp3026b*). Transcription levels are represented in log2 scale (mean ± standard error). Asterisks denote statistically significant differences from control according to one-way ANOVA followed by Dunnett's post hoc-test ($p < 0.05$).

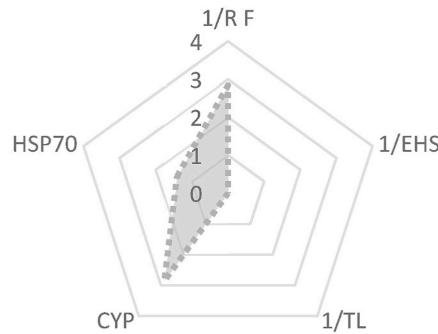
changes. The high doses of both IFO 180 and MGO WAF had a significantly higher IBR/n index values than those obtained for their corresponding controls, demonstrating the toxicity of IFO180 and MGO to *Acartia tonsa*.

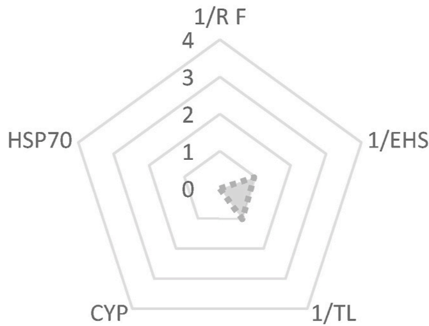
It was not surprising to observe MGO toxicity, as previous studies have highlighted the toxicity of MGO WAF to marine organisms. The larvae of the marine Pejerrey *Odontesthes argentinensis,* exhibited LC50 of 5.86% after 96 h exposure to MGO WAF (Rodrigues et al., 2010). Similarly, several studies showed that the observed toxicity of MGO on marine fish and crustaceans is due to the highly volatile BTEX compounds (benzene, toluene, ethylbenzene, and xylene) in the WAF (González-Doncel et al., 2008; Neff et al., 2000). Indeed, WAFs from crude oil derivatives (i.e., MGO) have higher BTEX content and additives (e.g., phenols and heterocyclic compounds containing toxic nitrogen

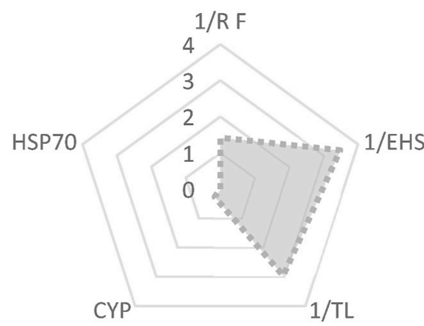












g)

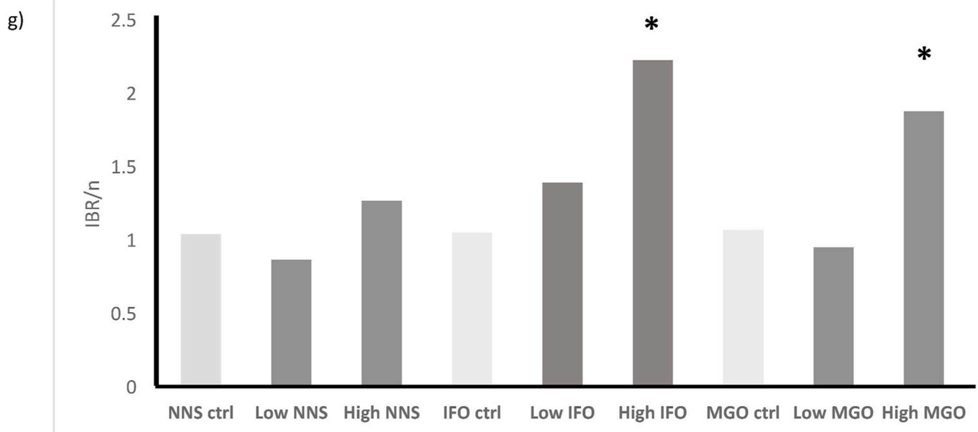


Fig. 6. (a–f) Star plots constructed using five endpoints according to their hierarchical biological level representativeness for each experimental group of *A. tonsa*. The biological parameters were percentage of reproductive females (RF), egg hatching success (EHS), total lipid content (TL), *cyp 3026b* transcription levels (*cyp*) and *hsp70* transcript levels (*hsp70*). g) IBR/n index calculated based on the surface values within the area delimited by dotted lines in star-plots. Asterisks denote significant differences between control and treated groups according to *Z*-test ($p < 0.05$).

and sulfur) than WAFs from crude oils (Saeed and Al-Mutairi, 1999; Rodrigues et al., 2010). It has been reported that MGO WAF prepared by mixing at 1:40 (oil: water) ratio, contained BTEX compounds up to 1008 μg/L. Therefore, aside from PAHs, further chemical analysis of other types of hydrocarbons is necessary to better understand the WAF toxicity profiles.

Several studies in copepods highlighted a decrease in the egg production rates due to toxic effects of oil WAF compounds (Hansen et al., 2015, 2017; Olsen et al., 2013). For example, copepods of the genus *Calanus* exposed up to 5.5 mg/L naphthenic crude oil, that is similar to the NNS crude oil tested in the present study, with mechanically and chemically dispersed conditions, showed reduced egg and nauplii production (Hansen et al., 2015a,b). The copepod *Paracartia grani* showed decreased egg production under exposure to high doses of naphthalene (Calbet et al., 2007). Accordingly, the results of the present study demonstrated that both IFO 180 WAF and MGO WAF showed a lower percentage of egg-laying females than the controls. Indeed, only two females laid eggs in the high dose of IFO 180.

The egg production rate of fecund females did not vary among the studied WAF conditions and controls, suggesting a possible compensatory mechanism in exposed fecund female reproductive capacity. A compensatory effect in egg-laying was also described for the harpacticoid *Nitocra spinipes* exposed to musk fragrances (Breitholtz et al., 2003). In *Calanus glacialis*, a reduction in the number of reproductive females was described after exposure to WAF prepared with 10 μg/L crude oil without affecting the cumulative produced egg number, suggesting that egg-laying was at a normal rate, similar to controls (Jensen and Carroll, 2010), as shown in the present study for *Acartia tonsa*. Similarly, *C. finmarchicus* females exposed to 16.5 mg/L arctic crude oil WAF showed a reduction in the number of egg-laying females, but the number of produced eggs in the exposed females was not different from that of control females (Olsen et al., 2013). Furthermore, as shown in the present study, the four-day recovery period was not enough for the *Acartia tonsa* females to recover from the observed toxicity effects, suggesting a long-term effect of the exposure conditions. This observation is consistent with effects detected in *Calanus* copepods, which showed signs of recovery after exposure to oil in fecundity after 20 days in clean water (Hansen et al., 2015, 2017).

The females exposed to the high dose of MGO WAF showed lower lipid content levels than the non-exposed control females. Linking both lipid and egg viability endpoints suggested that the short exposure to MGO WAF affected egg development, lipid metabolism and lipid accumulation in females. In copepods, lipids sacs, when present, are often close to developing oocytes (Lee et al., 2006; Hansen et al., 2017). We have shown that in *Acartia tonsa,* the ovary is the main organ for lipid accumulation. Thus, it is likely that the disruption of lipid levels caused by exposure to MGO WAF resulted in decreasing the percentage of egg-laying females and egg-hatching success. Such observation is consistent with previous works on *Calanus finmarchicus* females showing that exposure to naphthenic crude oil reduced lipid sac size and egg-laying rate. In addition, egg hatching was negatively affected by maternal transfer of PAHs in lipid sacs (Hansen et al., 2017). In the marine copepod *Centropages hamatus*, long-lasting effects on egg viability were detected after oil WAF exposure (Cowles and Remillard, 1983), suggesting that the biosynthetic pathway involved in oogenesis was affected.

Although the expression patterns of *catalase, gst* and *hsp90* genes in oil WAF exposed copepods were not significantly different from those of non-exposed control copepods, the medium dose of IFO 180 showed a significant downregulation of heat shock protein 70 gene (*hsp70*). Several studies showed up-regulation of heat shock proteins in response to environmental pollutants (Yoshimi et al., 2002; Rhee et al., 2009). However, fluoranthene, cadmium, nonylphenol, and octylphenol have shown to inhibit the expression of heat shock proteins (Werner and Nagel, 1997; Rhee et al., 2009), as shown in the present work. The downregulation of *hsp70* could result on additional effects on cellular processes as *hsp70* plays essential roles in stabilizing and refolding denatured proteins (González-Aravena et al., 2018). Since HSPs are long-lived proteins and they show many isoforms (Aruda et al., 2011; Tarrant et al., 2019), additional research at protein level is required to further elucidate downstream responses. We observed that the *cyp3026b* gene was up-regulated in *Acartia tonsa* after exposure to IFO 180 WAF. Such response was consistent with previous studies reporting that genes from the cytochrome P450 (*cyp*) clan 3 showed up-regulation in the copepods *T. japonicus* and *P. nana* after exposure to crude oil WAF (Han et al., 2014, 2015, 2017). Although little is known about the regulation of the *cyp* genes of the PAH detoxification process in invertebrates, there is some evidence of their involvement in the PAHs detoxification pathway (Han et al., 2014, 2015, 2017). Indeed, several core sequence motifs (*e.g.*, xenobiotic response element, aryl-hydrocarbon response element, and estrogen response element) were described in the promoter regions of *cyp* clan 3 members of *T. japonicus* and *P. nana* (Han et al., 2014, 2015, 2017). We advocate that *hsp70* and *cyp3026b* genes can be suitable biomarker genes for estimating the toxic effect of WAF exposure because they showed early responses after oil WAF exposure conditions. The other assessed genes involved in ROS and stress dependent responses (*hsp90*, *ferritin*, *gst*, and *catalase*) showed variability in their transcription profiles, but did not differ to levels determined in controls.

5. Conclusions

The results of the present work demonstrated that exposure to IFO 180 and MGO WAFs had harmful effects on *Acartia tonsa*. The NNS showed less toxicity in comparison to refined oil WAFs. The biological multi level approach applied in the present study, highlights the importance of integrating the effects elicited by environmental pollutants at different levels of biological organization. The combination of multilevel biological endpoints provided a comprehensive picture of how oils (crude and refined) affect *Acartia tonsa* individuals. Suppression in egg spawning was observed in treated females, but the egg-producing females still produced eggs at rates similar to controls, probably as a results of unknown compensatory mechanisms. However, in the case of high doses of MGO WAF, the females produced non-viable eggs which could be explained as a consequence of altered lipid levels in exposed females. In addition, transcriptional levels of *Cyp3026a* and *hsp 70* could be potential biomarkers of oil WAF exposure in *Acartia tonsa*. RNAseq and proteomic approaches will help in the identification of additional of CYP and HSP family isoforms participating in the WAF mediated responses in copepods. Metabolic, reproductive and transgenerational toxic effects detected in copepods exposed to refined oil WAF, raise concerns about the risks of copepod communities in regions with high maritime activities such as harbors and high maritime traffic routes. Such areas can have concentrations of PAHs at similar levels to those quantified in this study. Disruption in the copepod population can strongly impact the whole food web by transferring the deleterious effects to the polluted ecosystem's higher trophic levels.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2021.105378.

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