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Biomarker responses in mussels (*Mytilus trossulus*) from the Baltic Sea exposed to water-accommodated fraction of crude oil and a dispersant at different salinities

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ABSTRACT

Oil spills pose significant environmental risks, particularly in cold seas. In the Baltic Sea, the low salinity (from 0 to 2 up to 18) affects the behaviour of the spilled oil as well as the efficiency and ecological impacts of oil spill response methods such as mechanical collection and the use of dispersants. In the present study, mussels (*Mytilus trossulus*) were exposed under winter conditions (5 °C) to the water-accommodated fraction (WAF) of Naphthenic North Atlantic crude oil prepared by mechanical dispersion or to the chemically enhanced fraction (CEWAF) obtained using the dispersant Finasol OSR 51 at salinities of 5.6 and 15.0. Especially at the lower salinity, high bioaccumulation of polycyclic aromatic hydrocarbons was recorded in mussels in the CEWAF treatments, accompanied by increased biomarker responses. In the WAF treatments these impacts were less evident. Thus, the use of dispersants in the Baltic Sea still needs to be carefully considered.

1. Introduction

Preparedness for efficient response actions in case of oil spills includes good understanding of the potential biological effects of oil on local biota (Martinez-Gomez et al., 2010). The hazards of oil spills in cold seas are considered higher than in warm and temperate waters. This is not due only to the higher vulnerability of the environment and behaviour of oils in cold, ice-infested waters but also caused by problems related to oil response activities and logistics during and after the spill; these can be further escalated under extreme weather conditions often prevailing in the north (Jørgensen et al., 2019; NASEM, 2022).

The Baltic Sea is the largest semi-enclosed subarctic brackish-water basin in the world, surrounded by nine highly industrialised countries with >80 million inhabitants (HELCOM, 2018). Shipping has steadily intensified in the Baltic Sea, and between 2006 and 2016 an average of 1340 IMO registered ships were at sea each day, including hundreds of tankers carrying oil or other products potentially harmful to the environment. Despite the global geopolitical tensions in the oil market, the volumes of imported crude oil and transported mineral oil products have increased in the Baltic Sea; e.g., in Finland their transportation volumes were 9.8 and 12.6 million tonnes in 2022, respectively (Finnish Customs). Changes in oil transportation include the replacement of Russian crude oil by a lighter type imported from the North Sea.

The special environmental characteristics of the Baltic Sea (Snoeijs-Leijonmalm et al., 2017) signify that in case of an oil accident, small or large, the most efficient countermeasures and predicted ecological effects are likely not to be the same as in temperate, full marine environments. Low salinity (from 0 to 2 in the north and northeastern parts of the sea to ca. 18 in the southwest) and ambient surface water temperature varying greatly according to season and latitude (from 0 up to ca. 22 °C), pose critical questions on the behaviour of the spilled oil types, efficiency and ecological impacts of the currently available oil response methods (mechanical collection, use of dispersants, in situ burning), and the acute toxicity and sublethal biological effects of the oil – and the response methods – on marine organisms (Frantzen et al., 2016; Schmutz et al., 2021). In the Baltic Sea, the use of dispersants is currently not recommended by the Baltic Marine Environment Protection Commission (Helsinki Commission [HELCOM]) but the issue has

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become under discussion again as an alternative method for the mechanical collection approach currently in use. Therefore, it is crucial to examine the potential effects of the alternative response methods on the Baltic Sea biota and ecosystem, taking into careful consideration the above-mentioned quite unique properties of the sea area.

Polycyclic aromatic hydrocarbons (PAH) are a highly toxic group of compounds present in oils. They are readily taken up by organisms directly from the water through body surfaces, gills or through food, causing negative effects both on acute and long-term basis (Hylland, 2006; Martinez-Gomez et al., 2010). Biological effects of different types of oil and PAH on marine biota have been examined considerably during the recent decades by using various types of biomarkers, both in the laboratory and in the field (van Der Oost, 2003; Garmendia et al., 2011; Vethaak et al., 2017; Sanni et al., 2017; Beyer et al., 2017; Turja et al., 2020). PAH concentrations in water causing the effects depend on the oil type. For example, in the case of Naphthenic North Atlantic crude oil (NNA) – a similar oil type used also in the present study – the soluble fraction of PAH increases markedly also at low temperatures (Faksness et al., 2008). In the dispersed fraction of NNA, the most abundant PAH are the lighter ones such as methylnaphthalenes and related compounds, whereas heavy PAH are found mostly at very low concentrations and frequently below the limits of detection (Faksness et al., 2008). Moreover, when performing exposure experiments with oil their complex mixture must be carefully considered since toxicity is directly dependent on the procedures used to prepare the exposure solutions. Thus, using standardized protocols to prepare the test exposure solutions is critical for the interpretation of toxicity results generated and comparisons across other studies (Faksness et al., 2008; Hodson et al., 2019; NASEM, 2022).

Mussels (especially of the genus *Mytilus*) and other bivalve molluscs are widely used in biomonitoring programs to detect both the bioaccumulation of hydrocarbons and biological responses in relation to oil contamination (Cajaraville et al., 2000; Hylland et al., 2008; Turja et al., 2013). Their sessile, filter-feeding lifestyle and generally low enzymatic degradation rates of organic contaminants make them capable of accumulating high levels of different types of substances, including PAH (Beyer et al., 2017; Kasiotis and Emmanouil, 2015). The Baltic mussel *Mytilus trossulus* is a key species in the low-diversity Baltic Sea and is adapted to live in a brackish-water environment and can therefore be considered as an optimal test organism for laboratory exposure studies (Prevodnik et al., 2007; Tedengren and Kautsky, 1987).

In the present study, *M. trossulus* was exposed to a water-accommodated fraction (WAF) of NNA and the fraction obtained by the application of a commercial dispersant for chemically enhanced dispersion of oil (CEWAF) using an established preparation protocol (Singer et al., 2000; Bilbao et al., 2022). The experiments were performed at two different salinities roughly corresponding the extremes occurring in the different geographical areas of the Baltic Sea (Benito et al., 2019). The biomarkers applied here to examine the effects of oil exposure on cellular integrity are related to the antioxidant defence system (ADS) and general stress responses at cell and tissue levels. An increased production of reactive oxygen species (ROS) during biotransformation of different types of contaminants commonly leads to an imbalanced redox state within the cells where the oxyradicals formed are not sufficiently neutralized by the ADS, causing damage to macromolecules such as lipids, proteins, and DNA (Baussant et al., 2009; Regoli and Giuliani, 2014). Organisms use ADS enzymes including catalase (CAT) and glutathione reductase (GR) as antioxidants that provide cellular defence against endogenous and exogenous ROS (Livingstone et al., 1992). Membrane damage caused by ROS can be detected by measuring lipid peroxidation (LPO) (Box et al., 2007). Regarding detoxification of organic contaminants, the activity of another ADS related enzyme, glutathione *S*-transferase (GST) involved in Phase II (conjugation), is a widely used biomarker in organisms exposed to PAH (Kopecka et al., 2006; Richardson et al., 2008; Turja et al., 2014b). Exposure to oil compounds has also shown to cause neurotoxic effects, e.g., inhibition of acetylcholinesterase (AChE) enzyme activity (Maisano et al., 2017). Moreover, geno- and cytotoxic effects have been reported in response to oil contamination, including the formation of micronuclei (MN) and other nuclear deformities (Baršienė et al., 2012; Turja et al., 2020). Regarding tissue level biomarkers in mussels, replacement of cell types within the digestive epithelium resulting in increase of basophilic cell volume density (Vv_{BAS}), thinning of the digestive epithelium in digestive alveoli (atrophy index) and loss of digestive gland tissue integrity (connective tissue area per digestive diverticula area ratio, CTD) have been reported to occur in response to pollutant exposure (Cajaraville et al., 1992; Marigómez et al., 2006; Garmendia et al., 2011; Blanco-Rayón et al., 2019). In the current study, the biomarkers above measured in mussels exposed to WAF and CEWAF of NNA were examined in connection with tissue accumulation of PAH during the experiments.

2. Material and methods

2.1. Sampling and acclimatization of experimental mussels

M. trossulus were collected by scuba diving on October 26, 2016, close to the Tvärminne Zoological Station of the University of Helsinki (southern Finland, northern Baltic Sea) from the depth of 5–6 m. The mussels were transported in cooled thermo-insulated boxes with ambient seawater to the laboratory of the Finnish Environment Institute (Helsinki, Finland). The boxes were placed in a 10 °C room (ambient temperature at the collection site) and equipped with aeration. After cleaning and measuring the approximate shell length (2.0–2.5 cm), 200 mussels were placed in each 30 L experimental tank with 20 L of ambient seawater. Two strengths of artificial sea water (ASW) were prepared for the experiment: 5.6, the same as in the area of collection, and 15.0, corresponding to the higher-end salinities present in the southwestern Baltic Sea. The ASW media were prepared by adding Instant Ocean® salt mix to local tap water, leaving them overnight under efficient aeration to remove any chlorine before the use. During a two-week acclimatization period the water temperature was gradually reduced in each tank to reach the desired experimental one of 5 °C, corresponding to local winter conditions. During the acclimatization period the salinity was gradually increased for mussels in the higher salinity treatment tanks. The mussels were fed daily with a commercial mixture of algal cells (Instant Algae Shellfish Diet 1800®) in concentrations of 0.6–1.6 mg algal mixture individual^{-1} day^{-1}, corresponding to approximately 12.5 $\times$ 10^4–33 $\times$ 10^4 cells individual^{-1} day^{-1}. Feeding was considered important since it has been shown that even short-term starvation may negatively impact the sensitivity of toxicological assessments in mussels (Blanco-Rayón et al., 2019). In addition, when mussels are exposed to very low algal concentrations (or zero in the ASW) this eventually leads to reduced valve gape behaviors or complete closure of the shells, which obviously affects exposure to the contaminants introduced to the test medium (Riisgård et al., 2006).

2.2. Preparation of the exposure media and PAH concentration measurements

The mussels were exposed to WAF and CEWAF of NNA (see Table 1 for final oil and PAH concentrations in the exposure media at both salinities). NNA oil is characterized by a low viscosity and a high proportion of low molecular weight PAH compounds. The chemical profile of the tested NNA oil, toxicity assessments using several aquatic species and the characteristics of the dispersant are available as downloadable deliverables of the EU Horizon 2020 GRACE project (EU GRACE, 2019) and in related publications (Bilbao et al., 2022; Johann et al., 2020; Jørgensen et al., 2019). The 5 % WAF was prepared by adding 20 g of crude oil in 4 L of ASW having the experimental salinity of 5.6 or 15.0 in 10 L Marionette glass bottles equipped with a tap. For the 5 % CEWAF, first a premix of 20 g of crude oil and 2 g of the dispersant Finasol OSR 51

Table 1
Concentrations of different oil fractions, total oil and total PAH during the exposure experiments at the salinities of 5.6 and 15.0. The maximum (Max) values were obtained always at 0 h directly after water renewal and the minimum values (Min) immediately before water renewal at 48 h. Limit of detection = 0.050 mg L^{-1}; ND = not detected.

Exposure	Salinity	>C10-C20 fraction			>C21-C40 fraction			Total oil			Total PAH		
		Max (0 h)	Min (48 h)	Mean	Max (0 h)	Min (48 h)	Mean	Max (0 h)	Min (48 h)	Mean	Max (0 h)	Min (48 h)	Mean
CT	5.6	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.12	0.05	0.09
	15.0	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.11	0.06	0.09
WAF	5.6	0.08	ND	0.04	ND	ND	ND	0.08	ND	0.04	2.62	0.65	1.64
	15.0	0.06	ND	0.06	ND	ND	ND	0.06	ND	0.03	6.79	3.01	4.90
CEWAF	5.6	25.0	3.50	14.25	19.0	4.00	11.5	44.0	7.50	25.75	88.99	16.93	52.96
	15.0	0.97	0.12	0.54	0.85	0.21	0.53	1.82	0.33	1.07	18.08	5.25	11.67

was prepared in a separate glass bottle and then added to the Marionette bottle containing 4 L of ASW with salinity of 5.6 or 15. The premix was prepared separately for both experimental salinities. After the oil alone or oil-dispersant premix was added the solution was gently mixed in a shaker (200 rpm to avoid vortex) for 40 h, after which it was left to settle for 3 h. The resulting WAFs and CEWAFs were drained into 1 L glass bottles, carefully not to include any of the oil slick formed during the 3 h settling time. Preparation of WAF and CEWAF were made at 10 °C. During the preparation, the solutions were clearly different visually since the dispersant made the oil disperse to the whole water phase making it dark brown, whereas in the WAF bottles the water was clear with a visible oil slick on top of the water. According to Bilbao et al. (2022), the half-lives of these solutions were approximately 24 h and 30 h with and without dispersant, respectively. Due to the low stability of the WAF and CEWAF solutions they were freshly prepared every other day for the experiments, with the preparation procedure being synchronized with the periodic replacement of the exposure media.

2.3. Experimental set-up

The mussels were exposed to the 5 % WAF and 5 % CEWAF solutions at the two different salinities for 21 days at 5 °C by applying a semi-static scheme with a full renewal of experimental media every 48 h. Two hundred individuals were placed into two replicate 30 L tanks per treatment (including the control treatment without oil) with 19 L of ASW, and one litre of WAF or CEWAF was added on top with gently mixing. Temperature was kept constant during the experiment by using thermostats (Lauda) placed in a large water tank surrounding the experimental tanks. A continuous airflow system was established for the aeration of the water in the tanks. Mussel samples for the biomarker measurements were taken after 24 h (D1), 7 days (D7), and 21 days (D21). The volumes of water and added WAF and CEWAF were kept unchanged after the removal of one third of the experimental mussels from the test units at D1 and D7.

2.4. Chemical analysis

Quantification of petroleum hydrocarbons levels (fractions C10-C40) from water samples was performed by gas chromatography equipped with flame ionization detector (GC-FID; Shimadzu GC-2010 Plus AF) according to ISO 9377-2:2000 (ISO, 2000). Water samples (1 L) were extracted with *n*-hexane (30 mL) for 30 min using magnetic stirrer. Prior to the extraction step the pH of the samples was adjusted to 2. After extraction, the hexane layer was collected and purified with solid phase extraction (SPE) using Florisil cartridges (Chromabond Florisil, Macherey-Nagel). The hydrocarbon fractions C10-C40 were eluted from the SPE cartridges with *n*-hexane (10 mL). The extract was the concentrated to 1 mL using a gentle flow of nitrogen at 40 °C. The final amount of extract was calculated by weighing and an aliquot of the final extract was transferred to a vial for gas chromatographic analysis.

Quantification of PAH compounds from water and mussel samples was performed by a gas chromatographytriple quadrupole mass spectrometer (GC–MS/MS). Water samples (1 L) were extracted with 25 mL of *n*-hexane (Fluka). Prior the extraction, 10 μL of the mixture of internal standards (Fluoro-PAHs, Chiron) were added to the samples. The samples were extracted for 30 min using a magnetic stirrer. The *n*-hexane layer was collected, 1 mL isooctane was added as the solvent keeper, and the solvent was evaporated under a gentle flow of nitrogen until a final volume of 1 mL was reached. 20 μL of injection standard solution (deuterated PAHs, Dr. Ehrenstorfer) was added to the samples prior to the analysis. Pooled samples of the whole mussel soft tissue from each treatment were homogenized and 5–10 g of homogenate was taken for the analysis. 10 μL of the mixture of internal standards (Fluoro-PAHs, Chiron) was added to the homogenate prior the extraction. 5 mL of water and 10 mL of ethyl acetate were added, and the samples were shaken for 1 min followed by addition of a salt mixture consisting of 4 g $MgSO_4$ and 2 g NaCl. The samples were shaken for 1 min and then centrifuged for 10 min. 5 mL of the ethyl acetate layer was collected, and 200 μL of isooctane was added to the extract. Ethyl acetate was evaporated under a nitrogen flow and 1 mL of hexane was added. The extract was purified using a column containing glass wool, Na_2SO_4, and silica. The PAH compounds were eluted with hexane/dichloromethane (3:1, v/v). After elution, 0.5 mL isooctane was added as the solvent keeper and the solvent was evaporated under a gentle flow of nitrogen until a final volume of 0.5 mL was reached. 20 μL of injection standards (deuterated PAHs, Dr. Ehrenstorfer) was added to the samples prior to the analysis. Both water and mussel samples were analysed using uTrace 1310 GC Ultra gas chromatograph (Thermo Scientific) interfaced to a Thermo Scientific TSQ Quantum XLS Ultra mass spectrometer (Thermo Scientific) in the electron impact (EI) mode.

2.5. Biomarker measurements

2.5.1. Biochemical biomarkers

For the biochemical biomarkers, digestive glands ($n = 10$) of mussels (for CAT, GR, GST and LPO) were homogenized in 0.1 M potassium phosphate buffer with 0.15 M potassium chloride (pH 7.4), and gills (for AChE, $n = 10$) in 0.2 M sodium phosphate buffer (pH 7.0) containing 0.1 % Triton-X. Tissues were homogenized using TissueLyser (Qiagen) 2 × 30 Hz s^{-1}, after which the samples were centrifuged at 10,000 ×*g* for 15 min at 4 °C. Samples for LPO measurements were taken before the centrifugation and 4 % butylhydroxytoluene (BHT) was added to inhibit peroxidation. The supernatants were aliquoted and stored at −80 °C.

CAT activity was measured as CAT-mediated degradation of hydrogen peroxide (H_2O_2) at 240 nm (Claiborne, 1985). GST activity was estimated by measuring the formation rate of the conjugated substrate (chlorodinitrobenzene [CNDB]-glutathione [GSH]) at 340 nm (Habig et al., 1974). GR activity was measured according to Smith et al. (1988), based on the reduction of oxidized glutathione (GSSG) by GR. The product, GSH, reacts spontaneously with 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) forming 5-thio(2-nitrobenzoic acid) (TNB) detected spectrophotometrically. Levels of LPO were measured as the generation of thiobarbituric acid reactive substances (TBARS) at 535 nm (Ohkawa et al., 1979). Analyses of AChE activity were performed from gill

samples as described in Bocquene and Galgani (1998) with modifications as in Leiniö and Lehtonen (2005). All the enzymatic assays and the target tissue protein content (Bradford, 1976) used for the calculation of specific enzymatic activities were measured in 96-well microplates using the TECAN Infinite 200 (TECAN) spectrophotometer with Magellan software.

2.5.2. Geno- and cytotoxicity biomarkers

Gill tissue samples of mussels were analysed for the selected geno- and cytotoxicity parameters. Preparation of the slides and investigation of gill cells was carried out following the methods described earlier (Baršienė et al., 2004, 2006b). The stained slides were analysed under bright-field Olympus BX51 microscopes (Tokyo, Japan) using an immersion oil objective (100×). Two thousand cells with intact cellular and nuclear membranes per mussel were evaluated using blind scoring. The frequency of the different nuclear abnormalities is expressed as the number of occurrences per 1000 cells scored. The formation of micronuclei (MN), nuclear buds (NB), nuclear buds on filament (NBf), binucleated cell with nucleoplasmic bridge (BNb) and blebbed nuclei (BL) cells were assessed as genotoxicity endpoints, and 8-shaped nuclei, fragmented apoptotic (FA) and binucleated (BN) cells as cytotoxicity endpoints. Total genotoxicity (Gentox) and total cytotoxicity (Cytox) levels were assessed as the sum of the frequencies of the listed endpoints.

2.5.3. Tissue level biomarkers and gamete developmental stages

Whole mussels were fixed in seawater with 4 % formaldehyde, dehydrated in an ethanol bath series, paraffin embedded using a Leica ASP3005 tissue processor, sectioned at 5 μm with a Leica RM2125RTS microtome, and stained with haematoxylin-eosin (H/E). Vv_{BAS} and CTD were quantified by means of stereology as an indication of changes in cell-type composition, mean digestive epithelium thickness, and the relative area of interstitial connective tissue. The counts were made on three randomly selected fields in one digestive gland slide per mussel (6 individuals per sample). The slides were viewed at a 40 × objective (final magnification ca. 400 ×) using a drawing tube attached to a light microscope. A simplified version of the Weibel graticule multipurpose test system M-168 (Weibel, 1979) was used, and hits on basophilic cells (b), digestive cells (d), diverticular lumens (l) and interstitial connective tissue (c) were recorded. Vv_{BAS} was calculated according to Delesse's principle (Weibel, 1979) as $Vv_{BAS} = V_{BAS}/V_{EP}$, where V_{BAS} is the volume of basophilic cells and V_{EP} the volume of digestive gland epithelium. The CTD ratio was calculated as $CTD = c/(b + d + l)$. The severity of the atrophy index of the digestive alveoli was ranked using a numerical grading from 0 to 4 as described by Kim et al. (2006), where 0 = normal digestive diverticula with nearly occluded lumen, 1 = co-occurrence of normal and partially atrophied tubules of epithelium thickness greater than one-half of normal, 2 = digestive epithelium thickness half of normal, 3 = significantly atrophied tubules with digestive epithelium less than half as thick as normal, and 4 = an extremely thin digestive epithelium with nearly all the tubules affected.

Gamete developmental stages were determined as described by Ortiz-Zarragoitia et al. (2011) and were distinguished in mussel gonads ($n = 10$) as follows: resting stage (inactive or undifferentiated); early gametogenic stage (gametogenesis has begun but no ripe gametes visible); advanced gametogenic stage (gametogenesis still progressing and ripe gametes and developing gametes have approximately equal proportions); mature stage (gonad fully mature, follicles full of ova or sperm); spawning stage (active emission of gametes, some follicles appear empty); post-spawning stage (empty follicles and only residual gametes remain).

2.6. Data integration and statistical analysis

The IBR index (Beliaeff and Burgeot, 2002; Broeg and Lehtonen, 2006; Marigómez et al., 2013) was based on the integration of five biological responses, CAT, Gentox, Cytox, Vv_{BAS} and atrophy, to represent responses at different biological organization levels. The calculations were based on a multivariate graphic method, according to the following procedure: (1) calculation of the mean and standard deviation for each sample; (2) standardization of data for each sample: $x_i' = (x_i - x)/s$; where, x_i' = standardized value of the biomarker; x_i = mean value of a biomarker from each sample; x = general mean value of x_i calculated from all compared samples (data set); s = standard deviation of x_i calculated from all samples; (3) addition of the standardized value obtained for each sample to the absolute standardized value of the minimum value in the data set: $y_i = x_i' + |x_{min}'|$; (4) calculation of the radar plot triangular areas as $A_i = (0.59 \times (y_i \times y_i + 1)/2$, where "$y_i$" and "$y_{i+1}$" are the standardized values of each biomarker and its next one in the radar plot, respectively, and 0.59 is sin α (α: radial angle for a pentagonal radar plot; $\alpha = 2\pi/5$); and (5) calculation of the IBR index which is the summing-up of all the radar plot triangular areas ($IBR = \Sigma A_i$) (Beliaeff and Burgeot, 2002). Since the IBR value is directly dependent on the number of biomarkers in the data set, the obtained IBR value was divided by the number of biomarkers used to calculate IBR/n (Broeg and Lehtonen, 2006).

All data were tested for normality applying the Kolmogorov-Smirnov test and homogeneity of variance with Bartlett's test. One-way ANOVA (F-statistics) followed by Bonferroni corrected pairwise *t*-test were used for the normally distributed data and Kruskal-Wallis for the non-parametric data (H-statistics). SYSTAT™ 11 and IBM SPSS Statistics 24 statistical software were used for the analyses. Statistical differences in the IBR/n values were established using the *Z*-score method.

3. Results

3.1. General remarks

At the end of the experiment (after 21 days), mortality in the control groups was low at both experimental salinities, being 3.7 % at 5.6 and 2.9 % at 15.0. Compared to the control groups mortality was higher in the WAF exposed mussels (13.6 % at 5.6 and 10.6 % at 15.0) as well as in the CEWAF treatments (18.9 % at 5.6 and 11.9 % at 15.0).

3.2. Oil concentrations in the exposure media

By the time before adding the new CEWAF medium at 48 h from the start of the experiment, the total oil concentrations had reduced by 83 % and 82 % in the salinities of 5.6 and 15.0, respectively (Table 1). Concerning WAF, these calculations were not feasible due to the low levels and/or no detection of oil. Thus, in all cases the average concentrations calculated between 0 and 48 h may be used as the best proxies for true exposure concentrations in the ecotoxicological assessment.

3.3. Bioaccumulation of PAH

Tissue accumulation of PAH in the digestive gland of the exposed mussels was observed already on D1 both in WAF and CEWAF treatments, being markedly higher in the latter (Fig. 1). No differences in accumulation could be observed between the groups at the two experimental salinities. On D7 the accumulation was again higher in the CEWAF treatments, but the markedly higher tissue concentrations were observed at the salinity of 5.6. On D21 a very similar bioaccumulation pattern compared to D7 was recorded in all treatments.

Increases in tissue concentrations of single PAH were observed especially regarding methylated compounds, with 1- and 2-methylnaphthalenes standing out as the most significant ones (Fig. 1, Table 2). Altogether, in the CEWAF treatment on D7 and D21 the total tissue levels of methylated PAH reached up to ca. 6000 μg kg ww^{-1} at the salinity of 5.6. The share of total methylated PAH ranged from 62 to 75 % of total PAH, except in cases with very low bioaccumulation (e.g., 32 % in the D21 WAF group at the salinity of 5.6). In control mussels, the tissue levels of methylated compounds were very low, ranging from 3 to

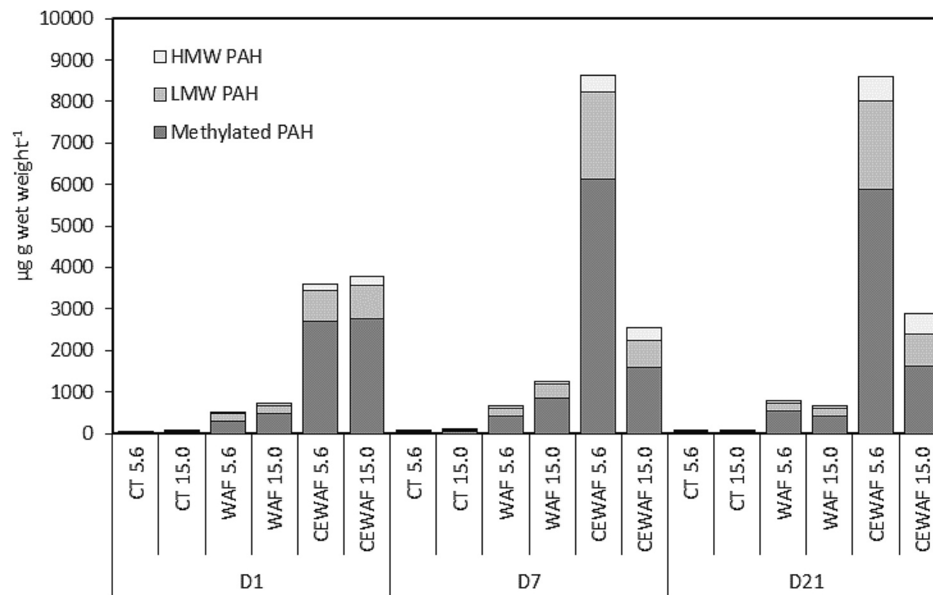

Fig. 1. Concentrations of PAH in the digestive gland of mussels exposed to WAF and CEWAF at the salinities of 5.6 and 15.0. HMW = high molecular weight PAH (≥four rings); LMW PAH = low molecular weight PAHs (<four rings) except methylated forms, which are shown separately.

Table 2
Tissue concentrations of the different PAH compounds (μg kg ww^{-1}) measured in *M. trossulus* in control individuals (CT) and in the different exposure treatments (WAF and CEWAF) during the experiment (days [D] 1, 7 and 21) at the different salinities (5.6 and 15.0). For CT, a mean value was calculated using all measurements from the different salinities and time points. Values below the limit of detection (1.0 μg kg ww^{-1}) have a value of 1, since all the values have been rounded up to the nearest 1.0 μg kg ww^{-1}.

	5.6 & 15.0	5.6						15.0					
	CT	WAF			CEWAF			WAF			CEWAF		
		D1	D7	D21	D1	D7	D21	D1	D7	D21	D1	D7	D21
1-methylbenzothiophene	1	1	2	2	10	41	43	1	1	2	4	17	42
1-methylnaphthalene	5	90	120	150	800	1700	1800	140	250	130	880	410	270
2-methylnaphthalene	11	210	280	340	1800	3700	3300	340	560	260	1800	850	540
3-methylphenanthrene	1	2	10	15	33	250	260	3	10	15	34	110	270
4-methyldibenzothiophene	1	1	5	7	33	120	130	2	4	7	16	53	120
9-methylphenanthrene	1	2	12	16	41	330	360	3	12	18	41	150	380
Anthracene	4	26	7	9	11	59	50	5	10	7	6	16	43
Acenaphthene	2	4	7	10	47	140	160	6	9	8	68	43	41
Acenaphthylene	1	1	3	4	34	100	130	2	4	3	44	27	25
Naphthalene	3	110	100	77	380	580	350	130	240	65	280	140	56
Phenanthrene	7	14	33	46	56	430	430	21	42	47	54	130	260
Fluorene	4	13	32	55	210	770	1000	23	61	48	340	300	360
Benzo[*a*]anthracene	1	1	2	2	7	16	20	1	4	1	16	14	29
Benzo[*a*]pyrene	1	1	1	1	6	5	8	1	1	1	7	6	12
Benzo[*b*]fluoranthene	1	1	2	2	7	12	18	32	2	1	10	12	23
Benzo[*e*]pyrene	1	1	1	1	5	12	18	1	1	1	10	14	28
Benzo[*ghi*]perylene	1	1	1	1	7	4	5	1	2	1	5	4	6
Benzo[*k*]fluoranthene	2	1	2	1	6	21	23	1	1	1	5	10	5
Dibenzo[*a,h*]anthracene	1	1	2	1	8	4	5	1	1	1	5	3	7
Dibenzothiophene	1	3	8	11	44	170	220	5	10	10	42	82	120
Fluoranthene	4	5	11	19	16	50	79	6	25	17	31	37	62
Indeno[1,2,3-*cd*]pyrene	1	1	1	1	8	2	3	1	2	1	4	2	3
Chrysene	1	1	2	2	9	31	53	1	1	1	21	27	57
Perylene	1	1	1	1	4	8	12	1	1	1	5	8	16
Pyrene	2	3	6	9	17	45	63	3	13	8	36	39	66
Triphenylene	1	1	1	1	11	33	60	1	1	1	23	40	57
Sum PAH	56	491	650	781	3610	8632	8599	728	1266	652	3787	2544	2898

31.8 μg kg ww^{-1} and share of total PAH being between 15 and 43 %.

Regarding the more detailed tissue accumulation pattern of the different PAH compounds measured, most of them showed increasing concentrations towards D21 of the exposure in every treatment (Table 2). However, with some PAH this trend could not be observed. Apart from the methylated PAH the most prominent compounds recorded in tissues were fluorene (FLU), phenanthrene (PHE), naphthalene (NAP), dibenzothiophene (DBT), acenaphthene (ACE) and acenaphthylene (ACY). Up to more than an order of magnitude higher concentrations were observed in tissue concentrations of all PAH in mussels exposed to CEWAF compared to WAF. In mussels exposed to WAF no patterns could be observed in the levels of accumulation of the different PAH between the two experimental salinities. On the contrary, tissue levels of many PAH, including the high tissue level FLU, PHE, NAP, DBT,

ACE and ACY, were 2–6 times higher in the CEWAF-exposed group at the lower salinity compared to the higher one.

3.4. Biomarker responses

For each of the experimental salinities, statistically significant differences in the measured biomarkers are shown (1) between the control treatments and the oil exposed groups at each sampling timepoint (D1, D7 and D21), and (2) within any treatment group at the different timepoints.

3.4.1. ADS responses and detoxification

At the salinity of 5.6, the highest CAT activity in mussels was detected in the CEWAF exposed group on D21 (Fig. 2A). In addition, the activity of the enzyme increased significantly in the control group from D7 to D21. At the salinity of 15.0 the activity decreased significantly from D1 to D7 in the control and WAF treatments (Fig. 2B). At the salinity of 5.6 no effects on GR activity could be observed (Fig. 2C). At the salinity of 15.0 the activity was significantly increased in the WAF exposed mussels on D21 compared to D1 (Fig. 2D). At the salinity of 5.6, GST activity was significantly increased in the CEWAF exposed mussels on D7 and D21 compared to D1 of the same group as well as compared to

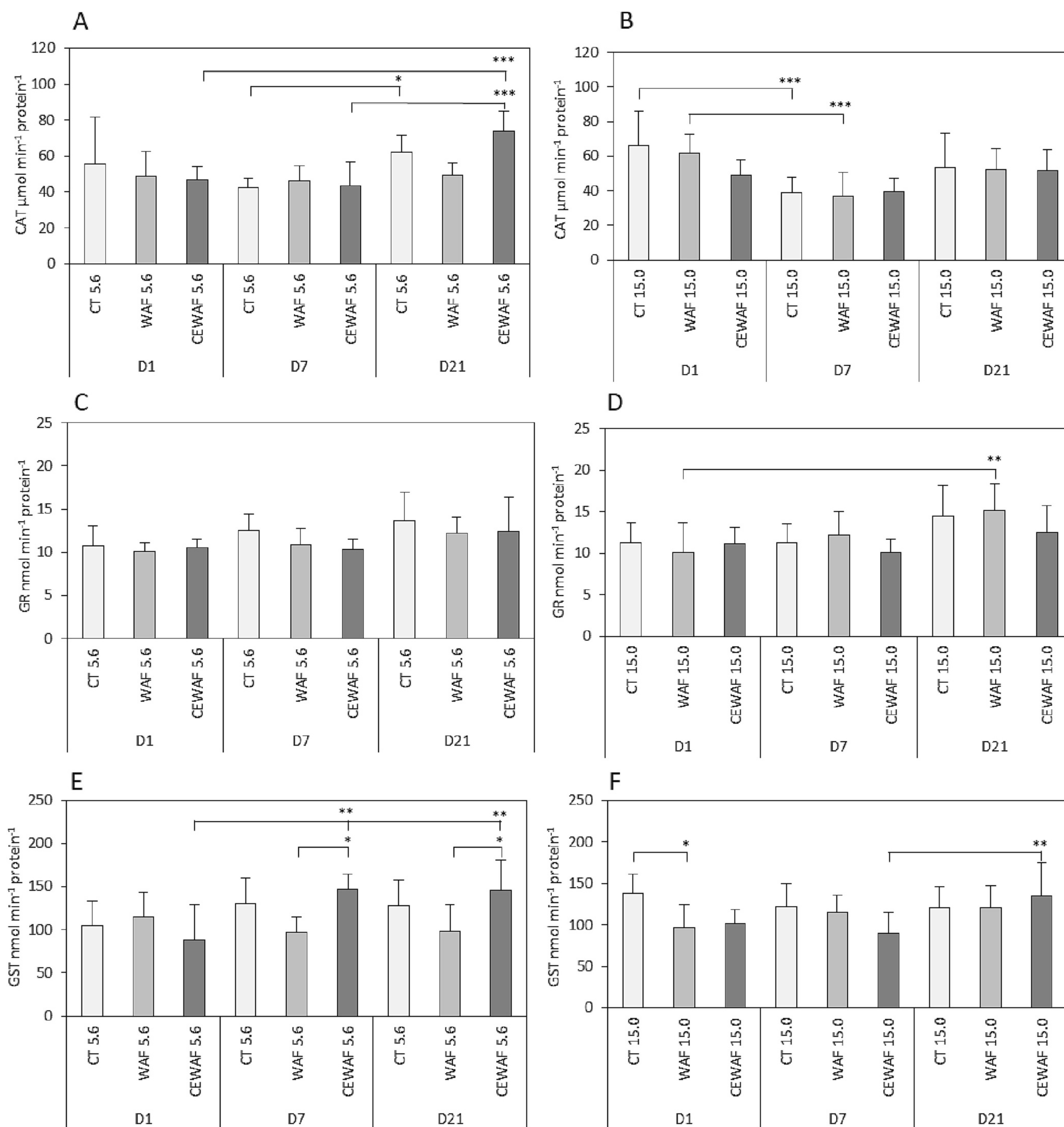

Fig. 2. ADS responses and detoxification (CAT [A–B], GR [C–D] and GST [E–F]) (mean ± SD) in *M. trossulus* during the exposure experiment at the different salinities and timepoints. Statistical significance: * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$.

WAF treated mussels on D7 and D21 (Fig. 2E). At the salinity of 15.0, lower GST activities were recorded in the WAF exposed mussels on D1 compared to the control group, with also the CEWAF treated mussels showing nearly a significant response (Fig. 2F). Also, at the salinity of 15.0, the CEWAF exposed mussels showed an increased GST activity on D21 compared to D7.

3.4.2. Oxidative damage

At the salinity of 5.6, the highest LPO level was recorded for the WAF exposed group of D7, being significantly higher compared to the same exposure group on D1 and D21, as well as to the mean level recorded for the CEWAF group on the same timepoint (Fig. 3A). At the salinity of 15.0 no effects on LPO could be observed (Fig. 3B).

3.4.3. Neurotoxicity

At both salinities the lowest AChE activities were observed at D21 in all treatments (Fig. 3C). At the salinity of 5.6, the activity had decreased markedly in the control group on D21 compared to D1 and D7, and in the WAF exposed group between D7 and D21. At the salinity of 15.0, the lowest AChE activities were recorded on D21 in all the treatments, while on D7 also the CEWAF exposed group showed significantly lower values compared to the control group (Fig. 3D).

3.4.4. Geno- and cytotoxicity

Total genotoxicity values (Gentox, ‰) were significantly lower in the control groups compared to the CEWAF exposed groups at every exposure timepoint at both salinities except for D1 at the salinity of 5.6, where no significant differences were found among the treatments (Fig. 4). The WAF exposed groups were significantly different from the control groups only on D21 at the salinity of 5.6 and on D7 at the salinity of 15.0.

At the salinity of 5.6, the total cytotoxicity values (Cytox, ‰) were significantly higher in both exposed groups compared to the control treatment on D21 (Fig. 4). At the salinity of 15.0, both exposed groups presented significantly higher values compared to the control group on D7, while on D21 this was observed only in the group exposed to CEWAF. Table S1 shows the individual geno- and cytotoxicity endpoints measured within each exposure group. Salinity had no effect on the geno- and cytotoxicity levels.

3.4.5. Tissue-level biomarkers

Regarding Vv_{BAS}, the control and CEWAF exposed mussels at the salinity of 15.0 showed significantly lower values on D21 compared to D1. When comparing treatments in the same sampling timepoint, mussels exposed to WAF exhibited significantly lower values than those exposed to CEWAF on D1 at the salinity of 15.0. Comparisons of the same experimental groups on the same timepoint at the different salinities showed that on D1 at the salinity of 15.0 the CEWAF exposed mussels showed higher Vv_{BAS} values than CEWAF exposed mussels at the salinity of 5.6. At the salinity of 15.0 on D21, significantly lower Vv_{BAS} values were observed in the control and CEWAF treatments compared to their counterparts at the salinity of 5.6 (Fig. 5).

The atrophy index exhibited significant differences at the different salinities in the D1 control group with mussels at 15.0 displaying higher values (Fig. 6). Mussels exposed to CEWAF presented significantly lower index values when compared to the control mussels on D1 at the same

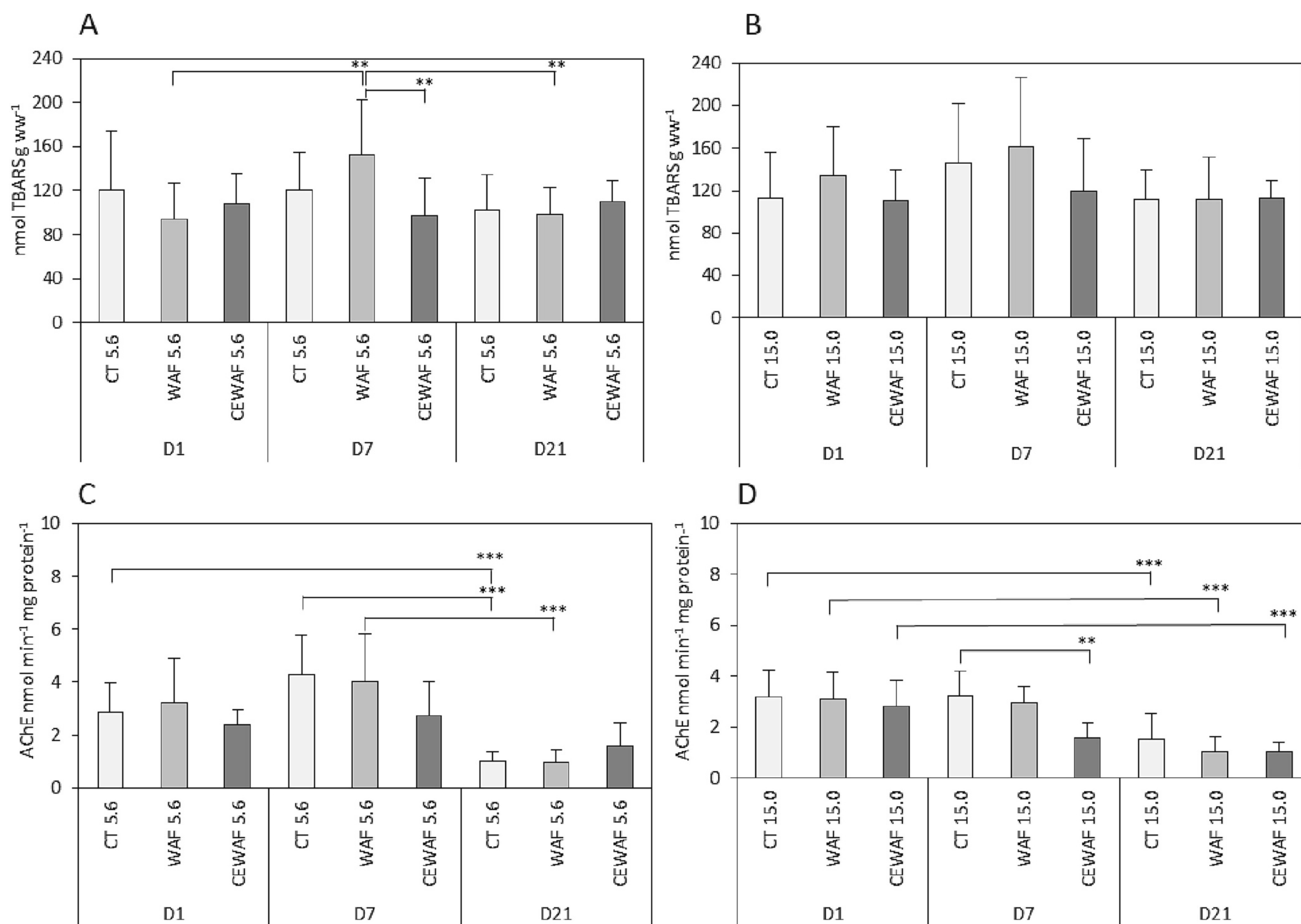

Fig. 3. Oxidative damage (LPO [A-B]) and neurotoxicity (AChE [C–D]) (mean ± SD) in *M. trossulus* during the exposure experiment at the different salinities, 5.6 (A–B) and 15.0 (C–D), and timepoints. Statistical significance: ** = p < 0.01, *** p < 0.001.

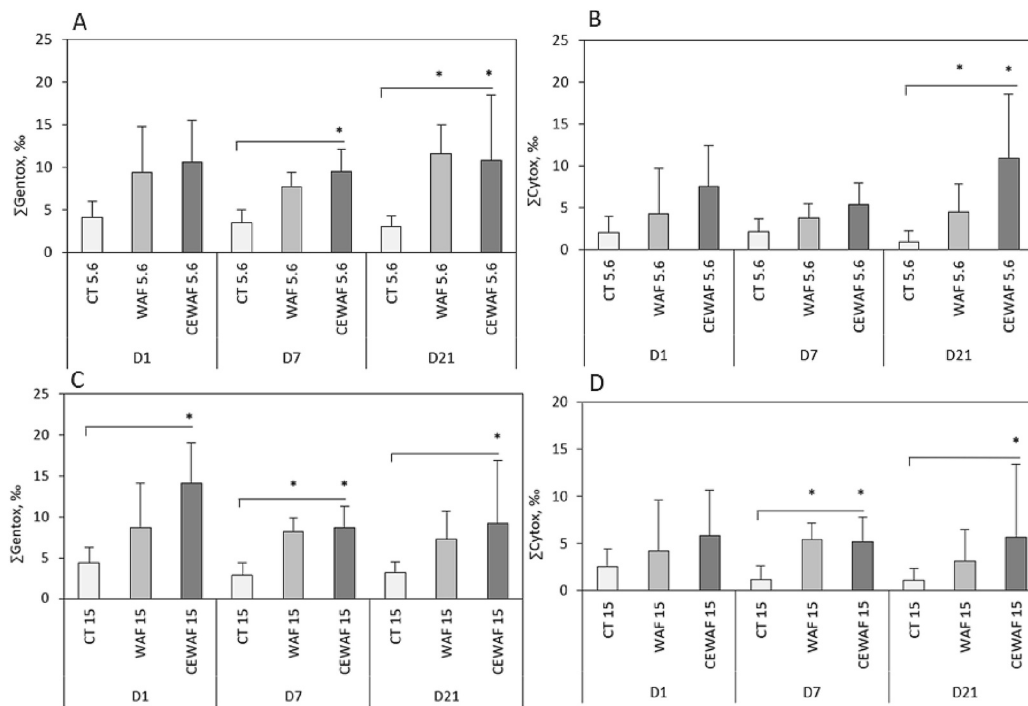

Fig. 4. Total genotoxicity (Gentox) and cytotoxicity (Cytox) levels (mean ± SD) measured in *M. trossulus* during the exposure experiment at the different salinities, 5.6 (A–B) and 15.0 (C–D), and timepoints. Statistical significance: * = $p < 0.05$.

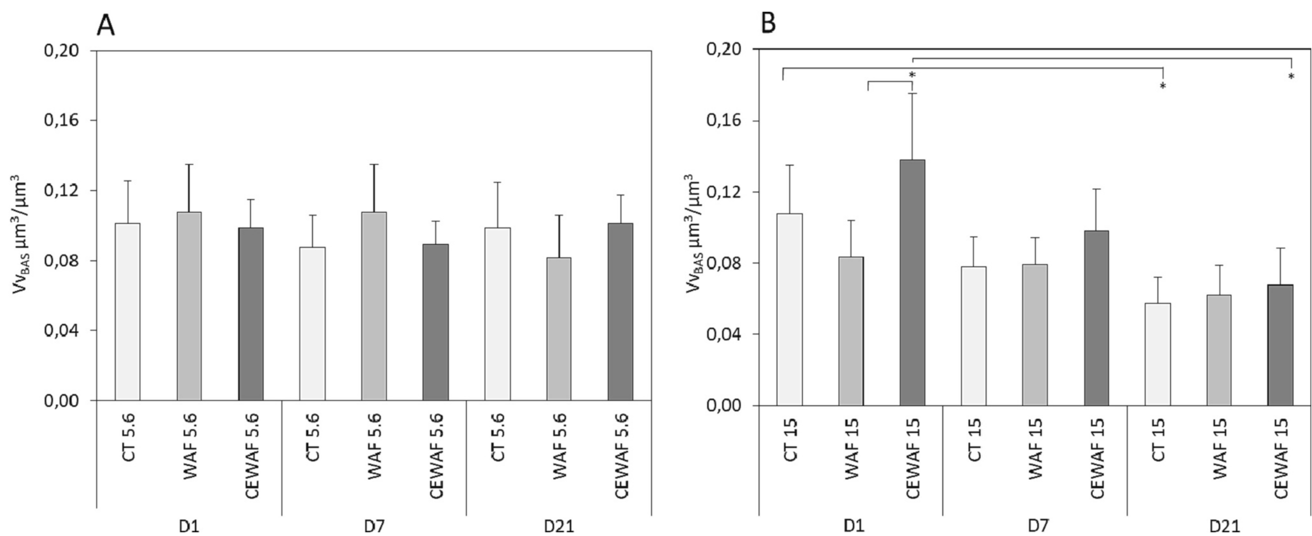

Fig. 5. Basophilic cell volume density (Vv_{BAS}) (mean ± SD) measured in *M. trossulus* during the exposure experiment at the different salinities, 5.6 (A) and 15.0 (B), and timepoints. Statistical significance: * = $p < 0.05$.

salinity. Control mussels also displayed a significant decrease in the atrophy index on D21 when compared to D1 controls at the salinity of 15.0.

Regarding the CTD ratio, all mussel groups at the salinity 15.0 experiment showed significantly lower values when compared to the same experimental groups at the salinity of 5.6 except for the D1 and D7 control groups (Fig. 7). Furthermore, when pooling the values of the control groups from the different timepoints and comparing their distribution using the Mann-Whitney *U* test the CTD values in the control groups at the 15.0 salinity were observed to be significantly lower.

3.4.6. Gonadal developmental stages

A marked fraction of the analysed mussels was going through the spawning process, or showed signs of an imminent or recent spawning, although great variability was observed among the specimens (Fig. 8). All the treatment groups showed a higher percentage of spawning mussels as the experiment advanced, except for the oil exposed groups at both salinities, which on D7 seemed to reach the maximum proportion of spawning and post-spawning individuals.

3.4.7. Integrated biomarker response index

At both salinities, the IBR index exhibited significant integrated stress signals in some of the groups of mussels exposed to WAF (all except D21 WAF at the salinity of 15.0) and in all the groups exposed to CEWAF when compared to the control groups at the respective salinities and timepoints (Fig. 9). The control group at the salinity of 15.0 on D1

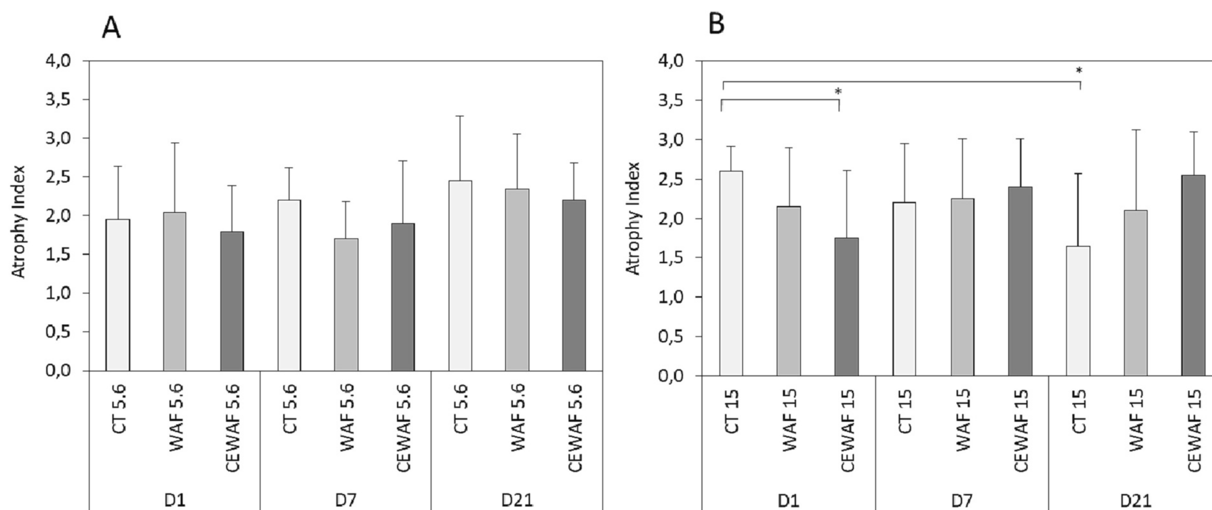

Fig. 6. The trophy index (mean ± SD) in *M. trossulus* during the exposure experiment at the different salinities, 5.6 (A) and 15.0 (B), and timepoints. Statistical significance: * = $p < 0.05$.

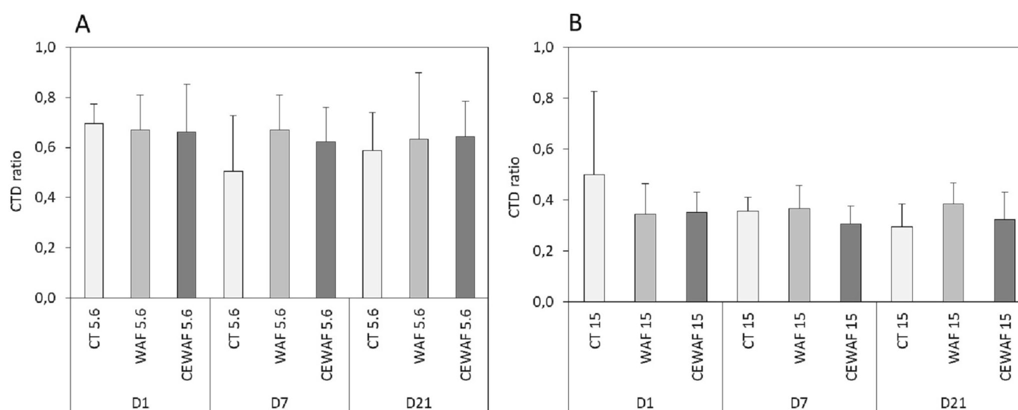

Fig. 7. CTD ratio (mean ± SD) in *M. trossulus* during the exposure experiment at the different salinities, 5.6 (A) and 15.0 (B), and timepoints.

was the exception as it also showed a stressed condition. Radar plots for a more detailed view of the IBR are available as supplementary material (Fig. 1S).

4. Discussion

4.1. General remarks

Compared to many other types of contaminants, examining the effects of exposure to oil is traditionally a highly complex issue. Various factors affect the behaviour of oil both in experimental situations as well as under natural conditions during releases of oil in the marine environment. Also, every oil is different, not only the main types of oil but different batches deriving from the same source can vary markedly in hydrocarbon composition, thus obviously affecting their toxicity (NASEM, 2022). In laboratory exposures the methods used in the preparation of the exposure medium can vary significantly, resulting in different concentrations and compositions of the compounds, or if the oil tested is present in water only as the "soluble" fraction, as microdroplets, or a mixture of these (Dupuis and Ucan-Marin, 2015). Considering the above, all oil exposure studies are in this sense unique and refer only to the materials and methods used (not to mention the test organisms), and this needs to be understood when extrapolating the results in oil spill risk assessments (Redman and Parkerton, 2015). The research accomplished in this study contributes to the knowledge on biological effects and accumulation dynamics of a specific crude oil in a sub-arctic environment at different salinities using a global model biomonitoring organism. It also focuses on the important question regarding environmental safety of using dispersants as a response method in oil spills.

4.2. Tissue accumulation of PAH

In the Baltic Sea, total soft tissue levels of PAH in mussels from areas considered less contaminated are usually <50 μg kg ww^{-1} (Benito et al., 2019; Turja et al., 2014a; Lehtonen et al., 2016). In areas affected by anthropogenic activities, including oil terminals and sites of intensive maritime traffic, tissue levels of PAH in mussels have been measured to range between ca. 100–200 μg kg ww^{-1} (Turja et al., 2014a; Lehtonen et al., 2019), which can, however, be considered low in comparison with many other impacted sea areas globally, and especially after major oil spills (Neuparth et al., 2012; NASEM, 2022). However, if the number of the analysed PAH compounds also included the methylated compounds, the background concentrations would be higher. The high interannual variability in seasonal abiotic and factors in the Baltic Sea such as temperature and primary production is also reflected in the background concentrations of PAH in the tissues of mussels (Lehtonen et al., 2019; Turja et al., 2020). In the present study, the concentration of PAH in the control mussels was in the range of 30–54 μg kg ww^{-1} at the beginning of the experiment, i.e., after a two-week acclimatization period in ASW.

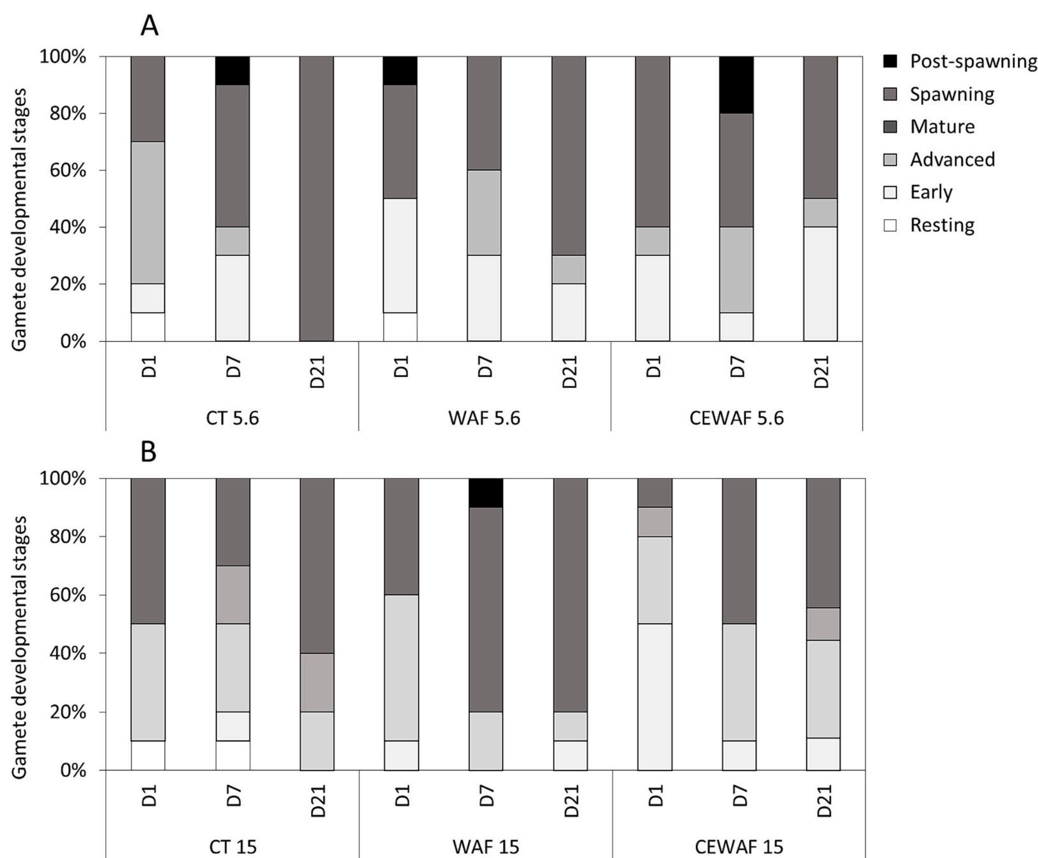

Fig. 8. Distribution of gamete developmental stages in *M. trossulus* during the exposure experiment at the different salinities of 5.6 (A) and 15.0 (B), and timepoints.

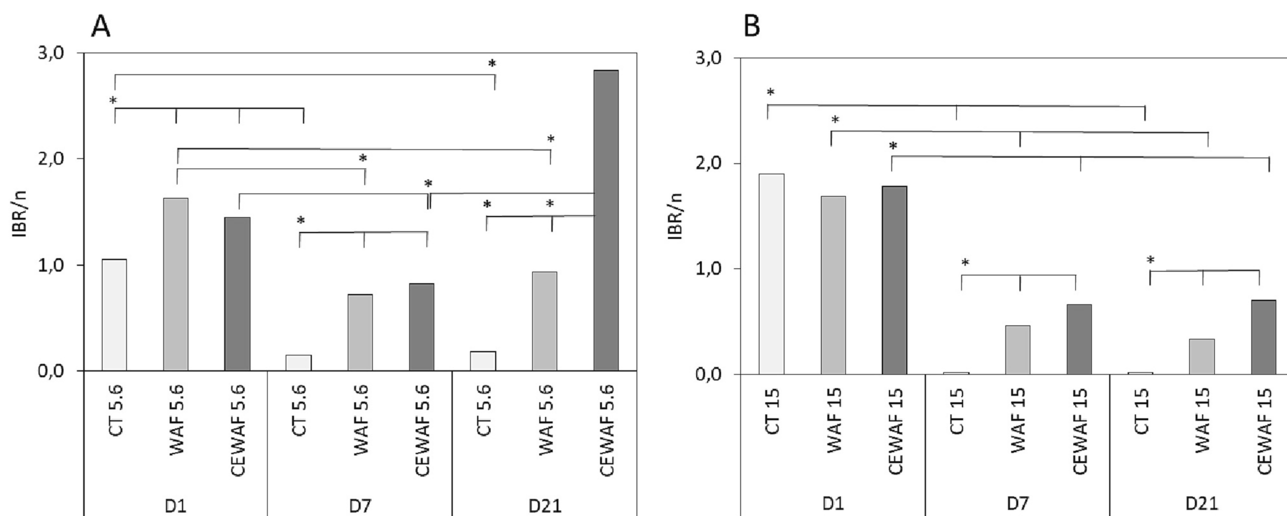

Fig. 9. Integrated Biomarker Response index (IBR/n) in *M. trossulus* during the exposure experiment at the different salinities of 5.6 (A) and 15.0 (B), and timepoints. Statistical significance: * $= p < 0.05$, ** $= p < 0.01$, *** $p < 0.001$.

During the experiment, exposures to WAF and CEWAF resulted in rapid tissue accumulation of PAH, and after reaching a certain level (by D7) the concentrations remained at this level as the exposure continued (D21). Schmutz et al. (2021) reported a similar pattern in crude oil exposed mussels with the highest PAH bioaccumulation observed after 3 and 7 days of exposure, followed by a gradual decrease after the end of the exposure in clean water (i.e., depuration period). In the current study, compared to the WAF treatments the dispersant used, Finasol OSR 51, increased the levels of PAH compounds in the CEWAF exposure media resulting in a markedly higher concentration at the salinity of 5.6 (88.99 μg L^{-1}) compared to the salinity of 15.0 (18.08 μg L^{-1}), which is in line with the product information claiming this dispersant being especially effective in brackish water. From D7 onwards the tissue concentration of PAH was ca. 3 times higher at the lower salinity (max. 8631.7 μg kg ww^{-1}) compared to the higher one (max. 2898 μg kg ww^{-1}). Thus, the CEWAF treatments in this experiment greatly differed

between the two salinities in terms of the levels of oil-derived compounds in the exposure media although the original amount of oil added was the same, resulting in marked differences in the bioaccumulation of PAH.

Alike to the study by Turja et al. (2020), methylated PAH were by far the dominant bioaccumulating compounds in the exposed mussels also in the present study. In the study above, methylphenanthrenes comprised 33–54 % of all the bioaccumulated PAH during the experiment. Methylated PAH are known to be very potent compounds inducing various toxic effects in aquatic organisms (Barjhoux et al., 2014; Lam et al., 2018; Rhodes et al., 2005). However, in bioaccumulation studies concerning PAH, methylated compounds have been much less frequently reported and the focus has been on the US EPA priority list of 16 PAH substances; this approach has been criticized as it greatly underestimates the environmental hazards of PAH (Andersson and Christine Achten, 2015). Accordingly, the above-mentioned authors proposed to include some of them – including 2-methylnaphthalene that showed the highest bioaccumulation in the present study – to the US EPA priority list, expanding it to 40 substances. Thus, more studies involving the various other types of PAH are obviously needed to make risk assessments regarding oil spills more realistic and reliable.

4.3. Biomarker responses

ADS responses are regulated through complex cellular pathways (Regoli and Giuliani, 2014). It has been shown that exposure to different PAH mixtures (including crude and diesel oil) results in enhanced, decreased, or unchanged ADS responses at transcriptional and/or catalytic levels in marine bivalves (Blanco-Rayón et al., 2019; Brooks et al., 2011; Frantzen et al., 2016; Luna-Acosta et al., 2017; Giuliani et al., 2013; Zanette et al., 2011). In the present study, ADS responses in mussels showed time-dependent changes in all treatments with generally more pronounced changes in the WAF and CEWAF treatments compared to the controls. CEWAF exposure at the salinity of 5.6 resulted in elevated CAT and GST levels towards D21 and the enzyme activities were higher compared to the WAF exposed mussels. However, in the WAF exposure at the salinity of 15.0, GST activity was first reduced (D1) and then elevated (D21) while the activity of CAT decreased between D1 and D7. It should be noted that the CEWAF exposure showed similar looking trends although they were not statistically significant. Baussant et al. (2009) and Turja et al. (2020) recorded corresponding biomarker responses with a somewhat low responsiveness of the ADS but increased genotoxic effects in mussels exposed to comparable oil concentrations. Specifically, *Mytilus edulis* exposed to the mechanically dispersed North Sea crude oil at nominal concentrations of 0.25, 0.06 and 0.015 mg L^{-1} (PAHs: 3.758, 0.812 and 0.154 μg L^{-1}, respectively) showed increased GST activity after exposure to the highest concentration while relevant genotoxic effects (elevated % DNA in Comet tail) were observed in all the oil exposed individuals at all the three concentrations used (Baussant et al., 2009). In the *M. trossulus* from the Baltic Sea, exposure to mechanically dispersed Arctic crude oil resulted in elevated CAT and superoxide dismutase activities in the digestive gland only at the highest oil concentration after four days of exposure while significantly elevated geno-and cytotoxicity parameters were detected at all exposure concentrations of 0.75, 0.120, 0.015 mg L^{-1} (PAHs: 3.555, 0.498, 0.04 μg L^{-1}) after 14 days (Turja et al., 2020). In the present study, LPO and AChE did not show any clear treatment related effects, except for D7 when the activity of AChE was decreased in the CEWAF treatment at the salinity of 15.0; however, this was followed by reduction in AChE levels in all treatment groups at both salinities on D21. Exposure to crude oil and PAH have been shown to affect the activity of AChE in fish (reviewed by Olivares-Rubio and Espinosa-Aguirre, 2021). In marine invertebrates, the decrease in AChE activity has been linked to the induction of other cellular mechanisms such as oxidative stress, LPO, DNA damage, mitochondrial malfunction, and histopathological damage (Deidda et al., 2021), and therefore in mussels it can also be interpreted as a response to general physiological stress over time (Maisano et al., 2017; Rank et al., 2007).

In the present study, significant geno- and cytotoxic effects were observed in WAF and CEWAF treated *M. trossulus*. In the control groups, background levels of the individual geno- and cytotoxicity parameters measured were detected (Davies and Vethaak, 2012; Stankevičiūtė et al., 2022), showing that exposure to the different salinities itself did not result in any significant alterations. Similarly, no relationship between salinity and MN frequencies in mussels collected from the North Sea, Wismar Bay, and the Baltic Sea (Lithuanian coast) has been observed (Davies and Vethaak, 2012). Effects of oil exposure on geno- and cytotoxicity biomarkers in mussels have been recorded in a number of previous laboratory studies (Caliani et al., 2022; Baršienė et al., 2010; Baršienė and Andreikėnaitė, 2007) and in organisms collected from areas polluted by oil and PAH (Parry et al., 1997; Baršienė et al., 2004, 2006a, 2012; Francioni et al., 2007; Hylland et al., 2008; Gorbi et al., 2008; Turja et al., 2014a); thus, the linkage of oil exposure to geno- and cytotoxic lesions is well-established. Caliani et al. (2022) observed higher MN, NB, and BN frequencies in the haemocytes of *Mytilus galloprovincialis* exposed to oil-polluted wastewater for 15 days, with NB being the most frequent nuclear abnormality identified. After an oil spill at a marine oil terminal in the Baltic Sea, Baršienė et al. (2012) reported an increase in the frequency of MN, NB, and FA cells in *M. edulis*. Sundt et al. (2011) observed elevated MN levels in *M. edulis* exposed to produced water (North Sea oil field) in both laboratory and field studies, as well as a link between MN frequency and PAH bioaccumulation. In the present study, significantly elevated frequencies of genotoxicity biomarkers including MN, NB, BL, and cytotoxicity markers such as FA cells were also reported. Katsumiti et al. (2019) conducted an in vitro study using the haemocytes of *M. galloprovincialis* to investigate WAF of NAA crude oil, both with and without the dispersant Finasol OSR 52, with WAF exhibiting the lowest cytotoxicity (MTT assay), followed by WAF + dispersant and the dispersant alone. The study also suggested that oxidative stress mediated by ROS was the primary mechanism involved in the toxicity of WAF, WAF + dispersant, and the dispersant itself.

In mussels, digestive gland is the key organ in digestion, energy storage, and metabolism (Marigómez and Baybay-Villacorta, 2003). The observed gradual decrease in Vv_{BAS} and CTD values during the experiment at the salinity of 15.0 suggests ongoing osmotic acclimatization processes in the digestive gland. At the same time, the reduced Vv_{BAS} values indicated a lower density of basophilic cells, which play a key role in digestion and metabolism. However, at the same time the lower CTD ratio suggested a higher level of digestive activity and thus a healthier status of the organ. Moreover, the atrophy index was decreased over time in the control treatment, indicating improved physiological condition of the mussels at the salinity of 15.0.

When compared to their respective control groups, effects of CEWAF and WAF exposure on the tissue level biomarkers in mussels can be observed on D7 and/or D21. Interestingly, these trends were more predominant at the salinity of 15.0, being concordant with a laboratory exposure study by Prevodnik et al. (2007) showing that even a small change in salinity (ca. 2) can cause highly significant effects in the scope for growth (SFG) in Baltic Sea mussels. In the study above, SFG was reduced in mussels exposed to petrol at the higher salinity compared to the control treatment, where the index was much higher than that observed at lower salinities, indicating a better energy balance and physiological condition. In addition, less oxidative damage, measured as the formation of protein carbonyls, was observed in the oil exposed mussels at the higher salinity. These findings above suggest that the low salinity conditions approaching the limits of the distribution of marine mussels may have a significant impact to mask any additional effects of pollutants on their growth and the physiological status of the digestive gland, which are already highly affected by the prevailing natural conditions.

Compared to the natural conditions prevailing in the Baltic Sea in

November–December, food availability probably caused mussels to invest their energy in reproduction to start spawning, which is confirmed by the gamete developmental stages described (Benito et al., 2019). The physiological stress caused by this process might have partially masked the extent of the response to oil (Benito et al., 2019; Cuevas et al., 2015). It is noteworthy that, at both salinities, the gamete developmental stages were different in the exposed groups when compared to the controls; on D21, the control mussels were mostly spawning, while on D7 and D21 the oil exposed mussels exhibited a similar share of spawning individuals, possibly indicating a differential gonadal development taking place under exposure to WAF and CEWAF. Reproductive impairments have previously been described in mussels exposed to oil (Baussant et al., 2011; Schmutz et al., 2021).

An integrative tool such as the IBR index is helpful to accentuate and simplify the single and diffuse stress signals in a holistic way (Brooks et al., 2011; Marigómez et al., 2013: Blanco-Rayón et al., 2019). On D1, the IBR was not consistent, most likely reflecting the transitional physiological changes reported to take place during the first days of toxicological experimentation using molluscs (Benito et al., 2019). However, elevated IBR values were recorded in the WAF and CEWAF treated groups on D7 and D21 at both salinities when compared to their respective controls, except for mussels exposed to WAF on D21 at the salinity 15.0. Conclusively, the WAF and CEWAF exposures generated a clear biomarker response in Baltic mussels, observed at least after one week's exposure and lasting at least until three weeks.

5. Conclusions

The results of the present study show that exposure to CEWAF of a specific crude oil (NNA) elicits a higher bioaccumulation of hydrocarbons in the tissues of Baltic *M. trossulus* compared to exposure to a WAF of the same oil, and, subsequently, elevated biomarker responses at the biochemical and cellular levels. These events were recorded at both studied experimental salinities, 5.6 (ambient) and 15.0 (experimentally increased). Higher concentrations of hydrocarbons were measured at the lower salinity media, this leading to markedly higher exposure levels in the CEWAF treatments. Acclimatization to the higher salinity caused apparent physiological adaptations in mussels, mirrored by significant changes in the different tissue level biomarkers. Baseline levels of most of the biomarkers measured were not affected by salinity; however, temporal variability occurred along the course of the experiment, probably due to laboratory conditions. Conclusively, the use of dispersants poses a risk of increased acute sublethal effects for marine organisms due to the subsequent manifold increase in hydrocarbon concentrations in water. Further studies are needed to examine (1) how long lasting are the effects of acute exposure (hours/days/weeks), and (2) a how long exposure is needed to cause significant higher level biological effects in the Baltic Sea organisms such as *M. trossulus*.

CRediT authorship contribution statement

Raisa Turja: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft, Visualization. **Denis Benito:** Methodology, Formal analysis, Investigation, Data curation, Writing – review & editing, Visualization. **Aino Ahvo:** Methodology, Investigation, Data curation. **Urtzi Izagirre:** Methodology, Investigation, Writing – review & editing. **Xabier Lekube:** Methodology, Investigation, Writing – review & editing. **Milda Stankevičiūtė:** Methodology, Investigation, Data curation, Formal analysis, Writing – review & editing. **Laura Butrimavičienė:** Methodology, Investigation, Resources, Writing – review & editing. **Manu Soto:** Methodology, Investigation, Resources, Writing – review & editing. **Kari K. Lehtonen:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Visualization.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2023.115100.

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