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Effects of microplastics alone or with sorbed oil compounds from the water accommodated fraction of a North Sea crude oil on marine mussels (*Mytilus galloprovincialis*)

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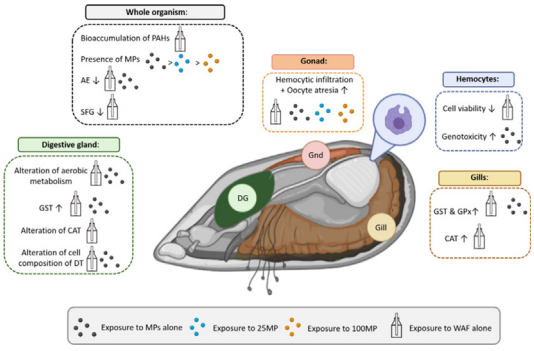
HIGHLIGHTS

- Mussels were exposed 21 d to 4.5 μm PS MPs alone or with sorbed WAF (MP25, MP100).
- MPs caused genotoxicity, changes in enzymes, cell composition, absorption efficiency.
- Mussels exposed to MP25 or MP100 did not accumulate PAHs in their tissues.
- Mussels exposed to WAF accumulated PAHs triggering molecular to individual responses.
- Exposure to MP, MP25, MP100 or WAF increased prevalence of oocyte atresia.

GRAPHICAL ABSTRACT



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ABSTRACT

Microplastics (MPs) can adsorb persistent organic pollutants such as oil hydrocarbons and may facilitate their transfer to organisms (Trojan horse effect). The aim of this study was to examine the effects of a 21 day dietary exposure to polystyrene MPs of 4.5 μm at 1000 particles/mL, alone and with sorbed oil compounds from the water accommodated fraction (WAF) of a naphthenic North Sea crude oil at two dilutions (25 % and 100 %), on marine mussels. An additional group of mussels was exposed to 25 % WAF for comparison. PAHs were accumulated in mussels exposed to WAF but not in those exposed to MPs with sorbed oil compounds from WAF (MPs-WAF), partly due to the low concentration of PAHs in the studied crude oil. Exposure to MPs or to WAF alone altered the activity of enzymes involved in aerobic (isocitrate dehydrogenase) and biotransformation metabolism (glutathione S-transferase). Prevalence of oocyte atresia and volume density of basophilic cells were higher and absorption efficiency lower in mussels exposed to MPs and to WAF than in controls. After 21 days MPs caused DNA damage (Comet assay) in mussel hemocytes. In conclusion, a Trojan horse effect was not observed but both MPs and oil WAF caused an array of deleterious effects on marine mussels at different levels of biological organization.

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1. Introduction

Plastics in oceans tend to accumulate according to their density and to fragment into smaller pieces eventually reaching dimensions lower than 5 mm called microplastics (MPs) (Andrady, 2011; Cole et al., 2011). In addition to these MPs, known as secondary ones, particles with original size lower than 5 mm are also introduced into the marine environment and are often indicated as primary MPs. MPs can be found in most parts of world's seas and oceans (Auta et al., 2017; Ying et al., 2020) and may cause disorders in marine organisms, including microalgae (Zhang et al., 2017), bivalves (Moore, 2008), crustaceans (Lee et al., 2013) and fish (Batel et al., 2016) among others (Andrady, 2011; Chae and An, 2017). MPs can be internalized by aquatic organisms with different feeding strategies from phytoplankton, zooplankton and invertebrates such as crabs and mollusks, to fishes, sea turtles, seabirds and cetaceans (reviewed by Pirsaheb et al., 2020) and they can alter their feeding activity, growth and reproduction or even cause mortality, as widely reported in copepods and bivalves (Browne et al., 2008; Van et al., 2012; Lee et al., 2013; Sussarellu et al., 2016; Oliveira et al., 2018).

Bivalve mollusks, and specially mussels, have been pointed as appropriate organisms to use both in field monitoring programs and laboratory studies to investigate the impact of MPs in the marine environment (Akdogan and Guven, 2019; Li et al., 2019). They are abundant, broadly distributed and sessile filter-feeders that accumulate contaminants in their tissues, reflect levels of environmental pollution and show significant responses to pollutants (Cajaraville et al., 2000; Vethaak et al., 2017). Furthermore, mussels have a key role in ecosystems and they are consumed by several species of higher trophic levels such as crabs, sea birds or seals, thus acting as vehicles of pollutants biomagnification (Beyer et al., 2017). Marine mussels can ingest MPs (Sendra et al., 2021) which have been reported to cause oxidative stress (Paul-Pont et al., 2016), immunological responses (Pittura et al., 2018; Green et al., 2019), alterations in gene expression patterns (Détrée and Gallardo-Escárate, 2017; Capolupo et al., 2018), genotoxicity (Gonzalez-Soto et al., 2019), neurotoxicity (Avio et al., 2015) and changes in the physiology of mussels (Wegner et al., 2012; van Cauwenberghe et al., 2015).

An additional risk of MPs to marine biota comes from their ability to adsorb persistent organic pollutants (POPs) from the environment due to their large surface to volume ratio and hydrophobicity (Bakir et al., 2016; Ahmed et al., 2021). Several laboratory studies have demonstrated that POPs such as polycyclic aromatic hydrocarbons (PAHs) sorbed to MPs can bioaccumulate in exposed animals such as fish (Rochman et al., 2013), clams (O'Donovan et al., 2018) and mussels (Avio et al., 2015; Gonzalez-Soto et al., 2019; Von Hellfeld et al., 2022) and enhance toxic effects of MPs. Moreover, in aquatic animals exposed to mixtures of MPs and PAHs the presence of the particles influences the biotransformation, bioaccumulation and toxicity of the latter (Oliveira et al., 2013). However, unrealistic high concentrations of POPs are usually used in laboratory studies (Burns and Boxall, 2018). To the best of our knowledge, there are no studies addressing the effects of complex mixtures of PAHs or other oil hydrocarbons sorbed to MPs on bivalves (De Sá et al., 2018). However, PAHs and other oil hydrocarbons are known to be ubiquitous pollutants, widely found in the sea, mainly due to the anthropogenic activity in land, maritime traffic, off-shore production of oil and gas and oil spills (Meador et al., 1995; Abdel-Shafy and Mansour, 2016) and thus, they can co-exist with MPs in natural environments.

Crude oil is a complex mixture that contains thousands of different chemicals (Carls and Meador, 2009) and it is one of the most complex and variable products to evaluate toxicologically (Singer et al., 2000). Toxicity of oil in an aquatic system is usually assessed through its water accommodated fraction (WAF). The WAF is a laboratory prepared medium where a test material difficult to dissolve, such as crude oil, is placed in a volume of water and mixed for hours at low energy avoiding the formation of small oil drops (Aurand and Coelho, 1996). Effects of different WAFs on mussels include alterations of enzyme activities, impairment of the lysosomal system, tissue damage, impact on the reproductive capacity, decrease of condition index and mortality (Cajaraville et al., 1991, 1992a, 1992b; Marigómez and Baybay-Villacorta, 2003; Counihan, 2018; Blanco-Rayón et al., 2019).

This work fills a knowledge gap by studying the impact of a complex mixture of petroleum hydrocarbons derived from WAF sorbed into MPs. The hypothesis of the work is that MPs can act as carriers of oil hydrocarbons and enhance their toxicity at different levels of biological organization to marine mussels *Mytilus galloprovincialis* through the so-called Trojan horse effect. For this purpose, the effects of dietary exposure to polystyrene (PS) MPs of 4.5 μm alone or previously incubated in two dilutions (25 % and 100 %) of WAF of a naphthenic crude oil were investigated in mussels, using a battery of biological responses at molecular, cellular, tissue and organism levels. In parallel, a group of mussels was exposed to a 25 % dilution of WAF for comparison.

2. Materials and methods

2.1. Obtention and preparation of MPs, WAF and MPs-WAF

As in previous experiments (Gonzalez-Soto et al., 2019), unlabeled 4.5 μm PS microspheres (Polyscience Inc., cat.# 17135-5; density 1.05 g/cm^3) were used. Hydrodynamic size (*Z*-average) and surface charge (Z-potential) were already reported by Katsumiti et al. (2021), being 5009 ± 188 nm and −53.7 ± 0.61 mV, respectively. PS beads were selected for this work because they are known to adsorb PAHs from the WAF (Martínez-Álvarez et al., 2022) and because they comprise the most common polymer type studied in the literature, thus allowing comparison with current literature.

The WAF of a naphthenic North Sea crude oil was selected as a complex mixture of persistent organic pollutants. The crude oil was kindly provided by Drifts laboratoriet Mongstad, Equinor, Norway (former Statoil) and contained 28.6 % w/v of PAHs. WAF was produced at a ratio of 200:1 sea water/crude oil (w/w) at 20 °C based on the protocol of Singer et al. (2000) with modifications. The sand filtered (particle size ≤ 0.2 μm) marine water was added to glass bottles 24 h prior to the addition of the oil, to achieve the desired temperature. The stirrer was switched on (at low speed, without vortex) once oil was in contact with the water. The bottles were covered with aluminum paper and kept in agitation for 40 h. The 25 % WAF dilution was obtained by diluting the WAF with sand filtered marine water.

For preparation of MPs with sorbed oil compounds from WAF, incubation of MPs with WAF was based on Batel et al. (2016) and Gonzalez-Soto et al. (2019). The MPs stock (89.8 μL, 4.9 × 10^8 particles/mL) was incubated with 25 % WAF and 100 % WAF for 24 h, to get MP25 and MP100, respectively. The vials were incubated at 300 rpm in an orbital shaker at 18 °C in darkness. Then, the suspensions were syringe filtered using sterile 0.45 μm pore sized filters (Merck Millipore, Darmstadt, Germany). The MPs were resuspended in 40 mL of Milli-Q water and added to the algae feed for each respective mussel treatment. MPs were mixed with microalgae (1:100000 MP/microalgae-day-aquarium during the first 7 days and 1:50000 MP/microalgae-day-aquarium after the mussel sampling at day 7) and dosed freshly to mussels as explained in Section 2.2 (Fig. 1) during the 21 days. Non-contaminated pristine MPs were incubated, filtered and resuspended in the same way but using Milli-Q water. The final concentration of MPs in mussel exposure tanks was assessed taking into consideration the amount of MPs recovered from the filters that was added to the algae tanks used to feed the mussels. Then, the volume of the MPs-algae suspension that ensured the desired concentration of MPs in mussel exposure tanks was dosed by constant dripping with the help of a peristaltic pump.

2.2. Sampling and maintenance of mussels

Roughly 600 mussels *Mytilus galloprovincialis* (3.5–4.5 cm in shell length) were collected in February 2018 in Mundaka, Urdaibai's Biosphere Reserve (43°24′04.9″N, 2°41′ 41.6″W), a relatively clean area (Puy-Azurmendi et al., 2010; Bellas et al., 2014) and maintained for acclimation in aquarium facilities at the Plentzia Marine Station (PiE) of the University

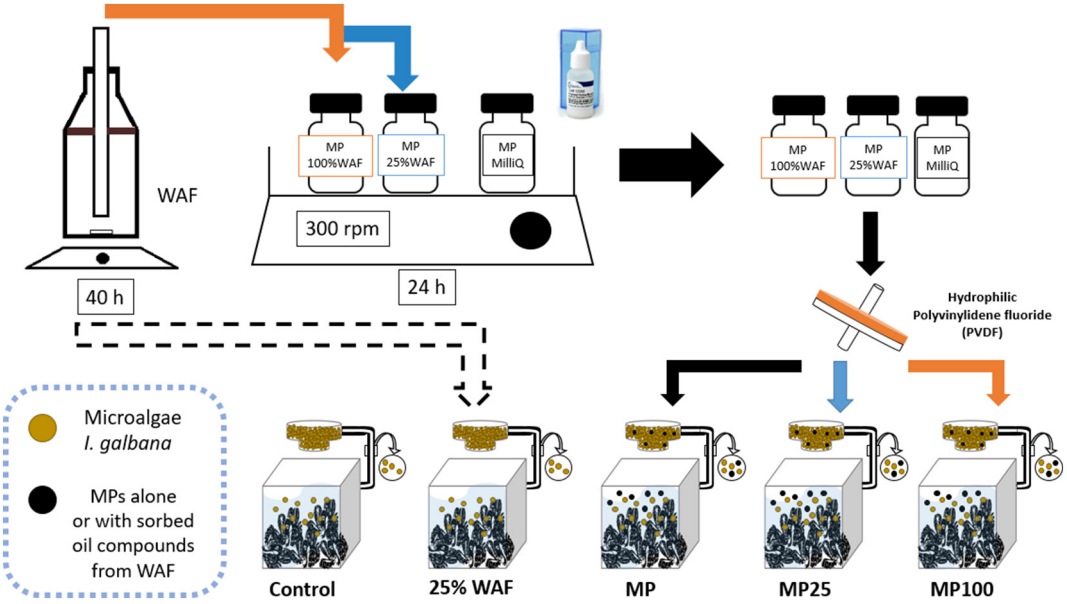


Fig. 1. Scheme for the preparation of WAF, MPs alone (MP) and MPs with sorbed oil compounds from WAF (MP25 and MP100).

of the Basque Country (UPV/EHU) for 7 days. Mussels were placed in a single large 300 L polypropylene tank with a recirculating filtered seawater system (particle size ≤ 3 μm) for 2 days without feeding. Then mussels were distributed in 5 glass aquaria (120 mussels per aquarium) with 40 L seawater and fed with a suspension of *Isochrysis galbana* microalgae cells (T-Iso clone) close to maintenance ration (Bayne et al., 1976) for another 5 days. This was achieved by dosing 36 × 10^9 cells of *I. galbana*/mussel-day with a multichannel peristaltic pump (Watson-Marlow, United Kingdom) over a period of 22 h/day as in Gonzalez-Soto et al. (2019). During both the acclimation and exposure periods, all tanks were rinsed every day with 10 L of seawater to remove feces and then water was renewed. During acclimation, water quality was checked daily in all tanks. Light regime was 12L/12D and room temperature was kept at 14 °C. Water parameters were checked using a multi parametric probe (mean ± standard deviation): salinity 28.56 ± 0.28 PSU, dissolved O_2 94.95 ± 2.90 %; 8.51 ± 0.25 mg/L, pH 7.3 ± 0.04 and temperature 12.3 °C.

2.3. Mussel exposure experiment

5 experimental groups were set: control, MPs alone (MP), MPs with sorbed oil compounds from WAF at a 25 % dilution (MP25) and MPs with sorbed oil compounds from 100 % WAF (MP100). Mussels were exposed through the diet to a mixture of MPs and microalgae, at 1000 MP particles/mL-day (0.058 mg/L), which is considered an environmentally relevant concentration (Eriksen et al., 2013; Lechner et al., 2014; Gonzalez-Soto et al., 2019). In the fifth group (WAF), mussels were exposed to WAF at a dilution of 25 %, considering that the hydrocarbon content of this dilution was equivalent to hydrocarbons sorbed to plastics incubated with 100 % WAF (Martínez-Álvarez et al., 2022). This approach of using the sorption percentage to select the dose of the "soluble" control has already been described (Chen et al., 2017). On the other hand, the 25 % WAF exposure could be compared to the plastics incubated with 25 % WAF (MP25) following the approach used by Paul-Pont et al. (2016), where the same concentration was used in the PAH alone exposure and in the exposure to MPs with sorbed PAHs.

During exposure, mussels were fed with *I. galbana* microalgae or with microalgae mixed with the MPs, in the same way than in the acclimation period. Aeration was used to keep both plastics and algae in suspension. Water from each aquarium was renewed every day, after algae/algae + MPs dosing finished. During exposure, light regime and room temperature were kept as in acclimation. Water quality was checked every two days in the control tank using a multi parametric probe (mean ± standard deviation): salinity 29.13 ± 0.8 PSU, dissolved O_2 90.02 ± 7.9 %; 8 ± 0.50 mg/L, pH 7.3 ± 0.05 and temperature 13.29 ± 0.52 °C. Mussels were dissected after 7 and 21 days of exposure.

Two different exposure times (7 and 21 days) were selected to elucidate toxicity mechanisms at different biological levels at short and middle term exposure times. 21 days exposure to MPs has been previously reported to be long enough to produce an impact at histological, physiological and/or organism levels, in agreement with some of the endpoints that were studied in this work (e.g.: Brate et al., 2018; Putri et al., 2021; Gonçalvez et al., 2022). Following sampling on day 7, dosing of algae was recalculated in order to maintain the same microalgae and MP rations.

2.4. Bioaccumulation of PAHs in mussel soft tissues

Whole mussels for chemical analysis (21 per experimental group) were stored at −40 °C and analyzed at the General Services SGiker of UPV/EHU. Measurements were done in 3 pools of 7 animals per each experimental group. Tissues were extracted using MARX microwave (CEM, Mathews, NC, USA) and cleaned through solid phase extraction (SPE Vacuum Manifold System, Millipore). The extracts obtained were concentrated through evaporation with nitrogen flow LV Evaporator (Zymarck, Hopkinton, MA, USA), filtered and encapsulated in chromatographic vials. Concentrations of 16 EPA PAHs were measured in an Agilent (6890) Gas Chromatograph with Mass Detector (Agilent 5975C) as described by Navarro et al. (2008) and given as ng/g dry weight. Calibration standards were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). In all cases, recovery of analytes was approximately 90 %, except for naphthalene that was 60 %. The detection limits (LOQ) of the analytical method were: 30 ppb for naphthalene, 1 ppb for acenaphthylene, 10 ppb for acenaphthene, and 0.5 ppb for the rest of PAHs.

2.5. Tissue distribution of MPs in mussels

Digestive gland, gills and mantle tissue of 10 mussels per experimental group were dissected out. Half of the digestive gland, half of the gills and whole mantle were processed for tissue localization of MPs using the N-butyl alcohol protocol as described in Gonzalez-Soto et al. (2019). The prevalence of mussels showing MPs in the digestive gland, gills and gonad were calculated as percentages.

2.6. Whole organism responses

After 21 days of exposure, 7 mussels from each treatment were collected to determine physiological parameters (clearance rate, respiration rate, absorption efficiency, scope for growth) and condition index as previously described (Gonzalez-Soto et al., 2019).

2.7. Gamete development, gonad index and histopathology of mussel gonad

Gonad tissues of 15 mussels per experimental group were dissected out and processed for histopathological analysis, determination of gamete developmental stages and gonad index as described in Gonzalez-Soto et al. (2019). The following alterations were recorded and prevalences given as percentages: occurrence of parasites, fibrosis in the connective tissue, hemocytic infiltration, oocyte necrosis and oocyte atresia (Ortiz-Zarragoitia and Cajaraville, 2006, 2010; Ruiz et al., 2014). Oocyte atresia was semiquantified based on the following scale: 1) ¼ of follicles in the tissue section showed signs of atresia, 2) about half the follicles in the tissue section showed signs of atresia, 3) ¾ of follicles in the tissue section showed signs of atresia and 4) all follicles in the tissue section showed signs of atresia (Kim et al., 2006).

2.8. Cellular biomarkers in mussel hemocytes

The hemolymph of 8 mussels per treatment (same individuals as in Section 2.7) was withdrawn from the posterior adductor muscle and cell viability, catalase activity and DNA damage were measured in hemocytes of individual mussels.

Cell viability was assessed by the neutral red uptake assay as described in Gonzalez-Soto et al. (2019). Cell concentration determined in the hemolymph of each animal using a Bright-Line™ Hemacytometer (Sigma Aldrich, St. Louis, USA) was used to normalize absorbance data. Catalase activity was determined as described in Gonzalez-Soto et al. (2019). Protein concentration (Lowry et al., 1951) was used to normalize absorbance data. Catalase activity was expressed as μmol H_2O_2/min*mg protein. The Comet assay was performed on mussel hemocytes following Raisuddin and Jha (2004) with modifications described in Gonzalez-Soto et al. (2019).

2.9. Enzyme activities in mussel tissues

Taking into consideration results on the bioaccumulation of PAHs, the presence of MPs in the groups exposed to MP25 and MP100 and the rest of results, enzyme activities were only determined in the control group and in groups exposed to MPs and to WAF.

Gills of 10 mussels and digestive glands of 15 mussels per experimental group (same individuals as in Section 2.7) were dissected out, frozen in liquid nitrogen and stored at −80 °C until analysis. Half of each gills were homogenized in 0.1 M KP buffer (pH = 6.5) to determine the activity of the enzymes glutathione *S*-transferases (GST). The second half of gills was homogenized in 0.1 M KP buffer (pH = 7.4) for catalase and glutathione peroxidase (GPx) activity determination. Digestive glands were divided in three portions. The first one was used for GST activity determination, the second one for palmitoyl-CoA oxidase (AOX1), catalase and GPx activity determination and the last portion was homogenized in 50 mM Tris(hydroxymethyl)-aminomethan buffer (pH = 7.8) for isocitrate dehydrogenase (IDH) activity determination. Tissues were homogenized in each buffer following a 1:10 proportion, tissue weight: volume.

AOX1 activity was determined as described in De los Ríos et al. (2013) and expressed as the oxidation of leuco-dichlorofluorescein in mU AOX1/mg prot. IDH activity was determined according to Ellis and Goldberg (1971) and expressed as the production of nicotinamide adenine dinucleotide phosphate (NADPH) in nmol/min/mg protein. GST activity was determined according to Habig et al. (1974) and expressed as the production of 1-chloro-2,4-dinitrobenzene (CDNB) conjugates with the thiol group of glutathione in nmol/min/mg protein. Catalase activity was determined as explained in Section 2.8. GPx activity was determined according to Flohé and Günzler (1984) and expressed as the consumption of NADPH in nmol/min/mg protein. Activity of IDH, GST and GPX were measured in microplates as in Lima et al. (2007). Each enzyme activity was normalized to protein concentration measured using the Bradford method (Bradford, 1976) with the Quick Start™ Bradford Protein Assay Kit from Bio-Rad, following manufacturer's recommendations.

2.10. Cell composition, quantitative structure and histopathology of mussel digestive gland

Paraffin sections of the digestive gland stained with hematoxylin/eosin described in Section 2.5 were used (from control mussels and mussels exposed to MPs and to WAF). Cell composition of digestive tubules was measured in terms of volume density of basophilic cells (VvBAS, given as $\mu m^3/\mu m^3$) and the structure of digestive tubules was quantified as mean epithelial thickness to mean diverticular radius (MET/MDR, μm/μm) and mean luminal radius to MET (MLR/MET, μm/μm) by using a stereological method (Bignell et al., 2012; De los Ríos et al., 2013). The analysis was performed as previously described in Gonzalez-Soto et al. (2019) in 10 mussels per experimental group. Then, parameters were calculated as reported by Bignell et al. (2012).

Prevalences of the following histopathological alterations were recorded for the same 10 mussels per experimental group: occurrence of parasites, fibrosis in the connective tissue, atrophy of digestive tubule epithelium, focal and diffuse hemocytic infiltrations, and accumulation of brown cells in connective tissue and in epithelium of the digestive tract (Bignell et al., 2012; Ruiz et al., 2014; Gonzalez-Soto et al., 2019). Results are given as percentages.

2.11. Data analysis

Statistical analyses were carried out using the statistical package SPSS 24 (IBM Analytics, Armonk, NY). When possible outliers were eliminated with the Chauvenet method. All data were tested for normality and homogeneity of variances using Kolmogorov-Smirnovs and Levene's tests, respectively. Normally distributed data, which met the assumptions of homogeneity of variances, were assessed via one-way ANOVA and the Tukey's post hoc was used for differences among treatments within the same day, while the Student's *t*-test was used to assess the differences between days within the same treatment. Data which did not met the above assumptions were analyzed by the one-way Kruskal-Wallis test. The Dunn's post hoc test was used to determine the differences among treatments within the same day and the Mann-Whitneys *U* test to determine the differences between days within the same treatment. Since histopathological data were expressed as percentages, the X^2 test was used (Ruiz et al., 2014). In all cases, significance was established at $p < 0,05$.

3. Results

3.1. Bioaccumulation of PAHs in mussels' soft tissues

PAH concentrations were close to detection limits in control mussels and in mussels exposed to MPs alone and similar to the PAH concentrations measured in mussels exposed to MP25 and MP100 (Table 1, Table S1). The total amount of PAHs recorded ranged from 142 ± 39.27 to 188 ± 40.29 ng/g dry weight (control group and MP100, respectively) at day 7 and from

Table 1
Bioaccumulation of Σ 16 EPA PAHs in mussel soft tissues (ng/g dry weight). Data is given as mean ± standard deviation for 3 pools of 7 mussels, except for controls at day 21 that were 2 pools.

	Control	MP	MP25	MP100	WAF
7 D	142 ± 39.27	146 ± 21.48	174 ± 36.06	188 ± 40.29	2306 ± 372.42
21 D	247 ± 59.89	156 ± 11.42	157 ± 19.58	169 ± 11.70	1229 ± 165.30

Table 2
Prevalence of mussels showing MPs in different tissues of control mussels and mussels exposed to MP, MP25 and MP100 for 7 and 21 days. Data are shown as percentages of 8–10 mussels per experimental group (one section per mussel). Not observed, n.o. No MPs were observed in Control and WAF groups.

	Gonad		Gill		Digestive gland	
	7D	21D	7D	21D	7D	21D
MP	n.o.	10	33.33	20	90	90
MP25	n.o.	n.o.	22.22	n.o	44.44	50
MP100	n.o.	n.o.	22.22	70	20	30

156 ± 11,42 to 247 ± 59,39 ng/g dry weight (MP and control group, respectively) at day 21 of exposure (Table 1, Table S1). Naphthalene was the main PAH detected in all the cases. Mussels exposed to WAF bioaccumulated PAHs to a great extent, especially at day 7. Main PAHs accumulated were phenanthrene, pyrene, fluorene, acenaphthylene, fluoranthene and crysene.Higher total PAH values were measured at day 7 (ΣPAHs 2306 ± 372.42 ng/g dry weight) than at day 21 (ΣPAHs 1229 ± 165.30 ng/g dry weight) (Table 1, Table S1).

3.2. Tissue distribution of MPs in mussels

MPs were found in different tissues of mussels exposed to MP, MP25 and MP100. 20 to 90 % of mussels treated with MPs presented MPs in the digestive gland (Table 2). MPs were mostly found in the lumen of the stomach mixed with the stomach content (Table 3, Fig. 2A). MPs were also observed in the lumen of ducts, though to a lesser extent. MPs were observed occasionally in the lumen of the digestive tubules, within the epithelium of the digestive tract and in the connective tissue of the digestive gland (Table 3 Fig. 2B;C). MPs were found in the gills of 20 to 70 % of treated mussels with the exception of mussels exposed to MP25 for 21 days, whose gills did not show any MP (Table 2). MPs were mostly found interspersed between gills filaments, and occasionally within hemocytes (Fig. 2D;E). In gonads, MPs were only found in one of the mussels exposed to MP for 21 days (Table 2). In this individual MPs were localized in the connective tissue and within gonad follicles (Fig. 2F).

Overall, the occurrence of MPs showed high variability among individuals of each exposure group. The highest amount of MPs was found in mussels exposed to MP, followed by mussels exposed to MP25 and mussels exposed to MP100 (Table 3). The amount of MPs in tissues of mussels decreased from day 7 to 21 in mussels exposed to MP, whereas it increased in mussels exposed to MP25. No time-dependent differences were detected in mussels exposed to MP100 (Table 3).

3.3. Whole organism responses

After 21 days of exposure, there were no significant differences in the condition index, clearance rate and respiration rate among treatments (Fig. S1).

Mussels exposed to MP and to WAF showed significantly lower absorption efficiency than controls and mussels exposed to MP25 (Fig. 3A) at day 21 of exposure. Mussels exposed to WAF showed lower scope for growth than control mussels and than mussels exposed to MP25 (Fig. 3B) at day 21 of exposure.

3.4. Gamete development, gonad index and histopathology of mussel gonad

In all treatments, 3 gametogenic stages were dominant at both exposure times: advanced gametogenesis, mature gonad and spawning gonad (Fig. S2). Gonad index (GI) was around 4 in all groups, with a significant increase in mussels exposed to MP25 compared to control mussels and mussels exposed to MP100 and WAF at day 7 of exposure (Fig. S2A). No significant differences among groups were recorded at day 21, but GI increased in controls in comparison with day 7 (Fig. S2A). In females, no significant differences in the GI were found (Fig. S2B) whereas in males a higher GI was observed in mussels exposed to MP25 compared to control mussels and mussels exposed to MP100 and WAF at day 7 of exposure. In addition, mussels exposed to MP100 for 21 days showed lower GI than mussels exposed to MP and the GI of the controls increased from day 7 to day 21 of exposure (Fig. S2C).

In general, low prevalences of inflammatory responses such as fibrosis and hemocytic infiltration were recorded in all exposure groups (Fig. S3, Table S2) although higher hemocytic infiltration was recorded in mussels exposed to MP25, MP100 and WAF after 21 days of exposure comparing to controls. The prevalence of hemocytic infiltration in controls decreased from day 7 to 21 (Table S2). At day 21 of exposure mussels exposed to MP, MP25, MP100 and WAF showed higher prevalences of oocyte atresia than controls (Table S2). Meanwhile, prevalence of oocyte atresia decreased significantly from day 7 to 21 in controls (Table S2). Oocyte necrosis was observed in a single mussel exposed to MPs at day 21 and a trematode parasite was found in another mussel exposed to MPs for 21 days (Table S2).

3.5. Cellular biomarkers in mussel hemocytes

Neutral red uptake was not significantly affected by any treatment at day 7 whereas it was significantly reduced in mussels exposed to WAF at 21 days in comparison to control mussels, to mussels exposed to MP and MP25 as well as to mussels exposed to WAF at 7 days (Fig. 4A).

Catalase activity in hemocytes was not altered after any treatment (Fig. S4).

Background levels of DNA damage were observed in control groups (Fig. 4B). No significant effects were recorded at day 7 of exposure, while after 21 days of exposure to MP, DNA damage was significantly higher than in the control group (Fig. 4B). DNA damage decreased in control mussels along the exposure time (Fig. 4B).

3.6. Enzyme activities in mussel tissues

At day 7, IDH activity in mussels exposed to MPs was significantly higher than in control mussels, whereas at day 21 of exposure it was lower than in controls and in mussels exposed to WAF. At the same time, activity in mussels exposed to WAF was significantly lower than in controls.

Table 3
Tissue distribution of MPs in the digestive gland of mussels exposed to MP, MP25 and MP100 for 7 and 21 days (10 mussels per experimental group and one section per mussel). Not observed, n.o.

		MP		MP25		MP100	
		7D	21D	7D	21D	7D	21D
Stomach	Lumen	35.22 ± 22.24	19.43 ± 9.74	4.33 ± 0.48	45.6 ± 21.02	1 ± 0	2.66 ± 0.66
	Epithelium	1 ± 0	1 ± 0	n.o.	1 ± 0	n.o.	n.o.
Ducts	Lumen	2.5 ± 0.22	n.o.	16 ± 0	n.o.	n.o.	n.o.
	Epithelium	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.
Digestive tubules	Lumen	1 ± 0	1 ± 0	n.o.	n.o.	n.o.	n.o.
	Epithelium	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.
Connective tissue		1 ± 0	1 ± 0	n.o.	n.o.	1 ± 0	n.o.
Total MPs in DG		32.8 ± 21.20	13.9 ± 8.44	2.9 ± 2.18	22.9 ± 15.92	0.2 ± 0.13	0.8 ± 0.51

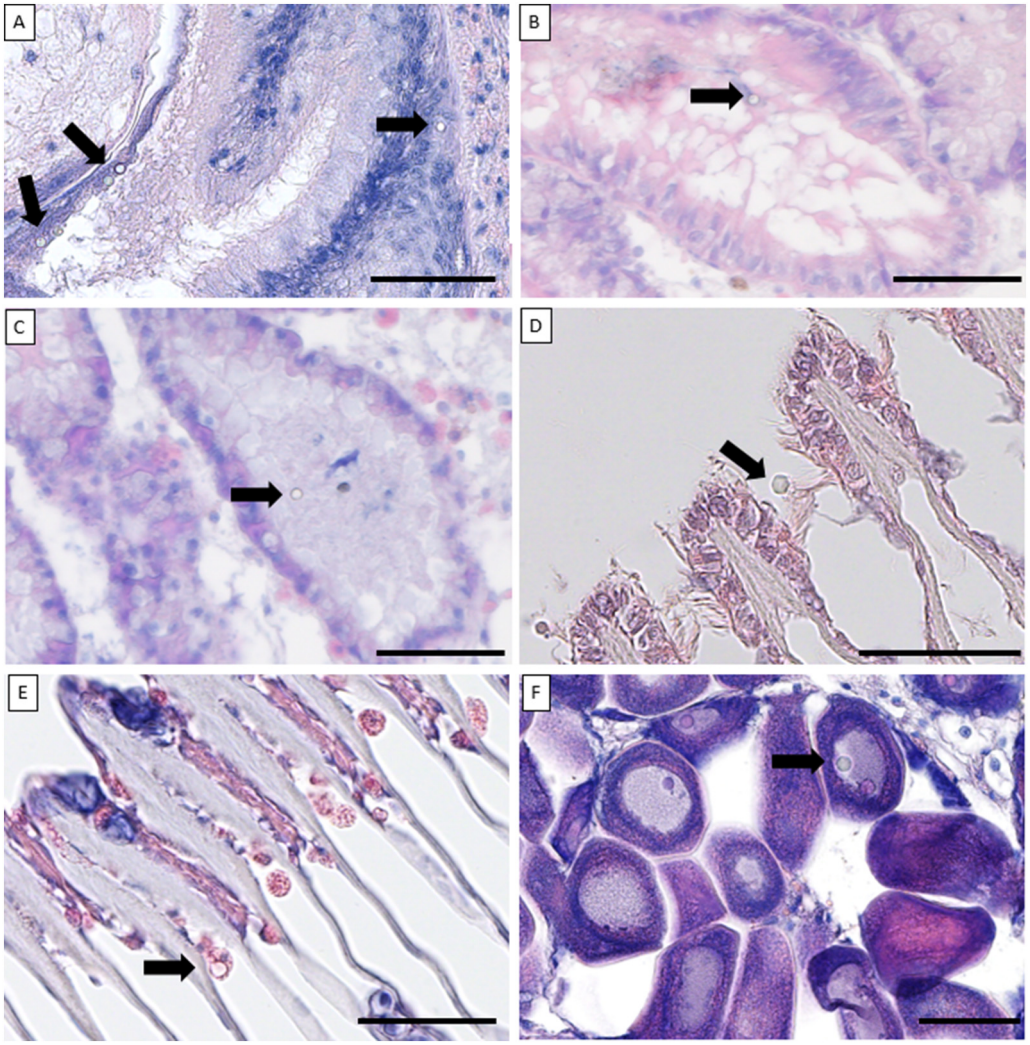


Fig. 2. Light micrographs of mussel paraffin sections showing the presence of 4.5 μm MPs (black arrows) in: A) stomach lumen and epithelium of the digestive tract of a mussel exposed to MPs for 7 days; B) lumen of a digestive duct in a mussel exposed to MPs for 21 days; C) lumen of a digestive tubule in a mussel exposed to MPs for 21 days; D) gill filaments of a mussel exposed to MPs for 7 days; E) gill filaments of a mussel exposed to MP100 for 7 days showing a MP within a hemocyte; E) gonad follicle of a mussel exposed to MPs for 7 days showing a MP within an oocyte. Scale bars: 50 μm.

IDH activity increased along the exposure time both in the control and in the WAF group (Fig. 5A).

AOX1 activity was not altered at days 7 and 21 of exposure but activity increased significantly along exposure time in the WAF group (Fig. S4B).

At day 7 of exposure, GST activity in the digestive gland of mussels exposed to MP was significantly higher than in control mussels. Meanwhile, at day 21 of exposure mussels exposed to WAF showed significantly higher activity than controls and mussels exposed to MP. Along the experiment, GST activity decreased significantly in all the groups (Fig. 5B). On the other hand, GST activity in gills of mussels exposed to MP and to WAF for 7 days was significantly higher than in controls. GST activity decreased significantly in all the groups from day 7 to 21; however, after 21 days, mussels exposed to WAF showed significantly higher activity than control mussels and mussels exposed to MPs (Fig. 5C).

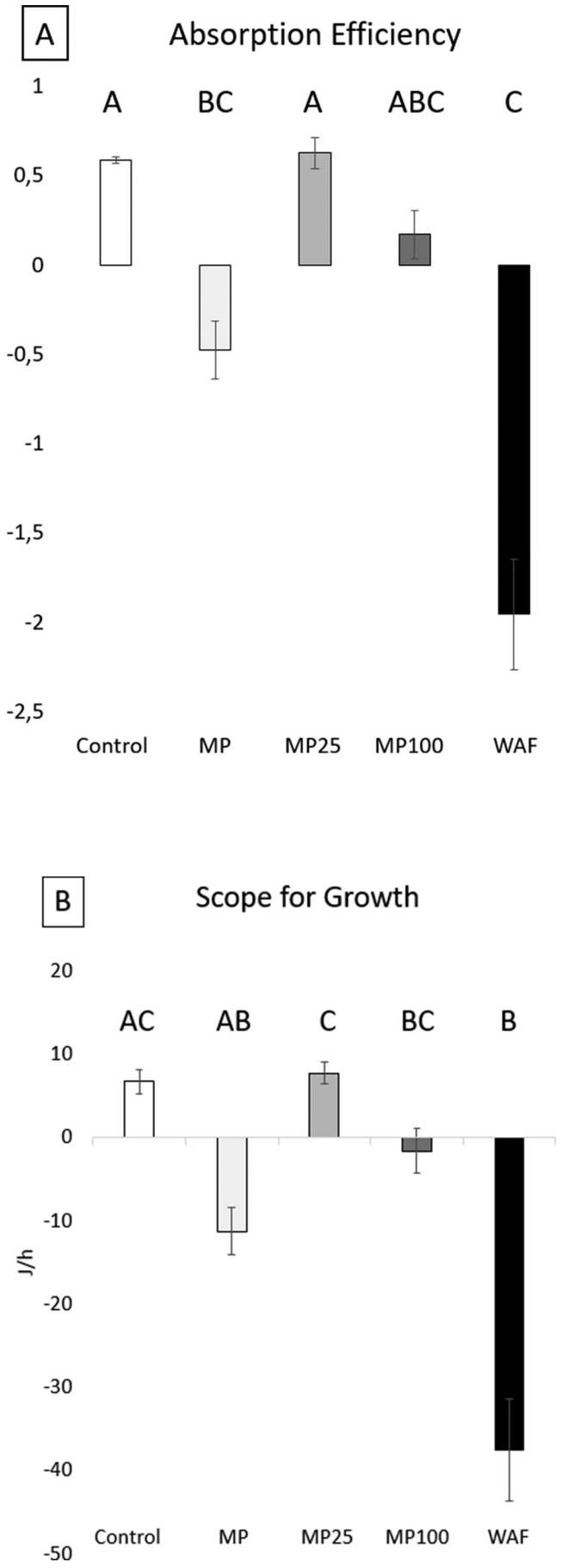
In the digestive gland, catalase activity was significantly lower in mussels exposed to WAF for 7 days than in controls while, after 21 days of exposure, mussels exposed to WAF showed higher activity than controls. Catalase activity increased significantly in the WAF group from day 7 to day 21 (Fig. 6A). On the contrary, no alteration was recorded in the activity of catalase in gills of mussels exposed for 7 days. At day 21 of exposure mussels exposed to WAF showed higher catalase activity than controls, though no differences were recorded along the time (Fig. 6B).

GPX activity was not altered significantly in the digestive gland of mussels exposed to MPs or to WAF (Fig. 6C). In gills, GPx activity was significantly higher in mussels exposed to MPs and to WAF than in controls, both after 7 and 21 days of exposure (Fig. 6D). GPx activity decreased significantly in all groups along the experiment (Fig. 6D).

3.7. Cell composition, quantitative structure and histopathology of mussel digestive gland

The volume density of basophilic cells (VvBas) was significantly higher in mussels exposed to MP and to WAF for 7 days than in control mussels (Fig. 7A). No significant differences were recorded at day 21 of exposure, as the VvBas in control mussels increased significantly from day 7 to day 21 of exposure (Fig. 7A).

No significant differences were found at day 7 of exposure with respect to controls in the two parameters indicative of the structural integrity of the digestive tubules, mean epithelial thickness to mean diverticular radius (MET/MDR) and mean luminal radius to mean epithelial thickness (MLR/MET) (Fig. 7B). MET/MDR was significantly lower in mussels exposed to WAF than in mussels exposed to MP and, on the contrary, MLR/MET was significantly higher in mussels exposed to WAF than in mussels exposed to MP (Fig. 7C). At day 21 of exposure, mussels exposed to WAF showed



significantly higher MET/MDR and lower MLR/MET than controls. During the exposure, MLR/MET significantly increased and MET/MEDR significantly decreased in control mussels while in mussels exposed to WAF MLR/MET decreased along the exposure time (Fig. 7B,C).

Digestive tubule atrophy increased in controls along the experiment whereas the prevalence of fibrosis decreased in mussels exposed to MP and to WAF along the experiment. Inflammatory responses such as hemocytic infiltration and accumulation of brown cells were observed in control mussels and in mussels exposed to MP and to WAF for 7 and 21 days (Fig. S5, Table S3). The parasites *Mytilicola intestinalis* and *Nematopsis* spp. were observed in the digestive gland of several individuals of all groups at both exposure times (Table S3).

4. Discussion

In the present study we investigated the effects of dietary exposure to PS MPs (4.5 μm) alone and with sorbed oil compounds from the WAF of a naphthenic North Sea crude oil in mussels *M. galloprovincialis* using a battery of biological responses. In parallel, mussels were also exposed to WAF alone. As far as we know, this is the first work addressing the effects of a complex mixture of hydrocarbons sorbed to MPs in bivalves. The study considers an environmentally realistic scenario in which widely distributed MPs could adsorb a mixture of oil hydrocarbons originating from different sources, including oil spills. Several studies have reported that plastics collected from beaches and oceans presented a complex mixture of POPs, including PAHs (Van et al., 2012; Rios et al., 2010; Zhang et al., 2015). Thus, our study is relevant because most of the experimental works on the impact of MPs with adsorbed or co-exposed organic or metallic pollutants have been performed with single model compounds, usually at doses that are known to be toxic (Burns and Boxall, 2018; Huang et al., 2021a; Sendra et al., 2021).

Chemical analysis showed that only mussels exposed to WAF alone bioaccumulated PAHs in their tissues and this accumulation was higher at day 7 of exposure than at day 21. On the contrary, mussels exposed to MPs with sorbed oil compounds from WAF (MP25, MP100) did not accumulate PAHs at any exposure time. This is opposed to what was observed in previous works where transfer of PAHs such as pyrene (Avio et al., 2015) or benzo(*a*)pyrene (Gonzalez-Soto et al., 2019) from MPs to mussels did occur. However, Paul-Pont et al. (2016) already showed that PS MPs exhibited a high sorption capacity for fluoranthene, but fluoranthene did not bioaccumulate in marine mussels. In mussels exposed to WAF alone phenanthrene was the PAH showing the highest concentration at both exposure times. However, in mussels exposed to MPs with sorbed oil compounds from WAF this was not observed, possibly because the sorption of PAHs into MPs was correlated with their initial concentration in the WAF, in which naphthalene was the main component (Martínez-Álvarez et al., 2022). In mussels exposed to WAF alone naphthalene represented <10 % of total bioaccumulated PAHs. Another explanation could be that the MPs were not retained inside mussels long enough to desorb the PAHs, as proposed by Burns and Boxall (2018). In addition, at examining the presence of MPs in mussels exposed to MP25 and MP100, the prevalence and the total number of MPs observed in both exposure groups were lower than in mussels exposed to MPs alone. This reduced presence of MPs in mussels when they have sorbed pollutants has been observed in previous studies (Gonzalez-Soto et al., 2019; Islam et al., 2021) and could be due to a behavioural response of valve closure or to decreased appetite and feeding, that could prevent mussels from the accumulation of associated pollutants sorbed into MPs. If animals can reduce feeding rate or clearance rate, increase the production of pseudofeces or speed up defecation, the toxic impact caused by MPs can be reduced (Huang et al., 2021b). However,

Fig. 3. A) Absorption efficiency (ratio) and B) Scope for growth (J/h) of control mussels and mussels exposed to MP, MP25, MP100 and WAF after 21 days of exposure. Data are given as mean values and standard errors of 7 mussels per experimental group. Letters indicate significant differences among treatments (Kruskal-Wallis followed by Dunn's post hoc test, $p < 0.05$).

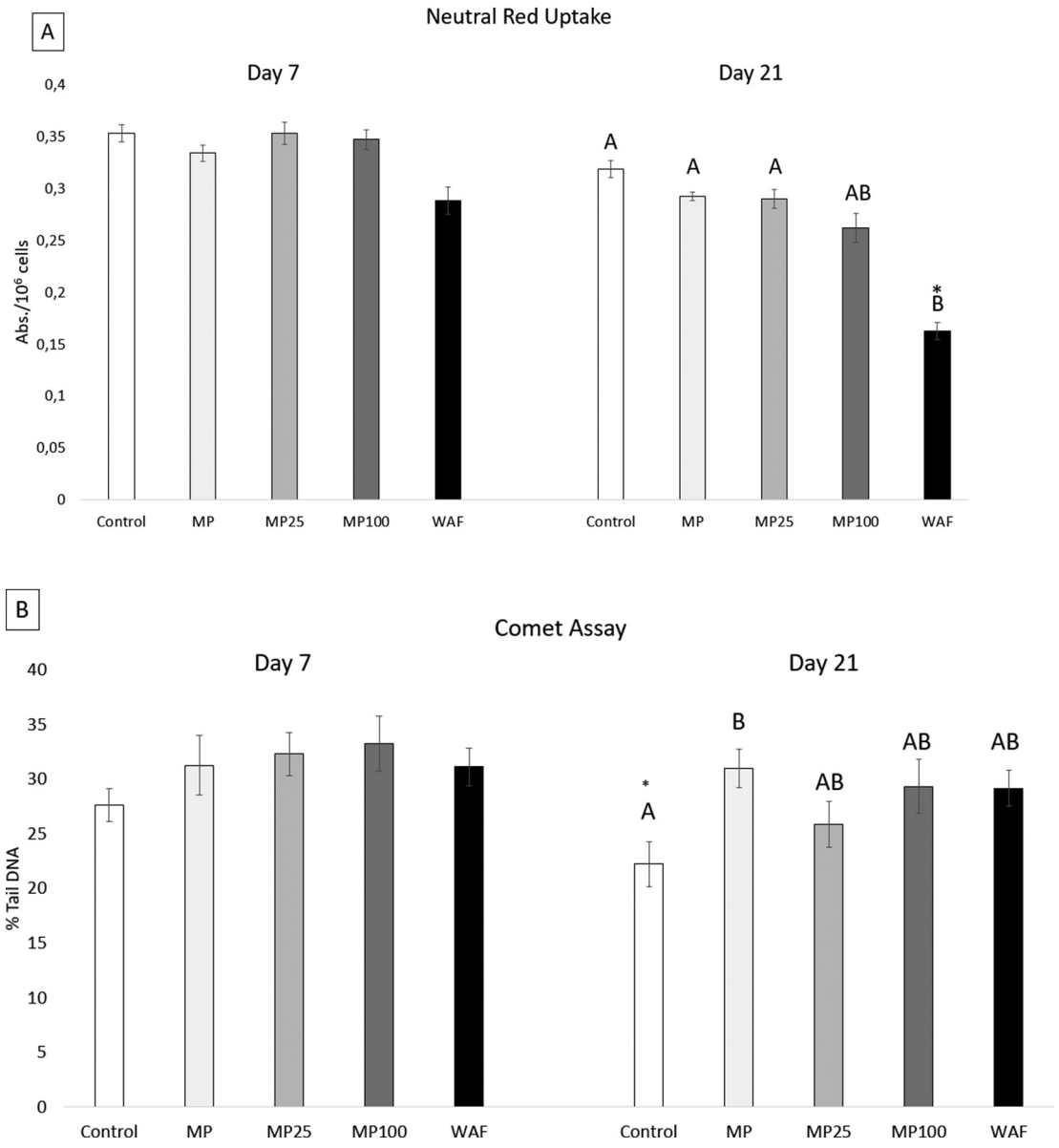


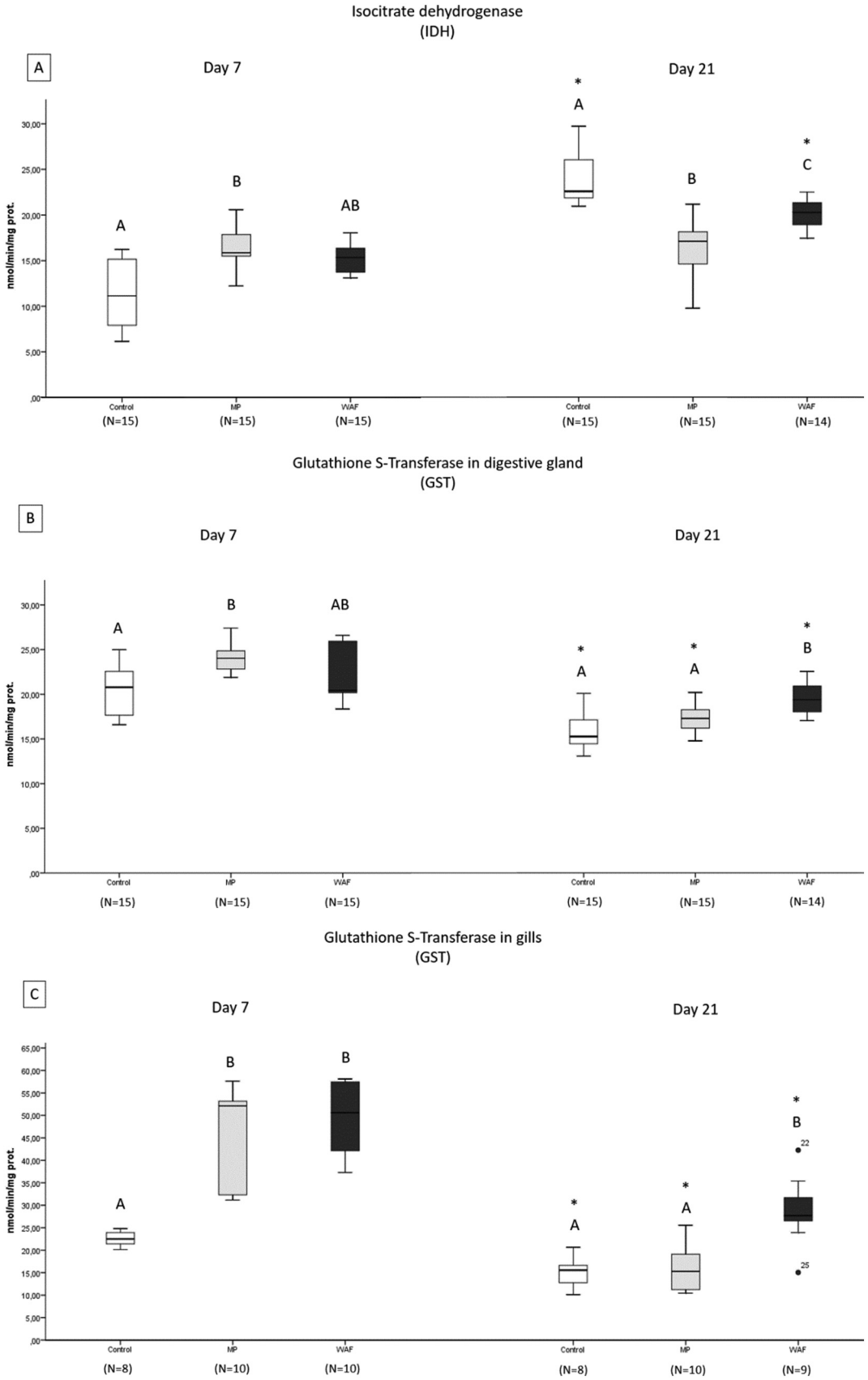
Fig. 4. A) Neutral red uptake (given as absorbance/10^6 cells); and B) DNA strand breaks (given as % tail DNA) in hemocytes of control mussels and mussels exposed to MPs, MP25, MP100 and WAF after 7 and 21 days of exposure. Data are given as mean values and standard errors (8 mussels per experimental group, 4 replicates per mussel in A; and 50 cells in 2 slides per mussel in B). Letters indicate significant differences among treatments within the same day (Kruskal-Wallis followed by Dunn's post hoc test, $p < 0.05$). Asterisks indicate significant differences between days within the same treatment (Mann-Whitney's *U* test, $p < 0.05$).

these mechanisms will have an impact on the energy budget of the animal (Wegner et al., 2012; Van Cauwenberghe et al., 2015; Santana et al., 2018).

In this work, clearance rate was not altered upon exposure to MP, MP25, MP100 or WAF, whereas absorption efficiency (AE) was reduced upon exposure to MPs or to WAF and Scope for Growth (SFG) decreased in mussels exposed to WAF at day 21. Further, in the gonad, prevalence of oocyte atresia increased at day 21 in mussels exposed to MP, MP25, MP100 or WAF and hemocytic infiltration increased in groups MP25, MP100 or WAF. As shown previously in mussels exposed to oil WAF or PAHs (Cajaraville et al., 1992a; Ortiz-Zarragoitia and Cajaraville, 2006), these two alterations suggest the activation of gamete resorption by hemocytes in order to cope with a higher energy demand, especially when energy available from food (i.e., SFG) was, in the present case, reduced to negative values. Similarly, the energy fraction allocated to reproduction seemed to be used to meet the high maintenance costs of stress situations provoked by exposure to MPs in oysters (Sussarellu et al., 2016; Gardon et al., 2018). Thus, gonads appeared to provide the energy to maintain animals' metabolism through the production of metabolites derived from germ cells phagocytosis, which could lead to detrimental effects on reproduction, with potential consequences at the population level (Gardon et al., 2018).

As to the occurrence of MPs in mussels along the exposure period, in mussels exposed to MPs alone the presence of MPs was higher at day 7 of

Fig. 5. Enzyme activities (given as nmol/min/mg protein) of A) IDH in digestive gland; B) GST in digestive gland and C) GST in gills of control mussels and mussels exposed to MPs and to WAF after 7 and 21 days of exposure. Box-plots show median value (horizontal line), 25 %–75 % quartiles (box) and standard deviation (whiskers). Dots denote outliers, values that do not fall in the inner fences. Letters indicate significant differences among treatments within the same day (one-way ANOVA followed by Tukey's post hoc or Kruskal-Wallis test followed by Dunn's post hoc, $p < 0.05$). Asterisks indicate significant differences between days within the same treatment (Mann-Whitney's U test, $p < 0.05$).


Isocitrate dehydrogenase
(IDH)
A
Day 7
Day 21
nmol/min/mg prot.
Control
MP
WAF
(N=15)
(N=15)
(N=15)
(N=15)
(N=15)
(N=14)
Glutathione S-Transferase in digestive gland
(GST)
B
Day 7
Day 21
nmol/min/mg prot.
Control
MP
WAF
(N=15)
(N=15)
(N=15)
(N=15)
(N=15)
(N=14)
Glutathione S-Transferase in gills
(GST)
C
Day 7
Day 21
nmol/min/mg prot.
Control
MP
WAF
(N=8)
(N=10)
(N=10)
(N=8)
(N=10)
(N=9)

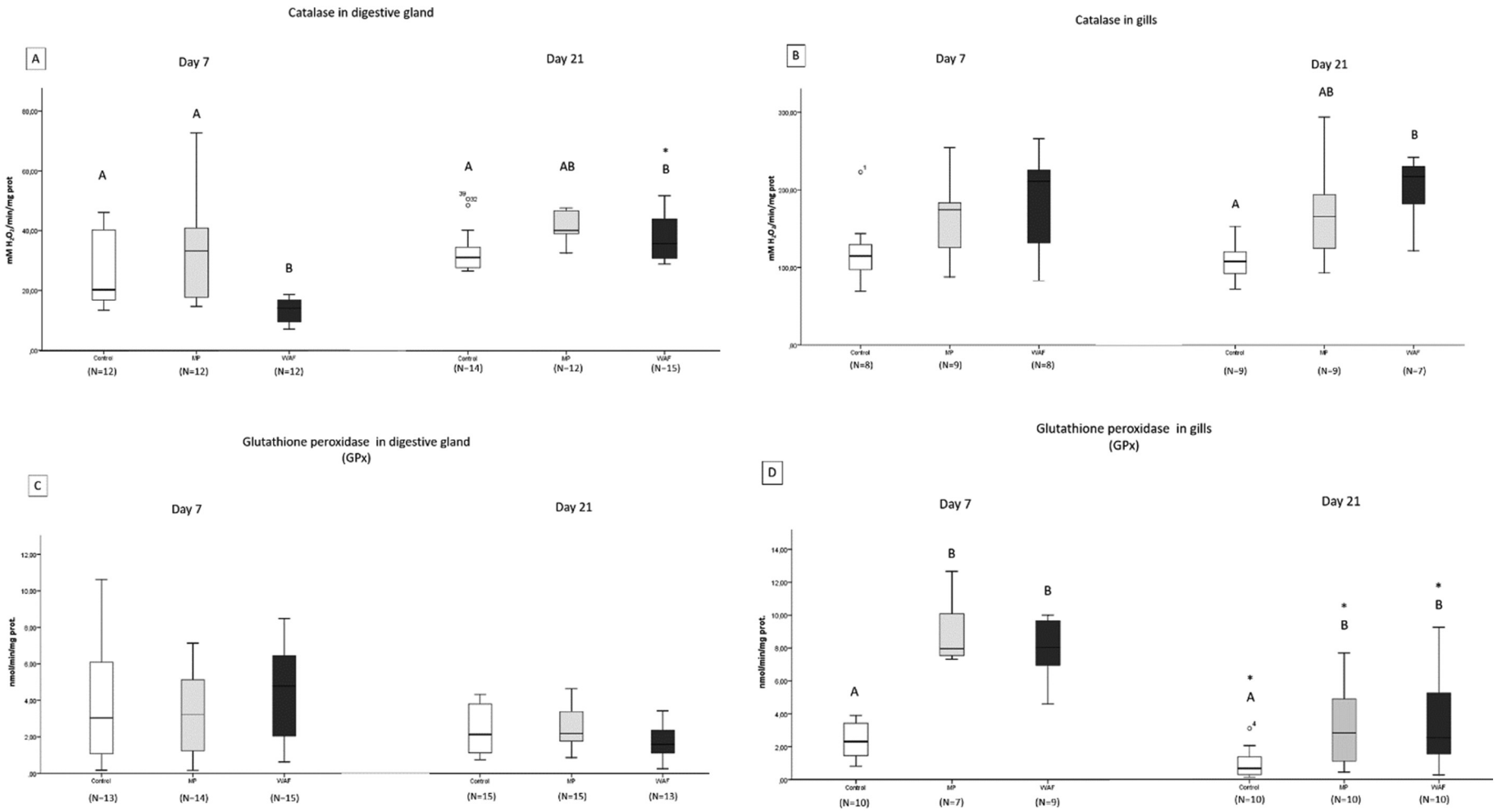


Fig. 6. Catalase activity (given as mM H_2O_2/min/mg prot) in A) digestive gland; B) gills; and GPx activity (given as nmol/min/mg protein) in C) digestive gland and D) gills of control mussels and mussels exposed to MPs and to WAF after 7 and 21 days of exposure. Box-plots show median value (horizontal line), 25 %–75 % quartiles (box) and standard deviation (whiskers). Dots denote outliers, values that do not fall in the inner fences. Letters denote statistical differences among means (one-way ANOVA followed by Tukey's post hoc or Kruskal-Wallis test followed by Dunn's post hoc, $p < 0.05$). Asterisk indicates significant differences between days within the same treatment (Mann-Whitney's U test, $p < 0.05$).

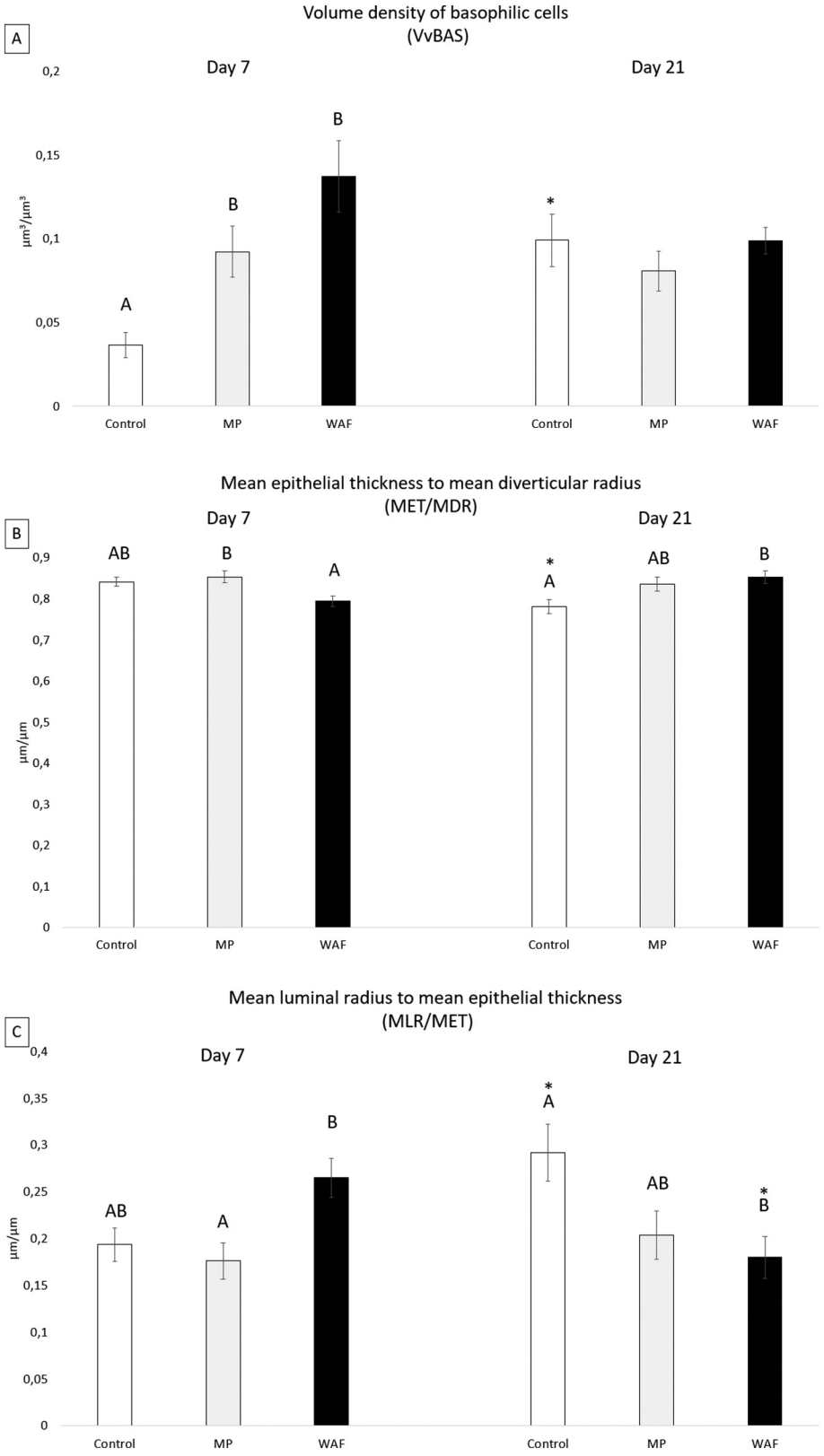


Fig. 7. A) Volume density of basophilic cells ($\mu m^3/\mu m^3$); B) Mean epithelial thickness to mean diverticular radius (µm/µm) and C) Mean luminal radius to mean epithelial thickness (µm/µm) of control mussels and mussels exposed to MPs and to WAF after 7 and 21 days of exposure. Data are given as mean values and standard errors of 10 mussels per experimental group. Letters indicate significant differences among treatments within the same day (one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$). The asterisk indicates significant differences between days within the same treatment (Mann-Whitney's U test, $p < 0.05$).

exposure than at day 21, while the opposite was observed in the case of mussels exposed to MP25 and almost no differences between days were recorded in mussels exposed to MP100. This again could indicate a differential modulation of MP uptake, accumulation and elimination in mussels exposed to pristine versus contaminated MPs. In agreement with previous works (Browne et al., 2008; Von Moos et al., 2012; Avio et al., 2015; Paul-Pont et al., 2016; Magni et al., 2018; Pittura et al., 2018; Gonzalez-Soto et al., 2019), MPs were mainly observed in the digestive tract of the digestive gland, with the only exception of mussels exposed to MP100 at day 21 of exposure, which showed a higher prevalence of MPs in the gills than in the digestive gland. Two routes have been proposed for the uptake of MPs in bivalves: through gills and mouth (Sendra et al., 2021). Consequently, it is not surprising that the digestive gland and gills are the organs most affected by MPs (Sendra et al., 2021).

Even though PS MPs were seen to translocate into mussel's hemocytes and then they could affect the immune function of these cells (Katsumiti et al., 2021; Sendra et al., 2021), hemocyte viability and catalase activity were not altered in mussels exposed to MP, MP25 or MP100. Only mussels exposed to WAF showed a lower viability of hemocytes after 21 days of exposure, in agreement with results of a previous work of *in vitro* exposure of hemocytes to the same oil WAF (Katsumiti et al., 2019). Meanwhile, damage in DNA was observed in hemocytes of mussels exposed to MPs alone after 21 days of exposure. DNA damage has been observed previously in bivalves after exposure to MPs alone (Ribeiro et al., 2017; Brandts et al., 2018; Gonzalez-Soto et al., 2019; Revel et al., 2019) and in hemocytes exposed to MPs and nanoplastics *in vitro* (Katsumiti et al., 2021). The fact that hemocytes of mussels exposed to MP25 or MP100 did not show increased levels of DNA strand breaks can be explained by the lower occurrence of MPs in tissues of those mussels compared to mussels exposed to MPs alone, as discussed above.

Overall, our results showed a lack of PAH accumulation in mussels exposed to MPs with sorbed oil compounds from WAF (MP25 and MP100), a reduced occurrence of MPs in the same two groups compared to mussels exposed to MPs alone, and no effects on mussel physiology and hemocyte functions in the same two groups. Therefore, further studies on enzyme activities in mussel tissues and on cell composition, quantitative structure and histopathology of mussel digestive gland were carried out only in mussels exposed to MPs or to WAF alone, in comparison to controls.

IDH activity was induced in mussels exposed to MPs for 7 days, suggesting increased energy needs. However, at 21 days IDH was lower in mussels exposed to MPs and to WAF compared to controls, due to the significant increase in IDH activity in the controls, maybe related to estabulation conditions or to seasonal changes. This enzyme has shown a bell-shaped response in previous studies, with a rise in the activity at the beginning of the exposure to get additional energy to overcome the toxicity (Oliveira et al., 2013) and a decrease in IDH activity at longer exposures, which implies a reduction of energy obtained through the aerobic pathway. This reduction could be connected with a decrease in feeding (Guilhermino et al., 2018) which agrees well with the reduced AE at day 21 in mussels exposed to MPs and to WAF and with the reduced SFG in the case of mussels exposed to WAF.

The activity of the phase II biotransformation enzyme GST was induced transitorily at day 7 both in digestive gland and gills of mussels exposed to MPs, whereas GST induction under WAF exposure lasted up to day 21 in both organs. The transitory response of GST to MP exposure could be related to the decreased occurrence of MPs in mussels exposed to MPs for 7 days in comparison to 21 days. The antioxidant enzyme GPx was not altered in the digestive gland but exposure to MPs and to WAF caused a significant induction of GPx in the gills at days 7 and 21. The lack of response of GPx activity in the digestive gland of mussels exposed to MPs has also been reported by Paul-Pont et al. (2016). Catalase activity was also induced in the digestive gland and gills of mussels exposed to WAF for 21 days. Different responses of enzyme activities in gills and digestive gland have been recorded in several works (Ribeiro et al., 2017; O'Donovan et al., 2018; Revel et al., 2019).

As mentioned before, induction of GST in mussels exposed to WAF lasted until day 21 in both gills and digestive gland but GST activity decreased at day 21 with respect to day 7. This decrease could be due to the decrease in the bioaccumulation of PAHs in mussel tissues, which was reduced almost to the half from day 7 to 21 of exposure. However, mussels exposed to WAF showed higher activity of catalase at day 21 compared to day 7 in the digestive gland. Similarly, the activity of the peroxisomal β-oxidation enzyme AOX1 increased in mussels exposed to WAF from day 7 to day 21 of exposure, but this increase was not enough to cause a significant difference with respect to the control. Both catalase and AOX1 activities are known to vary seasonally in mussels (Cancio et al., 1999) which could explain observed differences along the experiment. In line with our results, no alteration of AOX1 was observed in mussels exposed to MPs alone or in combination with pyrene (Avio et al., 2015), BaP (Pittura et al., 2018; Von Hellfeld et al., 2022) and cadmium (Von Hellfeld et al., 2022).

The cell type composition of the digestive tubules of the digestive gland was altered after 7 days exposure to MPs or to WAF, with a higher volume density of basophilic cells (VvBas) with respect to controls. After exposure to pollutants VvBAS may surpass 0.12 $\mu m^3/\ \mu m^3$ (Bignell et al., 2012), as observed in the present work in mussels exposed to WAF for 7 days, possibly related to the high PAH concentrations measured at day 7 in the WAF group. After 21 days of exposure there was no statistically significant difference among treated groups and controls, due to increased values of VvBAS in controls that could be associated again to estabulation conditions or to seasonal changes. For the exposure to MPs, increased values of VvBAS in mussels exposed for 7 days to 0.5 μm MPs have been reported but not in mussels exposed to 4.5 μm MPs (Gonzalez-Soto et al., 2019). This difference is probably related to the higher number of MPs found in the digestive ducts and tubules in this work than in Gonzalez-Soto et al. (2019) which could result in a higher impact on the cellular composition of the digestive tubules.

Higher MET/MDR and lower MLR/MET values were observed at 21 days of exposure to WAF in comparison to the control. These values might possibly be due to increased lysosomal activity related to uptake and elimination of accumulated pollutants (Zorita et al., 2006; Sforzini et al., 2018), which finally resulted in a reduced SFG in mussels exposed to WAF. Large digestive imbalances, accounting for negative AE (and hence, negative SFG) would be expected to occur associated to these detoxification mechanisms, because of the extensive losses of cell materials voided with the feces that were found to consist mainly of membrane lipids (Ibarrola et al., 2000a, 2000b). Finally, it must be taken into consideration that the impact of WAF in mussels´ digestive gland has been observed to be dependent on exposure time, oil type and food ration (Cajaraville et al., 1992b; Marigómez and Baybay-Villacorta, 2003; Blanco-Rayón et al., 2019).

In conclusion, MPs did not transfer sorbed oil compounds from the WAF of a naphthenic North Sea crude oil to mussels and effects compatible with a Trojan horse effect were not observed, which has implications for risk assessment of MPs and associated oil pollution in the marine environment. On the other hand, exposure to MPs or to WAF alone triggered responses at molecular, cellular, tissue and organism levels. Although in general WAF alone caused a greater impact in comparison to MPs alone on studied biological responses, toxicity of MPs should not be ignored, as they altered several enzyme activities, caused genotoxicity, increased oocyte atresia and basophilic cell volume in digestive tubules and decreased absorption efficiency. Future works are needed to elucidate the behavior of other microplastic polymers and sizes towards other types of oil with differing hydrocarbon composition as well as the impact on marine ecosystems under co-exposure scenarios, using a wide array of biological responses.

Data availability

Data will be made available on request.

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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CRediT authorship contribution statement

N.G.-S., E.B. and M.P.C. designed the experiment; N.G.-S. performed the experiment and laboratory work, data acquisition, treatment and statistical analysis, prepared the graphs and tables, and wrote a first draft of the manuscript; L.C. contributed to the histopathological analysis; E.N. supervised the laboratory work on physiological parameters; L.G. supervised the laboratory work on enzyme activities; E.B. and M.P.C. supervised all laboratory work, data treatment and analysis; interpretation and discussion of results; L.C., E.N., L.G., E.B. and M.P.C. revised the manuscript; M.P.C. designed and supervised the overall project, obtained funding and produced the final manuscript for submission.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.157999.

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