Chemosphere 336 (2023) 139273

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Assessment of the effects of non-phthalate plasticizer DEHT on the bivalve molluscs *Mytilus galloprovincialis*

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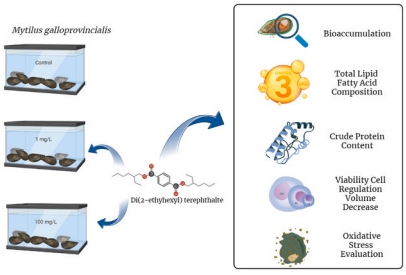
HIGHLIGHTS

- Plastics release plasticizers potentially harmful for the environmental health.
- The plasticizer DEHT increasingly contaminates aquatic environments.
- *Mytilus galloprovincialis* bioaccumulates DEHT in its tissues.
- DEHT affects lipid and protein metabolism of exposed mussels.
- DEHT impairs physiological parameters of exposed mussels.

GRAPHICAL ABSTRACT



ARTICLE INFO

Handling Editor: Willie Peijnenburg

Keywords:
Di(2-ethylhexyl) terephthalate

ABSTRACT

Due to their uncontrolled use, plastics has become an environmental concern, not only for their varying dimension but also for the potential release of substances such as phthalates (PAEs) and non-phthalates (NPPs) into the water. Phthalates are the most common plasticizers of concern, but non-phthalate plasticizers such as di

Abbreviations: (DEHT), di (2-ethylhexyl) terephthalate; (PUFA), polyunsaturated fatty acids; (DG), digestive gland; (PAEs), phthalic acid esters; (NPPs), non-phthalate plasticizers; (DBP), dibutyl phthalate; (DIBP), diisobutyl phthalate; (DEHP), di(2-ethylhexyl) phthalate; (BBP), n-butyl benzyl phthalate; (ROS), reactive oxygen species; (GC-MS), gas chromatography coupled to mass spectrometry; (FA), fatty acid; (GC-FID), gas chromatography coupled to flame ionization detector; (EC), environmental concentration; (FAMEs), fatty acid methyl esters; (NR), Neutral Red; (TB), Trypan Blue; (CMSF), calcium, magnesium free solution; (SOD), superoxide dismutase; (LPO), lipid peroxidation; (OMP), oxidatively modified proteins; (TBARS), 2-thiobarbituric acid-reacting substances; (TBA), thiobarbituric acid; (TCA), trichloroacetic acid; (MDA), malondialdehyde; (DNPH), 2,4-dinitrophenylhydrazine; (SD), standard deviation; (SE), standard error; (SFAs), saturated FAs; (MUFAs), monounsaturated FAs; (NMIFAs), non-methylene-interrupted fatty acids; (GST), Glutathione S-transferases; (CAT), Catalase; (BPA), bisphenol A; (PFOS), perfluorooctane sulphonate.

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https://doi.org/10.1016/j.chemosphere.2023.139273
Received 12 May 2023; Received in revised form 16 June 2023; Accepted 17 June 2023
Available online 19 June 2023

Mytilus galloprovincialis
Nutritional values
Cytotoxicity test
Biochemical parameters

(2-ethylhexyl) terephthalate (DEHT) have also been lately found in the marine environment. *Mytilus galloprovincialis* is a well-known bioindicator of aquatic environments due to its ability to accumulate a wide variety of xenobiotics, including plasticizers. Hence, aim of this study was to evaluate the potential bioaccumulation and effects of the NPP DEHT on *M. galloprovincialis*. To this purpose, following exposure to DEHT at 1 mg/l (DEHT1) and 100 mg/l (DEHT100), its accumulation in tissues and its effects on total lipids and fatty acid (FA) composition, protein content, cell viability, ability to recover volume and changes in biomarkers of oxidative stress were assessed. Mussels were able to bioaccumulate DEHT in their tissues, with a statistically significant increase compared to the control organisms. Differences in FA composition were observed after exposure, since C16:0, C18:0, C20:5ω-3 and C22:6ω-3 were significantly decreased from control to exposed groups. As a result, total SFA, MUFA and PUFA were affected in DEHT-exposed groups. Also, total protein varied following DEHT exposure, and significantly decreased in the DEHT100-group. Considering the physiological responses, both DEHT-exposed groups lost their ability to return to the original volume of digestive gland (DG) cells. On the other hand, oxidative biomarkers in the gills and DG were not significantly affected by the DEHT exposure. Overall, this study showed for the first time that DEHT exposure differentially affect mussels, in their lipid and protein metabolism, as well as cellular parameters.

1. Introduction

As a result of accelerated industrial development and increased manufacture and disposal of plastics in recent decades, plastic pollution has become a universally pressing problem, gaining significant attention from governmental bodies, scientific community, and not least, civil society. Indeed, alongside the global dimension of plastic pollution determined by product design, waste management, and structural issues (Barrowclough and Birkbeck, 2022), other broad-scale environmental and public health questions, such as climate change (Shen et al., 2020) and spread of antibiotic resistance and human pathogens (Bank et al., 2020; Arrigo et al., 2023), are inextricably linked to, and therefore potentially exacerbated by, the mismanagement of plastic pollution. Despite the recent and numerous efforts by governments, businesses, and civil society to support plastic reduction and circular economy approaches, the scale of plastic pollution is likely to increase over the coming decades, driven by the projected increase in human population size and plastic products consumption (Lau et al., 2020). Notoriously, plastic polymers are susceptible to either abiotic or biotic degradation pathways (Gewert et al., 2015). As a result, based on their size, nanoplastics (<100 nm), microplastics (<5 mm), mesoplastics (5–25 mm) and macroplastics (>25 mm) are now virtually ubiquitous worldwide (Stock et al., 2019). However, as plastic ages in the environment, the health and ecological hazard comes not only from the generation of plastic fragments of various sizes, which moreover act as a vector of many environmental pollutants (Wang and Wang, 2018; Wang et al., 2018; Ateia et al., 2020), but also from the release of chemicals by leaching or degradation of the plastic polymer itself (Gewert et al., 2015). Indeed, during manufacturing, a wide variety of chemical additives are incorporated into plastic polymers to impart peculiar functional properties (e.g., flexibility, processability, weathering resistance, heat and light stability etc.) (Yan et al., 2021; Schrank et al., 2019). These additives are generally not covalently bound to the polymer and can therefore leach out of the plastic and enter into the environment by wastewater treatment plants, runoff, drainage, and atmospheric deposition (Karbalaei et al., 2018; Beltifa et al., 2017; Liu et al., 2020).

Plasticizers, such as phthalic acid esters (PAEs) and non-phthalate plasticizers (NPPs), are among the most common additives recovered from plastic debris collected in various environmental comparts (Net et al., 2015; Xie et al., 2005, 2007). PAEs are typically used as softeners in PVC products, accounting for up to 60% by product weight (Arbeitsgemeinschaft and Umwelt, 2006). Like many other additives, they are not chemically bounded to the polymer structure, thus, having a high probability to be released into the environment, and endanger the health of biota by primarily interfering with the endocrine system (Engler, 2012; Net et al., 2015; Mankidy et al., 2013; Sedha et al., 2021). PAEs, such as dibutyl phthalate (DBP), diisobutyl phthalate (DIBP), di (2-ethylhexyl) phthalate (DEHP), n-butyl benzyl phthalate (BBP), have been identified as substances of very high concern, characterized by endocrine-disrupting properties, as well as, reproductive toxic effects (category 1 B chemicals, Regulation (EC) No. 1907/2006), and consequently they have been strictly regulated for production and use by EU (European Union, 2013; European Commission, 2007; European Commission, 2006). Over the last half century, manufacturers turned to the production of NPPs to overcome the legislation and safety issues related to PAEs. From the outset, NPPs, mainly including terephthalates, sebacates, benzoates and adipates, appeared to be a good alternative to PAEs, due to lower leaching rates and no use restrictions in PVC products (Jebara et al., 2021; Coltro et al., 2013). It is suffice to say that, coinciding with the declining market share of PAEs, DEHP especially experienced a 32% decrease in EU consumption between 1999 and 2014- (Cadogan, 2007; ECPI, 2018), NPPs accounted for ~12% and ~35% of the world plasticizer consumption respectively in 2005 and 2017, and they now account for 40% of the EU plasticizer market (Plastic Technology, 2016 ECPI). In the same market, terephthalates, with the main representative DEHT, have a market share of 12%, since they are increasingly used as a commercial alternative in a wide range of applications such as in plastic toys and childcare articles, films, vinyl products and beverage closures (ECPI, 2019a). Knowledge about the physicochemical properties of NPPs and their toxicity on human and environment is still limited and fragmented (Bui et al., 2016; Burgos-Aceves et al., 2021). Physicochemical similarities between PAEs and their alternatives exist for some cases. For example, DEHT is the structural isomer of DEHP, and it is characterized by a similar hydrophobicity and water solubility. Also, a lower toxic potential and the absence of category 1 b toxic effects can be confirmed. However, other non-standard effects, such as early developmental toxicity or endocrine disruption, should be addressed (Bui et al., 2016). Although NPPs do not exhibit the characteristics of a persistent chemical, it is likely that they may still be present, due to their continuous production and release into the surrounding environment, thus leading to continuous environmental and human exposure (Jebara et al., 2021).

Key land-water interfaces, such as coastal zones, rivers, lakes, estuaries, inland waters, lagoons, and wetlands, has always been considered as complex and dynamic nexus for plastic pollution as they represent habitats often densely populated, with major pollution sources (Lee et al., 2020;Kasavan et al., 2021; Gokul et al., 2023). In aquatic environments, organisms at every level of the food chain have been reported to uptake and bioaccumulate plastic debris (Mcneish et al., 2018; Carson et al., 2013; Zicarelli et al., 2023; Pastorino et al., 2023; Savuca et al., 2022; Nelms et al., 2019). Accordingly, the complex suite of hydrophobic contaminants with which plastic is associated could transfer to organisms via direct ingestion of plastic debris or indirect bioaccumulation (bioconcentration from water, including sediment porewater) of chemicals leached from microplastics (Cássio et al., 2022; Campanale et al., 2020; Aliko et al., 2022; Gugliandolo et al., 2020; Paluselli et al., 2018, Jebara et al., 2021). Most field and laboratory studies investigated the toxicity of micro- and macrodebris on various

invertebrates and fish species and revealed that the type of polymer, its size, shape, and concentration are important variables in the determination of final toxicity, at all levels of biological organization-i.e., from sub-organismal (e.g., molecular, cellular and tissue effects) and organismal level to ecologically relevant levels (e.g., population and ecosystems). Overall, aquatic organisms demonstrated to be very susceptible to plastic debris ingestion, with both lethal and sub-lethal effects, such as altered feeding behaviour, impaired growth and fertility, oxidative stress, inflammation, immunotoxicity, neurotoxicity, and changes in metabolic pathways and gene expression (Hodkovicova et al., 2022; Bucci et al., 2020;Mincarelli et al., 2023; Banaee et al., 2023; Liu et al., 2020; Choi et al., 2021; Von Moos et al., 2012; Martyniuk et al., 2023; Vo and Pham, 2021). However, a literature overview pointed out that studies on chemical contaminants associated to plastics (i.e., plastic additives) and released in the aquatic environment are still scarce. Very few laboratory studies have shown lethal and sublethal effects in organisms exposed to plastic with sorbed environmental contaminants (Browne et al., 2013; Lithner et al., 2012; Rochman et al., 2014). Also, few recent findings have been published on the toxicity of plastic additives in freshwater fish. PAEs (i.e., DBP, DEHP and BBP) have been reported to disrupt skeletal development of zebrafish larvae (Pu et al., 2020; Qian et al., 2020). In the goldfish, bisphenol A impaired the gonadal maturation in male and female by reducing the expression of hypothalamic-pituitary-gonad-related genes (Wang et al. (2019) and altered cardiac function by inducing structural remodelling and oxidative stress (Filice et al., 2021). Chen et al. (2020) reported that another class of plastic additives, namely organophosphate flame retardants, affected sperm velocity and morphology in adult male Chinese rare minnows, probably by altering the activity of Na+/K + ATPase rather than the hormone system. Even more limited studies assessed the accumulation degree and the toxicity of PAEs on invertebrate organisms, such as the sentinel *Mytilus* spp. (Impellitteri et al., 2023a,b; Multisanti et al., 2022;Albergamo et al., 2016). In this respect, Xu et al. (2021) pointed out that DEHP negatively impacted osmotic regulation and energy metabolism. Mincarelli et al. (2021), showed that the combination of stressors, such as DEHP and heat stress, could affect the timing and breeding season success in *M. galloprovincialis*. In another work, Mincarelli and coworkers (2023) pointed out the importance of evaluating the sex and the gametogenesis stage of mussels when studying the (altered) stress response-related gene expression of *M. edulis* exposed to DEHP. Recently, Andreyeva et al. (2023) demonstrated that DEHP accumulated primarily in hepatopancreas and induced immunosuppressive effects in haemocytes of mussels including shift in haemolymph composition and inhibition of reactive oxygen species (ROS) production levels. Non-specific general response to DEHP also involved the modulation of the antioxidant complex in digestive gland. However, to the best knowledge of the authors, data on bioaccumulation and toxicity of NPPs in *M. galloprovincialis* are practically absent. Hence, aim of this study was to provide a detailed framework on the bioaccumulation and toxic effects of the most common NPP, namely DEHT, on *M. galloprovincialis* by performing a controlled laboratory experiment and exploiting a multidisciplinary approach. Specifically, adult mussels were exposed to environmentally relevant doses and the bioaccumulation of DEHT was first evaluated by gas chromatography coupled to mass spectrometry (GC-MS). Then, alteration in lipid and protein metabolism was evaluated by screening the total protein and lipid in whole organisms, and the fatty acid (FA) composition by means of gas chromatography coupled to flame ionization detector (GC-FID). The general stress response to DEHT exposure was explored by determining the cell viability of haemolymph and digestive gland of the mussels through suitable staining assays. Also, antioxidant and lipid peroxidation biomarkers as well as protein oxidative damage was investigated in both gills and digestive gland of the experimental bivalves. Hopefully, findings from this work will help to elucidate for the first time the bioaccumulation extent and the toxicity of NPP additives on the invertebrate *M. galloprovincialis*, to predict their adverse outcomes at organism and population level, and to pave the way toward the study of the toxicity of DEHT-based leachates released from plastic products into the aquatic environment.

2. Materials and methods

2.1. Experimental design

The study was conducted during the months of November and December 2022 and the specimens *Mytilus galloprovincialis,* (Lamarck, 1819) was selected to investigate the toxicity of the plasticizer DEHT. A total of n = 150 adult mussels with a mean length of 4.6 ± 0.38 cm and a mean weight of 8.2 ± 2.1 g, were collected at the shellfish farm FARAU SRL Frutti di Mare located at "Lago di Faro", a coastal lagoon in Messina (Italy, 38°15′39.95″N 15°37′01.9″E) which is renowned for the shellfish culture, in accordance with the guidelines of Directive (2010)/63/EU on animal testing. The environmental background DEHT contamination of the site was considered low, since despite the anthropic pressure mainly related to the presence of several farming activities, the area is part of the "Laguna di Capo Peloro" natural reserve and it is continuously monitored. Collected mussels were transported to the laboratory in aerated natural seawater and acclimated for one week in artificial saltwater (Premium REEF-Salt, Tropical Marine Centre, Chorleywood, UK) at constant temperature (18.2 ± 0.12 °C) and aeration. Following the acclimatisation period, they were randomly subdivided into six continuously aerated glass tanks (20l) and two experimental groups, and a control group were created in duplicates (n = 25 specimens per tank). The experimental groups were separately exposed to two different concentrations of DEHT (purity ≥96%, Sigma-Aldrich, Steinheim, Germany), namely DEHT1 (1 mg/l, environmental concentration, EC) and DEHT100 (100 mg/l, 100xEC). On the other hand, the control groups did not receive any exposure treatment. The exposure time lasted 14 days. During the study period, mussels were constantly kept under natural light condition and were not fed; while the saltwater was renewed three times per week (at day 2, 4, 6, 8, 10, and 12), with the same concentrations of DEHT being added immediately afterwards. No mortality was observed during the entire exposure period. The DEHT concentration of 1 mg/l was chosen based on the DEHT levels recently detected in seawaters from the Mediterranean area (0.78 mg/l, Jebara et al., 2021; 0.68–1.21 mg/l, Gugliandolo et al., 2020), where the concentration 100 mg/l, which corresponded to 100 times the environmental concentration, was also considered in relation to previous work (Stara et al., 2021). A 14-day exposure was chosen considering that DEHT is not a highly persistent and accumulative plasticizer (Bui et al., 2016) and on the basis of previous study (Martyniuk et al., 2023).

2.2. Sample collection

For the chemical analysis n = 16 organisms were randomly selected from each group, and their whole body was dissected, pooled, freeze-dried, grounded into a fine powder, and stored at −80 °C until the bioaccumulation study was performed, as well total lipid, protein and FA composition were assessed. Additionally, n = 8 water samples (50 mL) from each experimental/control group were collected in glass bottles before and after each water exchange and stored at + 4 °C until further analysis. For the biological analysis, n = 4 organisms were randomly selected from each experimental group and pooled for assessing cell viability and the ability to regulate volume decrease. In this case, haemolymph was collected from the anterior adductor muscle of mussels from each experimental/control group, using a glass syringe with a 23-gauge needle, according to Bolognesi and Fenech (2012). After haemolymph collection, each mussel pool was sacrificed on an ice flake and digestive glands (DGs) were dissected and collected. For antioxidant biomarkers and oxidative protein damage, n = 6 mussels were randomly selected, and their gills and DGs were collected and stored at −80 °C until use.

During the exposure study and the subsequent experimental procedures, every effort was made to significantly reduce the potential background DEHT contamination of solvents, reagents, and laboratory materials. All samples were processed using glassware and stainless-steel instruments, that were previously washed with acetone, rinsed with hexane, and dried at 120 °C for at least 4 h. Sodium sulphate was heated for 4 h at 140 °C and, after cooling, kept in a tightly sealed glass vial. Organic solvents were tested for background levels after concentration and GC–MS analysis. Only the batches of solvents marked by negligible levels of contamination were used throughout the analytical procedure.

2.3. Determination of DEHT in mussel and water samples

2.3.1. Sample preparation

The determination of DEHT content in experimental and control mussels, as well as in saltwater, was carried out according to the method proposed by Jebara et al. (2021). In brief, 0.1 g of every powdered sample was spiked with a known amount of deuterated standard (DEHT-d4 in nonane, 100 μg/mL, Cambridge Isotope Laboratories Inc., Andover, MA, USA) and mixed with 0.2 g of anhydrous sodium sulphate (Sigma-Aldrich) and 0.4 g of solid sorbent magnesium silicate (Florisil®, 60–100 mesh, coarse powder, Fluka, Sigma-Aldrich). The obtained mixture was added on the top of a glass Pasteur pipette which was previously prepared with 0.1 g of Florisil and glass wool, and subsequently sealed at the top with additional glass wool. The extract was eluted with a solution of *n*-hexane: acetone (1:1 *v/v;* >99.9% grade, Sigma-Aldrich) and evaporated to dryness by a rotating evaporator (Buchi V700, BUCHI labortechnik AG, Switzerland). DEHT in saltwater samples was determined using a C18 cartdrige (Supelclean C18, 3 mL, 500 mg sorbent, Supelco), previously conditioned with 5 mL ethyl acetate (>99.9% grade, Sigma-Aldrich) and 5 mL methanol (>99.9% grade, sigma-Aldrich). After passing the samples, the cartridge was washed with 4 mL deionised water (Sigma-Aldrich), followed by 6 mL of a methanol: water solution (35%, v/v). DEHT was eluted with 5 mL ethyl acetate and evaporated to dryness by a rotating evaporator (Buchi V700, BUCHI Labortechnik AG, Switzerland).

2.3.2. Targeted GC-MS analysis

All dried extracts were re-suspended in n-hexane and analysed by a gas chromatography system (GC-2010, Shimadzu, Japan) equipped with an autosampler (HT300A, HTA, Italy) and coupled to a single quadrupole mass spectrometer (QP-2010 Plus, Shimadzu, Japan). Chromatographic separations occurred on a SLB-5MS capillary column (30 m × 0.25 mm i. d. × 0.25 μm film thickness, Supelco, USA). The oven temperature program was: from 60 °C to 190 °C at 8 °C/min (5 min hold), from 190 °C to 240 °C at 8 °C/min (5 min hold), and from 240 °C to 315 °C 8 °C/min. The injection port was at 260 °C and was provided with a narrow inlet liner (0.75 mm ID, Agilent Technologies). Sample injection took a place in splitless mode, with sampling time of 60 s, then split ratio 1:15. Injection volume was 1 μL. Carrier gas (He, 210.0 Kpa, pressure control mode) was used at a linear velocity of 30 cm/s. The MS setup was: EI source temperature; 200 °C; ionization energy and emission current, 70 eV and 250 μA; interface temperature and electron multiplier voltage, 300 °C and 1.0 kV. Acquisition was performed both in full scan (mass range 40–400 *m/z*) and selected ion monitoring (SIM) by monitoring three characteristic mass fragments of the analyte (Table 1). Data acquisition and processing were performed by GCMS solution software. Identification of DEHT occurred by comparison of its retention time and mass spectrum with that of the corresponding commercial standard (DEHT, purity >99%, Aldrich Chemical, Chicago, IL, USA). The quantitative procedure was carried out in SIM mode, considering the relative base peak ion (Table 1) and exploiting the internal standard normalization. Triplicate measurements were conducted for every sample, alternated with analytical blanks (solvent) and, in the case of mussel pools, results were expressed on a converted fresh weight (fw) basis. During the entire study period, trace contents of DEHT (1.07 μg/kg) were detected in these blanks, and they were constantly subtracted from the analysis of sample extracts.

Table 1
Retention time (t_R), molecular weight, monitored ions of the target analyte.

Analyte	t_R (min)	Molecular weight (g/mol)	Monitored ions (*m/z*)
DEHT	37.5	390.57	149, 112, 261

Underlined ions were considered for quantitative analysis.

2.4. Total lipid and FA composition

For the lipid extraction from pooled mussels, the method recommended by the Organization for Economic Cooperation and Development test guidelines (OECD, 2012) was followed. Briefly, every powdered sample (~4 g) was homogenized with 40 mL of a mixture chloroform: methanol (1:2, v/v, HPLC grade, Carlo Erba), and then placed in an ultrasonic bath for 30 min. The mixture was added with 10 mL of NaCl 0.73%, shaken and centrifugated at 6300×*g* for 10 min (Awel Industries, France, MF 20-R). The lower chloroform phase was transferred to a flask; while the upper phase was extracted again according to the same procedure. Then, the two extraction phases were pooled, filtered with anhydrous ammonium sulphate, and dried. After determining the extraction yield (%) gravimetrically on a fw basis, the lipid extract was recovered by 1 mLn-hexane, added with 1 mL of sodium methoxylate and heated at 100 °C for 15 min. After cooling down the mixture, 1 mL of boron trifluoride/methanol (14%) was added, and again heated under the same conditions. Approximately 1 mL of *n*-hexane and 4 mL of a saturated sodium chloride solution were added to the cooled mixture. The organic layer with fatty acid methyl esters (FAMEs) was collected and analysed by a gas chromatograph (GC) equipped with a split/splitless injector and a flame ionization detector (FID) (Dani Master GC1000, Dani Instrument, Milan, Italy). A Supelco SLB-IL100 capillary column, 60 m × 0.25 mm ID, 0.20 μm film thickness (Supelco, Sigma Aldrich, USA) was employed. The following operating conditions were used: oven temperature program 60 °C for 2 min rate 13 °C/min, 150 °C at rate 2 °C/min, and 240 °C for 10 min, injector and detector temperatures were respectively 220 °C and 250 °C; helium at a linear velocity of 30 cm/s (constant). The injection volume was 1 μL, with a split 75.0 mL/min. Data acquisition and management was performed using Clarity Chromatography Software v4.0.2. All samples were analysed in triplicate along with analytical blanks. FAMEs of nutritional interest were identified by direct comparison with the reference retention times of compounds present in the commercial standard mixture (FAMEs reference standards C4–C24, Supelco (Bellefonte, PA, USA). For every sample, individual FAME percentages were calculated with respect to the total chromatogram area in triplicate analysis.

2.5. Total proteins

Crude protein was determined from every powdered sample (~1 g) consisting of pooled mussels, by using the Kjeldahl method already proposed in our previous work on marine organisms (Costa et al., 2019). For every sample, the nitrogen content obtained through the assay was used to calculate the crude protein on a dry weight (dw) basis according to the formula:

$$Protein\ (\%, dw) = \%\ nitrogen \times 6.251$$

The protein content was subsequently expressed as %, fw. All samples were analysed in triplicate along with analytical blanks and results were expressed in terms of converted fw.

2.6. Cell viability assays

Haemolymph and DGs from pooled mussels were considered to

evaluate the effects of DEHT on cell viability. The tests were conducted using two different colourimetric assays, namely the Trypan Blue (TB, Sigma-Aldrich) exclusion method and Neutral Red (NR, Sigma-Aldrich) retention assay. The first method mentioned entails staining the cells with Trypan blue, which has the ability to enter only non-viable cells with compromised cell membranes. This is because, under normal circumstances, viable cells would prevent the uptake of Trypan blue dye. The percentage of unstained cells represents the percentage of viable cells in the cell suspension. This assessment helps determine the extent of damage caused to the cells. Additionally, the stability of the lysosomal membrane was assessed using the neutral red retention assay (NR). According to Torre et al. (2013), this assay relies on living cells' ability to absorb and bind the neutral red dye in their lysosomes, after incubating the cells for 15 min. The extent of dye retention provides insights into the stability of the lysosomal membrane. To calculate the percentage of cell viability, the following formula was used:

$$Cells\ viability\ (\%) = \frac{number\ of\ viable\ cells}{total\ number\ of\ cells}\ x\ 100$$

2.7. Regulatory volume decrease

DG cells were isolated according to the method of Pagano and coworkers (2017). The DGs was cut in an isosmotic solution in a calcium, magnesium free solution (CMSF), then transferred in a glass tube with 0.01% collagenase (CLS IV, 175 U/mg, Sigma-Aldrich) and placed in a thermostatic bath for 60 min at 18 °C. The cellular suspension was filtered through 200 μm and 50 μm filters, resuspended with a physiological solution and then centrifuged for 10 min at 4 °C, at 400×*g* (Awel Industries, France, MF 20-R). The supernatant was removed, and cells were resuspended twice with a physiological solution. The samples were transferred again into the thermostatic bath (18 °C) for another hour. The drop of cells sample was put on a slide and observed under a light microscope (Carl Zeiss Axioskop 20, Wetzlar, Germany) connected to a digital camera (Canon 550D). The sample was gently washed with an isotonic solution and three images were taken in sequence. Subsequently, the sample was gently washed with a hypotonic solution (800 mOsm), for the first 10 min, the images were taken every 1 min and then every 5 min during the last 20 min. Cells area from exposed mussels was compared with the control group with the software ImageJ (NIH, Bethesda, MD, USA). Images were taken of 15 cells from each experimental group.

2.8. Determination of antioxidant and lipid peroxidation biomarkers and oxidatively modified proteins

Gills and digestive gland (n = 6 for each experimental and control condition) for each experimental and control condition) were homogenized in cold 100 mM Tris/HCl buffer (pH 7.2), containing a mixture of protease inhibitors to assess superoxide dismutase (SOD) activity, lipid peroxidation (LPO) and oxidatively modified proteins (OMP).

SOD activity was determined by using the method described by (Marklund and Marklund, 1974), modified according to (Filice et al., 2023). The inhibitory effect of SOD on the auto-oxidation of pyrogallol at pH 8.20 was assayed spectrophotometrically at 420 nm and 25 °C. The reaction was run in 50 mM Tris-HCl, 1 mM EDTA, 0.2 mM pyrogallol and monitored every 30 s for 5 min. One unit of SOD activity was defined as the amount of the enzyme that inhibits 50% of pyrogallol auto-oxidation. Results were expressed in U/mg protein. LPO was determined by measuring the concentration of 2-thiobarbituric acid-reacting substances (TBARS), as reported in (Filice et al., 2021). A reaction mixture containing sample homogenate (10% w/v), 2-thiobarbituric acid (TBA, 0.8%), and trichloroacetic acid (TCA; 20%) was kept in a water bath at 100 °C for 10 min and then centrifuged at 5000×*g* for 10 min (Sigma Laborzentrifugen, Germany, 1–15). TBARS levels were determined in the supernatant by assessing malondialdehyde (MDA) concentration at 540 nm (MDA extinction coefficient: 156,000 M^{-1} cm^{-1}); TBARS values were reported as nmoles MDA/mg protein. OMP was evaluated by measuring the carbonyl groups content according to the 2,4-dinitrophenylhydrazine (DNPH) method, described by Levine et al. (2000). Aliquots of homogenates were incubated for 1 h at room temperature with 10 mM DNPH in 2 M HCl; proteins were then precipitated with 2 volume of TCA and centrifuged at 5000×*g* for 10 min (Sigma Laborzentrifugen, Germany, 1–15). Pellet was washed thrice with ethanol-ethyl acetate (1:1; v/v) to remove DNPH excess and then dissolved in 6 M guanidine in 2 N HCl. The concentration of carbonyl groups was measured spectrophotometrically at 370 nm (aldehydic derivates) and at 430 nm (ketonic derivates) using the extinction coefficient of 22,000 M^{-1} cm^{-1}. Results were expressed as nmol/mg protein.

2.9. Statistical analysis

Experimental data from the determination of the DEHT content, the total lipid content, the FA composition, the total protein content, the cell viability and the RVD were expressed as mean ± standard deviation (SD) of triplicate analysis. The statistical analysis was conducted by using the software Graphpad Prism, version 5 (Graphpad Software Ldt., USA, 2003). After running a Shapiro–Wilk test to verify the normal distribution of experimental data, the one way-ANOVA was applied for every independent variable to produce an F-statistic, i.e., the ratio of the variance calculated among the means to the variance within the samples. The one way-ANOVA was followed by a Tukey's honestly significant difference (HSD) post-hoc test for multiple comparative analyses between experimental and control groups. Statistical significance was always accepted at $p < 0.05$.

Oxidative stress data were expressed as means ± standard error (SE) of individual experiments performed in duplicate; significance was assessed by one way-ANOVA followed by Tukey's HSD post-hoc multiple comparative test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Levels of DEHT in experimental water and mussel samples

Levels of DEHT detected by GC-MS in water and mussel samples are reported in Table 2.

Water from the control tank was considered DEHT-free, as DEHT was below the instrumental limit of detection (<LOD, where LOD = 0.04 μg/kg). In both exposure treatments, the detected DEHT concentrations were >92% of the nominal concentration throughout the exposure period, and thus significantly different from each other (DEHT1:0.95 mg/l and DEHT100: 96.36 mg/l, $p < 0.05$). Mussels maintained in DEHT-free saltwater exhibited no bioaccumulation of such NPP. Mussels coming from the DEHT1 treatment bioaccumulated an average of 123.69 mg/kg of this plasticizer, whereas DEHT100 mussels bioaccumulated DEHT to an average concentration of 595.13 mg/kg, which was significantly different from each other ($p < 0.05$).

Table 2
Concentration of DEHT revealed by GC-MS in experimental waters (mg/l) and control and treated pools (i.e., DEHT1 and DEHT100) of *M. galloprovincialis* (mg/Kg, fw). Data are presented as mean ± SD of triplicate analysis. LOD = instrumental Limit of Detection of DEHT (0.04 μg/kg).

Sample	Test groups		
	Control	DEHT1	DEHT100
Water	<LOD	0.95 ± 0.02^{a}	96.36 ± 11.54^{b}
Mussels pool	<LOD	123.69 ± 23.52^{a}	595.13 ± 41.99^{b}

a–b: different superscript letters in the same row indicate significantly different values ($p < 0.05$ b y post hoc Tukey's HSD test).

3.2. Total lipid and FA composition

Total lipid and the FA profile obtained from control and test mussel pools are reported in Table 3. Total lipids were equal to 5.52% fw in the control group. On the other hand, DEHT exposure caused a strong lipid increase, since DEHT1 and DEHT100 groups showed respective lipid contents of 8.75% and 8.41% fw. Statistical analysis showed a significant difference ($p < 0.05$) for both DEHT1 and DEH100 groups compared to the control condition (Table 3). Regardless of the control or experimental condition, the FA composition of *M. galloprovincialis* was characterized by a high content of polyunsaturated FAs (PUFA, 36.71–39.88%, $p < 0.05$), followed by an intermediate level saturated FAs (SFAs, 27.28–32.19%, $p < 0.05$) and a lower amount of monounsaturated FAs (MUFAs, 16.11–17.39%, $p > 0.05$). The most abundant fatty acids revealed in the soft tissues of mussels, were palmitic acid (C16:0, 19.33–22.90%, $p < 0.05$), stearic acid (C18:0, 4.67–6.01%, $p < 0.05$), palmitoleic acid (C16:1 ω-7, 3.36-5.84.47-6.41, $p < 0.05$), eicosapentaenoic acid (C20:5 ω-3, 12.86–14.59%, $p < 0.05$) and docosahexaenoic acid (C22:6 ω −3, 14.92–15.91%, $p > 0.05$) (Table 3). DEHT exposure affected the lipid metabolism of *M. galloprovincialis* with respect to the control condition. Conversely, non-significant changes were observed between DEHT1 and DEHT100 mussels, which were basically similar in both lipid content and FA composition. Overall, an increase in total lipid from 5.52% in control mussels to 8.75–8.41% ($p < 0.05$) in exposed organisms was reported. Also, a general decrease in SFAs, particularly reflected in the variation of palmitic acid from 22.90% (control) to 20.73–19.33% (DEHT exposure, $p < 0.05$), and a general increase in PUFAs, especially shown in the variation of ω3 FAs from 30.82% (control) to 33.96–33.38% (DEHT exposure, $p < 0.05$), were highlighted. Regarding the latter, most of PUFAs were characterized by an upward trend after DEHT exposure (e.g., the stearidonic acid [C18:4 ω-3] increased from 1.24% to 1.59–1.71%, $p < 0.05$), except for arachidonic acid (C20:4n-6), which slightly decreased from 4.35% to 4.04–4.01% ($p > 0.05$). Interestingly, the plasticizer DEHT did not induce significant changes in MUFAs (Table 3).

Table 3
Total lipid (%, fw) and FA profile (%of total FAs, fw) of control and treated pools (i.e., DEHT1 and DEHT100) of *M. galloprovincialis*. Data are presented as mean ± SD of triplicate analysis.

Analyte (%)	Test groups		
	Control	DEHT1	DEHT100
Total lipid	5.52 ± 0.53^{a}	8.75 ± 0.36^{b}	8.41 ± 0.94^{b}
C14:0	2.07 ± 1.36^{a}	2.63 ± 0.22^{a}	2.35 ± 0.19^{a}
C16:0	22.90 ± 0.35^{a}	20.73 ± 0.76^{b}	19.33 ± 0.42^{b}
C17:0	1.21 ± 0.02^{a}	$0.98 \pm 0.04^{a,b}$	0.93 ± 0.08^{b}
C18:0	6.01 ± 0.52^{a}	$4.81 \pm 0.29^{a,b}$	4.67 ± 0.39^{b}
SFA	32.19 ± 0.84^{a}	29.15 ± 0.91^{b}	27.28 ± 0.97^{b}
C16:1 ω-7	4.47 ± 0.11^{a}	5.84 ± 0.25^{b}	6.41 ± 0.19^{c}
C17:1	1.12 ± 0.26^{a}	1.25 ± 0.08^{a}	1.05 ± 0.05^{a}
C18:1 ω-9	5.81 ± 0.24^{a}	3.36 ± 0.15^{b}	3.53 ± 0.25^{b}
C18:1 ω-7	2.23 ± 0.41^{a}	2.30 ± 0.07^{a}	2.20 ± 0.11^{a}
C20:1 ω-11	1.51 ± 0.51^{a}	1.69 ± 0.14^{a}	1.54 ± 0.10^{a}
C20:1 ω-7	0.97 ± 0.06^{a}	1.11 ± 0.08^{a}	1.03 ± 0.05^{a}
C20:1 ω-9	1.87 ± 0.18^{a}	$1.84^{a} \pm 0.17$	1.60 ± 0.13^{a}
MUFA	16.11 ± 0.54^{a}	17.39 ± 0.14^{b}	17.36 ± 0.01^{b}
C18:2 ω-6	0.92 ± 0.07^{a}	1.01 ± 0.01^{a}	0.96 ± 0.05^{a}
C18:3 ω-6	0.06 ± 0.01^{a}	0.11 ± 0.01^{a}	0.10 ± 0.01^{a}
C18:3 ω-3	0.48 ± 0.04^{a}	0.41 ± 0.04^{a}	0.44 ± 0.04^{a}
C18:3 ω-4	0.18 ± 0.01^{a}	0.27 ± 0.02^{b}	0.29 ± 0.01^{b}
C18:4 ω-3	1.24 ± 0.12^{a}	1.59 ± 0.10^{b}	1.71 ± 0.17^{b}
C20:2 ω-6	0.26 ± 0.03^{a}	0.36 ± 0.04^{a}	0.38 ± 0.03^{a}
C20:3 ω-6	0.18 ± 0.01^{a}	0.14 ± 0.01^{b}	0.31 ± 0.03^{c}
C20:4 ω-6	4.35 ± 0.39^{a}	4.04 ± 0.41^{a}	4.01 ± 0.20^{a}
C20:5 ω-3	12.86 ± 0.47^{a}	14.59 ± 0.67^{b}	14.08 ± 0.15^{b}
C22:5 ω-3	1.26 ± 0.05^{a}	1.35 ± 0.08^{a}	1.40 ± 0.10^{a}
C22:6 ω-3	14.92 ± 0.99^{a}	15.91 ± 0.82^{a}	15.66 ± 0.20^{a}
PUFA	36.71 ± 0.20^{a}	39.88 ± 0.39^{b}	39.34 ± 0.74^{b}
Σω3	30.82 ± 0.32^{a}	33.96 ± 0.14^{b}	33.38 ± 0.47^{b}
Σω6	5.71 ± 0.29^{a}	5.59 ± 0.37^{a}	5.81 ± 0.24^{a}
ω6/ω3	0.18 ± 0.01^{a}	0.16 ± 0.01^{a}	0.17 ± 0.01^{a}

a–c: different superscript letters in the same row indicate significantly different values for a given parameter ($p < 0.05$ b y post hoc Tukey's HSD test); same superscript letters in the same row indicate not significantly different values for a given parameter ($p > 0.05$ b y post hoc Tukey's HSD test.

3.3. Total protein

Total protein from control and test mussel pools are described in Table 4. The mean protein content of *M. galloprovincialis* in the control group was equal to 15.95% and decreased after DEHT exposure, as DEHT1 and DEHT100 mussels had respectively mean protein levels of 14.76% and 11.46%. Statistical analysis showed a significant decrease ($p < 0.05$) for the DEHT100 group compared to the control group and for the DEHT1 group compared to the DEHT100 group .

3.4. Cell viability

After exposure of *M. galloprovincialis* to 1 mg/l and 100 mg/l of DEHT, haemolymph cells showed a high stability of the lysosomal membrane and high viability (≥93%) (Tables 5–6), thus resulting non significantly different ($p > 0.05$) from the control group (≥95%). Similar considerations could be made for DG cells coming from control mussels and organisms exposed to 1 mg/l of the plasticizer (≥97%, $p > 0.05$). DG cells from DEHT1 mussels showed a significantly lower viability respect to the control group after staining with NR (i.e., 98.5% vs. 97.1%, $p < 0.05$), and there is also significance between the two exposure groups DEHT1 and DEHT100 staining with NR (i.e., 97.1% vs. 97.9%, $p < 0.05$).

3.5. Regulatory volume decrease

Under physiological conditions, DG cells *M. galloprovincialis* can regulate their volume in the presence of hypotonic solution, by swelling and gradually returning to their original volume over time. In Fig. 1, DG cells in the control group slowly reached their maximum volume after 10 min of exposure to the hypotonic solution. Conversely, DG cells from exposure groups (DEHT1 and DEHT100) showed a lower degree of swelling and did not return to their initial volume. Specifically, the final volume of DEHT1 cells was even higher than their initial volume, and the volume of DEHT100 cells was even lower than control cells. Overall, statistical analysis showed that DEHT1 and DEHT100 mussels has a significantly lower ability to regulate their DG cell volume with respect to the control condition ($p < 0.05$). Moreover, this ability appeared to be significantly more impaired with increasing exposure levels.

3.6. Oxidative status evaluation

The activity of antioxidant enzymes, as well as the levels of oxidative products, are typically used as indexes of an altered oxidative homeostasis, thus reflecting the general health status of an organism (Filice et al., 2023; Lombardo et al., 2022). Accordingly, the activity of SOD and the levels of LPO and OMP in gills and DG of *M. galloprovincialis* exposed to the plasticizer were measured and reported in Figs. 2 and 3. In both gills and DG, SOD activity showed a slight, not significant ($p > 0.05$), concentration-dependent increase in DEHT-exposed animals

Table 4
Total protein (%, fw) of control and treated pools (i.e., DEHT1 and DEHT100) of *M. galloprovincialis*. Data are presented as mean ± SD of triplicate analysis.

	Test groups		
	Control	DEHT1	DEHT100
Total protein (%)	15.95 ± 1.03^{a}	14.76 ± 0.29^{a}	11.46 ± 0.08^{b}

a–b: different superscript letters in the same row indicate significantly different values for a given parameter ($p < 0.05$ b y post hoc Tukey's HSD test).

Table 5
Effects of DEHT1 and DEHT100 exposure on the viability of haemolymph and digestive gland (DG) cells of *Mytilus galloprovincialis* assessed by the Trypan Blue exclusion method. Data are expressed as % of viable cells (mean ± SD of ten duplicates analysis).

Trypan Blue exclusion method (%)	Test groups		
	Control	DEHT1	DEHT100
Haemocytes	95.4 ± 2.1[a]	93.6 ± 4.9[a]	93.6 ± 4.9[a]
DG Cells	97.2 ± 0.5[a]	98.0 ± 0.3[a]	97.5 ± 0.4[a]

a–c: different superscript letters in the same row indicate significantly different values for a given parameter ($p < 0.05$ b y post hoc Tukey's HSD test); same superscript letters in the same row indicate not significantly different values for a given parameter ($p > 0.05$ b y post hoc Tukey's HSD test).

Table 6
Effects of DEHT1 and DEHT100 exposure on the viability of haemolymph and digestive gland (DG) cells of *Mytilus galloprovincialis* assessed by the Neutral red retention assay. Data are expressed as % of viable cells (mean ± SD of ten duplicates analysis).

Neutral red retention assay (%)	Test groups		
	Control	DEHT1	DEHT100
Haemocytes	96.9 ± 1.6[a]	97.0 ± 2.9[a]	96.9 ± 2.9[a]
DG Cells	98.5 ± 1.5[a]	97.1 ± 1.8[b]	97.9 ± 1.4[a,c]

a–c: different superscript letters in the same row indicate significantly different values for a given parameter ($p < 0.05$ b y post hoc Tukey's HSD test); same superscript letters in the same row indicate not significantly different values for a given parameter ($p > 0.05$ b y post hoc Tukey's HSD test).

(gills: ctrl = 31.27 U/mg prot, DEHT1 = 33.67 U/mg prot, DEHT100 = 38.61U/mg prot; DG: ctrl = 14.05U/mg prot, DEHT1 = 16.71U/mg prot, DEHT100 = 16.05 U/mg prot).

No differences were observed in the levels of TBARS, and aldehydic and ketonic derivatives, in the three experimental conditions.

4. Discussion

Due to the high production volumes and numerous applications, plastics of various sizes have been detected in a wide range of aquatic environments and have already been shown to bioaccumulate through the food chain, thus, causing adverse toxic effects. In this scenario, additives, such as PAEs and NPPs, represent emerging pollutants that are intrinsically linked to plastic pollution, but have not yet been adequately addressed for the range of sublethal effects they can induce in aquatic organisms, including the sentinel *M. galloprovincialis*.

In this study, we demonstrated for the first time that the NPP DEHT can bioaccumulate in *M. galloprovincialis* and consequently induce alterations in lipid and protein metabolism and certain physiological processes, without affecting oxidative stress processes. Although defined as a not-highly persistent and accumulative plasticizer (Bui et al., 2016), the first evidence of DEHT bioaccumulation in aquatic organisms dates back to a study by Liu et al. (2019), which found very low levels of DEHT in the tissues of Chinese water snake and common carp from a pond polluted with e-waste (3.2 μg/kg and 12 μg/kg respectively) (Liu et al., 2019). More recently, DEHT was revealed at higher concentrations in Mediterranean coastal waters (range 0.25–0.93 mg/ g,k Jebara et al., 2021 and range 0.68–1.21 mg/l, Gugliandolo et al., 2020) and sediments (range:0.67–2.86 mg/kg dw, Jebara et al., 2021; Souaf et al., 2023), as well as in biota. Indeed, the sea grass *Posidonia oceanica* (0.32–11.1 mg/kg dw, Jebara et al., 2021; Souaf et al., 2023), the gilthead sea bream (14.9–37.7 mg/kg, dw), the shark *Hexanchus griseus* (liver: 2.40 mg/kg dw, Salvo et al., 2020) and *M. galloprovincialis* (0.58–5.68 mg/kg dw, Souaf et al., 2023, dw) reasonably bioaccumulated such NPP at higher concentrations than abiotic matrices. Clearly, these previous field studies highlighted much lower DEHT levels than those found in mussels exposed to 1 mg/l and 100 mg/l of DEHT under laboratory conditions (respectively, 123.69 mg/kg and 595.13 mg/kg on a fw basis), but they still give an idea of the ubiquitous presence of this plasticizer also in the Mediterranean area, and the urgent need to explore its toxic effects on aquatic life at environmental realistic concentrations.

Macronutrients, such as lipids and proteins, are involved in many vital functions of aquatic organisms. They have already proved to be useful trophic markers for tracing predator-prey relationships and defining the food webs of the marine environment (De Troch et al., 2012; Kelly and Scheibling, 2012), since some of them can only be obtained from peculiar food resources (e.g., essential nutrients such as essential FAs). However, they were claimed to be also good biomarkers of ecosystem health (Maazouzi et al., 2008) and indicators of stress in response to xenobiotic exposure (Sánchez-Muros et al., 2013; Gonc alves et al., 2016; Filimonova et al., 2016). On this basis, results from this study confirmed that DEHT exposure caused evident alterations in lipid of *M. galloprovincialis*. Although lipids vary depending on the diet and the surrounding environmental conditions from which the mussel

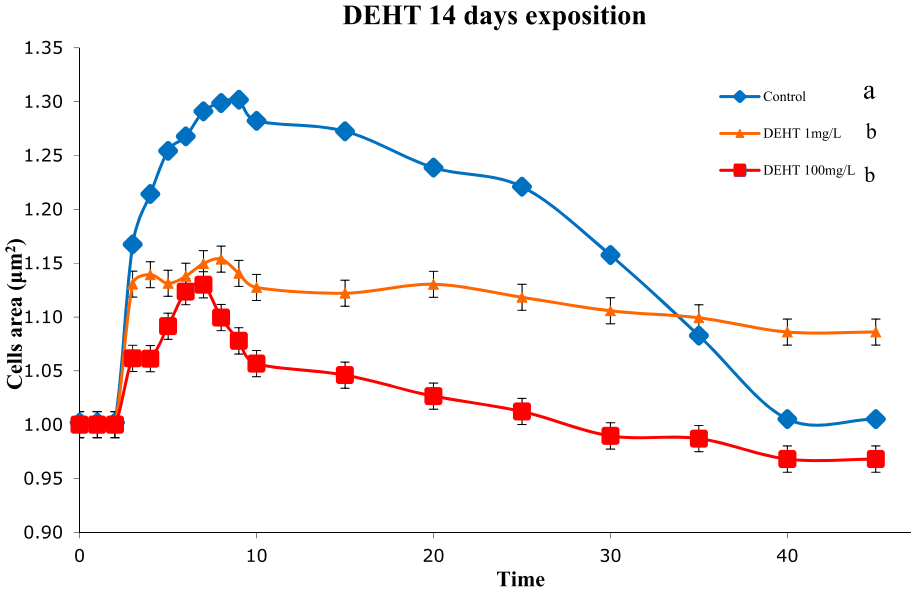

Fig. 1. Effects of DEHT1 and DEHT100 exposure on the regulatory volume decrease of DG cells of *Mytilus galloprovincialis*. Data are expressed as mean cells area ±SD of triplicate measurements.
a–b: different superscript letters in the same row indicate significantly different values for a given parameter ($p < 0.05$ b y post hoc Tukey's HSD test); same superscript letters in the same row indicate not significantly different values for a given parameter ($p > 0.05$ b y post hoc Tukey's HSD test).

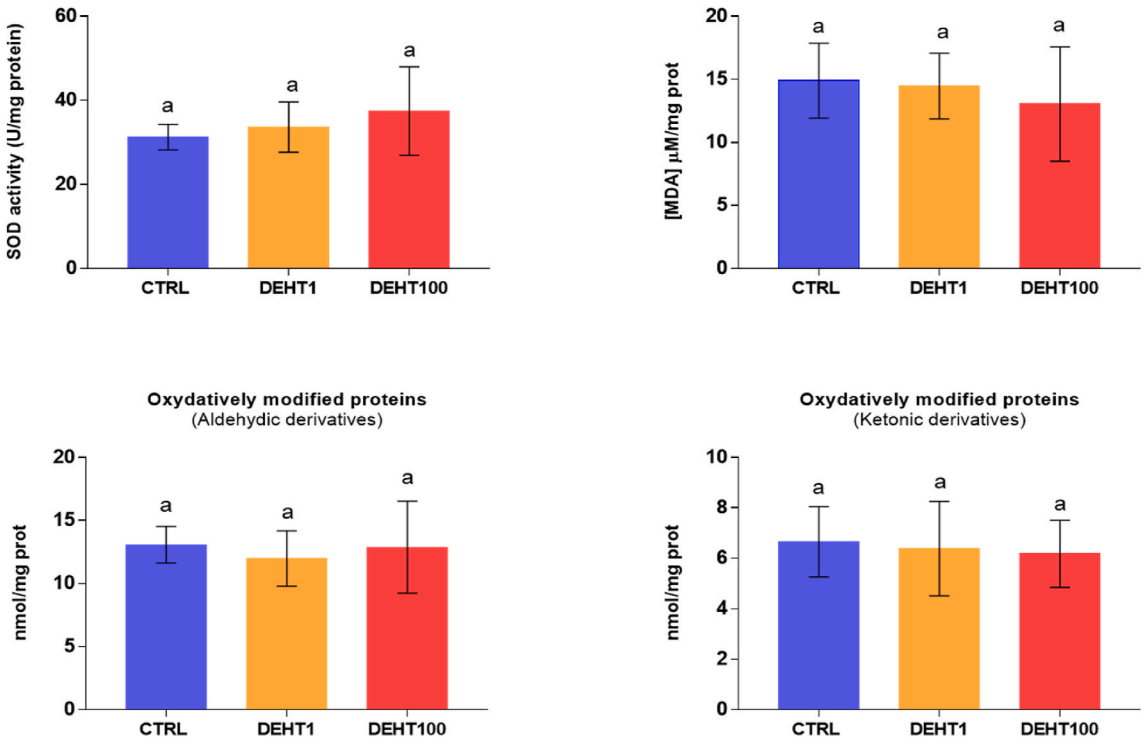

Fig. 2. Effects of DEHT1 and DEHT100 exposure on the SOD activity, TBARS, and OMP of the gills of *M. galloprovincialis*. Data are expressed as mean ± S.E. of absolute values of individual experiments performed in duplicate.
a–b: different superscript letters in the same row indicate significantly different values for a given parameter ($p < 0.05$ b y post hoc Tukey's HSD test); same superscript letters in the same row indicate not significantly different values for a given parameter ($p > 0.05$ b y post hoc Tukey's HSD test).

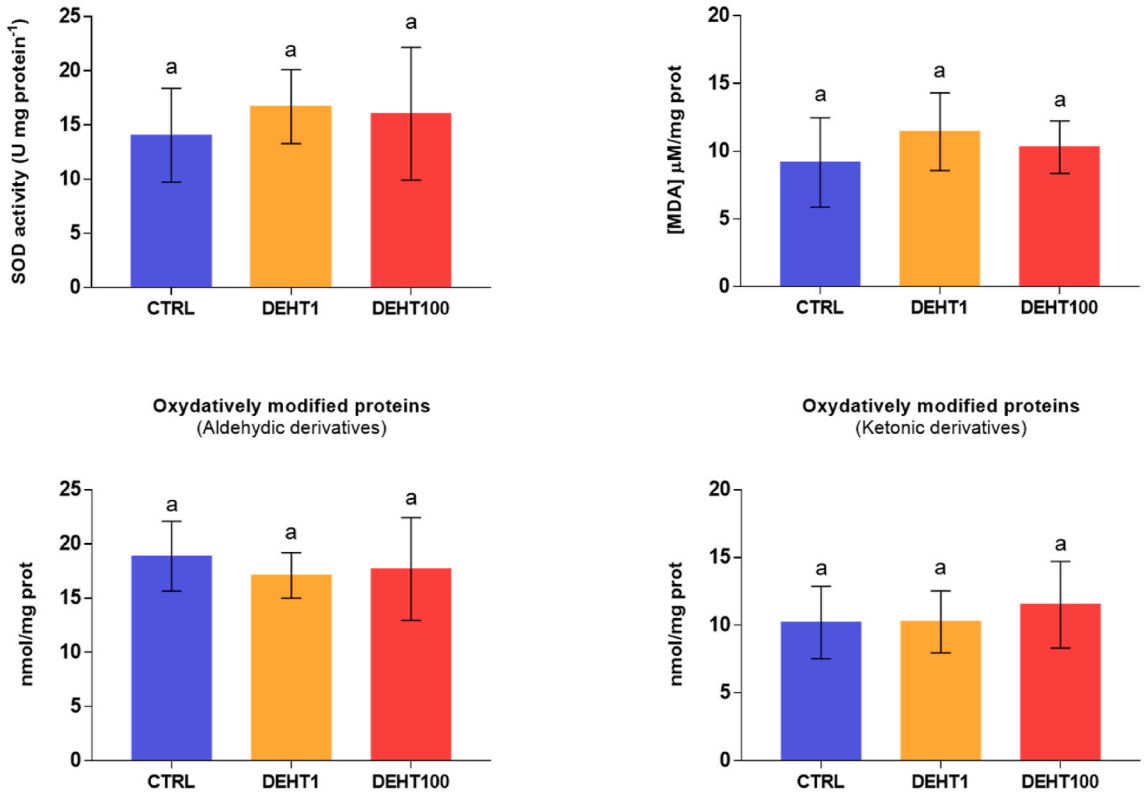

Fig. 3. Effects of DEHT1 and DEHT100 exposure on the SOD activity, TBARS, and OMP in the DG of *M. galloprovincialis*. Data are expressed as mean ± S.E. of absolute values of individual experiments performed in duplicate.
a–b: different superscript letters in the same row indicate significantly different values for a given parameter ($p < 0.05$ b y post hoc Tukey's HSD test); same superscript letters in the same row indicate not significantly different values for a given parameter ($p > 0.05$ b y post hoc Tukey's HSD test).

comes, specimens from the control group showed total lipid and FA profile quite comparable to those displayed in previous works on mussels from the same collection site (Costa et al., 2015,2016), especially with respect to the major SFAs, MUFAs and PUFAs. However, after laboratory exposure to both experimental DEHT concentrations, mussels were characterized by an increase in total lipids and their FA profile showed peculiar variations depending on the FA class (i.e., decreased SFAs, increased PUFAs, and unchanged MUFAs). The interpretation of these findings is a challenging task, since, to the best knowledge of the authors, there has been no previous attempt to investigate lipid and FA profile changes after exposure of aquatic organisms to DEHT. However, similarly to this study, Henderson and Sargent (1983) pointed out that the rainbow trout fed with a DEHP-based diet (2%) showed a reduction in the content of palmitic acid in liver, muscle and adipose, as well as an increase in the eicosapentaenoic and docosahexaenoic acids and a slight decrease in arachidonic acid in the same tissues. Similar variations were also indicated by Morris et al. (1982), which exposed *Gammarus duebeni* to lypophilic contaminants including PAE plasticizers. Despite the decrease observed in the content of palmitic acid, the crustacean body showed an increase in the percentage level of palmitoleic acid, eicosapentaenoic and docosahexaenoic acids (Morris et al., 1982). Overall, it may be speculated that, similarly to PAEs, also NPPs may impact the organism's lipidome by altering the expression of genes involved in lipid metabolism (e.g., desaturase and elongase genes) and stress response (Pradhan et al., 2018; Saad, 2021; Wei et al., 2023).

For example, bivalves are capable of the *de novo* synthesis of certain FAs, namely non-methylene-interrupted fatty acids (NMIFAs), such as the dihomo-γ-linolenic acid (C20:3 ω-6) which is synthesized from γ-linolenic acid (C18:3 ω-6) by activating the elongase activity (Freites et al., 2002; Zhukova, 1991). On this basis, the increase in the dihomo-γ-linolenic acid reported in mussels exposed to 100 mg/l of DEHT could be due to a potential impairment of the elongation pathway induced by the NPP plasticizer. However, according to our results, organisms exposed to an environmental concentration of DEHT (1 mg/l) showed levels of such FA comparable to the control mussels, probably due to the well-known phenomenon of hormesis (Xu et al., 2021).

DEHT exposure induced significant change also in the protein metabolism of *M. galloprovincialis*. Mussels under physiological conditions (control group) showed a much lower protein content than that reported in literature for other *Mytilus* species. For example, *M. edulis* from China had total protein varying from 42.23% to 45.62%, although on a dw basis (Chi et al., 2012). No other studies were found on the variation of protein content in *M. galloprovincialis*; the study reported by Chi et al. (2012) represents a point of comparison that is distant from ours, both in terms of species and origin of the study. However, total protein tended to progressively decrease from the control group to DEHT100 group, with intermediate levels found in DEHT1 mussels.

Similarly, to lipid, the comparison of our data with previous literature is somewhat troublesome, since, to the best knowledge of the authors, there are no previous studies focusing on protein changes after exposure of aquatic organisms to DEHT. However, similarly to this study, *Daphnia magna* exposed to a concentration of DEHP ranging from 0 to 811 μg/l show a variable decrease in protein content, ranging from 241 μg/daphnia to 128 μg/daphnia (Knowles et al., 1987).

To better understand the health status of the model organism, cell viability tests are performed on haemolymph and digestive gland cells (Impellitteri et al., 2022). The uptake of NR by bivalve hemocytes occurs through mechanisms such as pinocytosis or passive diffusion across cell membranes. This phenomenon is often used to assess the effect of stressors on the stability of lysosomal membranes in bivalve cells. Indeed, lysosomes are of great importance, and extensive research has been devoted to investigating their role as a target organelle for monitoring the aquatic environment in which these animals live. Effective regulation of this process is likely to contribute to the ability of certain organisms to tolerate stressful and polluted environments. A cell is considered viable if lysosomal stability and viability exceed 80%. Viability tests conducted on mussels exposed to DEHT showed that there was a slight decrease in viability, although this was not significant. Only digestive gland cells showed high significance ($p \leq 0.01$) in the DEHT100 group compared to the control. The study by Choy and co-workers (2021) examined the exposure of these organisms to polyethylene terephthalate microfibres and showed a dose-dependent increase in the presence of apoptotic and necrotic haemocytes in exposed mussels compared to controls. This is an indication that the presence of plasticizers influences haemocytes, as in our case, since they are the first cells involved in cellular immune responses. Furthermore, exposure to microplastics >0–80 μm (Von Moos et al., 2012) and to polystyrene microplastics (González-Soto et al., 2019) showed that there is a significant reduction in lysosomal membrane stability, as in our case, evidenced by NR staining, ranging from 99% in the control to 97%, with a significance of $p \leq 0.01$. The trypan blue dye exclusion test is used to determine the number of viable cells in a cell suspension. It relies on the fact that living cells have intact cell membranes that prevent certain dyes, like trypan blue, from entering, while dead cells do not. However, in our case, the trypan blue method data showed no significant changes in cell viability among the tested groups, with an average viability of over 90%. This finding is not uncommon, as another study investigating the impact of microplastics (polystyrene, 35–50 μm) on our experimental model also found no significant changes in cell viability after trypan blue staining (Impellitteri et al., 2023a,b). It is worth noting that although the integrity of the cell membrane may remain intact, the cell's overall viability, including its ability to grow or function, could still be compromised.

The DG of *M. galloprovincialis* plays a critical role in the absorption and processing of essential nutrients needed for the animal's survival. The hepatopancreas will therefore be the organ most exposed to xenobiotics and it is consequently susceptible to damage. Exposure to terephthalate reduces the ability of these cells to regulate their own cell volume when exposed to a hypotonic solution. Even at the environmental concentration of DEHT, the cells could not reach the maximum volume achieved by the control group, nor could they return to their original volume, remaining with an area greater than 1 (exactly 1.09 μm^2), significance $p < 0.05$. Even the cells exposed to the 100-fold higher concentration showed an irregular pattern and their volume was significantly reduced at the end (0.97 μm^2) compared to the control group ($p < 0.01$). These tests assess the ability of cells to regulate their volume in response to a hypotonic solution, which is important for determining cell functionality and health. Similar tests have been conducted on other contaminants in the aquatic environment, such as pesticides, fungicides (Ravi et al., 2023; Tresnakova et al., 2023; Stara et al., 2021), heavy metals (Torre et al., 2013; Banaee et al., 2022), additives (Freitas et al., 2020) and pharmaceuticals (Pagano et al., 2022; Porretti et al., 2022), to confirm how these substances can affect cell functionality.

Marine bivalves dispose of an efficient antioxidant defence system which allows them to face oxidative stress when exposed to pollutants (Freitas et al., 2020; Curpan et al., 2022; Filice et al., 2023; Lombardo et al., 2022). In *M. galloprovincialis*, the influence of plastics pollutants on antioxidant enzyme activity, as well as on the expression levels of oxidative biomarkers is, to date, poorly investigated. The few available studies suggest effects which strictly depend on the specific substance used, along with the intensity and duration of the treatment. Data mainly refer to changes in the activity of antioxidant enzymes, such as Glutathione S-transferases (GST) and Catalase (CAT), while information on lipid peroxidation and protein carbonylation are lacking or even absent. In cells of the digestive gland from *M. galloprovincialis*, exposure to either bisphenol A (BPA), or perfluorooctane sulphonate (PFOS), induces a different modulation of GST and CAT (Balbi et al., 2017). Similarly, exposure to sub-lethal concentration of plastic leachates revealed the capacity of various polymers to differently affect the activity of antioxidant enzymes, as well as the levels of lipid peroxidation in gills and DG of *M. galloprovincialis* (Capolupo et al., 2021). A recent

paper by Andreyeva and coworkers reports that in the DG of *M. galloprovincialis* the exposure to DEHP (4.0 mg/l) did not influence SOD activity following 24 h exposure period but induced a 1.8-fold increase after 48 h of treatment (Andreyeva et al., 2023). In our experimental conditions, the exposure to either 1 mg/l or 100 mg/l of DEHT did not affect the activity of the SOD enzyme in gills and DG of *M. galloprovincialis*, as well as the levels of oxidative products. This allows us to hypothesise that at the concentrations and the exposure time of DEHT used in this study, *M. galloprovincialis* does not suffer from oxidative stress. The possibility that the basal levels of antioxidant enzymes are enough to avoid a DEHT-induced oxidative status needs to be further investigated.

5. Conclusion

In conclusion, this study highlights the negative effects of DEHT exposure on the sentinel species *Mytilus galloprovincialis*, including changes in physiological and biochemical parameters, as well as alterations in nutritional parameters such as fatty acid profiles, total lipids, and total proteins. The presence of terephthalate in Mediterranean waters and biota is increasing, which is concerning as it can accumulate in organisms higher up in the food chain, such as humans. This emphasizes the urgent need to reduce plastic pollution in our aquatic ecosystems and to better understand its impact on marine life and ultimately on human health. As plasticizers can alter the nutritional value of seafood, which is an important source of nutrients for humans, this kind of pollution can have significant implications for human health.

Further studies will undoubtedly be necessary, including on other species, to better understand the toxic effects of this compound, not only on its own, but also in combination with other chemical compounds, because unfortunately there is no single plasticizer in the water, but a variety that can act in synergy or in opposition to each other.

Credit author statement

Miriam Porretti: Concenptualization, Formal analys, Writing - original draft; **Federica Impellitteri**: Formal analys, Writing-Editing; **Alessia Caferro**: Formal analysis; **Ambrogina Albergamo**: Writing - review & editing, Validation; **Federica Litrenta:** Formal analysis; **Mariacristina Filice:** Formal analysis; **Sandra Imbrogno:** Supervision; **Giuseppa Di Bella**: Conceptualization, supervision; **Caterina Faggio**: Conceptualization, supervision and revisions.

All authors have read and agreed to the published version of the manuscript.

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

The authors do not have permission to share data.

Acknowledgements

The authors would like to express their gratitude to “FARAU SRL Frutti di Mare” Company (Messina, Italy) for their valuable practical support and provision of facilities at the mussel farm. A special acknowledgement is extended to Mr. Giuseppe Donato.

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