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The hazardous impact of true-to-life PET nanoplastics in *Drosophila*

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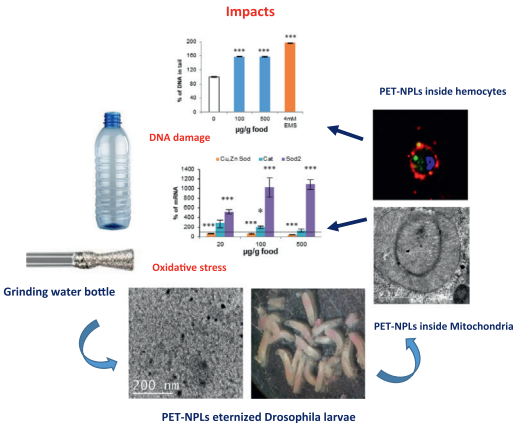
HIGHLIGHTS

- PET nanoplastics resulting from plastic bottle degradation were tested in *Drosophila*.
- PETNPLs' journey, after ingestion, was followed by TEM and confocal microscopy.
- The induction of different hazardous effects was evaluated.
- Changes in the expression of genes regulating different pathways were observed.
- Among the hazards, the induction of oxidative stress and genotoxicity stand out.

GRAPHICAL ABSTRACT



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ABSTRACT

Plastic pollution is a continuously growing problem that can threaten wildlife and human beings. Environmental plastic waste is degraded into small particles termed micro/ nanoplastics (MNPLs) that, due to their small size, can be easily internalized into the exposed organisms, increasing the risks associated with their exposure. To appropriately determine the associated health risk, it is essential to obtain/test representative MNPLs' environmental samples. To such end, we have obtained NPLs resulting from sanding commercial water polyethylene terephthalate (PET) bottles. These true-to-life PETNPLs were extensively characterized, and their potential hazard impacts were explored using *Drosophila melanogaster*. To highlight the internalization through the digestive tract and the whole body, transmission electron microscopy (TEM) and confocal microscopy were used. In spite of the observed efficient uptake of PETNPLs into symbiotic bacteria, enterocytes, and hemocytes, the exposure failed to reduce flies' survival rates. Nevertheless, PETNPLs exposure disturbed the expression of stress, antioxidant, and DNA repair genes, as well as in those genes involved in the response to physical intestinal damage. Importantly, both oxidative stress and DNA damage induction were markedly increased as a consequence of the exposure to PETNPLs.

1. Introduction

Plastics are indispensable materials in our current way of life. One of their good properties, like durability, became a problem with the management of plastic waste. Their extended use, and the fact that an important amount of about 40 % corresponds with the only one-use material (PlasticsEurope, n.d.), suppose a steady increase in their waste levels.

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Since the ability to recycle is very limited, a big proportion ends up in the environment resulting in an important environmental concern. In the environment, plastic suffers a slow but constant physicochemical/biological degradation producing countless mall fragments each time smaller until reaching micro and nano ranges. Such fragments constitute the so-called micro/nanoplastics (MNPLs) (Akhbarizadeh et al., 2020; Liu et al., 2022). At these sizes, MNPLs easily enter the organisms through feeding, drinking, and breathing, cross biological barriers, and diffuse among tissues and organs (Paul et al., 2020; Huang et al., 2022; Alaraby et al., 2022a). At this point, MNPLs cease to be an environmental concern to become a health concern.

Although MNPLs have been reported to be present in human feces (Schwabl et al., 2019), placenta tissues (Ragusa et al., 2021), and blood (Leslie et al., 2022), the quantification of such exposure levels is conflictive because the lack of standard protocols to adequately quantify such exposures. Nevertheless, these results point out the urgent need of getting a good estimation of the potential health hazards posed by MNPLs exposure. It is something obvious that recently a large number of studies have been carried out trying to identify their health hazards but, the majority of these studies have used pristine polystyrene MNPLs as a model. In spite of the advantages of such a PS model, they are not enough representative of the expected environmental secondary MNPLs to which humans are exposed. Consequently, there is a lack of data with more true-to-life representative MNPLs to get sound data to be used for risk assessment evaluations.

To solve these problems, different studies have been carried out to get MNPLs from real plastic goods. These true-to-life MNPLs can result very useful to determine the risk associated with environmental exposures. Different experimental approaches have been proposed such as the use of UV-laser ablation to obtain nanoplastics from polyethylene terephthalate (PET) bottles (Magrì et al., 2018) and the mechanical grinding of PET water bottles (Rodríguez-Hernández et al., 2019). Mechanical milling is another process used to obtain mainly the MPLs fraction (Pignattelli et al., 2021; Lionetto et al., 2021). As observed, most of the studies used PET as a source to get MNPLs; this is due to the extended use of this polymer as textile fibers and single-use beverage bottles and packaging goods (El-Sherif et al., 2022), which supposes a consequent environmental impact (Bai et al., 2022). In fact, we have recently published a protocol to produce PETNPLs by sanding water bottles using a diamond burr (Villacorta et al., 2022). This approach succeeds in producing uniform samples of PET-NPLs of around 100 nm, which have been evaluated in the current study.

Although *in vivo* studies using mammals seem to be the best approach to generate sound data to be used for risk assessment, they present serious limitations related to ethical considerations and manipulation difficulties, including elevated costs in terms of money and time. In this scenario, the use of more simple organisms can represent a good alternative to the use of rodents. Among these model organisms *Drosophila melanogaster* stand-out. This model has many experimental advantages, such as a short life cycle (less than two weeks), easy manipulation, and ethical acceptance. Furthermore, their genome contains genes sharing homology with 75 % of the genes involved in human pathologies (Yamaguchi et al., 2021). Accordingly, it is assumed that represents a good model to extrapolate the obtained findings to humans (Alaraby et al., 2016; El Kholy et al., 2021a, 2021b). Recently, *Drosophila* has been used to evaluate the potential risks associated with polystyrene nanoplastics exposure (Alaraby et al., 2022a).

Taking into account the aforementioned advantages of *Drosophila*, we have explored the risk associated with true-to-life PETNPLs determining their toxicity, uptake, oxidative stress induction, interaction with the intestinal barrier, genotoxic, and mode of action by determining changes of expression in genes involved in different pathways.

2. Materials and methods

2.1. Obtention and characterization of PET nanoplastics

The used true-to-life PETNPLs were obtained using the protocol contained in our recent work (Villacorta et al., 2022). Shortly, PET water plastic bottles were used as a source. PET powder was obtained by sanding bottle pieces with a diamond rotary burr, sieved, and dispersed by stirring in pre-heated trifluoroacetic acid. After centrifugation, the supernatant was removed, and the pellet was resuspended in sodium dodecyl sulfate and sonicated. The emulsion was allowed to settle, and the supernatant was washed with Milli-Q water and pure ethanol, to discard SDS, and left to dry. The obtained PETNPLs powder was resuspended in Milli-Q, sonicated, aliquoted in cryotubes, and immediately frozen in liquid nitrogen.

PETNPLs were characterized by using a Malvern Zetasizer Nano-ZS zen3600 instrument (Malvern, UK), transmission electron microscopy (TEM; Jeol 1400; Jeol Ltd., Tokyo, Japan), and a Hyperion 2000 microspectrometer (Bruker, Billerica MA, USA). Zetasizer was used to detect the hydrodynamic size and the zeta potential of PETNPLs. TEM images were analyzed with ImageJ software to evaluate PETNPLs' average size as well as the degree of dispersity. The chemical composition and functional groups of PETNPLs were determined using Fourier transform infrared spectroscopy (FTIR). All the detailed information about these methods is fully detailed in our recent paper (Villacorta et al., 2022).

2.2. Investigation of health impacts of PETNPLs exposure

The Canton-S strain (wild-type) of *Drosophila melanogaster* was used in all the experiments aiming to determine the biological effects of PETNPLs exposure. This strain has been kept in our laboratory for a long and maintained in conditions of 21 °C, 60 % humidity, and (12 h/12 h light/dark).

2.2.1. Toxicity

The toxic effects of PETNPLs were determined as changes in the egg-to-adult survival. A total of 250 eggs (50 per replicates) were seeded in food vials containing 4 g of instant medium (Carolina Biological Supply, Burlington, NC, USA), previously wetted with 10 mL of various concentrations (0.0, 8, 40, and 200 μg/mL, equivalent to 0.0, 20, 100 and 500 μg/g of food) of PETNPLs. Treatment vials were kept at conditions of 25 °C, 60 % humidity, and 12/12 h light/dark periods. Milli-Q water was used to wet the untreated medium for negative control. Once all flies emerged, they were counted and preserved in 70 % alcohol for further investigation. Emergence rates in the control and in the exposed vials were considered for the statistical analysis.

2.2.2. PETNPLs uptake

Detecting the internalization of our material of study is essential to give sound value to further hazard determination studies. To such end, two different methodologies: TEM and confocal microscopy have been used. These approaches can permit us to follow the PETNPLs' journey from ingestion to its interaction/crossing the intestinal barrier, and reaching hemolymph, as the general larval body compartment.

2.2.2.1. TEM investigation. To highlight the ability of PETNPLs to cross the intestinal barrier, as an important issue that should be explored, fifth-day treated larvae (500 μg/g of food) were collected and dissected in phosphate buffer (PB; 0.1 M, pH 7.4), following the different steps outlined our previous protocol (Alaraby et al., 2015). Accordingly, samples from the isolated intestines were processed to obtain ultrathin sections (100 nm in thickness) that were placed on non-coated 200 mesh copper grids. The intestinal sections were contrasted with uranyl acetate and visualized by TEM (Jeol 1400,100 kV) equipped with a CCD Gatan ES1000w Erlangshen Camera (Gatan Inc., Pleasanton, CA, USA).

2.2.2.2. Confocal microscopy investigation. Drosophila larvae were treated with Nile red-stained PETNPLs (500 μg/g of food). To stain PETNPLs, the procedure described by Villacorta et al. (2022) was followed. PETNPLs (1 mg/mL) was centrifuged at 16100 RCF for 25 min and, after removing the supernatant, 1 mL of Nile red solution (0.50 % in DMSO) was added to stain PETNPLs pellet. The mixture was stirred for 24 h (avoiding light exposure) at 200 rpm. To remove the excess stain, the stained pellet was washed several times with ethanol in 0.10 M PBS, pH 7.40. On the fifth

day, treated larvae with stained PETNPLs were dissected in 1 % PBS and mounted upon a microscopic slide with one cavity. For confocal visualization, Hoechst 33342 (excitation of 405 nm and emission collected at 415–503) was used to stain nuclei, while Cellmask (excitation of 633 nm and emission collected at 645–786) was used to stain cell membranes. To visualize Nile red-stained PETNPLs, an excitation wavelength of 514 nm and emission collected at 546–628 were used. Confocal images were acquired with a Leica TCS SP5 AOBS spectral confocal microscope (Leica Microsystems, Germany) equipped with an HCX PL APO lambda blue 63.0 × 1.40 OIL UV objective (1.4 NA).

2.2.3. Gene expression changes detection

The detection of primary molecular changes, like changes in the expression of defined genes, is considered a sensitive biomarker for any disturbance in the cellular hemostasis induced by the exposure to exogenous agents. Herein, a wide battery of genes covering different levels of responses to PETNPLs exposure was investigated. Representative genes of general stress (*Heat-shock-protein-70* "*Hsp7*" and *Heat-shock-protein-83* "*Hsp83")*, oxidative stress (*Superoxide dismutase-2 "Sod2", Catalase* "*Cat", and Cu/Zn Superoxide dismutase* "*Cu/Zn Sod"*), intestinal genes/physical stress (*Dual oxidase* "*Duox*" and *Prophenoloxidase 2* "*PPO2*"), and repair gene (*8-oxoG glycosylase1* "*Ogg1*") were included.

To processed, we followed our previously protocol (Alaraby et al., 2020). Briefly, 30 third-instar larvae (~50 mg) were homogenized in TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) to obtain pure mRNA. To convert mRNA into complementary cDNA, the Transcriptor First Strand cDNA Synthesis Kit (Roche) was used, and amplified by real-time RT-PCR using a Light Cycler 480 (Roche, Basel, Switzerland). The mixture of each gene (10 μL) contained 4 μL of cDNA (10 ng/μL, 5 μL of SYBER Green mix, and 1 μL of 10 μM gene-specific primers (forward and reverse). The used RT-PCR conditions were: pre-incubation for 5 min at 95 °C (1 cycle), and the amplification was repeated 45 times (10 s at 95 °C, 15 s at 61 °C, 72 °C for 25 s). β-actin was used as reference gene.

2.2.4. Intracellular oxidative stress (ROS) induction

Oxidative stress is one of the effects associated to nanoparticles exposure (El Kholy et al., 2021b), and by extension also to MNPLs. To determine these effects, larvae' hemocytes were used as a suitable target. To proceed, about 60 exposed/unexposed fifth day-old larvae were collected, and the hemocytes from hemolymph were extracted as previously described (Alaraby et al., 2020). Hemocytes from untreated larvae were used as a negative control. Such hemocytes, treated with 0.5 mM H_2O_2, were used as the positive control. Hemocytes from exposed larvae, and those from unexposed larvae were treated with 5 μM 6-carboxy-2,7′-dichlorodihydro-fluorescein diacetate (DCFH-DA) and incubated for 30 min at 24 °C. When DCFH-DA internalize into the cytoplasm is deacetylated by nonspecific esterases and oxidized by intracellular ROS to the green fluorescent product 2′,7′-dichlorofluorescein (DCF). Thus, the presence/intensity of fluorescent signal in hemocytes is indicative of the intracellular levels of ROS. They were detected by a fluorescent microscope with an excitation of 485 nm and an emission of 530 nm (green filter). The effects were quantified with the ImageJ program.

2.2.5. Genotoxicity of PETNPLs

DNA damage is considered as an indicative biomarker of the strong hazardous effects of a defined exposure. To determine such effects, hemocytes from larvae exposed to different doses of PETNPLs were used to determine DNA damage levels by using the comet assay, as previously developed (Alaraby et al., 2021). Hemolymph containing about 10,000 cells was processed using the cited protocol and dropped on the hydrophilic surface of ice-cold Gelbond® films (GBF) (Life Sciences, Lithuania). The experiment was done in triplicate. After the standard steps: lysis, electrophoresis (20 min at 20 V and 300 mA), neutralization, staining (SYBERGold), washing, and drying overnight, the levels of DNA damage were measured using the Komet 5.5 Image-Analysis System (Kinetic Imaging Ltd., Liverpool, UK) fitted with an Olympus BX50 fluorescence microscope. As a measure of the DNA damage, the percentage of DNA in the tail was used, in three replicates of 100 randomly selected cells per dose. In addition to the negative control, 4 mM EMS was used as the positive control.

2.3. Statistical analysis

Firstly, to check the normality and the homogeneity of the obtained data the Kolmogorov-Smirnov & Shapiro-Wilk test and Levene's test were applied. The IBM SPSS Statistics 21 package was used to analyze the data. Data showing normal distribution and equal variance were analyzed with the Student *t*-test and the one-way ANOVA. Those data with skewed distribution and unequal variance were analyzed with nonparametric approaches (Mann-Whitney *U* test). For viability data, the one-way ANOVA was used, while all remain data were analyzed by using the paired *t*-test except for gene expression data, where the nonparametric Mann-Whitney U test was used. Significant differences were considered at the $P \leq 0.05$ level. Data were calculated as mean ± standard error (SE).

3. Results

3.1. Characterization of the obtained PETNPL samples

By using TEM we can observe that our true-to-life PETNPLs from water bottles pieces show a small size and uniform dispersity (Fig. 1a), particles having an irregular shape (Fig. 1b) and an average size mean of 40.08 ± 4.94 nm (Fig. 1c). Interestingly, our PETNPLs show low levels of aggregation. When the hydrodynamic size was determined by DLS, PETNPLs showed an average size of 139 ± 1.22 nm, and a polydispersity index of 0.15). The good dispersity of PETNPLs was further confirmed by the zeta potential value which was found to be −29.90 ± 0.44 nm, bearing a negative charge. Both hydrodynamic size and zeta potential values are presented in Fig. 1d. To confirm the chemical composition of particles produced from water bottle, FTIR methodology was used. The different peaks and values obtained (Fig. 1e, f), clearly confirm the affiliation of these particles to PET, which consists of repeated units of the monomer ethylene terephthalate ($C_{10}H_8O_4$).

3.2. PETNPLs toxicity

Toxicity experiments are valuable tools to determine the range of doses to be used in the further experiments. Toxicity was determined as the total of emerged adult from a number of seeded eggs (egg-to-adult survival). From the total of 250 egg/dose (50 egg/vial), the emerge adults were etherized, counted, and kept in 70 % alcohol for potential future determinations. The emerged adults from each dose were compared with those of the negative control, for the statistical analysis. As indicate in Fig. 2, the different evaluated doses of PETNPLs (4, 20, 100, and 500 μg/g of food) failed to reduce the survival rate of *Drosophila melanogaster*. In addition, changes in the average time were also evaluated, but no differences were observed. Potential morphological alterations in the emerged adults were also checked, but without significant findings. In conclusion, PETNPLs did not exert toxic effects on *Drosophila*.

3.3. PETNPLs internalization

A good determination of the potential hazard associated to PETNPLs exposure requires to determine if the agent is able to internalize in our organism model. To solve this important issue, two experimental approaches namely (i) TEM and (ii) confocal microscopy have been used.

TEM methodologies permit to follow the complete journey of PETNPLs through the different compartments of the larvae body. Representatives images of this journey are observed in Fig. 3.

Thus, we visualize step by step their presence inside the midgut lumen of *Drosophila* larvae up to intestinal enterocytes; reaching cytoplasm and cellular organelles. In the midgut lumen, PETNPLs appear near the peritrophic membrane, with analogue function that mammalian mucus

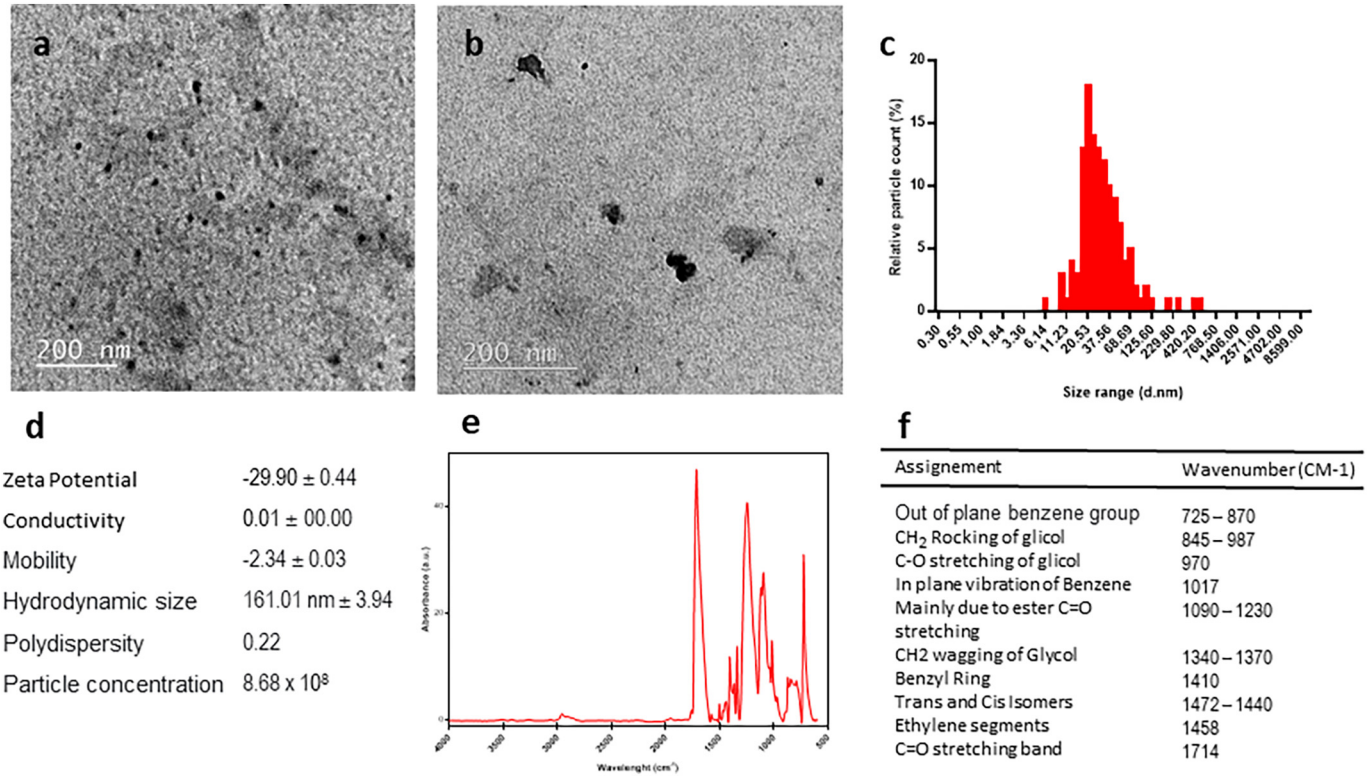

Fig. 1. TEM images (a and b) show the shape, morphology, and dispersity of PETNPLs. Histogram of size frequencies showing a dry mean size of 40.08 ± 4.94 nm (c). Table reporting Zetasizer data including hydrodynamic size (161.01 ± 3.94 nm) and *Z*-potential (−29.90 ± 0.44) (d). Chemical composition of PETNPLs measured by FTIR, showing the major functional groups of polyethylene terephthalate (e and f).

shield (a). Interestingly PETNPLs succeeded to interact with the symbiotic bacteria constituting the gut microbiota, and detected as dense contrasted vacuoles (b). PETNPLs are able to overcome the intestinal barrier and its impenetrable chitinous wall layer (peritrophic membrane) invading its cells components: microvilli, cytoplasm, and mitochondria (c-e). The existence of PETNPLs inside enterocytes cytoplasm introduce morphological and structural changes to mitochondria (f).

When PETNPLs are stained with Nile red, they can be easily visualized by using confocal microscopy (Fig. 4). Due to the membranous texture of *Drosophila* larvae cuticle, Nile red stained PETNPLs can be easily observed in the abdomen region by naked eye (a). Confocal microscopy is a suitable tool in showing not only the entrance of stained MNPLs in biological tissue, but their localization. With this technique, PETNPLs with green signal were detected in the intestinal enterocytes (b, c), also in the celomic compartment (hemolymph) whether at bright field (d) and fluorescent (e) and, what it is especially relevant, inside the hemocytes (f), as cellules equivalent to human lymphocytes.

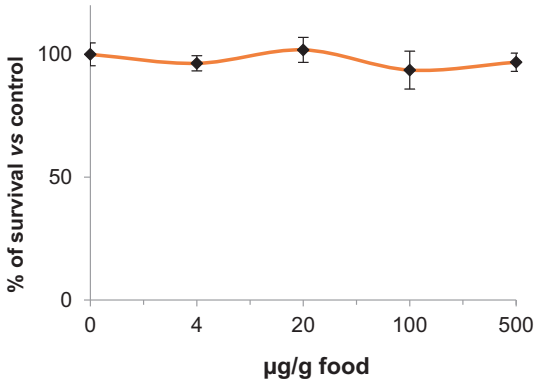

Fig. 2. The rate of survival of *Drosophila melanogaster* after exposure to different doses of PETNPLs, in comparison with untreated flies.

3.4. Molecular induced response as gene expression changes

The early toxic effects of true-to-life PETNPLs can be detected by identify changes in the expression of genes involved in different target pathways. Thus, in Fig. 5 we show those changes induced in a group of selected genes, grouped according their functional role.

Regarding those involved in a general stress response (*Hsp70* and *Hsp83*), the expression of *Hsp70* was significantly down-regulated in *Drosophila* larvae after exposure to PETNPLs. When genes involved in the antioxidant defense were evaluated (*Cu/Zn Sod*, *Sod2,* and *Cat*), the expression of *Cu/Zn Sod* was significantly down-regulated in a dose dependent manner. Contrarily, *Sod2* expression was significantly increased as the PETNPLs exposure increase. The DNA repair gene (*Ogg1*), showed a significant overexpression at all the doses of PETNPLs. Finally, when the genes involved in the response to the physical stress induced on the intestinal barrier (*PPO2* and *Duox*) were evaluated, the expression of both genes showed significant changes with the different doses of PETNPLs. Nonetheless *Duox* appeared as more sensitive, presenting higher values with all the tested doses. As a conclusion of these experiments, the obtained data gives us valuable information on the mode of action of the used true-to-life PETNPLs.

3.5. Oxidative stress induction by PETNPLs

PETNPLs exposure was able to induce significant high levels of intracellular ROS in the hemocytes of the exposed larvae, as observed in Fig. 6. The fluorescent signal of DCF, as indicator of the action of intracellular ROS, was quantified and the results are indicated as related to the background levels observed in the hemocytes of untreated larvae. The effects are dose-dependent and are remarkable the high levels of

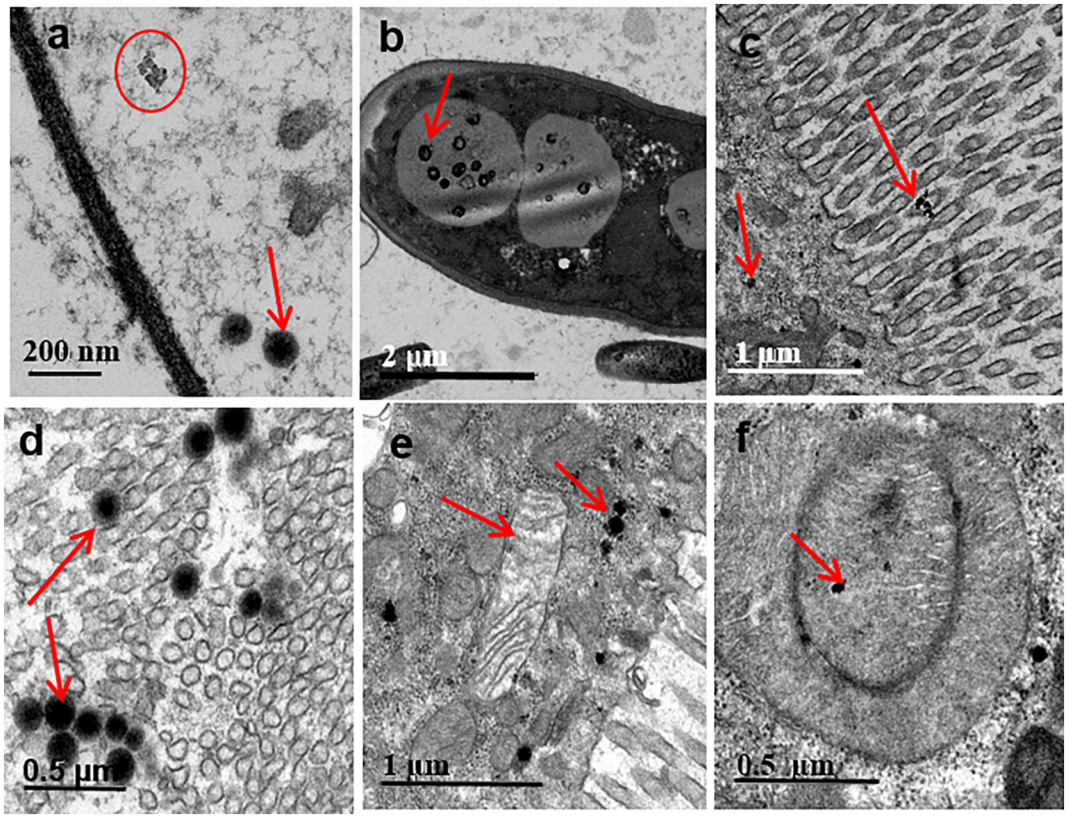

Fig. 3. Internalization and interaction of PETNPLs with intestinal barrier as detected by TEM. (a) PETNPLs appear in the intestinal lumen surrounding the peritrophic membrane, (b) affecting microbiota as several vacuoles inside bacteria, (c, d) appearing within the microvilli and in the cytoplasm of enterocytes, (e, f) surrounding or inside mitochondria from enterocytes.

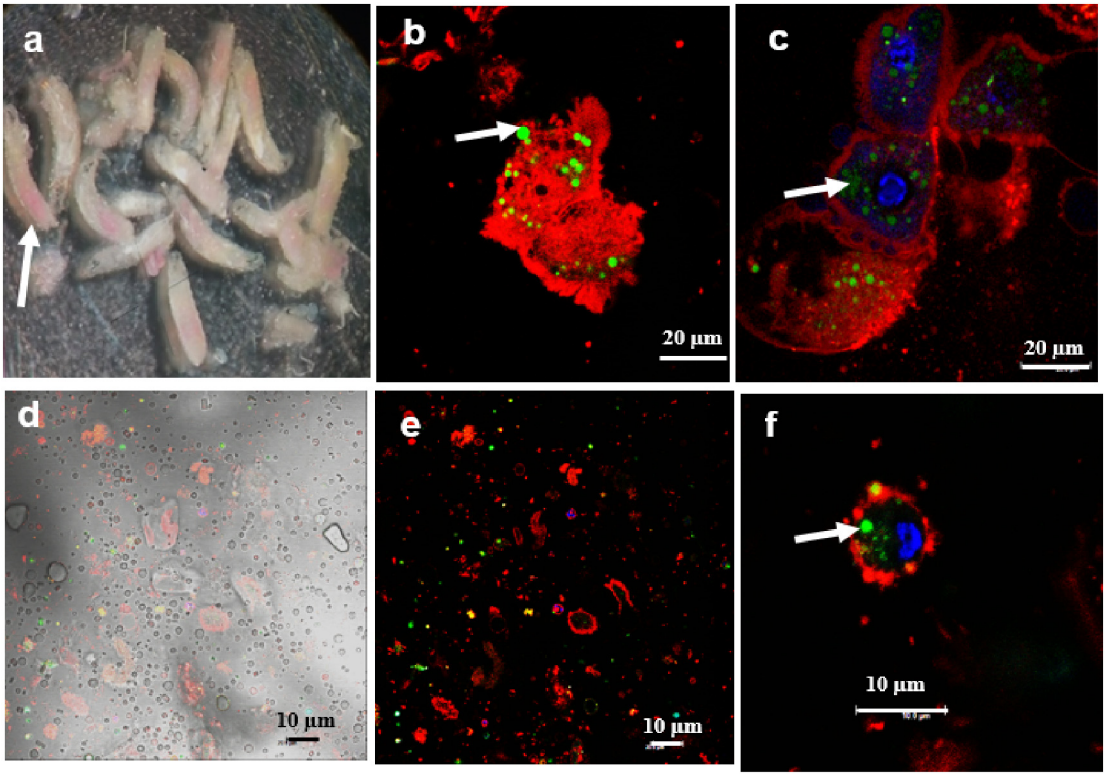

Fig. 4. Internalization of PETNPLs as detected by confocal methodology (a-f). While larvae treated with Nile red stained PETNPLs have red intestinal tract (a), PETNPLs were detected in the intestinal enterocytes (b, c), as well as in (hemolymph), whether at bright (d) or fluorescent field (e), and inside the hemocytes (f).

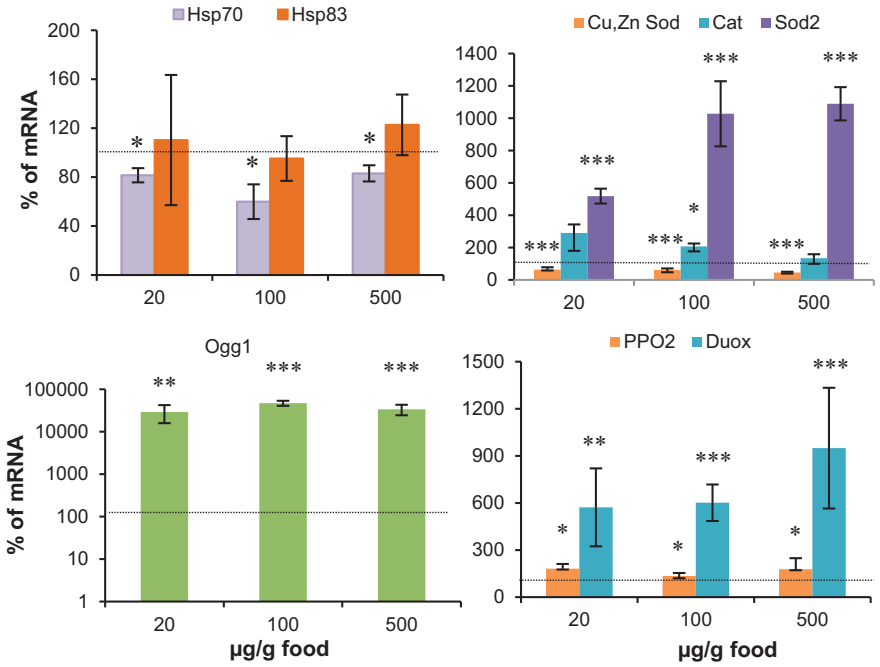

Fig. 5. The expression of different genes including stress genes (*Hsp70, Hsp83*), oxidative stress (*Cat, Sod2, Cu/Zn Sod*), DNA repair (*Ogg1),* and intestinal physical stress genes (*Duox, PPO2*) after exposure to different doses of PETNPLs. The horizontal dotted line correspond to the control values. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Mann-Whitney *U* test).

intracellular ROS detected in the larvae exposed to the highest dose (500 µm/g food). These results clearly indicate that the used true-to-life PETNPLs are able to induce oxidative stress disfunction in the exposed larvae of *Drosophila*.

3.6. DNA damage induction by PETNPLs

Among the different hazardous effects caused by environmental agents, the genotoxic damage stands out. To determine if the used true-to-life PETNPLs were able to induce DNA damage, the comet assay was used. This assay mainly detects single strand DNA breaks directly induced, or as consequence of DNA repair processes involving a base/nucleotide excision pathways. Due to the sensitivity of the assay, only the two highest doses were evaluated. Using the comet assay, it was observed that PETNPLs exposure (100 and 500 µm/g food) were able to induce significant levels of DNA damage. The significant results obtained with the used positive control indicates\ the proper design of the experiments (Fig. 7).

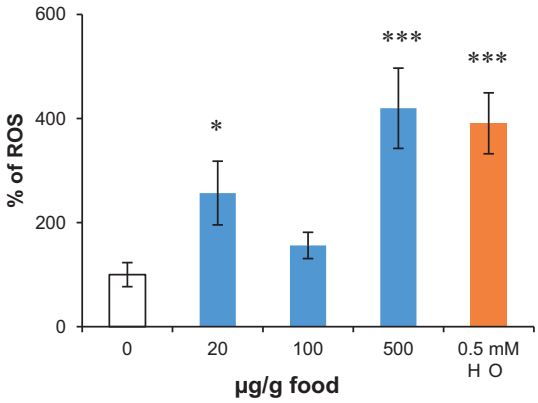

Fig. 6. The intracellular levels of ROS observed in *Drosophila* larvae treated with different doses of PETNPLs. *$P < 0.05$, ***$P < 0.001$ (*t*-test).

4. Discussion

To obtain adequate answers about the potential health hazards posed by the exposure to environmental MNPLs, it is relevant to work with environmental representative samples. This can be reached by mimicking in the lab the natural degradation processes of plastic wastes. From this point of view, we had succeeded in producing reliable uniform PET plastic particles at the nano scale (PETNPLs) *via* grinding samples from PET water bottles (Villacorta et al., 2022). These true-to-life MNPL samples represent a valuable material to get sound data on MNPLs toxicity, useful for future risk assessment studies of MNPL exposures. At this point, it should be remembered that practically all the available studies on the risk associated to MNPL exposures have been carried out using pristine polystyrene nanoplastics, which are far from being considered as representative of those MNPLs found in the environment, resulting from the degradation of plastic goods. The generation of true-to-life MNPLs starts a new research period characterized by the use of MNPLs samples from environmental origin,

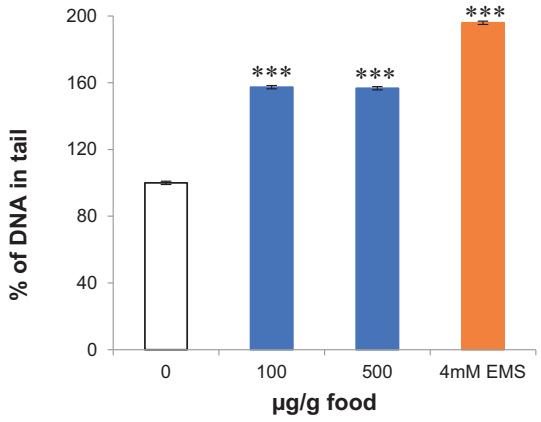

Fig. 7. The levels of DNA damage observed in *Drosophila* larvae hemocytes treated with different doses of PETNPLs and measured in the comet assay. ***$P < 0.001$ (*t*-test).

to obtain more realistic data on the potential harmful effects of environmental MNPLs.

Drosophila has shown high abilities to unveil the hazard impacts of nanoscale materials (Alaraby et al., 2016), including MNPLs (Alaraby et al., 2022a, 2022b). Herein, the survival rate of *Drosophila* flies was not affected after exposure to different concentration of PETNPLs. This lack of systemic toxicity is similar to that observed in *Drosophila* exposed to PSNPLs (Alaraby et al., 2022a), as well to PSMPLs (Demir, 2021), or to PETMPLs (Shen et al., 2021). In spite of this lack of toxicity, other effects were reported such as impaired climbing behavior (Demir, 2021) and decreased oviposition in exposed females (Shen et al., 2021). As a conclusion, exposure to MNPLs, either PETMNPLs or PSMNPLs, do not produce toxic effects such as those affecting the egg-to-adult viability. This would indicate that the harmful effects of MNPLs must be detected at a more subtle level. In this sense, the detected changes of expression in genes involved in the general stress response would point out in a such direction. Interestingly, these effects were also observed when *Drosophila* larvae were exposed to PSNPLs (Alaraby et al., 2022a) and were also reported in *Daphnia* after exposures to PSNPLs (Liu et al., 2018).

Ingestion is considered one of the most relevant exposure routes for MNPLs; consequently, knowing their interactions with the digestive tract components is of paramount relevance. It has been reported that, in snails, prolonged exposure to PET microfibers inhibited food intake and excretion, causing villi damage in the stomach and intestine (Song et al., 2019), and this type of gut damage has been shown to reduce nutrient absorption in *Daphnia* (Kim et al., 2021). Interestingly, gut damage induction was observed in *Drosophila* exposed to PSMPLs (Zhang et al., 2020). To deepen the knowledge of the effects of nanoplastics on the gut components, we have followed up PETNPLs on their intestinal journey after ingestion in the *Drosophila* larvae. Interestingly, and due to the semitransparent nature of the larvae cuticle, we could easily visualize the stained PETNPLs not only in the intestinal tract but also in the celomic compartment, suggesting their easy translocation through the intestinal barrier. The use of both, TEM and confocal microscopy, succeeded in showing PETNPLs distribution in the lumen, inside symbiotic bacteria, and attached to the peritrophic membrane. Further PETNPLs internalized the intestinal barrier being found to be distributed in microvilli, cytoplasm, and mitochondria. The translocation through the barrier was confirmed by detecting their internalization in the hemocytes (equivalent to lymphocytes) present in the hemolymph (equivalent to blood). Once internalized into the intestinal barrier PETNPLs caused morphological and structural changes to enterocyte mitochondria, and induced a highly significant overexpression of physical-stress intestinal gene markers (*PPO2* and *Duox*) in a dose dependent manner. Gut mucosa damage, inflammation, increased permeability, microbiota dysbiosis, and metabolism disruption have been reported in different animal models associated with MNPLs exposure (Lei et al., 2018; Qiao et al., 2019; Li et al., 2021). It has been suggested that in the intestinal tract MNPLs can experience changes due to the acidic environment, the digestive enzymes, and microbiota. Thus, cracking and deterioration on the surface of PET microfibers were observed in terrestrial snails after their excretion, which was associated with the observed damage in their gastrointestinal walls (Song et al., 2019). In *Drosophila*, this partial degradation of MNPLs in the intestine was also reported for PSNPLs (Alaraby et al., 2022a). Among the gut effects induced by PETNPLs, our results point out to damage the intestinal microbiota. This hazard could suppose the induction of microbiota dysbiosis which could affect the *Drosophila* health status. Unfortunately, no studies have been conducted until now in *Drosophila* evaluating the effects of MNPLs on gut microbiota (Chiang et al., 2022). Nevertheless, as reported in the previous quoted review, *Drosophila* has proven to be a good model to study the effects of environmental pollutants on the gut microbiota. This lack of information on the potential effects of MNPLs in the *Drosophila* gut microbiota is a relevant gap requiring urgent efforts.

Oxidative stress induction is considered one of the effects associated to MNPLs exposure (Ferrante et al., 2022), and there is a strong association between oxidative stress induction and mitochondrial damage (Alimba et al., 2022). Furthermore, mitochondria has been reported as a target of MNPLs exposure (Cortés et al., 2020; Liu et al., 2022). In this context, our results indicating variable changes in the expression of antioxidant enzymes (*Cu/Zn Sod* and *Sod2*) in PETNPLs exposed larvae would support their role in the oxidative stress Induction. Furthermore, our TEM figures showing PETNPLs internalization and, specially, the significant increases in the intracellular ROS levels definitely support the induction of oxidative stress induced by true-to-life PETNPLs in our *in vivo* model.

When checking for hazard effects of environmental pollutants, genotoxicity became an indispensable biomarker. It is well known that DNA damage can drive very relevant health consequences such as gene/chromosome mutation, carcinogenesis, and aging, among others (Mohamed et al., 2017). Genotoxicity is considered a surrogate biomarker of carcinogenesis, playing a major role in the initiation and progression steps. In spite of the relevance of this biomarker, very few studies have evaluated the potential genotoxic effects of MNPLs (Tagorti and Kaya, 2022). Our results reporting a highly significant up-regulation of the repair gene (*Ogg1*), were a signal that repair was acting against the induced DNA damage observed at all studied doses of PETNPLs. This signal was confirmed when the comet assay detected significant levels of DNA breaks in the hemocytes of exposed larvae. Both approaches confirm that our true-to-life PETNPLs are genotoxic in *Drosophila* and, consequently, their exposure can be considered to suppose a genotoxic risk for any exposed organism, including humans. The *Ogg1* overexpression was demonstrated against different nanosized materials (Alaraby et al., 2020, 2021), including nanoplastics (Alaraby et al., 2022a, 2022b) confirming its sensitivity as genotoxicity biomarkers. Interestingly, our results agree with a recent study using nanoplastic particles from real-life food containers by using a grinding process. The obtained nanoplastics from polypropylene and PET goods were considered genotoxic when evaluated using the comet assay in both Caco-2 and HepG2 cells (Roursgaard et al., 2022). Similarly, when PET microfibers were tested in mussel hemocytes, increased levels of DNA damage have also been reported (Choi et al., 2022). In that study DNA damage was determined according to the percentage of hemocytes containing fragmented DNA (sub-G_0/G_1) as measured by flow cytometry.

5. Conclusion

Our study reports relevant *in vivo* information on the potential hazard associated with MNPLs exposure. The relevance of the obtained data is based on the tested material used, which was obtained from the degradation of plastic goods (water plastic water) to reach the nano size. Thus, the use of these true-to-life NPLs produces data more easily extrapolated than those obtained using pristine samples of MNPLs. This is an important achievement of our study. In addition, it must be emphasized the important ability of this material to internalize cells and to cross the intestinal barrier. This ability to internalize was associated with significant changes in the expression of genes involved in the response to general stress induction, oxidative damage induction, and physic damage in the intestinal barrier. These effects were corroborated by detecting increases in the levels of intracellular ROS as well as in the amount of DNA breaks, as detected by the comet assay. We consider that the use of true-to-life MNPLs constitute an important step forward to obtain sound data on the health risk associated with the exposure to environmental MNPLs.

CRediT authorship contribution statement

RM, AH, and MA planned the experiments. MA, DA and AV performed the experiments. MA did the statistical analysis of the obtained data as well as prepared figs. MA, AH, and RM wrote the final manuscript.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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