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Assessing genotoxic effects of plastic leachates in *Drosophila melanogaster*

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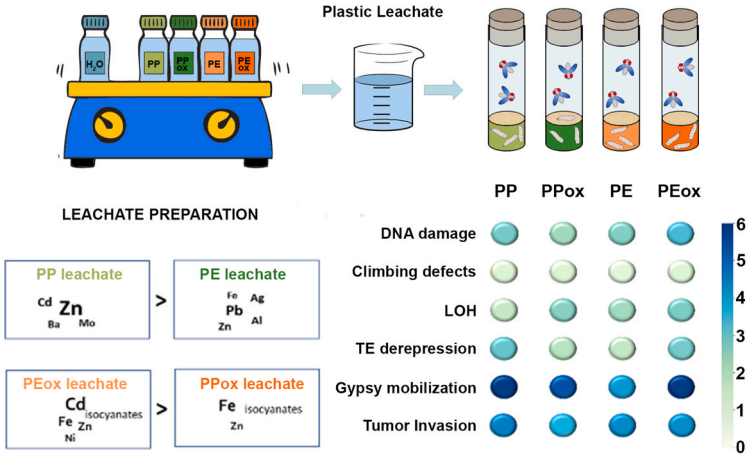
HIGHLIGHTS

- Plastic leachates (from both virgin and oxodegradable PP and PE) induce DNA damage in *Drosophila*.
- Exposure to plastic leachates promote Loss of Heterozygosity (LOH) at the tumor suppressor *warts* gene.
- Plastic leachates induce Transposable Element (TE) expression and mobilization.
- Plastic leachates synergize with oncogenic Ras^{V12} to promote tumor progression and invasion.

GRAPHICAL ABSTRACT



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ABSTRACT

Plastic polymers were largely added with chemical substances to be utilized in the items and product manufacturing. The leachability of these substances is a matter of concern given the wide amount of plastic waste, particularly in terrestrial environments, where soil represents a sink for these novel contaminants and a possible pathway of human health risk. In this study, we integrated genetic, molecular, and behavioral approaches to comparatively evaluate toxicological effects of plastic leachates, virgin and oxodegradable polypropylene (PP) and polyethylene (PE), in *Drosophila melanogaster*, a novel *in vivo* model organism for environmental monitoring studies and (eco)toxicological research. The results of this study revealed that while conventional toxicological endpoints such as developmental times and longevity remain largely unaffected, exposure to plastic leachates induces chromosomal abnormalities and transposable element (TE) activation in neural tissues. The combined effects of DNA damage and TE mobilization contribute to genome instability and increase the likelihood of LOH events, thus potentiating tumor growth and metastatic behavior of Ras^{V12} clones.

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Collectively, these findings indicate that plastic leachates exert genotoxic effects in *Drosophila* thus highlighting potential risks associated with leachate-related plastic pollution and their implications for ecosystems and human health.

1. Introduction

The abundant presence of plastic has lately become a pressing environmental issue (Kibria et al., 2023). In this last period plastics have generated more concern due to the increasing awareness of the possible adverse effects upon organisms, once released into the environment. The use of degradable materials was a potential solution for alleviating the environmental repercussions stemming from plastic consumption (Bahramian et al., 2016; Spierling et al., 2018). One method employed to render plastics degradable involves integrating pro-degradant additives, such as oxodegradable compounds, to facilitate degradation.

Most attention is posed to the marine environment where the huge amount of plastic litter can generate a toxic impact upon biota, especially after a temporal dimora in seawater which provokes plastic fragmentation and leaching. Terrestrial and freshwater environments are still less explored though the effects of these novel contaminants become more and more evident (Kallenbach et al., 2022). Anthropogenic materials enter the soil ecosystem from various routes such as municipal wastewater, fragmentation of incorrectly disposed materials by UV radiation and elevated temperature, agricultural actives and settled on the soil surface where became available to soil organisms (Chae and An, 2018).

It is well known that many chemicals (e.g. additives, plasticizers), used in plastic fabrication and manufacturing to improve plastic technical characteristics (Hahladakis et al., 2020), can be released in the environments (Bridson et al., 2021). The plastic leachate, containing a cocktail of several additives or hazardous chemicals became then available to organisms, also via food (Cverenkárová et al., 2021). Actually, soil represents a sink for these novel contaminants and given the potential cross-contamination among environmental compartments (water, soil, crops), a pathway of human health risk (Penserini et al., 2023). The cocktail of substances in plastic production is often indistinguishable and provokes adverse effects still poorly described. The difficulty in their identification, both for the trace amounts occurrence and for their unknown nature, together with the unpredicted results of their effect in mixture represent a challenge for the (eco)toxicologist in the description of the hazard represented by plastic leachates (Gunaalan et al., 2020; Hahladakis et al., 2020). It is therefore very urgent to study the effects of these hidden contaminants and evidence their potential toxic effect also upon terrestrial organisms.

Drosophila melanogaster is one of the best invertebrates for modeling higher organisms and for more than 100 years has played a starring role to study diverse biological processes (Enomoto et al., 2018; Johnson and Cagan, 2010). Over the last few years, the European Centre for the Validation of Alternative Methods (ECVAM) recommends *Drosophila* as a pioneering *in vivo* model organism for environmental monitoring studies and (eco)toxicological research (Chifiriuc et al., 2016). Comparative genome analysis reveals that at least 50% of fly genes are conserved in humans (Rubin et al., 2000). Moreover, among the human disease-associated genes, 75% have a *Drosophil*a ortholog (Reiter et al., 2001). This functional conservation implies that diseases resulting from disruption of conserved cellular pathways should be easily modeled in fruit flies at genetic and molecular levels, making *Drosophila* an excellent choice for testing potential adverse health effects of exposure to new environmental contaminants such as plastics. In addition, several ecologically relevant endpoints are available in *Drosophila* for studying the behavioral, neurological, and genetic impacts of toxicants (Peterson and Long, 2018; Rand et al., 2023).

We previously evaluated the effects of polypropylene (PP), polyethylene (PE) and polystyrene (PS) virgin polymer leachates on plants (*Sorghum saccharatum, Lepidium sativum, Sinapis alba,* and *Vicia faba*), crustacean (*Daphnia magna*), and bacteria (*Vibrio fischeri*) (Schiavo et al., 2018), evidencing Reactive Oxygen Species (ROS) increment especially linked to PS exposure and a marked genotoxic effect with PP (*V. faba*). Similar effects related to exposure of freshwater organisms to polymer leachates were also reported in recent literature (Gunaalan et al., 2020; Nava et al., 2022; Schwarzer et al., 2022).

Besides, the leachates of the same polymers additivated to be oxo-degradable seemed to mainly affect *D. magna* survival (PS and PP) while PS notably reduced the crustacean reproduction rate (Schiavo et al., 2020), however there are still data scarcity on this matter (Sciscione et al., 2023).

Apart from a recent review (Demir and Demir, 2023), few works have been published about the effects of *D. melanogaster* exposure to micronanoplastics (MNPs), mainly focused on ingestion, while no studies have been performed, to the best of our knowledge, upon plastic polymer leachates effects. In particular, PS MNPs ingestion showed damage in the gastrointestinal tract, locomotor dysfunction, epigenetic gene silencing (Demir, 2021; Matthews et al., 2021; Zhang et al., 2020), and altered gene expression playing a role in the impairment of several functions such as response to general stress and intestinal damages (Alaraby et al., 2022). The exposure to PET MNPs increased the male fly's lifespan (Liang et al., 2022) while impaired fertility and offspring size (Jimenez-Guri et al., 2021), along with declined oviposition in females and reduced triglyceride and glucose content in *Drosophila* males (Shen et al., 2021).

This study aims to deepen our knowledge on the adverse effect of plastic leachates (virgin and oxodegradable PP and PE) on *Drosophila melanogaster*. Different toxicological relevant endpoints were evaluated, including developmental (lifespan and developmental timing), behavioral (feeding intake and negative geotaxis), and genetic toxicity (DNA damage in neuroblasts and SMART test) endpoints. Additionally, we investigated the mobilization of Transposable Elements (TEs) using the *gypsy*-TRAP reporter system (Li et al., 2013). Transposable elements (TEs), discovered in maize in the late 1940s by Barbara McClintock (McClintock, 1950), are repetitive mobile elements, widely recognized as major players in genome structure, function, and evolution (Arkhipova, 2018; Bourque et al., 2018; Chuong et al., 2017; Fedoroff, 2012; Feschotte and Pritham, 2007; Finnegan, 1989; Piacentini et al., 2014; Pimpinelli and Piacentini, 2019). Transposable elements are activated in response to a variety of environmental stressors and toxicants, including ionizing radiation, heat stress, nutrient deficiencies, and chemical pollutants (Cappucci et al., 2019, 2022; de Oliveira et al., 2021; Fanti et al., 2017; Jardim et al., 2015; Miousse et al., 2015). This responsiveness makes changes in TE activity valuable as new biomarkers for assessing both exposure to environmental pollutants and the adverse effects of toxicants on organisms. Finally, the effects of plastic leachates on tumor invasion and metastasis were evaluated using the Mosaic Analysis with a Repressible Cell Marker (MARCM) genetic system developed by Pagliarini and Xu (2003).

Although no relevant toxicity, as measured by significant changes in developmental times or longevity was observed, the results of this study showed that exposure to plastic leachates induces genome instability, transposable element dysregulation and impaired locomotor behavior. We also found that exposure to plastic leachates promotes loss of heterozygosity (LOH) at the *warts* locus and potentiates tumor progression and metastatic behavior of non-invasive clones expressing the activated oncogene Ras^{V12}. Collectively, these data indicate that plastic leachates can exert genotoxic effects in *Drosophila*, thus setting the stage to further investigate the toxicological impact of leachate-related plastic pollution

on terrestrial organisms.

2. Materials and methods

2.1. Preparation of plastic leachates

The plastic particles were kindly supplied by a petrochemical factory (that asked to be not mentioned). Virgin polymers: Polypropylene (PP) and Polyethylene (PE) were provided as round pellets (mean size 5 mm ± 0.3), while the oxodegradable ones (PPox and PEox) were provided as fragments with an irregular shape with a jugged surface (mean size 1.6 mm ± 0.45) declared to contain a proto-degradant additive (formulation not reported). In general, the pro-degradant additives contain metal salts of carboxylic acid or dithiocarbamates based on Co^{2+}, Fe^{2+}, Mn^{2+}, or Ni^{2+} (AISBL et al., 2013) that promote the oxidation of polymeric chains, triggered by UV radiation and/or heat, followed by fragmentation. Additional stabilizers (e.g., lead salts, organotin, Zn/Ca complex), in order to avoid rapid plastic degradation of the product when in use (commercial product), could be present. Each polymer was added to Milli-Q water in a liquid-to-solid ratio of 10 (L/S = 10) according to Italian and European Standard procedure (EN 12457–2:2002). This liquid-to-solid ratio was proposed by European standards (CEN and European Committee for Standardization, 2002) and was previously applied to test plastic leachate toxicity (Bejgarn et al., 2015; Lithner et al., 2012; Schiavo et al., 2018, 2020).

The suspension was rotary shaked for 24 h at 1 rpm, 20 ± 2 °C in the dark and leachates were obtained by pellets/fragments sedimentation (1 h) followed by a filtration on filter papers Whatman Grade through 0.22 μm H-PTFE filters (Whatman Puradisc). All collected samples were stored in darkness at 4 °C. Metal content analysis of the leachates was conducted using ICP/MS, and organic content analysis was performed using GC/MS, as previously described in Schiavo et al. (2018) (Table S1 and Fig. S1). Additionally, quantitative assessment of the organic content was carried out on the same concentrated GC samples using Ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) analysis with equipment from Waters Inc. Also, Acquity IClass UPLC coupled to a Xevo G2-XS QTof high-resolution mass spectrometer, operated through Masslynx 4 software, was employed for enhanced qualitative analysis. This analytical approach allowed better evaluating the differences among samples and identifying compounds prone to derivatization, a fundamental information for structure elucidation.

2.2. Drosophila strains

The Oregon-R wild-type strain was maintained in our laboratory (Sapienza, University of Rome, Italy) for at least 10 years. *ey-FLP1; act > y^+>Gal4, UAS-GFP*; *FRT82B, tub-Gal80* and *FRT82B, UAS-Ras^{V12}* transgenic lines, used to generate *Ras^{V12}* tumor clones, were previously described (Pagliarini and Xu, 2003). The *gypsy*-TRAP lines were generously provided by Josh Dubnau (Stony Brook University, USA) (Li et al., 2013). Additional strains including *P{GawB}elav^{C155}* (#458), *y^1, w*; Dp (3;Y)BL2, P{HS-lacZ.scs}65E*(#57371) and *$st^1in^1kni^{ri-1}p^pwts^{3-17}$/TM3, Sb^1* (#7052), were obtained from Bloomington *Drosophila* Stock Center (http://flystocks.bio.indiana.edu). Flies were cultured at 25 °C on a standard medium supplemented with 0.75% (vol/vol) Propionic Acid as an antifungal agent.

2.3. Leachate exposure and toxicity

Drosophila larvae or adult flies were grown on standard *Drosophila* medium supplemented with each of the leachates at a final concentration of 15% (vol/vol). The leachates were added as % of the total water used to avoid the risk of high medium dilution. The leachate concentration was selected starting from previous experiments reported by Schiavo et al. (2020) and taking into account the plastic amount that is likely found in contaminated areas and that can be ubiquitously reached in future scenarios (Liwarska-Bizukojc, 2021). The negative control groups were grown on a standard medium containing the only solvent (Milli-Q water) used for preparing the leachates and were included each time when test samples were analyzed.

For developmental timing assays, larval pupariation rates (Rand et al., 2019) were evaluated in 200 ml glass bottles (55 × 105 mm) containing 30 ml of control or leachate-supplemented medium. First instar Oregon-R larvae were collected from embryos laid within a 3–4 h period from a mating population of about 400 flies. L1 larvae were then transferred to glass bottles (n = 100/bottle) and allowed to develop at 25 °C. Newly formed pupae were counted daily until day 10 after larval hatching. Pupal eclosion rates were determined as previously reported (Rand et al., 2014). Flies that successfully eclosed were scored starting from 10 days after L1 larvae were seeded on fresh food medium. The pupariation and eclosion assays were performed in triplicate and reported as percent pupariation (number of pupae/number of L1 larvae) or eclosion (number of adult flies eclosed/number of pupae) expressed as the mean and standard deviation. Comparisons between developmental times and relative % were performed by Log-rank (Mantel-Cox) test.

For lifespan analysis, control and treated flies were reared at 25 °C on standard sugar-yeast medium supplemented with leachates (15% v/v) or vehicle only. To estimate the longevity of each experimental group, about 300 flies were collected within 24 h post-eclosion. Flies were transferred to a fresh medium three times a week and dead flies were counted daily. The survival rate was calculated as the percentage of total surviving flies. For each treatment, at least two biological replicates were pooled. The survival rate was estimated using the Kaplan–Meier method and plotted as survival curves.

Quantitative evaluation of food intake in L3 larvae or adult flies raised on leachate-supplemented media was performed through a dye-consumption assay (Keita et al., 2017; Wong et al., 2009). Four experimental groups of ten L3 larvae (5 males and 5 females) or eight 3-day--old mated flies (four males and four females) were placed into vials containing standard medium supplemented with 1% (w/v) of Blue 1 food dye tracer (Blue Dye no. 1, Merck) and 15% (v/v) of plastic leachates (or solvent only for control groups). L3 larvae or adult flies were fed on labeled medium for 3 h and then homogenized in 150 μL of PBS1X (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). The homogenate was centrifuged at 12000 rpm for 1 min and the absorbance of the supernatant solution was measured at 629 nm (Thermo Scientific Multiskan GO Microplate Spectrophotometer) to quantify the volume of food consumed. Statistical analysis of data from four independent biological replicates was performed using one-way ANOVA test followed by Dunnett's post hoc test.

2.4. Cytological analysis of mitotic chromosomes

Mitotic chromosome preparations were obtained from larval brains, according to Pimpinelli et al. (2000). Newly hatched larvae were grown on a standard medium supplemented with each of the leachates (15% v/v) and aged to the late third instar stage. The negative control group received the standard medium containing the only solvent used for preparing the leachates. Third instar larval brains were dissected in 0.7% NaCl, incubated in hypotonic solution (0.5% sodium citrate dihydrate) for 8 min, and then fixed for 30 s in acetic acid/-methanol/distilled water (5.5:5.5:1). Fixed brains were squashed in 45% acetic acid and frozen in liquid nitrogen. After 3 washes for 5 min with PBS1X, slides were stained with DAPI (4,6-diamidino-2-phenylindole, 0.01 mg/mL) and mounted in the antifading medium (23.3 mg/mL of DABCO (1,4-Diazabicyclo-(2,2,2) octane) in 90% glycerol–10% PBS1X). All images were captured at 100× magnification using an Eclipse Fluorescence microscope (E1000 Nikon) equipped with a CCD camera (Coolsnap). Statistical analysis of chromosomal abnormalities was obtained by one-way ANOVA test followed by Dunnett's post hoc test

(GraphPad Prism Software version 8.00). A *p-value* $\leq$ 0.05 was considered statistically significant.

2.5. Confocal microscopy of adult brains

Drosophila adult brains, dissected from 2 to 3 day-old leachate-treated and control flies, were fixed and stained essentially as previously described (Maggiore et al., 2022). Briefly, brains were dissected in PBS1X and fixed at room temperature in 4% paraformaldehyde in PBS1X for 30 min. After fixation, brains were washed three times in PBT (PBS1X 0.1% Triton X-100) for 10 min, stained with 1 μM TOTO-3 iodide (642/660) (Invitrogen) to visualize DNA and mounted in antifading medium. Confocal images were captured using a Leica DMIRE (Leica Microsystems, Hiedelberg, Germany) and a Zeiss LSM 780 (Zeiss, Berlin, Germany) microscope. Brain imaging analysis was performed using Zen Software (version: ZEN 2009 Light Edition) and Adobe Photoshop CS6. To count GFP-positive cells, at least 9 adult brains were sectioned by confocal microscopy. For each section, GFP-positive cells were counted and averaged between each brain. Statistical analysis was performed using one-way ANOVA test followed by Dunnett's post hoc test (GraphPad Prism version 8.00). A *p-value* $\leq$ 0.05 considered as statistically significant.

2.6. Measurement of eye pigment

Quantification of eye pigments was performed as previously described (Cappucci et al., 2022). 2-3 day-old adult male heads (n = 40 heads for each experimental group) were homogenized in 2 mL of methanol acidified with 0.1% HCl and centrifuged at 12000 rpm for 5 min. After centrifugation, the absorbance of red eye pigments in the supernatant solution was measured at 480 nm using a Multiskan GO Spectrophotometer (Thermo Scientific). Statistical analysis was performed on four independent biological replicates using one-way ANOVA test followed by Dunnett's post hoc test (GraphPad Prism version 8.00). A *p-value* $\leq$ 0.05 considered as statistically significant. Representative images of BL2 fly eyes were acquired using a Nikon camera D5000 mounted on a stereomicroscope.

2.7. Climbing assay

The climbing assay was performed as previously described (Laneve et al., 2017). Briefly, for each leachate treatment, groups of 10 flies were CO_2 anesthetized and collected into empty glass tubes (9.5 cm × 2.5 cm) following a recovery time of 2 h. Each assay consisted of ten trials in which flies were gently tapped down and assayed for their capacity to cross an 8 cm line mark after 10 s. For each experimental group, n $\geq$ 100 flies were assayed. All average data are presented as mean ± SEM and compared with one-way ANOVA test followed by Dunnett's post hoc test. Statistical tests were performed using GraphPad Prism Software. A *p-value* $\leq$ 0.05 was considered statistically significant.

2.8. Analysis of flies with wts tumor clones

The analysis of *wts* tumor clones was performed in two biological replicates by counting about 200 flies for each treatment. The tumor frequency was calculated as the number of epithelial tumors per number of *wts*/+ flies. Data were analyzed using one-way ANOVA test followed by Dunnett's post hoc test (GraphPad Prism version 8.00). Pictures of adult flies were acquired using a Nikon D5000 digital camera mounted to Wild M38 stereomicroscope (Wild Heerbrugg).

2.9. RNA Isolation, and qRT-PCR analysis

Adult flies newly emerged from larvae grown on a leachates-supplemented medium, were transferred into new vials containing the same diet and aged 10 days. For each sample, total RNA was purified from 30 adult heads using Qiazol reagent (Qiagen) according to the manufacturer's instructions. The RNA concentration and purity were assessed using a NanoDrop 1000 spectrophotometer (Thermo Scientific). Following DNAse treatment, 5 μg of total RNA was reverse transcribed using oligo dT and SuperScript™ Reverse Transcriptase III (Invitrogen). qPCR reactions were carried out with QuantiFast SYBR Green PCR Kit (Qiagen) and the relative abundance of transposable elements transcripts was determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) using, as a control, *rp49* transcript which exhibited a stable expression pattern across all experimental conditions tested in this study. The BestKeeper software (https://www.gene-quantification.de/bestkeeper.html) was used to calculate *rp49* expression stability with respect to other reference genes. qRT-PCR experiments were performed in at least three independent biological replicates, with three technical replicates for each. The primer sequences and amplification sizes are listed in Table S2. Statistical significance was determined by One sample *t*-test using GraphPad Prism version 8.00. A *p-value* $\leq$ 0.05 was considered statistically significant.

3. Results and discussion

3.1. Toxicity assessment

Toxicity evaluations were performed to verify the lacking of effects upon *Drosophila* vital parameters and regular development due to plastic leachate dose used in experiments. Toxicity was assessed as individual mortality (%) along test exposure (80 days), as pupariation and eclosion rates (%) and as food intake of both larvae and adults (Rand et al., 2014, 2023).

The surviving individuals from each exposure were compared with those of the negative control, for the statistical analysis. As indicate in Fig. 1A, the different polymer leachates did not reduce the mean lifespan of *Drosophila*. Flies treated with plastic leachates showed no statistically significant differences also in developmental times (50% pupation rate was 7.14 ± 0.04 days for H_20; 7.16 ± 0.04 days for PP; 7.25 ± 0.04 days for PPox; 7.04 ± 0.04 days for PE and 7.28 ± 0.05 days for PEox; 50% eclosion rate was 11.95 ± 0.06 days for H_20; 11.95 ± 0.05 days for PP; 11.82 ± 0.06 days for PPox; 11.79 ± 0.06 days for PE and 11.86 ± 0.06 days for PEox), although adult emergence appeared to be slightly biostimulated in the case of PE leachates (Fig. 1B). Potential morphological alterations in the emerged adults were also checked, but without significant findings. No significant differences in food intake were also observed (Fig. 1C and D). The biostimulating effects of PE leachates could be linked to the occurrence in PE leachate of thermoregulators chemicals with potential estrogenic effect (Yang et al., 2011) as already observed in *D. magna* (Schiavo et al., 2020) with the same test material. The PE virgin polymer leachate analyzed by GC/MS (Table S1 and Fig. S1) were similar to the process blank samples showing, at used experimental conditions, the lack of added organic chemicals. While additives in plastic leachates may fall below analytic detection limits (Franzellitti et al., 2019), it remains imperative for future research to intensify efforts in chemically characterizing to establish precise cause-effect relationships.

3.2. Exposure to plastic leachates induces chromosomal aberrations and pericentromeric heterochromatin decondensation

To investigate the effects of plastic leachate exposure on genome stability, we analyzed metaphase chromosomes from third-instar larval brains. Developing larval brains are actively dividing tissues, undergoing rapid and synchronized rounds of cell division. The high proliferative rate of these tissues makes them particularly suitable for studying chromosome segregation, chromosome behavior and chromosomal abnormalities, in a reliable and reproducible manner. For mitotic chromosome preparations, third-instar larval brains were dissected and, following hypotonic lysis and formaldehyde fixation, squashed to

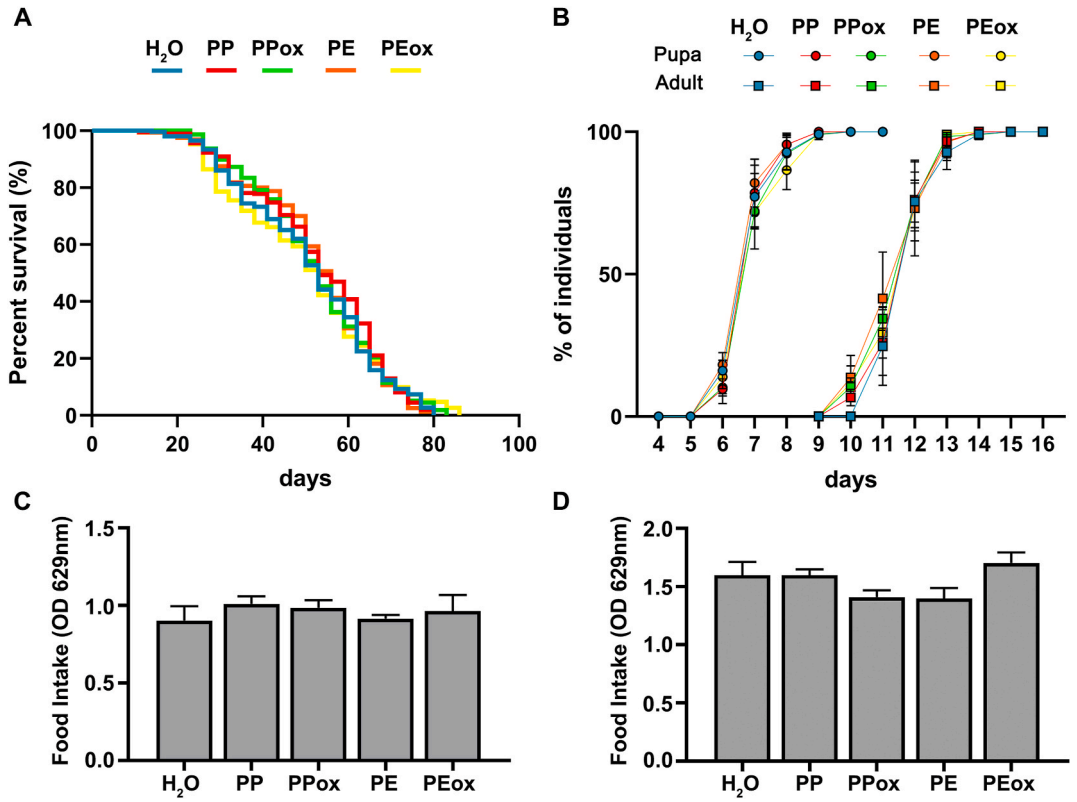

Fig. 1. Plastic leachates do not affect mean lifespan,developmental timing, or food intake. (A) Survival curves of leachate-treated and control flies. The log-rank (Mantel–Cox) test with Bonferroni correction did not show significant differences between the experimental groups. **(B)** Development timing of leachate-treated flies. Pupariation and eclosion rates of larvae reared from the L1 stage on the indicated supplemented medium. Graphs show the percentage of larvae that pupated or eclosed over time. The log-rank (Mantel–Cox) test with Bonferroni correction did not show significant differences between the experimental groups. **(C and D)** Food intake in larvae **(C)** and adult **(D)** was quantified by spectrophotometric absorbance measurements (OD 629 nm); one-way ANOVA test followed by Dunnett's post hoc test did not show significant differences between the experimental groups.

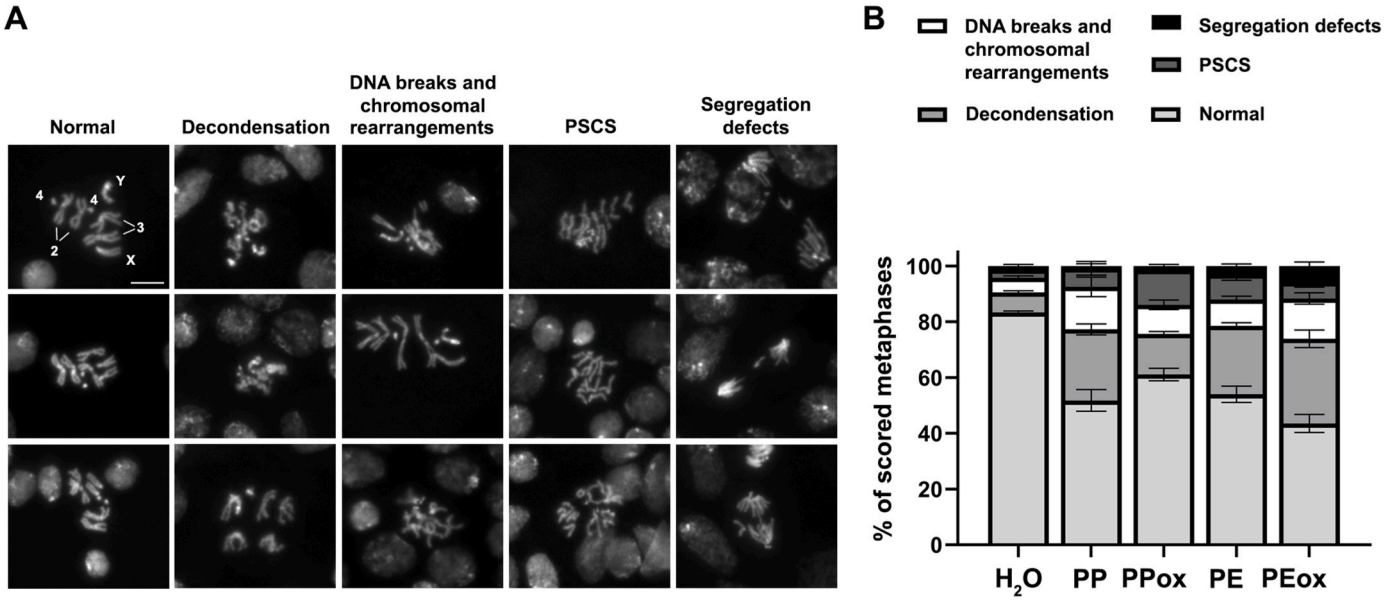

Fig. 2. Plastic leachates induce genome instability in larval brains. (A) Representative images of DAPI-stained mitotic chromosomes from larval brains dissected from plastic leachate-treated larvae. The scale bar indicates 5 μm. **(B)** Quantitative analysis of mitotic defects scored in leachate-treated and control larval brains. Statistically significant differences between the control and treatment groups were determined by one-way ANOVA test followed by Dunnett's post hoc test. At least 100 metaphases were scored in each of three independent experiments.

analyze metaphase chromosomes. The results of the cytological analysis showed a broad spectrum of aberrant chromosome configurations for all the plastic leachates tested. The abnormal chromosome configurations (Fig. 2A) can be grouped into four categories, the frequencies for which were summarized in Fig. 2B: in the first class, the chromosomes undergone an extensive and generalized chromatin decondensation (H_2O: 7.1% ± 0.63, PP: 25.5% ± 1.98, ***$p < 0.0001$; PPox: 14.6% ± 0.85, **$p = 0.0017$; PE: 24.5% ± 1.12, ***$p < 0.0001$; PEox: 30.4% ± 3.15, ***$p <$ 0,0001); the second class of metaphases exhibited DNA breaks and complex structural chromosomal rearrangements involving two or more chromosomes (H_2O: 5.3% ± 0.72, PP: 15.3% ± 3.5, ***$p = 0.0005$; PPox: 10.4% ± 1.67, *$p = 0.0392$; PE: 9.5% ± 1.18, $p = 0.0986$; PEox: 14.5% ± 2.05, ***$p = 0.001$); the third and fourth classes were characterized by premature sister chromatid separation (PSCS) (H_2O: 3.0% ± 0.2, PP: 6.6% ± 2.48, $p = 0.0753$; PPox: 12.5% ± 0.82, ***$p = 0.0002$; PE: 9.0% ± 2.1, **$p = 0.0046$; PEox: 5.8% ± 1.75, $p = 0.183$) and abnormal chromosome segregation (H_2O: 1.2% ± 0.58, PP: 0.8% ± 0.87, $p = 0.9388$; PPox: 1.4% ± 0.59, $p = 0.9978$; PE: 3.0% ± 0.77, $p = 0.1232$; PEox: 5.8% ± 1.43, ***$p = 0.0004$), respectively.

Collectively, these results indicated that plastic leachates have the potential to cause significant, consistent, and treatment-specific chromosomal abnormalities, leading us to speculate that the genotoxic effects of leachates are probably due to the toxic effects of chemical mixtures leached from plastics on epigenetic modification, chromatin structure, DNA damage signaling, and chromosome segregation (Langie et al., 2015). Although with different concentrations and leaching patterns, chemical analyses of plastic leachates showed the presence of heavy metals, such as Al, Pb, Zn, Cd, Fe, and Cr (Table S1) (Schiavo et al., 2021) which could significantly contribute to DNA damage through two different mechanisms: favoring double-strand breaks (DSBs) formation as well as inhibiting DNA repair pathways (Morales et al., 2016; Wu et al., 2016). The analysis of organic compounds released from plastic leachates also revealed in PEox leachates the presence of 2,6-diisopropylphenyl isocyanate (Fig. S1) which could further contribute to genome instability by forming isocyanate-DNA adducts (Beyerbach et al., 2006). In addition, nickel ions, as well as several other metal ions, may inhibit proper condensation of pericentromeric heterochromatin (Imbra et al., 1989; Lin et al., 2021) leading to defects in chromosome cohesion and segregation (Kellum et al., 1995). To verify whether leachates can impact the heterochromatin condensation state, we used a Position Effect Variegation (PEV) assay (Elgin and Reuter, 2013) that allowed us to evaluate the condensation status of constitutive heterochromatin by quantifying the expression levels of a mini-*white* variegating transgene inserted into Y pericentromeric heterochromatin in the BL2 reporter line (Lu et al., 1996). Given that PEV is a heterochromatin-mediated gene silencing phenomenon, it is reasonable that all environmental factors impairing the degree of heterochromatin compaction may act as suppressors of PEV-based gene silencing. Thus, the red eye pigment levels in BL2 male flies can serve as a convenient readout of heterochromatin levels *in vivo*. For the PEV assay, the optical density of red eye pigments extracted from BL2 transgenic males (hatched from larvae grown on leachate treated medium) was quantified and compared to that of control BL2 males (reared on untreated medium) (Fig. 3A and B). The results demonstrated that plastic leachates (with the only exception of PEox) act as suppressors of position effect variegation thus suggesting that they may effectively interfere with the heterochromatin structural organization.

Leachate-induced DNA damage can cause loss of genome integrity, holding important ecological and evolutionary implications. For instance, DNA damage and genomic instability might accumulate over time and potentially be passed through the germline to the offspring, affecting future generations of the organisms (Cappucci et al., 2019; Fanti et al., 2017). Moreover, in a natural context, the genotoxicity induced by plastic leachates could be exacerbated by the combination with other environmental stressors, leading to even more severe consequences (Cao et al., 2021; Chang et al., 2022; Liess et al., 2016; Pirotta et al., 2022). Given these concerns, it is very important to continue investigating the effects of plastic pollution on DNA and genomic stability to better understand their potential implications for both human health and the health of ecosystems.

3.3. *Exposure to plastic leachates impairs locomotor behavior in adult flies*

Genomic instability in the nervous system significantly contributes to the onset of neurodegeneration (Welch and Tsai, 2022) which in turn may lead to a premature loss of climbing ability, a typical symptom of neurodegenerative phenotypes. To evaluate the climbing impairment following leachate exposure, we quantified the locomotor performance of Oregon-R wild-type flies grown on a standard medium supplemented with each of the leachates (15% v/v), for the entire developmental period, from the embryo to adulthood. Flies fed on culture medium supplemented with an equal volume of leachate solvent (H_2O) were considered as controls. As shown in Fig. 4, the climbing behavior of 10-day-old flies was significantly impaired in PP, PPox, and PEox-treated flies whilst no effect was found in PE-treated flies that exhibited a climbing ability comparable to untreated controls. The Performance Index was 8.12 ± 0.29 for control flies, 6.43 ± 0.39 for PP (**$p = 0.0085$), 6.37 ± 0.45 for PPox (*$p = 0.016$), 7.24 ± 0.58 for PE ($p = 0.4912$) and 6.35 ± 0.47 for PEox (*$p = 0.024$) treated flies. Surprisingly, flies treated with PE, despite exhibiting a high degree of genomic instability did not show climbing defects, thus suggesting that genomic instability certainly contributes to neurodegeneration but is not the main cause of it. Further experiments will certainly be needed to deepen at the molecular level the potentially neurotoxic impact of plastic leachates on the nervous system homeostasis.

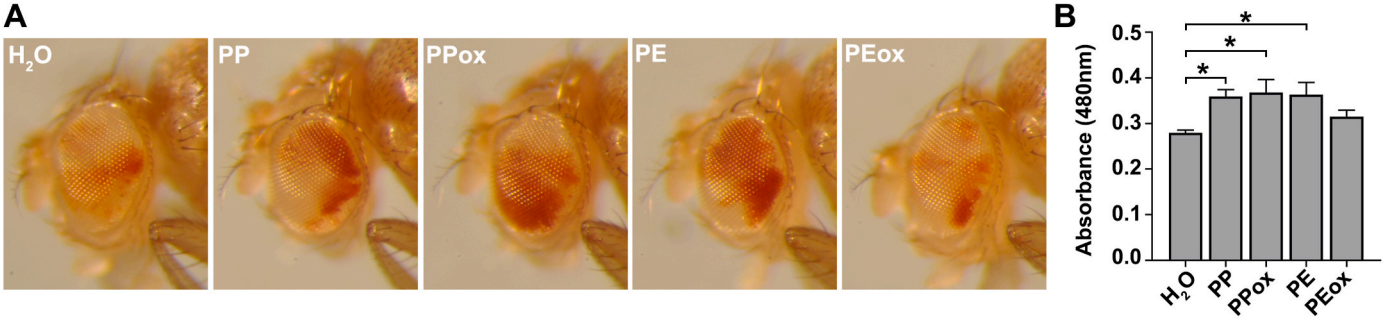

Fig. 3. Plastic leachates suppress BL2 PEV. (A) Representative bright-field microscopy images of variegated eye patterns in randomly selected BL2 flies from each experimental group. **(B)** Quantitative analysis of eye pigment levels was performed on 40 BL2 male heads for experimental group, in each of four biological replicates (n = 160 flies). Statistically significant differences between the control and treated groups were determined by one-way ANOVA test followed by Dunnett's post hoc test (*p-*value* PP = 0.0401; *p-*value* PPox = 0.0216; *p-*value* PE = 0.0307; p-*value* PEox = 0.5367).

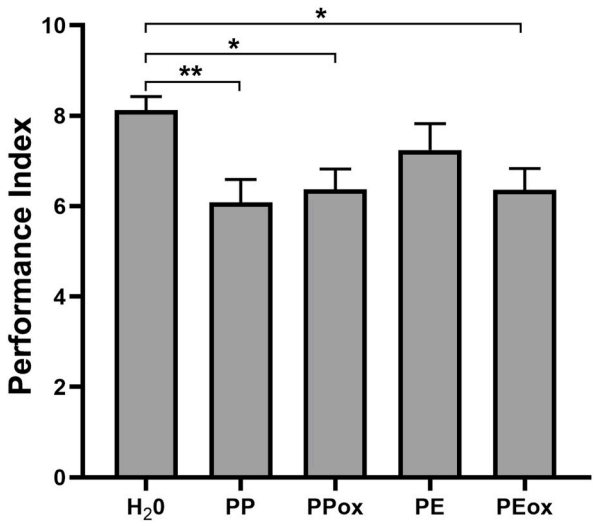

Fig. 4. Plastic leachates reduce locomotive behavior. Quantitative analysis of climbing defects in 10-day-old control and treated flies. Climbing abilities were presented as the average performance index (PI) ± SEM of two independent biological replicates. Statistically significant differences between the control and treatment groups were determined by one-way ANOVA test followed by Dunnett's post hoc test.

3.4. Plastic leachates promote loss of heterozygosity (LOH)

Starting from the evidence that treatments with plastic leachates induced DNA damage and defective chromosome segregations, we aimed to investigate whether leachates could cause loss of heterozygosity (LOH) using the Somatic Mutation And Recombination Test (SMART) based on LOH occurring at the tumor suppressor *warts* (*wts*) gene, the *Drosophila* homolog of the mammalian tumor suppressor gene *LATS1* (Eeken et al., 2002; Gnocchini et al., 2022; Sidorov et al., 2001; Xu et al., 1995). LOH occurs when diploid cells, heterozygous for the recessive lethal $wts^{3\text{-}17}$ allele ($wts^{3\text{-}17}$/+), lose the wild-type allele of the gene and manifest the mutant *wts* phenotype, characterized by tumorous outgrowths easily detectable on the adult fly cuticle (Nishiyama et al., 1999). The loss of heterozygosity for the *wts* genetic marker in heterozygous individuals, allowed us the quantification of DNA damage in the adult tissues by visual scoring of epithelial *wts* homozygous tumors. To explore whether plastic leachates might induce LOH at *warts (wts)* locus, we mated $wts^{3\text{-}17}$/*TM3, Sb*^{1} females to wild-type males on growth media containing each of the leachates and scored the $wts^{3\text{-}17}$/+F1 offspring for the presence of *wts* homozygous clones on the adult cuticle (Fig. 5A). In the LOH test, the $wts^{3\text{-}17}$/+ progeny was identified by the absence of *Stubble* bristle phenotype which only occurs in the +/*TM3, Sb*^{1} progeny that inherited the third chromosome balancers TM3. The results of LOH assay revealed that plastic leachate treatments strongly promote LOH at the *wts* locus, increasing the *wts* tumor burden in treated flies when compared to untreated control flies (H_2O: 14.7%, PP: 26.1%, PPox: 39.4%, PE: 35.4%, PEox: 41.7%) (Fig. 5B).

There were no significant differences in *wts* tumor incidence between males and females. No *wts* tumors were found in *TM3, Sb*/+ siblings. The reciprocal cross (wild-type females x $wts^{3\text{-}17}$/*TM3, Sb* males) gave us comparable results, so we decided to unify the data. Within the plastic leachates, the highest tumor frequency was induced by both PPox and PEox leachates. This frequency was considerably higher than those obtained from virgin PP and PE. Tumors induced by plastic leachates mainly arose on the abdomen, wings, and notum. However, we found tumors also in other body parts, including eyes, head, or legs (Table 1).

The results clearly show the mutagenic and recombinogenic potential of plastic leachates *in vivo* and strongly support the growing evidence of the harmful genotoxic effects of leachate-related plastic pollution (Oliviero et al., 2019; Schiavo et al., 2020, 2021). Leachate-induced loss of heterozygosity, in fact, could have a significant impact on evolutionary dynamics of populations and species in their natural contexts. LOH occurs at higher rate than that of point mutations, and can impact large portions of the genome, leading to genomic instability and potentially contributing to the development of cancer or other genetic diseases (Nichols et al., 2020). In addition, as a direct consequence of a reduction in the number of heterozygous loci, LOH can significantly limit the evolutionary potential of natural populations by reducing their ability to cope with environmental changes and increasing their susceptibility to additional stressors (Frankham et al., 2019). Overall, leachate-induced loss of heterozygosity can have profound negative effects on the evolutionary dynamics and long-term viability of natural populations.

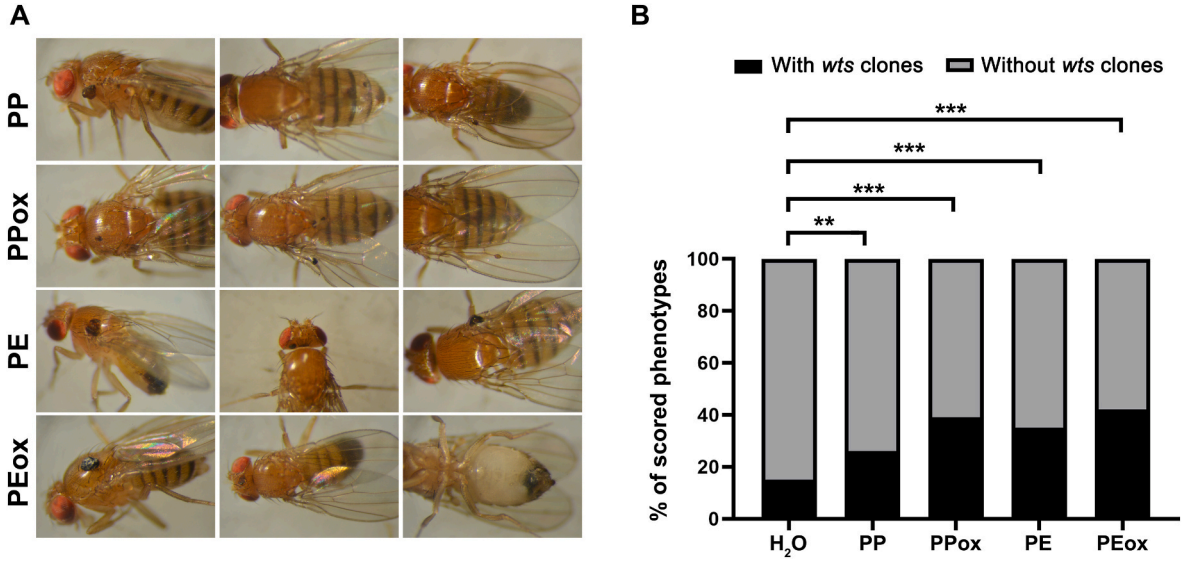

Fig. 5. Plastic leachates induce LOH at the *warts (wts)* locus. (A) Representative images of tumor *wts* homozygous clones in *wts*/+ flies treated with different plastic leachates. **(B)** Quantitative analysis of obtained results. Statistically significant differences between the control and treatment groups were determined by determined by Fisher's exact test (***p-value* PP = 0.004; ****p-value* PPox < 0.0001; ****p-value* PE < 0.0001; ****p-value* PEox < 0.0001).

Table 1
Organ-specific frequencies (%) of *wts* clones induced by plastic leachate treatments.

Treatment	Tot. Flies	N. of *wts* clones	Head (%)	Eye (%)	Notum (%)	Wing (%)	Abdomen (%)	Leg (%)
H_2O	204	30	13.3	0	53.3	26.7	6.7	0
PP	222	58	3.5	6.9	34.5	27.6	24.1	3.4
PPox	188	74	2.7	2.7	19	27	45.9	2.7
PE	192	68	2.9	2.9	20.6	35.3	38.3	0
PEox	240	100	10	0	6	36	46	2

3.5. Exposure to plastic leachates triggers transcriptional activation and mobilization of transposable elements

To evaluate the effects of plastic leachates on transposable element activity, we analyzed the expression profiles of specific TEs in adult fly heads. Total RNA from 10-day-old fly heads was reverse transcribed by an oligo(dT) primer and qRT-PCR experiments were performed to quantify transcript levels of different families of transposable elements. We analyzed three retroviral-like Long Terminal Repeat (LTR) retrotransposons (*copia, gypsy,* and *roo*), two LINE-like non-LTR retrotransposons (*I-element* and *R2*), and one DNA transposon (*1360*). The findings of this analysis revealed a statistically significant increase in the transcript levels for most transposable elements tested (Fig. 6).

To verify whether TE derepression following leachate treatments was also associated with physical TE mobilization, we used a *gypsy*-TRAP reporter system that allows to identification of cells with *de novo* insertions of the *gypsy* retrotransposon, through the expression of the GFP as reporter (Li et al., 2013). As shown in Fig. 7A, the *gypsy*-TRAP system consists of an *elav-Gal4* construct, used to drive pan-neuronal expression of the recombinant mCD8GFP protein and a *Gal80* transgene that, under

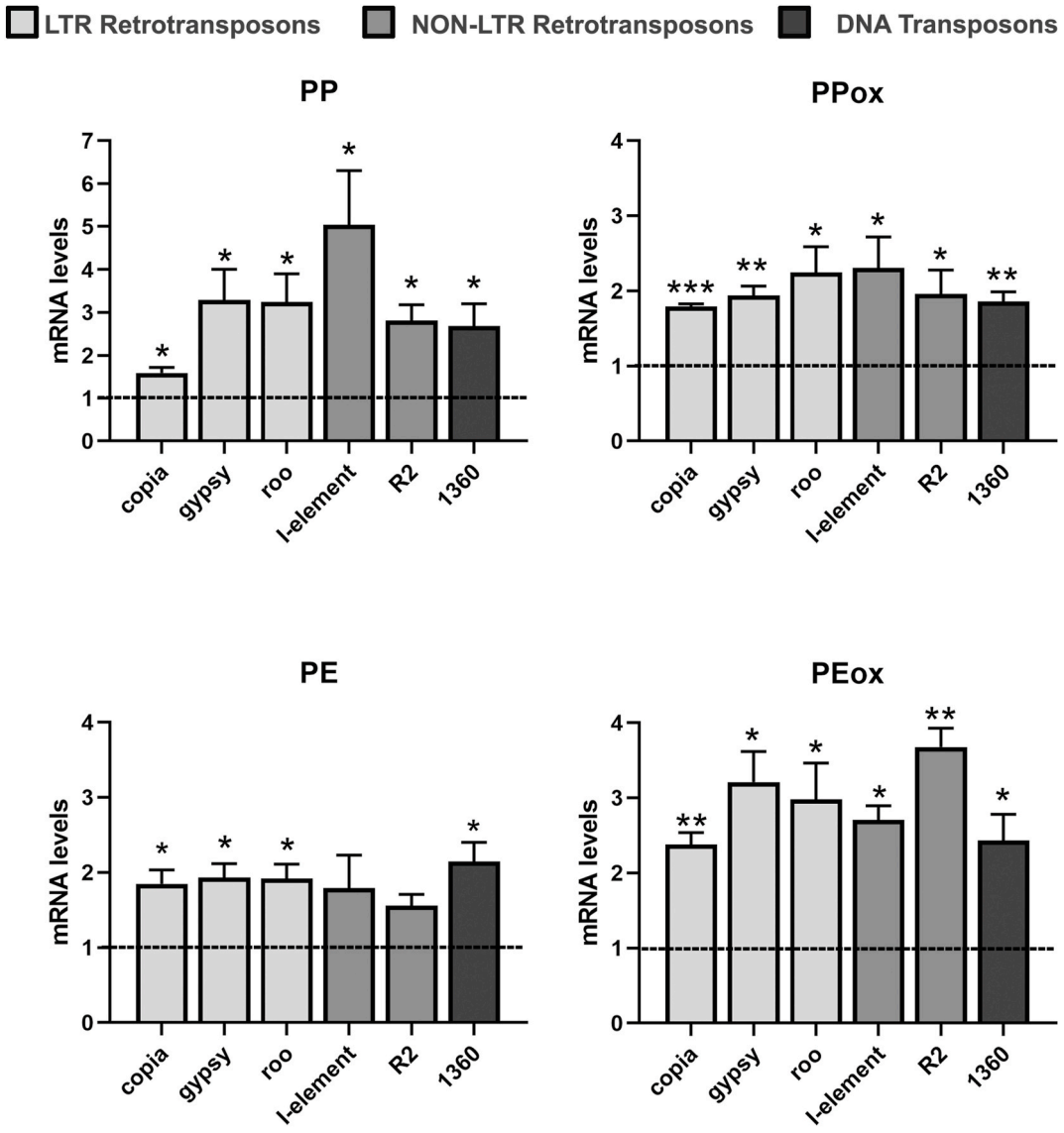

Fig. 6. Plastic leachates induce upregulation of transposable elements. qRT-PCR analysis of transposable element expression in 10-day-old fly heads; transposable elements transcript levels were normalized relative to the *rp49* gene. Data are displayed as fold change relative to untreated control flies and are mean of at least three independent biological replicates ± SEM. (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, one sample t-test). The horizontal black dashed line (set to 1) indicates the fold change control value.

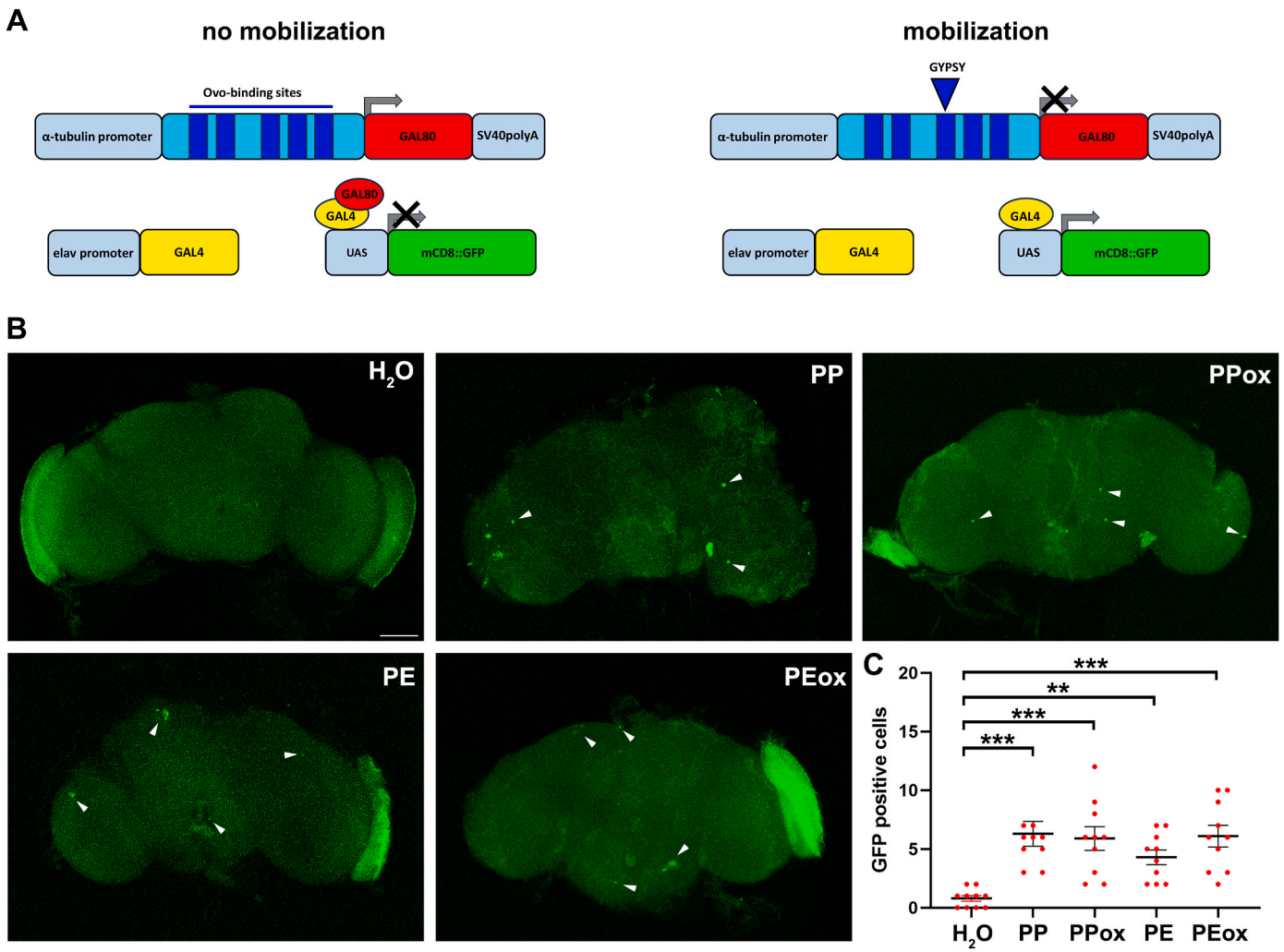

Fig. 7. Leachate-induced gypsy mobilization. (A) Schematic illustration of the gypsy-TRAP transposition assay. Gypsy insertion into the Ovo-binding site disrupts Gal80 expression and results in elav-Gal4-driven expression of GFP. **(B)** Representative images showing GFP-positive cells (arrowheads), representing gypsy transposition events. The scale bar indicates 100 μm. **(C)** Quantitative analysis of the active mobilization of *gypsy* in adult brains. Statistically significant differences between the control and treatment groups were determined by one-way ANOVA test followed by Dunnett's post hoc test (***p-value PP = 0.0003; ***p-value PPox < 0.0001; **p-value PE = 0.0047; ***p-value PEox < 0.0001). Red dots indicate the number of GFP-positive cells in each brain.

the control of an *α-tubulin* promoter, inhibits Gal4 activity in all tissues. The *α-tubulin* promoter and the coding sequence of *Gal80* are separated by a 500 bp fragment containing *Ovo* binding sites that are known as genomic hotspots for *gypsy* insertions. If endogenous *gypsy* retrotransposes into *Ovo* hotspot sequences, Gal80 expression is impaired and Gal4 can drive the expression of mCD8GFP reporter transgene, thus allowing to map *in vivo* cells with new integration events of *gypsy* retrotransposons. Using the *gypsy*-TRAP reporter system, we found a significant increase of GFP-positive cells in adult brains dissected from leachate-treated flies (Fig. 7B) as compared to untreated control flies. To rule out that these effects were due to loss of heterozygosity at the *Gal80* sequence, we used a similar system composed of mutated *Ovo* binding sites; in this case, we did not find any increase in GFP expression following exposure to leachates compared to untreated controls (Fig. S2).

It would be extremely important to clarify and deepen the molecular circuits through which leachates can trigger transcriptional activation and mobilization of transposable elements *in vivo*. It is well known that transposable elements are stable structural components of *Drosophila* heterochromatin (Pimpinelli et al., 1995), therefore an extensive decondensation of pericentric heterochromatin could potentially affect the epigenetic silencing of TE sequences. However, in our specific scenario, defective heterochromatin condensation, as evaluated through the PEV assay, may contribute to the epigenetic de-silencing of TEs but certainly does not represent its primary cause. This is evident from the observation that flies treated with PEox showed active mobilization of *gypsy* (Fig. 7B and C) while not exhibiting any significant defects in heterochromatin condensation (Fig. 3). Although the molecular basis of TE activation after leachate exposure remains to be elucidated, these results strongly indicate transposable element activation as a sensitive endpoint for the detection of toxic effects caused by plastic pollution. As suggested for the first time by Barbara McClintock (1984), specific environmental stimuli can drive bursts of activity of transposable elements which in turn lead to an increase of genetic variability allowing rapid adaptive phenotypic and genotypic responses (Cappucci et al., 2019; Casacuberta and González, 2013; Fanti et al., 2017; Klein and O'Neill, 2018; McClintock, 1984; Pimpinelli and Piacentini, 2019; Song and Schaack, 2018). Since then, the activation of transposable elements has been documented in many species in response to different types of environmental stressors including temperature (Cappucci et al., 2019; Fanti et al., 2017), UV rays (Jardim et al., 2015), radiofrequency electromagnetic fields (RF-EMFs) (Cappucci et al., 2022), ionizing radiation (de Oliveira et al., 2021), restraint stress (Cappucci et al., 2018), air pollution, persistent organic pollutants, pesticides, and metals (see

(Miousse et al., 2015) for a review), thus suggesting that transposable element activation could potentially be integrated into safety and risk evaluations and function as novel biomarker of environmental stress exposure (Miousse et al., 2015).

3.6. Plastic leachates promote metastatic behavior of tumor clones expressing the oncogene Ras^{V12}

It is widely recognized that DNA damage, loss of heterozygosity (LOH), and transposable element activation are common genetic events that substantially contribute to cancer development and progression (Hanahan and Weinberg, 2011). *Drosophila* has proven to be a valuable model system for studying important pathways involved in human cancer biology (Enomoto et al., 2018; Miles et al., 2011; Mirzoyan et al., 2019). Many cancer-related genes and oncogenic signaling pathways in humans are conserved in flies enabling the development of genetically engineered *Drosophila* models useful for dissecting the genetic and molecular mechanisms underlying cancer initiation and progression (Mirzoyan et al., 2019; Richardson et al., 2020; Saavedra and Perrimon, 2019).

To evaluate the potentially carcinogenic effects of plastic leachate components in *Drosophila*, we used the MARCM (Mosaic Analysis with a Repressible Cell Marker) genetic mosaic system (Pagliarini and Xu, 2003) which allowed us to generate epithelial tumor clones overexpressing the activated oncogene *Ras* (Ras^{V12}) in the eye-antennal imaginal disc and to analyze their metastatic behavior by exploiting the concomitant expression of GFP. To assess whether plastic leachate treatments can promote tumor overgrowth and invasion, we crossed females *ey-FLP1; act > y$^+$>Gal4, UAS-GFP*; *FRT82B, tub-Gal80(MARCM 82B tester line)* to transgenic males carrying the *UAS-*Ras^{V12}construct, distal to an FRT site (*FRT82B, UAS-*Ras^{V12}). The tumor somatic clones overexpressing Ras^{V12} were generated through a site-specific event of somatic recombination involving the FRT (Flippase Recognition Target) sequences and the recombinase enzyme Flippase (FLP) which, expressed in the developing eye, under the control of the *eyeless* promoter, induced Ras^{V12} overexpression, exclusively in homozygous recombinant cells that received the *UAS-*Ras^{V12} transgene and simultaneously lost the construct encoding the Gal80 inhibitor (Pagliarini and Xu, 2003).

As shown in Fig. 8A, in untreated control larvae, tumoral clones overexpressing the activated Ras^{V12} displayed hyperplastic growth and rarely metastasized in other tissues. Conversely, leachate-treated larvae exhibited tumor overgrowth and metastatic behavior of GFP-positive Ras^{V12} cells (Fig. 8A). The invasion percentage for leachate-treated larvae was of 100% for PP (***$p < 0.0001$), 82.9% for PPox (***$p < 0.0001$), 96.6% for PE (***$p < 0.0001$), 95.5% for PEox (***$p < 0.0001$), and 22.2% for untreated controls (Fig. 8B). Compared to untreated control larvae, in leachate-treated larvae, we also found an increased mean number of metastases (H_2O: 2.2 ± 0.7, PP: 24 ± 1.6, ***$p < 0.0001$; PPox: 6.1 ± 1.3, *$p = 0.0374$; PE: 12.2 ± 2.7, ***$p < 0.0001$; PEox: 15.1 ± 2.4, ***$p < 0.0001$) (Fig. 8C) and, except PP, a significant overgrowth of the cephalic tumor (H_2O: 0.043 ± 0.007, PP: 0.059 ± 0.009, $p = 0.3806$; PPox: 0.089 ± 0.006, ***$p < 0.0001$; PE: 0.096 ± 0.004, ***$p < 0.0001$; PEox: 0.093 ± 0.007, ***$p < 0.0001$) (Fig. 8D). Collectively, these results demonstrate that plastic leachate exposure exacerbates tumor growth and metastasis, suggesting a synergistic effect between leachates and oncogenic Ras signaling.

The genomic instability induced by plastic leachate exposure could synergize with Ras^{V12} to promote tumor growth and invasion by different mechanisms. Genomic instability could result in the accumulation of secondary mutations in genes involved in metastasis-related

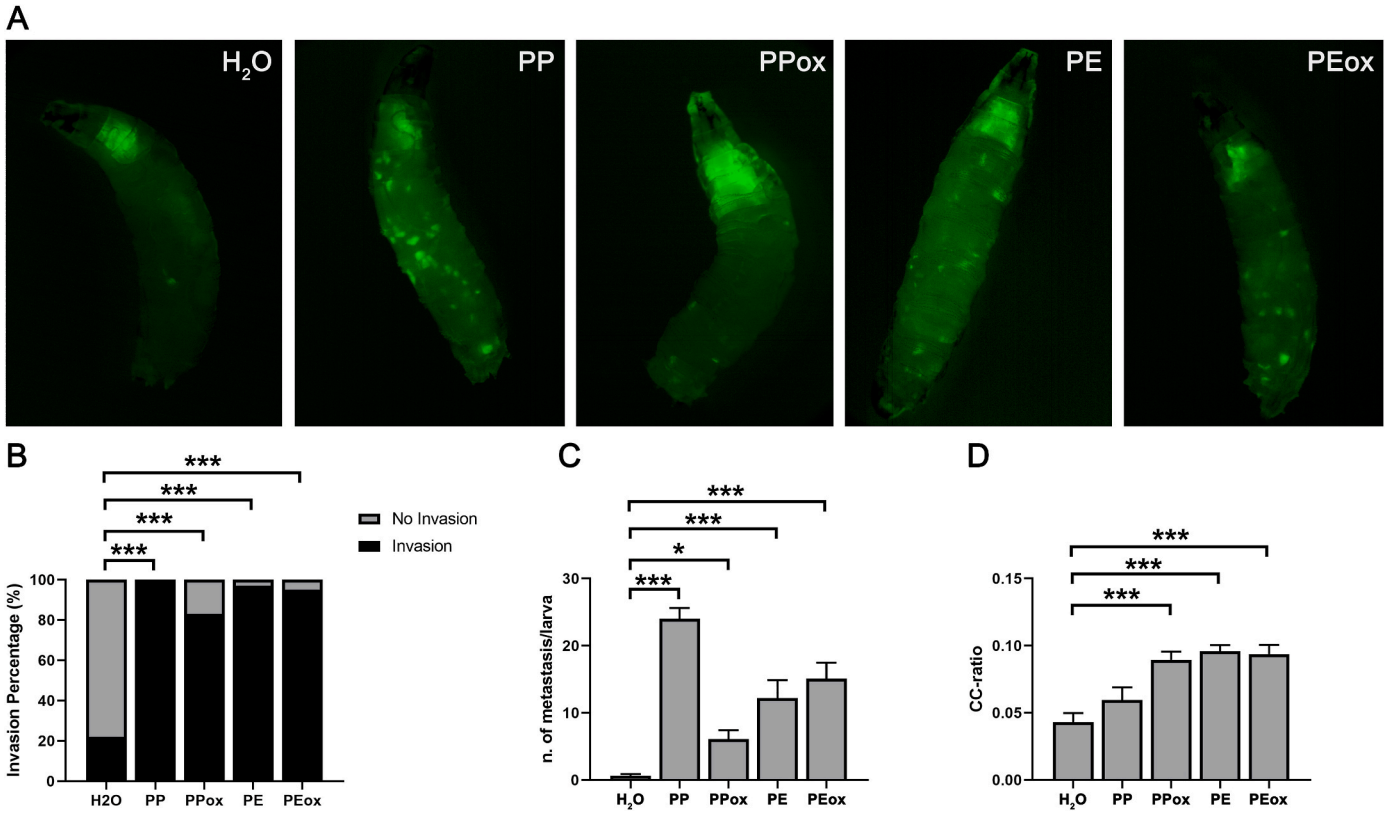

Fig. 8. Plastic leachates promote tumor growth and invasion potential of Ras^{V12} tumoral clones. (A) Fluorescence micrographs of GFP-labeled Ras^{V12} larvae are shown with indicated treatments. Compared with untreated control larvae, plastic leachates promote metastatic behavior of Ras^{V12} clones **(B and C)** and induce overgrowth of Ras^{V12} cephalic complexes (CC) consisting of eye-antennal discs, brain, and ventral nerve cord **(D)**. The CC-ratio is expressed as a ratio between the GFP-positive Cephalic Complexes and the overall body larval area. Statistical significance was determined by Fisher's exact test **(B)** or one-way ANOVA test followed by Dunnett's post hoc test **(C and D)** (***p-value < 0.001; *p-value < 0.05).

processes, such as cell adhesion, migration, or invasion (Yates and Campbell, 2012). Furthermore, chromosomal instability could generate genetic heterogeneity within the tumor cell population, providing a substrate for the selection of metastatic driver mutations (Bakhoum and Landau, 2017). Finally, we cannot exclude a potential impact of plastic leachates on epigenetic regulatory mechanisms, resulting in the transcriptional suppression of metastasis-suppressor genes or the activation of metastasis-promoting genes (Patel and Vanharanta, 2017). Overall, these results provide an interesting avenue for further investigation into the interplay between plastic pollution, genomic instability, and cancer.

3.7. Multilevel comparison of toxicity among different plastic leachates

In this study, we combined genetic, molecular, and behavioral approaches to comparatively evaluate the toxic effects of plastic leachates, using *Drosophila melanogaster* as a model system (Fig. 9). The results of this study demonstrated that plastic leachates induce DNA damage and extensive heterochromatin decondensation. Accordingly, leachate exposure strongly promoted loss of heterozygosity (LOH) of *warts (wts)* gene producing significant frequencies of *wts* tumors. Consistent with the high degree of genomic instability in neuronal tissues, leachates also impaired the locomotor behavior of adult flies. Most importantly, plastic leachates induced transcriptional activation and mobilization of transposable elements and enhanced the oncogenic potential of Ras^{V12} in promoting tumor growth and invasion. Concerning virgin polymers, PP exerted a higher effect with respect to PE regarding TE mobility while the opposite was evidenced for LOH. The remaining endpoints respond similarly. Although some specific toxic elements were identified such Zn in PP and Pb and Ag in PE, the mixture effects should be the most probable responsible for the observed effects. A different effect with respect to virgin leachates was evidenced in the two oxo polymers, with a slight decrease in the effects with PPox and increased values with PEox exposure. A common additive was evidenced in the two oxo polymers, in particular compounds from the isocyanate family (Fig. S2), which has already been shown to contribute to the toxic action (Li et al., 2023), in combination with metals already evidenced in polymer leachates (Turner, 2018) and here occurring in larger amount. Notably, the combined effects seemed to enhance the toxic action of PE and mitigate those of PP. Collectively, these findings indicate that plastic leachates exert genotoxic effects in *Drosophila* and set the stage to further investigate the harmful biological effects of leachate-related plastic pollution on terrestrial organisms.

4. Conclusions

Once in the environment, plastic particles undergo to leaching process that makes available chemical substances, generally toxic, utilized in polymer fabrication and manufacturing. In addition, the effects linked to plastic weathering accelerate the release of the primary leachable chemicals, but also of the pollutants likely adsorbed along the MP lifespan in the environments. This study corroborates previous data about the toxicity of PP and PE plastic leachates both as a virgin and additivated to be oxodegradable, showing genotoxic and transmissible effects linked to exposure via food without particle ingestion.

Actually, the toxic action already evidenced in virgin leachates and mainly linked to metal mixtures, is enhanced in the case of PE while was slightly reduced in the case of PP, underlining the need to often consider the complexity of chemical joint action.

Drosophila resulted a suitable model organism for studying hidden effects of MP showing that, although developmental times and longevity are unaffected by leachate assumption, severe and transmissible damages were instead evidenced at the genomic level. The integration of specific genotoxic endpoints in the ecotoxicological battery of test, surely will improve the confidence in the risk assessment.

Moreover, results showed the need to better explore the threat represented by plastic leachate contamination, highlighting, in particular, the mixture effect of known and unknown or undetectable chemicals.

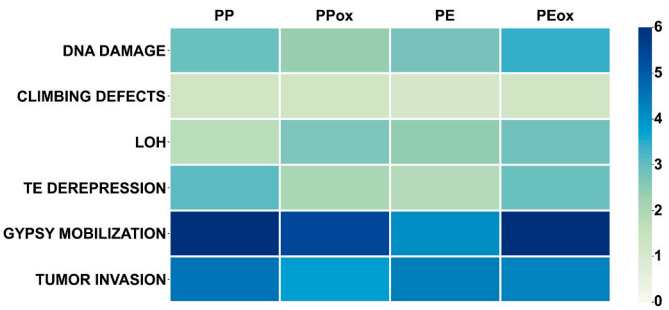

Fig. 9. Multilevel comparison of toxicity among different plastic leachates. Heatmap values indicate absolute ratios of each toxicity endpoint of leachate-exposed groups versus untreated controls.

CRediT authorship contribution statement

Ugo Cappucci: Visualization, Validation, Investigation, Funding acquisition, Formal analysis. **Mirena Proietti:** Validation, Investigation, Formal analysis. **Assunta Maria Casale:** Validation, Investigation, Formal analysis. **Simona Schiavo:** Validation, Investigation, Formal analysis. **Salvatore Chiavarini:** Validation, Investigation, Formal analysis. **Sara Accardo:** Validation, Investigation, Formal analysis. **Sonia Manzo:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Lucia Piacentini:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

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

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.142440.

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