Evaluating the Toxicity of Microplastics and Nanoplastics to *Drosophila melanogaster*

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December 2020

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master in Chemical Engineering

Abstract

The degradation of plastic waste into microplastics (MPs), plastic particles less than 5 mm, and nanoplastics (NPs), less than 100 nm, is a growing concern. Despite being the largest sink for plastic pollution, research on the effects of MP/NPs in terrestrial animal models is scarce. Here, we use the fruit fly (*Drosophila melanogaster* Oregon R strain*)* to study the toxicity of MP and NPs. The toxicity of dialyzed polystyrene particles (1 µm and 20 nm spheres) was assessed via dietary exposures to a range of concentrations (0.01 to 100 ppm). In a first experiment, flies were exposed from larval to adult stage (13 days). Uptake of both particle sizes in the gastrointestinal tract of larvae was observed, with considerably different depuration times, 1 and 24 h for 20 nm and 1 µm particles, respectively. In adults, only 1 µm particles accumulated to detectable levels and nano computed tomography imaging showed intestinal damage for both particles. Both MPs and NPs significantly affected locomotion. Individual larval crawling decreased after ≥50 ppm MP exposure ($F = 11.12$, $P < 0.001$). In a group setting, both MP ($F = 15.6$, $P < 0.001$) and NP ($F = 14.89$, $P < 0.001$) had an impact. Only NP impacted adult climbing at ≥50 ppm concentration ($F = 4.49$, $P = 0.005$). Mortality, development, and fertility were not significantly affected. A second experiment with adult flies (8 days) focused on daily behavior. We observed no effect on circadian rhythms but an increase in daily activity after MP ($F = 5.11$, $P = 0.003$) and NP ($F = 4.13$, $P = 0.012$) exposure. Overall, dietary exposures to clean spherical polystyrene micro- and nanoplastics caused low toxicity but significant sublethal effects in the fruit fly.

Résumé

La dégradation des déchets de plastique en microplastiques (MP), particules de moins de 5 mm, et en nanoplastiques (NP), particules de moins de 100 nm, est une préoccupation environnementale importante. Bien que la majorité de la pollution plastique touche les systèmes terrestres, les recherches sur les effets des MP et des NP dans les animaux terrestres sont rares. Pour étudier la toxicité des MP et des NP, nous avons utilisé la mouche à fruits (*Drosophila melanogaster* souche Oregon R). Nous avons évalué la toxicité des particules de polystyrène dialysées (sphères de 1 µm et 20 nm) en exposant les mouches à de la nourriture ayant diverses concentrations (0,01 à 100 ppm) de MP et NP. Dans la première expérience, les mouches ont été exposées du stade larvaire au stade adulte (13 jours). Nous avons observé l'ingestion des deux tailles de particules par les larves, et des temps d'épuration considérablement différents, soit 1 et 24 h pour les particules de 20 nm et de 1 µm, respectivement. Chez les adultes, seules les particules de 1 µm se sont accumulées à des niveaux détectables dans les intestins. La nanotomographie a montré des dommages aux tissus intestinaux. Les MP et les NP ont aussi eu un effet sur la capacité locomotrice des mouches. Le rampement individuel des larves a diminué après une exposition ≥ 50 ppm MP ($F = 11,12$, $P < 0,001$). Quand le rampement des larves a été évalué en groupe, les MPs ($F = 15,6$, $P < 0,001$) et NPs ($F = 14,89$, $P < 0,001$) ont eu un impact. L'escalade des mouches adultes était seulement affectée par ≥ 50 ppm NP ($F = 4.49$, $P = 0.005$). Ill avait aucun effet sur la mortalité, le développement et la fertilité. Une expérience subséquente faite avec des mouches adultes (8 jours) a porté sur le comportement quotidien. Aucun effet sur les rythmes circadiens n'a été observé, mais il y avait une augmentation de l'activité quotidienne suite à une exposition aux MP ($F = 5.11$, $P = 0.003$) et aux NP ($F = 4.13$, $P = 0.012$). Bref, les expositions alimentaires à des microplastiques

et à des nanoplastiques sphériques de polystyrène propres sont peu toxiques pour les mouches à fruits, mais produisent des effets sublétaux notables.

Acknowledgements

First and foremost, I would like to thank my supervisor Nathalie Tufenkji for providing me with the opportunity to complete this project, for her guidance, and for her positivity and enthusiasm throughout my thesis. I would also like to thank all the members of the Biocolloids and Surfaces Laboratory who readily provided their expertise and support during my experimental work, and for making my time at McGill truly memorable. In particular, I would like to thank, Elvis Genbo Xu, Oluwadamilola Pikuda, Eva Roubeau Dumont, Laura Rowenczyk, Shawn Chahal, Heidi Jahandideh, Brian Nguyen, Jeff Farner and Nicholas Lin. I am also grateful to our summer students, Victoria Meola, Mingrui Guo and Rachel Cheong for their help in this project. Thanks to Paul Lasko at McGill University for providing the *D. melanogaster* and Eric Déziel and Marie-Christine Groleau at Institut National de la Recherche Scientifique for advice on *Drosophila* rearing and handling.

Lastly, a special thanks to my family for their unconditional support, love and patience.

This project was funded by the Canada Research Chairs Program, the Canada Foundation for Innovation, the Natural Sciences and Engineering Research Council of Canada (NSERC) through its Discovery, Canada Graduate Scholarships (CGS) and the Collaborative Research and Training Experience (CREATE) programs, le Regroupement des Écotoxicologues du Québec, the Eugene Ulmer-Lamothe discretionary award, and the SURE program at McGill University. This research was performed using infrastructure of the Integrated Quantitative Biology Initiative, Canadian Foundation of Innovation project 33122.

Contribution of Authors

Sara Matthews performed all the experimental design, experimental work, data analysis, and writing tasks except for those delineated below. Oluwadamilola Pikuda, Rachel Cheong, Mingrui Guo provided help in setting up the developmental toxicity assays and processing videos of larval crawling and climbing assays. Victoria Meola assisted in performing fertility assays and performed preliminary daily locomotor assays during protocol development. Rui Tahara and Hans Larsson shared their expertise in X-ray computed tomography (CT) and performed the CT image acquisition. Eva Roubeau Dumont performed the statistical analysis in R. Elvis Genbo Xu shared his expertise in toxicological experiments, providing guidance in designing study, and aided in performing experiments. Nathalie Tufenkji provided guidance in the study design, throughout the project and reviewed all written work. Chapter 2 of this thesis is a manuscript was submitted to the journal *Environ. Sci.: Nano*. All co-authors participated in reviewing and editing the manuscript. The authorship of the manuscript is as follows:

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Chapter 1: Introduction

Plastics are everywhere. Looking around a single room, almost every item you see will have some plastic component. From electronics, to construction materials, to fabrics and even personal care products, they all contain plastic. As a result, the demand for this material has rapidly increased, with global production reaching 359 million tonnes in 2018.[1] In turn, plastic pollution also grew and has become one of the most serious environmental issues today.[2] While the negative impact of bulk plastic waste on wildlife is easily seen and well documented,[3] its degradation products, smaller plastic pieces known as microplastics (MPs) and nanoplastics (NPs), are harder to detect.[4] These fragments compose the vast majority (94%) of plastic pollution and can be carried to even the most remote locations globally via ocean currents and winds.[5] Inevitably, a wide variety of species, including humans, will be exposed to these fragments which could not only cause toxicity on their own but act as carriers for other contaminants.[6] Therefore, studies on a wide variety of organisms and models is important to understand the potential adverse biological impacts of MPs and NPs.

So far, researchers have largely focused on the effects of MPs on marine organisms, with some studies identifying MPs in various animals in the field, and other using laboratory-scale experiments to measuring their effects.[5] Work with filter feeders (e.g. *Daphnia magna)*, mussels, algae and oysters have shown that NPs can readily pass through biological barriers and accumulate in tissues and organs of exposed organisms, triggering physiological distress, diminished reproductive fitness and early mortality.[7] In contrast, work involving terrestrial species has been largely limited to earthworms. [8–10] While earthworms are a useful model, being a good indicator of soil health,[11] there is species to species variability in response to any toxicant. Therefore, the

number of terrestrial models still needs to be expanded to understand the impact of a substance on an ecosystem as a whole.

Drosophila melanogaster (fruit fly) offers an ideal *in vivo* terrestrial model to rapidly assess the toxicity of MPs and NPs. This species is found in abundance worldwide and plays an important role in the maintenance of terrestrial ecosystems.[12–14] There is also significant conservation of genetics, cellular signaling pathways and functions of their nervous[15] and digestive systems[16] with that of vertebrates and mammalian models. Therefore it is both an environmentally relevant model and a powerful tool for investigating the underlying cellular mechanisms of toxicity that could be conserved in vertebrates. It also offers the distinct advantages of being inexpensive, easy to maintain, and short-lived, making it well-suited for high-throughput experiments.[16] In this work, we used *D. melanogaster* as a new terrestrial model to identify the effects of MP/NP by measuring a variety of endpoints after exposure in controlled laboratory conditions.

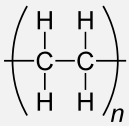
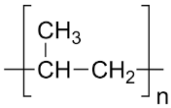
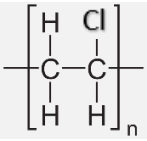
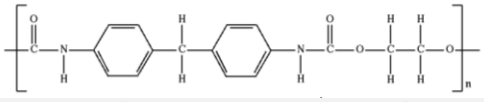
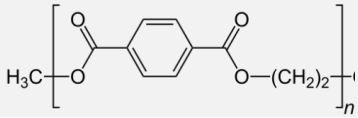
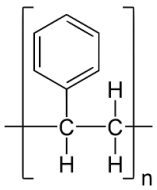
In this first chapter, I review plastic material and additives, their fate in the environment and their documented effects, with emphasis on terrestrial systems. A brief overview of the life cycle and physiology of *D. melanogaster* is also given to define terminology and explore its use in toxicity testing.

1.1 Plastics: Production, use and fate

Plastics are defined as synthetic polymers of high molecular mass that can be easily molded into a desired shape.[17] In general, plastics are separated into two categories: thermoplastics and thermosets. Thermoplastics are those that can be melted, shaped and hardened repeatedly and include polyethylene (PE), polyethylene terephthalate (PET), polypropylene (PP), polystyrene (PS) and polyvinyl chloride (PVC).[17] Thermosets trade this feature for additional heat-resistance and structural integrity, as a cross-linking reaction between the polymer chains creates a permanent

three-dimensional network.[17] The most common thermoset is polyurethane (PUR) which is primarily used in insulation. Plastics are typically synthesized via polymerization reaction of monomers (**Table 1.1**) that is initiated by a reactive species, and then cured.[17] During these processes, additional components can be added to fine tune material properties like strength, flexibility, chemical resistance, transparency and colour, or facilitate certain aspects of production. These substances include flame retardants, pigments, plasticizers, heat stabilizers, UV absorbers, antioxidants, antistatic agents, and slip compounds.[17,18] Nearly all additives, with the exception of some organic additives, are not chemically bound to the polymers.[17] Therefore, there is potential for them to migrate from the plastic into the surrounding environment, food, or into animal tissues, potentially carrying out negative effects of their own (**Table 1.2**.).

Table 1.1. Plastic types and uses listed in descending order of demand based on production and usage in Europe as described in Plastics – The Facts 2019[1]

Resin type	*Demand (Mt/year)*	*Primary uses*	*Monomer subunit*
Polyethylene	~ 15.2	Food packaging	
Polypropylene	~ 10	Food packaging	
Polyvinyl-chloride	~ 5	Building materials (ex. window frames, profiles, floor and wall covering, pipes, etc.)	
Polyurethane	~ 4	Insulation for building, pillows, mattresses, foams	
Polyethylene terephthalate	~ 3.9	Packaging, i.e. bottles	
Polystyrene	~ 1.8	Food packaging	

Given the endless combination of monomers and additives, plastic properties have been modified to suit a wide range of products.[17] While some like electronics and building materials have a lifetime of decades, packaging, which comprises 39.9% of resins produced in 2018, lasts less than a year.[1] With this rapid turnover, it is estimated that 6.3 billion tonnes of waste has been generated between 1950 and 2015, with the vast majority (79%) going to landfills or released directly into the natural environment where it can persist for hundreds of years. [2]

Table 1.2. Common additives in plastic materials modified from Hansen et al.[19] with general information on toxicity from the Agency for Toxic Substances and Disease Registry[20] or other primary sources. Lowest observed adverse effect level (LOAEL) are for 14 days of exposure periods in mice or rats for unless indicated otherwise.

Type of Additive	Typical amount in % w/w	Substances	Notes on toxicity
Plasticizers	10-70	Bisphenol A	Endocrine disruptor LOAEL: 25 ng/kg/day (11 days)[21]
		Phthalates (ex. Di(2-ethylhexyl) Phthalate (DEHP)	Endocrine disruptor with respiratory, immune and developmental effects. LOAEL 10-500 mg/kg/day
		Citrate esters	Less toxic alternative to phthalates, high doses can cause changes in thyroid function. LOAEL 400 mg/kg/day (28 days) [22]
Flame retardant	3-25	Brominated flame retardants. (ex. Polybrominated biphenyls)	Shown to effect thyroid, liver and brain development. LOAEL 130 mg/kg/day
	0.7-3	Phosphate esters (ex.Tri-n-butyl phosphate (TnBP), Tricresyl phosphate (TCP)	Long term exposure to some types can cause lesions to brain, kidney and ovaries in rats. [20] (TnBP) LOAEL 411 mg/kg/day (TCP) LOAEL 360 mg/kg/day (16 days)
Stabilizers, antioxidants and UV stabilizers	0.05-3	Irgafos 168 phosphate	Degradation products have been shown to inhibit cell growth [23]
Heat stabilizers	0.5-3	Cadmium or lead compounds. Nonylphenol	Cadmium causes hematological changes LOAEL 42 mg/kg/day Lead is toxic to every organ system. LOAEL <10 ug/dL of blood Nonylphenol is an endocrine disruptor. [24] LOAEL 50 mg/kg[25]
Slip agents, lubricants and anti-statics	0.1-3	Fatty acid amides, fatty acid esters, metallic stearates and waxes.	Generally, very low toxicity. Zinc LOAEL 200 mg/kg/day (15 days) Erucamide LOAEL > 1000 mg/kg/day (90 days) [26]
Curing agents	0.1-2	Peroxides, formaldehyde, hydrazine. 4,4'-Methylenebis(2-chloroaniline) (MBOCA)	These chemicals are irritants which cause injury to the gastrointestinal and respiratory system. Formaldehyde LOAEL 80 mg/kg/day (4 weeks) MBOCA LOALE 50mg/kg/day (3 months)
Biocides	0.001-1	Arsenic and organic tin compounds, triclosan.	Arsenic has systemic effects. LOAEL 119 mg/kg/day (19 days)

			Organic tin causes respiratory depression and damages the liver and kidney LOAEL 10.6 mg/kg/day (12-16 days)
Soluble colorants	0.25-5	Azocolorants.	
Organic pigments	0.001-2.5	Cobalt(II) diacetate	Cobalt causes systemic effects. LOAEL 12.4 mg/kg/day (3 weeks)
Inorganic pigments	0.01-10	Cadmium, chromium and lead compounds	Cadmium causes systemic effects LOAEL 12 mg/kg/day (12 days)

1.2 Plastic pollution: The emergence of microplastics and nanoplastics

MPs and NPs are defined as plastic pieces with diameters of 100 nm – 5 mm and < 100 nm, respectively, and can be either primary or secondary in origin.[27] Primary MPs and NPs originate directly from commercial products, most commonly as exfoliating beads in facial scrubs[28] and industrial pellets.[29] Secondary MPs and NPs are unintentional products of the degradation of larger plastics due to environmental processes (i.e. photodegradation, thermal oxidation, hydrolysis, biodegradation, mechanical abrasion).[30] They are defined by their irregular shapes (e.g. fragments, fibers, sheets, etc.) and come from various sources including car tires,[31] agricultural films,[32] clothes[33] and larger plastic debris.[27]

Regardless of their source, these plastics are eventually transported to environmental sinks such as oceans, sediments and soils.[27] Primary MPs and NPs are mainly released into waste water streams with < 5% being released into the effluent of waste water treatment plants (WWTP), and the remainder partitioning into biosolids, which are later sent to landfills (14-30%) or applied to agricultural soil (42-55%).[27] Since secondary MPs and NPs are degradation products, they are directly generated within the environment from waste[27] or agricultural plastics (i.e. plastic seed casings, ground covers, mulch, labels and wraps).[34] Once at large, these miniscule low density particles are transported by winds and water currents to even the most remote locations globally and, consequently, have become ubiquitous in nature.[5] Over time, the weathering process will also cause changes in physical and chemical properties which will influence plastic persistence and

bioavailability in the environment, the migration of additives and monomers from the material, and adsorption of pre-existing contaminants.[18,27,35–38]

Occurrence in aquatic environment

The marine environment receives an estimated 4.8-12.7 Mt of plastic per year[18] and it is estimated that over 250 Mt will have accumulated in oceans by 2025.[39] New sampling data is continuously being generated through independent research and large scale initiatives such as the Global Microplastics Initiative run by Adventure Scientists.[40] In a review done by Shim *et al.* on MPs in the marine environment, concentration of MPs in oceans ranged from 4.8×10^{-6} particles/m^3 in the eastern equatorial Pacific to 8.6×10^3 particles/m^3 off the Swedish coast, reflecting the spatial variation caused by the ocean currents.[5] The most common plastic types found were PP, PE and PVC, and fibers were the most common shape.[41] However, most of these studies only account for plastics ≥300 μm in diameter, while the majority of microbeads in personal care products are < 300 μm, leaving what is potentially the largest portion of particles, nanoplastics, unaccounted for in field studies.[42] In controlled laboratory studies, NPs concentrations on the order of 10^8 particles/mL^{-1} or particles/$g_{product}^{-1}$ have been measured in commercial products (i.e. facial scrubs[28]), from plastic products subjected to realistic weathering conditions (i.e. polystyrene coffee cup lid[43] and teabags[44]) or accelerated weathering (i.e. laser ablation of PET films[45]).

Occurrence in terrestrial environments

Despite the fact that the terrestrial environment is the largest sink for plastics, data on the concentrations of MPs and NPs in this environment is more limited because of the technical challenge of identifying small organic polymers in complex soil media.[8] Studies have been limited to quantifying MPs that are > 45 μm and are summarized in **Table 1.3**. Concentrations vary greatly based on land use and can reach concentrations that far exceed those found in aquatic

environments. Soil near a waste facility in Sydney, Australia, had MP concentrations as high as 67 500 mg/kg,[46] while a rural farmland in Germany detected less than 1 particle/kg.[47] This is low in comparison to other agricultural soils in the Swiss floodplains (55.5 mg/kg)[48] and Dian Lake, China (42 960 particles/kg)[49] because these German farmlands did not utilizes modern agricultural treatments such as the application of biosolids, soil conditioners (e.g. polyurethane foam and polystyrene flakes), or plastic films.[47] Biosolids (or sludge) capture the majority of MPs passing through WWTP, concentrating MPs to over 15 000 particles/kg in some cases.[50] Therefore, sludge application is a significant contributor to the accumulation of MPs in agricultural soil. One study estimated that the MP loading on agricultural lands can be as high as 300 000 tonnes annually in the US from the application of WWTP sludge alone.[51] Depending on the sludge treatment, like lime stabilization, which involves elevated pH and mechanical mixing, a higher proportion of these particles will be of a smaller size.[50]

Table 1.3. Concentration of microplastics (45 µm – 5 mm) in various soil types. Due to its use in agriculture, sewage sludge was included in the list.

Soil type	Location	Concentration	Ref.
Industrial	Australia	67 500 mg/kg	Fuller et al. 2016[46]
Sludge	Ireland	4196-15 385 particles/kg	Mahon et al. 2017[50]
	Syracuse, USA	4000 fibers/kg wet weight	Zubris and Richards 2005[52]
	Los Angeles, USA	5000 particle/ kg wet weight	Carr et al 2016 [53]
	Unknown	196 to 15 385 particles/ kg dry weight	Mahon et al. 2017 [50]
Suburban soil	Buenos Aires, Argentina	3 g/m^2*	Ramos et al 2015[54]
Agricultural	Shanghai, China	62-78 items/kg	Liu et al. 2018[55]
	Swiss floodplains	55.5 mg/kg	Scheurer et al. 2018[48]
	Dian Lake, China	42 960 particles/kg	Zhang et al. 2018[49]
	Germany	0.34 particles/kg	Piehl et al. 2018[47]
Fertilizer	Un-named plant	0-895 particles/kg	Piehl et al. 2018[47]
	Unknown	0-146 particles/kg dry weight	Weithmann et al. 2018[56]

***Plastic pieces were in meso and/or macro size range were included in counts**

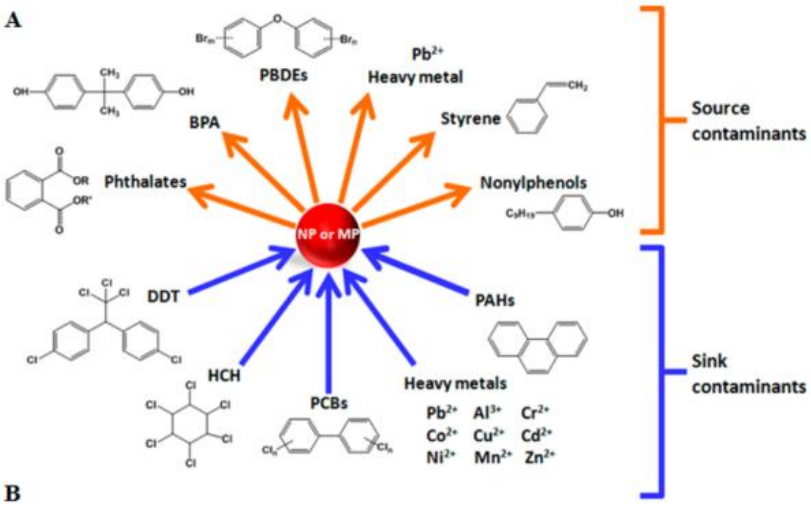
Interaction with other substances

In natural environments, a wide range of natural substances, like dissolved organic matter (DOM), proteins and DNA, as well as other contaminants will be present. Depending on their properties,

these substances can adsorb to the surfaces of NP and MPs.[27] Weathering processes can also roughen and oxidize plastic surfaces, modifying their surface properties and in turn adsorption affinities and surface capacity (**Figure 1.1**).[27] MPs can accumulate high concentrations (10^6 greater than dissolved concentrations) of persistent organic pollutants (POPs) and heavy metals, some of which are known to act as endocrine disruptors, carcinogens and mutagens.[27] Laboratory studies have shown efficient transfer of POPs from MPs, although field studies, where animals are already contaminated by POPs, show negligible effects from MPs.[57] Microbes can also colonize the surface of MPs and accelerate biodegradation and sedimentation.[35,37,38]

As previously discussed, plastics contain monomers and chemical additives that are more readily released with degradation. Generally, higher temperatures, contact times and fat content in the media increases migration levels of additives and monomers.[18] Released compounds can also be transformed via thermal degradation, with halogenated polymers releasing toxic products such as hydrogen cyanide, dioxins, and hydrogen fluoride upon heating.[18]

All these interactions, along with other particle properties such as shape, size, aggregation, will likely change the toxicity of plastic particles, and any associated chemicals or substances, as they modify environmental concentrations and bioavailability.



Plastic type	Wu et al., 2001	Muller et al., 2001	Mato et al., 2001	Pascall et al., 2002	Teuten et al., 2005	Karapanglioti and Klontza, 2007	Fries and Zarfl, 2008	Rochman et al., 2012	Lee et al., 2013(a)	Wang et al., 2014	Huffer and Hofman, 2015	Wang and Wang, 2016	Wang and Wang, 2017	Average ranking	Glass transition temperature *, T_g (°C)
PE			1	1	1	1			2	1	3	1		1.4	-125
HDPE	2	1					2	1						1.5	-90
LDPE		2					1	2						1.7	-110
PS				2					1	3	1	2		1.8	95-100
POM						2								2.0	-30
PP			2		2	3		3	3					2.6	0
PVC	1			3	3			5		2	2	3		2.7	87
PA											4			4.0	47
PET								4						4.0	69

Figure 1.1. (**A**) Contaminants associated with plastic debris in the environment and (**B**) relative ranking of sorption capacity as a function of plastic types, a score of 1 indicating the highest adsorption capacity. Reprinted with permission from Alimi et al. (2018).[27] Copyright 2018, American Chemical Society.

1.3 Toxicity of MPs and NPs

Being ubiquitous in the environment, virtually all organisms, including humans, are exposed to MP/NPs. In fact, MPs have been found in the guts, stomachs and tissues of many marine organisms from algae to whales[58], and recently in human feces.[4] One study estimated the human consumption of MPs could range from 39 000 to 52 000 particles per year in the US. [59] When intake via inhalation was also considered, that number more than doubled to 121 000 particles per year. [59]

Therefore, determining the toxic effects of MPs and NPs on ecosystems and human health has become a priority.

Toxicity of MP/NPs on aquatic life

The toxicity of MPs in aquatic organisms has been extensively studied, having been assessed in many aquatic organisms including filter feeders (e.g. *Daphnia magna*, mussels, algae, oysters), fish, coral, sea cucumbers, and crustaceans. [6,60,61] Several field and laboratory studies have observed MPs uptake into organisms but little evidence of accumulation as many species of fish and vertebrate clear MPs within 24 hrs. [61] While translocation through the intestinal wall is still unclear for larger particles (> 5 µm), there is stronger evidence for particles < 5 µm to enter the circulatory system and migrate to other tissues, most commonly the liver.[61] Averaging the results of multiple studies, lowest-observed-effect concentrations (LOECs) and no-observed-effect concentrations (NOECs) tended to occur at lower particles concentrations for larger diameters, but it is difficult to make comparisons as LOEC are rarely observed for large plastics (**Figure 1.2**).[61] At the range of concentrations of MPs studied, reduced survival was rarely observed while growth inhibition and changes in reproduction were more common as the ingestion of plastic reduced energy intake.[60] This in turn can impact population in the long-term, as Martins and Guilhermino[62] observed with *Daphnia magna* exposed to 1-5 µm polymer (type unspecified) at 0.1 mg/mL. In this study, it took three generations for the population to recover after exposure of the parental generation, and continuous exposure resulted in complete extinction after two generations.[62] Trophic transfer has only ever been demonstrated in laboratory studies. However these studies used high concentration and minimal egestion periods, which is more favorable for trophic transfer.[61] More significant to their toxicity is the ability for MPs to act as vectors for heavy metals and organic pollutants, with studies showing increased toxicity and accumulation of pyrene,

polychlorinated biphenyls (PCBs), organobromine compounds (PBDEs), chrome and benzo[a]pyrene when co-exposed with PE MPs.[60]

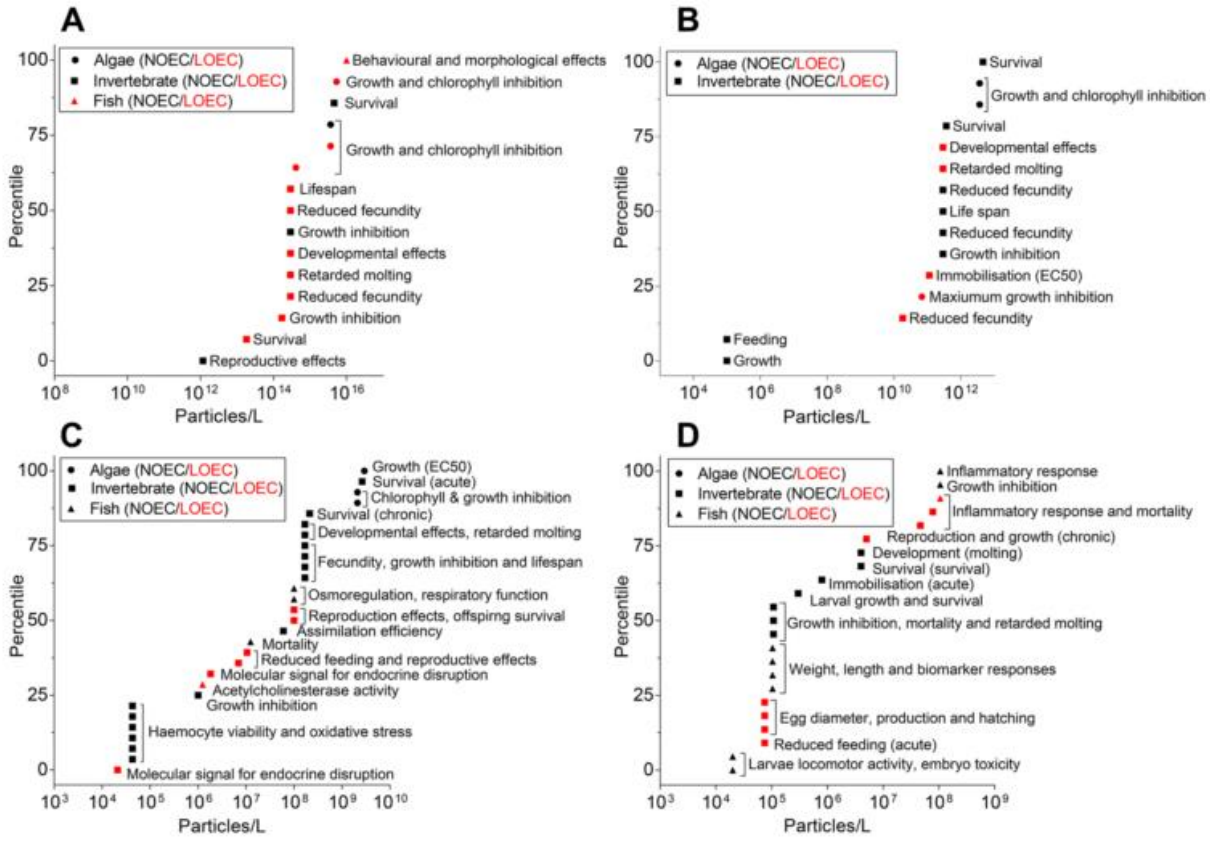


Figure 1.2. Cumulative microplastic ecotoxicity endpoints distributions for tests using particles sizes of (**A**) 0.01 to 0.1mm, (**B**) 0.1 to 1mm, (**C**) 1 to 10mm, and (**D**) >10mm. Red and black symbols represent lowest-observed-effect concentrations (LOECs) and no-observed-effect concentrations(NOECs), respectively. A cumulative distribution can be interpreted as where along the X-axis a NOEC/LOEC is likely to fall. Reprinted with permission from Burn & Boxall [61]. Copyright 2020 Society of Environ Toxicol Chem.

NPs had similar effects to MPs, with little impact on mortality but significant sublethal effects. In mussels, studies show that PS NPs remained in the body for longer times, were transported to the digestive glands and decreased filtering activities in the presence of food in less than 48 hours.[60] In *Artemia franciscana* (shrimp), acute exposure to 40-50 nm carboxylated (PS-COOH) and

aminated (PS-NH_2) PS caused sub-lethal effects such as decreased food intake due to massive sequestering of PS-COOH in gut lumen, and irregular motility due to adsorption of PS-NH_2 to sensorial antenna and appendages.[63] *Daphnia magna* also suffered no lethal effects from various NPs at high concentrations (50 mg/L) however, polystyrene nanospheres (20 nm) were transferred from parents to offspring two generations after exposure.[64] While the mechanism is unclear, it is suggested that uptake is mediated by appendage movements and the flow of water through the brood chamber. [64] Mattsson et al. [65] observed trophic transfer of 52 nm aminated-PS from algae, to zooplankton to fish, with the last exhibiting abnormal feeding and swimming activity. They attributed this to the NPs crossing the blood-brain barrier (BBB), supported by identification of PS in brain tissues, but there was no indication that blood vessels were emptied prior to homogenization, and so it could be that the plastic they observed was localized in blood vessels. [65] Their theory is supported by one study that showed uptake of positively charged PS NPs in an *in vitro* model of BBB, but more research is required to confirm translocation through BBB for unmodified or carboxyl PS.[66] Positively charged NPs have also been shown to cross the cell membrane, promoting ROS production[67] and expression of stress related genes.[68] From a review of comparative studies of NPs and MPs, it is unclear if size significantly contributes to toxicity of plastic particles in aquatic species.[60] Overall, MP and NP toxicity appears to be varied in aquatic organisms.

Toxicity of MP/NPs in terrestrial life

In contrast to aquatic systems, studies on the toxicity and impact of MPs and NPs on terrestrial life have been very limited. While studies have been gradually increasing since 2015 (**Figure 1.3A**), our review only found 80 studies that evaluated the impact of MP/NPs on terrestrial organisms which are summarized in **Table 1.4** excluding microbe studies. Approximately a third used a

species of earthworms and the remainder was a mix of common and widespread species of insects (i.e. *Folsomia candida, Proisotoma minuta, Porcellio scaber* and *Hypoaspis aculeifer),* mice, birds, and snails (**Figure 1.3B).** Plastics were often purchased or produced from commercial products, with sizes ranging from 20 nm to 3 cm, and used in chronic exposures (> 48 hours). Only 4 publications on NP toxicity in terrestrial organisms were found prior to this thesis project, however it has since increased to 17 in the last year (2020). PS and PE spheres were dominant, likely due to the commercial availability of monodispersed micron spheres, and exposure concentrations ranged from the order of 800 particle/mL to 60% (w/w), corresponding to an approximate range of 0.004-600 000 ppm. While concentrations as high as 60% MPs have not been detected in soils, it is possible for locations near landfills, or intensive use of plastic mulch and biosolids on farmlands to create such hotspots. Very high daily intake of plastics has also recently been observed in a field study of Eurasian clippers in Wales.[69] Based on number of MPs (0.5-5 mm) in regurgitates and fecal samples, it was estimated that they consume over 100 MPs per day.[69]

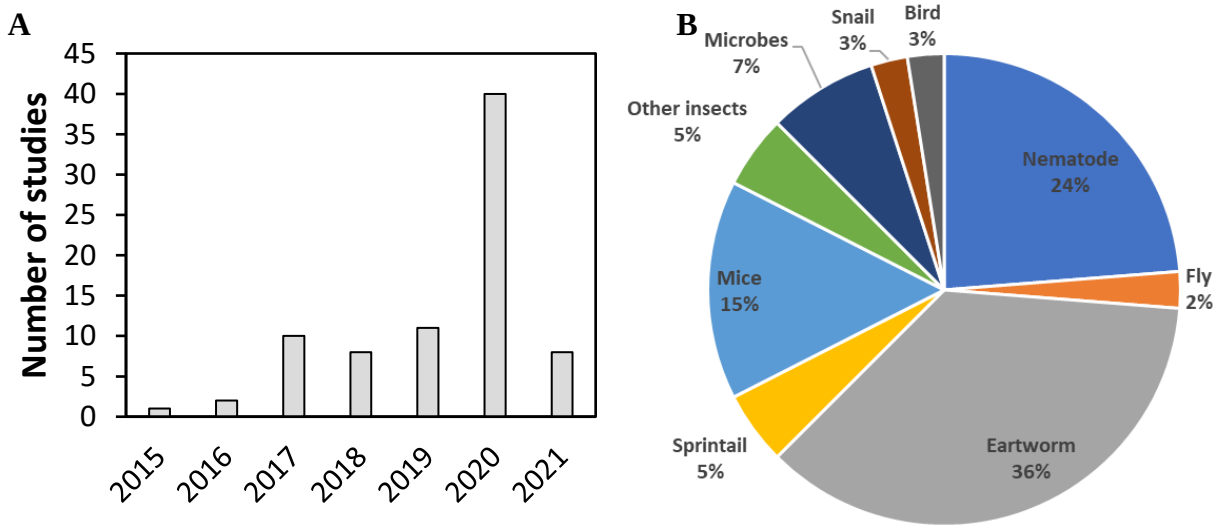


Figure 1.3. Overview of toxicity studies of micro- and nanoplastics on terrestrial species by **A)** year and **B)** animal model. Total of 81 studies as of Oct. 13, 2020.

While there is variation in responses based on plastic type, size and species. In general, mortality was rarely observed with only 2 studies reporting increased mortality or reduced lifespan in earthworm and *C. elegan*s, respectively.[70,71] Earthworms needed to be exposed to ≥ 28% MPs (< 150 µm) for 60 days to produce mortality rates of 8-25%.[70] *C. elegans* were exposed to a much lower concentration (1 mg/L), however, particles were smaller (≤ 5 µm) and unwashed, making the potential presence of preservatives and additives a confounding variable.[71] More common were a variety of sublethal responses. Earthworms were found to ingest, concentrate and transport PE MPs via their casts while experiencing only minor negative effects on their growth and reproduction.[70] Another study revealed an immune system response and gut damages at concentrations ≥125 mg/kg_{soil} after 56 days.[72]

Other insects could also transport PVC MPs and were more significantly affected in term of growth and reproduction.[34] Zhu et al.[73] fed soil oligochaete *Enchytraeus crypticus* oatmeal containing 0-10% (dry weight) PS NPs (50-100 nm) for 7 days, which had no effect on mortality, but inhibited growth, increased reproduction - a response to low toxic challenges - and caused an alteration in microbiome.[73] Tosetto et al.[74] exposed insects known as beachhoppers to marine-contaminated MPs for 24-72 hours and found decreased survival and jump height, and an increase in weight after 120 h exposure to 3.8 % 38-45 µm PE spheres.[74] These changes in growth, reproduction and active behavior could be due to a reduction in energy availability, as consumption leads to false satiation and extensive, energetically costly, digestive times.[74] The attachment of MP/NPs to cuticle and setae, important mechanosensory receptors, could disrupt the sensory feedback necessary for coordinated movement.[75]

In mice, despite the low bioavailability of PS MPs (0.22-.1.7%) there is still some uptake into various tissues[76] which explain the disturbance in energy and lipid metabolism, signs of oxidative stress, altered liver enzymes and gut microbiota.[77,78] Evidence of neurotoxicity is inconclusive in mice. Deng et al.[78] observed changes in acetylcholinesterase (AChE) activity in liver after 28 days exposure to 0.1mg/day of 5 or 20 µm PS. While Rafiee et al. [79] observed no behavioral changes in mice after 5 weeks exposure of up to 10 mg/kg body weight/day of 20 and 50 nm PS.[79] However, this difference could be attributed to the different particle sizes, with larger particle potentially requiring longer exposure times to trigger neurotoxicity.

Table 1.4. Summary of toxicity studies of micro and nanoplastics in terrestrial organisms.

Plastic	Details	Size	Species	Exposure Conc.	Duration	Assessment points	Results	Ref.
High-density polyethylene (HDPE)	Produced by cutting plastic bags	0.92 mm^2	*Lumbricus terrestris*	0.35 wt %	28 days	Accumulation, mortality or weight change	No evidence of Zn accumulation, mortality, weight change, or retention of MPs in their gut.	Hodson et al. 2017[80]
	Commercial (unwashed)	30-70 µm	*Caenorhabditis elegans*	2.21×10^5-16.9×10^5 particles/mL	72 h	Mortality and reproduction and RNAi screening	Changes in nucleotide excision repair and transforming transcription growth factor-β.	Kim et al. 2020[81]
	Cut from threaded bottle caps (washed)	4 mm	*Enchytraeus crypticus*	2-8 % (w/w)	3 days	Avoidance, mortality and oxidative stress	Mortality increased with concentration from 1-14%. Animals always moved to unspiked region of soil or chose lower MP concentration. Exposure caused enhanced oxidative stress even without chemical leaching or particle consumption.	Pflugmacher et al. 2020[82]
Low-density polyethylene (LDPE)	Produced by milling commercial pellets	250 and 1000 µm	*Eisenia fetida*	62-1000 mg/kg	28 days	Oxidative stress and energy metabolism biomarkers. Molecular changes with FTIR and NMR.	No mortality or weight variation. Increase in one molecule associated with the oxidative stress system, however other techniques register no changes in molecular profiles.	Rodriguez-Seijo et al. 2018[83]
	Commercial pellets	5 mm and 0.25-1 mm	*Eisenia fetida*	9-50 mg/kg of soil 17.9 or 2442 ng/g of chlorpyrifos	14 days	Avoidance, uptake, transport of contaminants	Worms avoided MPs but had greater contact time with contaminated soil. No evidence of MP uptake. MPs are not carriers of pesticides.	Rodriguez-Seijo et al. 2019[84]
	Produced by milling bulk plastic	<150 µm	*Lumbricus terrestris*	7-60% w/w	14 and 60 days	Mortality, growth, reproduction.	Mortality was higher and growth reduced for >28% (w/w). MPs < 50µm were concentrated in cast.	Huerta et al. 2016[70]
	Produced by milling bulk plastic	<150 µm	*Lumbricus terrestris*	7-60% w/w	14 days	Number, composition and structure of burrows.	More MPs and organic matter in the burrow walls. Greater bioturbation in 7% treatment.	Huerta et al. 2017[85]
	Commercial	<150 µm	*Lumbricus terrestris*	0-7 % w/w	14 days	Concentration and localization of MP, glyphosate and aminomethylphosphonic acid	Glyphosate and MPs only concentrated in top 1 cm of soil. Only glyphosate was detectable in burrows.	Yang et al. 2019[86]

	Commercial, rinse with ethanol and then DI water	<400 µm	*Eisenia fetida*	0.1-1.5g/kg of soil	28 days	Transport, intake, oxidative stress and neurotoxicity response	Ingestion in dose-response manner. Surface damage at 1.5 g/kg on worms. Egested particles are <100 µm. Catalase activity and malondialdehyde content increased at 1 g/kg. Acetylcholine esterase activity increase at 1 and 1.5 g/kg.	Chen et al. 2020[87]
	Produced by cutting and milling bulk plastic	250-1000 µm	*Eisenia andrei*	62.5 - 1000 mg/kg	28 and 56 days	Reproduction, growth, body weight, histology	No effect on mortality, reproduction or body weight. MPs found in gut of worms on contaminated soil but not on agar positive controls. Serious signs of inflammation and fibrosis in gut > 125 mg/kg.	Rodriguez-Seijo et al. 2017[72] (Right)
	Shredded from mulch films	550-1000 µm	*Eisenia fetida*	0.25% (w/w) MP; 0.02 and 2.0 mg/kg atrazine	28 days	ROS production and gene expression	Accumulation of ROS species and increased expression of stress related genes in single and combined exposure, but effects were greater in the latter.	Cheng et al. 2020[88]
Polyethylene (PE)	Plastic bag film, facial cleanser. Milled and washed	183 µm, 137 µm	*Porcellio scaber*	4 mg/g of food, 0.4% w/w	14 days	Food ingestion rate, defecation rate, food assimilation rate and efficiency, body mass, mortality, and energy reserves	No effects	Kokalj et al. 2018[89]
	Commercial powder	32-63 µm, 63-250 µm, 125-500 µm	*Chironomus riparius* (Harlequin fly)	1.25-20 g/kg of sediment	10 days	Ingestion and development	Ingested 32-63 µm particles which led to reduction in larval growth and delayed imagoes emergence at > 2.5 g/kg.	Silva et al. 2019[90]
	Commercial Washed with octane and pentane and dried.	< 500 µm	*Folsomia candida* (soil springtail)	0.1-1% (w/w dry soil)	28 days	Avoidance, reproduction and gut microbiota	Avoidance at 0.5 and 1% (59 and 69% avoidance respectively). Reproduction inhibited over whole range, being reduced by 70% at 1% MP exposure. Change in gut microbes at 0.5%.	Ju et al. 2019[91]
	Commercial & weathered for 2 months	38-45 µm	*Platorchesti a smithi*	3.8% (w/w) of PE 0.007 µg/g of polyaromatic hydrocarbons (PAH)	24, 72 and 120 hr	Survival, weight, behaviour (jumping, relocation)	MP clear after 48 hrs. Decreased survival and jump height, and increased weight at 120 hrs.	Tosetto et al. 2016[74]

	Commercial (unwashed)	32-63, 63-125, 125-250 and 250-500 µm	*Lumbriculus variegatus*	0.51, 3.2 and 20 g/kg dry sediment	48 hrs and 28 days	Reproduction, biomass and cellular responses.	No effect on reproduction and biomass. Ingested greater numbers of smaller particles. Activation of antioxidant and detoxification mechanisms and change in energy reserves for PEs > 125 µm.	Silva et al. 2021[92]
	Commercial fluorescent (unwashed)	180-212 or 250-300 µm	*Eisenia andrei*	1000 mg/kg dry soil	21 days	Reproduction	No effect on female organs but affect on male coelomocyte viability and damage to male organs. Particle were fragmented by earthworm digestive activity.	Kwak and An 2021[93]
	Commercial, mix of shapes	35 µm	*Physalaemus cuvieri* (tadpoles); tambatinga fish; Swiss mice	60 mg/L for tadpoles, fed up the chain	7 days	MP in livers	Accumulation in liver at all trophic levels. tadpole: 18,201.9 particles/g; fish: 1.26 particles/g; mice 57.07 particles/g. Change in mice behavior.	Da Costa Araujo and Malafaia 2021[94]
	Commercial (washed)	< 300 µm	*Eisenia Foetida*	2 and 10 mg/kg Cd 0-30% (w/w) PE	28 days	Growth, reproduction, avoidance, oxidative stress, sperm quality	Co-exposure induced higher response in avoidance, weight loss and reproduction. Oxidative stress. DNA damage occurred in dose dependent manner. MPs increased Cd concentration in worms.	Huang et al. 2021[95]
	Commercial (unwashed)	10-150 µm	*Mus musculus*	6-60 µg/day	5 weeks	Gut microbiome	Increase in *Staphylococcus* and decrease in Parabacteroides. Immune response with increase in interleukin-1α and decrease CD4+ cells. Inflammation in intestine.	Li et al. 2020[96]
	Commercial (unwashed)	45-53 µm	*Mus musculus*	100 mg/kg/day with and without PAE contamination (~70µg/g of MP)	30 days	PAE accumulation, transcriptomic analysis, gut microbiome	Phthalate ester (PAE) contaminated MPs increased gut permeability and inflammation compared to MP or PAE alone. 703 genes related to oxidative stress, immune response, lipid metabolism and hormone metabolism were altered. Change in gut microbiome composition.	Deng et al. 2020[97]
	Commercial, modified to add hydroxy groups to surface	40-48 µm	*Mus musculus*	0.125, 0.5, 2 mg/day/mouse	90 days	Body weight, ,accumulation, blood analysis, reproduction and development.	Reduced body weight gain and increased neutrophils in blood stream. MP like material in mast cell membrane and organelles of stomach and spleen cells. Reduced number of live births, altered sex ratio and body weight in subsequent pups.	Park et al. 2020[98]

Polyethylene terephthalate (PET)	Produced by cutting longer fibers	1257 µm length 76 µm diameter	*Achatina fulica* (African Giant snail)	0.01-0.71 g/kg soil dry weight	28 days	Egestion, histology of gastrointestinal tract, liver and kidneys. Oxidative stress proteins.	Fiber are ingested and excreted within 48 hrs. Crack and deterioration in fibers after passing through digestive track. 0.14-0.71 g/kg caused villi damage but did no effect liver and kidney. Elevated oxidative stress in liver.	Song et al. 2019[99]
HDPE, PET and PVC	Cut and milled from bulk plastic	< 2 mm	*Eisenia fetida* and *Caenorhabditis elegans*	0.1-1% (w/w) in mixed water organic output mixture	*C. elegans:* 24-72 h, worms 28 -56days	Mortality, reproduction, avoidance	No impact on earthworm growth mortality or avoidance. No impact on nematode mortality and reproduction. Little evidence of MP effect on microbial diversity but could be due to high variability in soils amended with mixed water organic output.	Judy et al. 2019[100]
PET and urea-formaldehyde (UF)	Washed and sieved commercial UF . Cut up PET bottle.	<100 µm and 100-200 µm	*Folsomi candida Proisotoma minuta*	7.5 mg	7 days	Particle transport	Larger species distributed more particles to further distances, 3 vs 2 cm. No difference between size for transport.	Maab et al. 2017[101]
Polyester	Laundering textiles	361 µm length 40 µm diameter	*Lumbricus terrestris*	0.1 and 1 w/w%	35 days	Mortality, weight, depuration, avoidance and gene expression.	No mortality or avoidance. 1% treatment lowered cast produced by 1.5-fold. Increase in expression of cellular stress genes.	Predergast-Miller et al. 2019[102]
	Fibers	Short(12–2.87 mm) Long (4–24 mm)	*Enchytraeus crypticus, Folsomia candida, Porcellio scaber and Oppia nitens.*	0.02% to 1.5% (w/w)	3-4 weeks	Intake, reproduction and energy levels.	All species ingested fibers with a greater portion of short fibers being ingested. No effects on lipid, feeding, and protein contents of *P. scaber*. Only low concentration of long fibers caused small decrease in survival and decrease in reproduction of *E. crypticus*. No effects on other species.	Selonen et al. 2020[103]
Polystyrene	Commercial (unwashed)	100 and 500 nm, 1-5 µm	*Caenorhabditis elegans*	1 mg/L	3 days	Lifespan, mortality, behavior, neuron, oxidative stress.	1 µm group had lowest body weight and shortest lifespan. Increased body bending and head thrashing, increased crawling speed. Damage to cholinergic and GABAergic neurons. Elevated GST-4 (oxidative stress).	Lei et al. 2018[71]
	Commercial, carboxylated fluorescent (ultraclean)	20 nm	*Achatina fulica*	0,10 and 100 ppm indirectly through mung bean leaf (14 days growth)	14 days	Foraging speed and growth rate. Gut microbiome viability, Histology of digestive glands, proventriculus and stomach	No effect on leaf weight and shoot length. Slightly lower leaf weight and root diameter. Lower snail growth, feeding speed decreased. Gut microbiome viability decreased with time (by 56% after 14 days). Damage to proventriculous and stomach.	Chae & An. 2020[104]

	Commercial, carboxylated, fluorescent	2 and 15 µm	*Culex pipiens* (Mosquito)	800 particles / ml	Until 4 instar	Concentration of MPs in gut	MPs transferred ontogenically from larvae to adults via malpighian tubules, the only structure not reorganized during metamorphosis. Greater transference for smaller particles.	Al-Jaibachi et al. 2018[105]
	Commercial (unwashed)	58 µm	*E. foetida*	0-2% (w/w)	30 days	Growth (weight) and mortality	Concentration > 1% significantly reduced weight and increased mortality (40% death at 2%).	Cao et al. 2017[106]
	Commercial (unwashed)	50-100 nm	*Enchytraeus crypticus*	0.025, 0.5 and 10% (w/w)	7 days	Mortality, reproduction, growth, gut microbiome	No change in mortality. Significant decrease in weight and microbiome diversity at 10%. Increased reproduction at 0.5%	Zhu et al. 2018[76]
	Cut from commercial expanded foam	2-3 cm	*Tenebrio. molitor Litopenaeus vannamei*	50 and 100% of diet (bran)	32 days	Survival, flame retardant in body and feces, offspring, weight.	No effects on survival overall, but in pairwise analysis PS with bran had better survival than PS alone. No significant accumulation of flame retardant as 90% is excreted.	Brandon et al. 2020[107]
	Commercial	5 and 20 µm	*Mus musculus*	0.01, 0.1 and 0.5 mg/day (drinking water)	1, 2, 4, 7, 14, 21, 28 days	Tissue distribution, accumulation, health risk	No change in mortality or weight. Significant tissue accumulation in liver, kidney and gut. Decreased lipid metabolism, increased ROS. Some evidence of neurotoxicity, although no difference between sizes.	Deng et al. 2017[78]
	Commercial (washed) neutral, positive and negative charged. Fluorescent	50 nm	*Mus musculus*	125 mg/kg of bodyweight	6 h	Bioavailability and biodistribution	Negatively charged particles had greatest uptake in kidney (37.4 µg/g tissue), heart (52.8), stomach wall (98.3) and small intestine wall (94,4). No increase of plastics in liver, lung, brain. No plastic detected in blood. Bioavailability estimate is 0.2-1.7%.	Walczak et al..2015[108]
	Commercial, pristine and fluorescent	5 µm	*Mus musculus*	100 and 1000 µg//L (drinking water)	6 weeks	Microbiota diversity and metabolism. Intestinal damage and function.	Reduce mucus secretion and damage in intestinal barrier. Changes in composition of gut microbiota and influenced their metabolic functions.	Jin et al. 2019[109]
	Commercial	0.5 and 50 µm	*Mus musculus*	100 and 1000 µg//L (drinking water)	5 weeks	Body weight, liver enzymes, gut secretion and microbiota	Decreased body weight, particularly liver and fat, at 1000 µg/L. Altered liver enzymes, gut microbiota mucin secretion in gut.	Lu et al. 2018[77]
	Commercial (uwnashed)	40 nm	*Mus musculus*	1-10 mg/kg of body weight/day	5 weeks	Behavioral, body weight, mortality.	No statistically significant differences but exposed rats had greater number of entries into open arms. No effect on body weight or mortality.	Rafiee et al. 2018[79]

	Commercial (unwashed)	30 nm	*Caenorhabditis elegans*	1-1000 µg/L	8 days	Lifespan, locomotor behavior, gene expression and ROS production	Reduced lifespan at highest concentration. At >1 µg/L decreased locomotion and activated oxidative stress. Insulin receptor and FOXO transcriptional factor expression regulated autophagy induction during exposure. Atg8/LC3 regulate NP induced ROS production.	Qiu et al. 2020[110]
	Commercial (unwashed)	0.5 and 1 µm	*Caenorhabditis elegans*	10×10^7 beads/mL	5min to 24 h	Pumping rates and egestion	Beads ingested within 5 min and egested completely in 30-40 min with high food availability. 1 µm were egested less rapidly than 0.5 µm beads.	Fueser et al. 2020[111]
	Commercial fluorescent and non fluorescent (unwashed)	0.5 µm	*Bombyx mori (silkworm)*	0.125 µg non fluorescent or 0.125 mg fluorescent/g of diet	10 or 21 days	Infiltration in gut tissues, cellular ROS response	Presence of particles in intestinal lumen, midgut epithelium, Malpighian tubules and haemocytes. Erratic movements and chemotaxis defects. Negative effects on survival and fitness. No effect on development or redox status.	Parenti et al. 2020[112]
	Commercial fluorescent (unwashed)	1 µm	*Caenorhabditis elegans, Acrobeloides nanus, Plectus acuminatus*	10×10^7 beads/mL	21-49 days	Population growth, carrying capacity, PS ingestion	All three nematodes ingested PS but number was species dependent. PS decreased carrying capacity of *C elegans*. *A. nanus* population grew faster.	Mueller et al. 2020[113]
	Commercal (unwashed)	30 nm	*Caenorhabditis elegans*	0.1, 1, 10 and 100 µg/L	24 hrs	Resistance to fungal infection of *Candida albicans*, oxidative stress activation and immune response	More severe toxicity on lifespan and locomotor behavior from fungal infection when exposed to NPs. More severe activation of oxidative stress and suppression of innate immune response.	Li et al. 2020[114]
	Commercial (unwashed)	5-5.9 µm	*Mus musculus*	0.01, 0.1 and 1 mg/day	6 weeks	Male reproduction	Decrease in number and motility of sperm, increase in sperm deformity rate. Decrease in sperm metabolism enzymes. Decrease in testosterone. Antioxidants alleviated effect suggesting damage is induced via oxidative stress	Xie et al. 2020[115]
	Commercial (unwashed)	100 nm	*Caenorhabditis elegans*	0.1,1,10 and 100 µg/L	6 days	Locomotor behavior and oxidative stress	>1 µg/L exposure induced severe lipid accumulation and increased expression of lipid sensors	Yang et al 2020[116]

	Commercial fluorescent (unwashed)	100 nm and 1.3 µm	*Eisenia fetida*	100 and 1000 µg/kg of soil	14 days	Uptake and accumulation, histopathological changes, oxidative stress and DNA damage	Decrease in mortality with 100 nm, increase with lower concentration of 1.3 µm PS. Increase in growth for all exposure. Higher accumulation of 1.3 µm PS than 100 nm. Damage to intestines, increase in oxidative stress and DNA damage. Larger PS had more toxic effects than 100 nm.	Jiang et al. 2020[117]
	Commercial (unwashed)	0.5 µm	*Wistar rats*	0.5,5 and 50 mg/L	90 days	Cardiovascular system	Structure damage and apoptosis to myocardium. Oxidative stress could induce fibrosis-related signaling pathway.	Li et al. 2020[118]
	Commercial (unwashed)	42 and 530 nm	*Caenorhabditis elegans*	0.01,1,1,10 and 100 mg/L (liquid media) or mg/kg (soil)	24 hrs	Offspring number	Offspring decrease at 100mg/L and 10 mg/kg in liquid and soil media respectively. Nematodes were more sensitive to larger particles with EC50 of 14.23 mg/kg compared to >100 mg/kg for 42 nm PS. Soil type had great impact on toxicity.	Kim et all. 2020[119]
	Commercial (unwashed)	100 nm	*Caenorhabditis elegans*	1,10 and 100 µg/L	6 days	ROS production, locomotor behavior, gene expression in neurons.	Low-dose exposure (>1 µg/L) to NP PS induce neuronal JNK MAPK signalling pathway and increased ROS production. Identified neuronal receptors in intestine related to NP toxicity sensitivity	Qu et al. 2020[120]
	Commercial (unwashed)	100 nm	*Caenorhabditis elegans*	0.1,1,10 and 100 µg/L	6 days	Response to RNAi knockdown of Gα subunits during NP exposure	Gα subunits (EGL-30 and GPA-10) were involved in the control of response to nano PS by regulating the downstream insulin, p38 MAPK, and/or Wnt signaling pathways.	Yang et al. 2020[121]
	Fluorescent and non-fluorescent commercial	0.1-10 µm	*Caenorhabditis elegans*	0.01-10 mg/mL	96 hours	Reproduction and oxidative stress	No oxidative stress. Effects correlated well with surface area of beads per mL with 50% inhibition at 55.4 cm^2/mL independent of bead size. Effects are not explained by additives but could be related to material density and its effects on food availability.	Mueller et al. 2020[122]

	Commercial (unwashed)	~120 nm	*Caenorhabditis elegans*	1 and 10 µg/mL	24 hrs	Reproduction, locomotion and oxidative stress	Affects on reproduction, locomotion and oxidative stress regardless of surface properties. Uncharged PS triggered greater metabolic disturbances compared to charged PS.	Kim et al. 2020[123]
	Commercial (unwashed)	0.5, 4 and 10 µm	*Mus musculus*	1 mg/day	28 days	Male reproduction	After 24 h, accumulation of all sizes in testicular cells. After 28 days, reduction in sperm quality and testosterone, testicular inflammation and disruption in blood-testis barrier	Jin et al. 2021[124]
	Commercial (unwashed)	100 nm	*Caenorhabditis elegans*	1 µg/L	6 days	ROS production, locomotion, gene expression	Increase ROS and decrease in mobility. mir-354 gene in intestine acted to regulate the toxicity of NPs by activating TGF-beta signaling pathway.	Wang et al. 2020[125]
	Commercial (unwashed)	1 µm	*Caenorhabditis elegans*	0, 0.1, 1, 10, and 100 µg/L	72 hrs	Body length, reproduction, locomotion, ROS production, gene expression	At concentration > 10 µg/L significant reduction in body length, reproduction and locomotion. At 100 µg/L significant ROS production and intestinal damage.	Yu et al. 2020[126]
	Commercial fluorescent (unwashed)	1 and 5 µm	*Caenorhabditis elegans*	10×10^{7}-10×10^{10} particles/m^2	96 hrs	Intake, lifespan, defecation rhythm, gene expression	Intake of both particle sizes at all concentrations and lifespan decreased. Effects were greater at lower (2.4×10^{7}) rather than highest concentration. Similar trend for protein expression of related genes. Defecation rhythm most strongly affected by 1 µm MP at 2.4×10^{8} particle/m^2.	Shang et al. 2020[127]
	Commercial (unwashed)	100 nm	*Caenorhabditis elegans*	0.1-100 µg/L	6 days	Gene expression in neurons and intestine	NanoPS exposure in the range of µg/L significantly increase expressions of genes encoding ERK MAPK signaling pathway. Modulated insulin signaling-mediated communication between neurons and intestine.	Qu et al. 2020[128]
	Commercial (unwashed)	30 nm	*Caenorhabditis elegans*	1-1000 µg/L	24 hrs	Brood size and locomotion, ROS production, gene expression	NP exposure enhanced the toxicity of microgravity stress on nematodes. Induced ROS production and activation of mitochondrial unfolded protein response. Reproduction only reduced at 1000 µg/L, locomotion reduced at > 1 µg/L.	Zhao et al. 2020[129]

	Commercial (unwashed)	25 µm	*Apis mellifera L. (bees)*	0.5,5 and 50 mg/L and 50mg/L MP + 500 µg/mL tetracycline	14 days	Mortality, body weight, microbiome, gene expression.	Low mortality and no change in body weight gains. Decrease in α-diversity of bee gut microbiome and change in expression antioxidant and immune system genes. The addition of tetracycline dramatically increased lethality of MPs.	Wang et al. 2021[130]
Polystyrene (PS) and Polyethylene (PE)	Commercial (unwashed)	0.47-300 µm	*Lobella sokamensis* (springtails)	4-1000 mg/kg	N/A	Behavioural	Movement was restricted as MPs went into bio-pores (small holes constructed by insect) and blocked paths.	Kim and An 2019[131]
	Commercial, washed with methanol and oven dried	<300 µm PE, <250 µm PS	*Eisenia fetida*	0-20% w/w	14 days	Oxidative stress proteins, concentration of PAH and PCB	20% of either particle size increased catalase and peroxidase, and inhibited superoxide dismutase and glutathione-s-transferase. No effect on amendment rates. Concentration of PAH and PCB was reduced in presence of MPs.	Wang et al. 2019[132]
	Milled from commercial pellets	< 150 µm	*Eisenia fetida*	0.03-0.9% (w/w) soil	42 days	Sensitivity to Cadmium, growth rate, mortality, particle retention	Co-exposure produce higher negative effects then either alone. Decreased growth and greater mortality. MP retained at 4-67.2 particles/g of earthworm which increased Cd accumulation.	Zhou et al. 2020[133]
Polyvinyl chloride (PVC)	Commercial	80-250 µm	*Folsomia candida Hypoaspsis aculeifer*	5000 particles per plate	7 days	Plastic displacement	Collembolans and mites all transport and disperse plastics. Presence of predators increase transport.	Zhu et al. 2018[134]
Plastics	Field study	0.5-5 mm	*Cinclus cinclus* (Eurasian clippers)	N/A	N/A	Trophic transfer.	Plastic found in 50% of regurgitates and 45% of faecal samples. 95% were fibers and concentration increased with urban land cover. Polyester, PP, PVC and vinyl chloride copolymers.	D'Souza et al. 2020[69]
	Field study	< 100 µm	*Cryptopygus antarcticus*			Abundance in animal	Detected traces of PS (< 100 µm) in gut of collembolans associated with larger piece, indicating ability to digest PS foam.	Bergami et al. 2020[135]
	Field study	>0.7 µm	*Birds of prey (8 species)*			Abundance in animals	MPs found in all 63 individual birds with an average 11.9 per bird. Microfibers were most abundant. Most common types were cellulose and PET.	Carlin et al. 2020[136]

Potential mechanism of toxicity and effects on toxicity of other contaminants

The mechanism of toxicity is the cellular process by which a substance produces its negative effects. It begins with the delivery of a toxicant from the point of exposure, via inhalation (lungs), ingestion (digestive tract), absorption (skin) or injection (blood), to its final target. There the substance will react with the target, or targets, in a way that triggers cellular dysfunction. Given the vast multitude of biological structures, there are many possible mechanisms that lead to toxicity, even for one toxicant, that can differ depending on exposure route and dosage. For example, ingestion of single large dose of mm sized polystyrene in a small animal like mice can cause an immediate obstruction in the stomach or in the intestines, leading severe internal damage or, eventually, starvation. In contrast, long-term exposure to small numbers of these same polystyrene pieces is unlikely to cause such an obstruction. Instead nefarious effects could emerge from inflammation triggered by the creation of reactive oxygen species (ROS), transfer adsorbed chemical pollutants, and disruption of the gut microbiome.[137] Thus, the same substance demonstrates toxicity via two very different mechanisms. Beyond the polymer structure itself, MP and NP toxicity is further complicated not only by the variety of shape, sizes and additives within the material itself, but also by potential interactions with background contaminants, and environmental conditions that can induce aggregation or changes in surface chemistry. As a result, the toxicity of MPs and NPs is multifaceted.

First considering the simplest model of MP/NPs, that is as a matrix of organic polymers without additives, most plastic are chemically inert and nonbiodegradable. This means is it unlikely to react with other molecules and proteins present in the body or be degraded into other potentially harmful products in significant quantities. This leaves physical interactions as the main mechanism for

toxicity of particles which will be sensitive to the relative size of the particle to the organism, and the environmental conditions (i.e. temperature, pH, ionic strength). For small organisms such as insects and nematodes, MPs are on the same scale as their digestive tracts. Therefore, MPs can cause pseudo-satiation or blockages and reduce energy availability to an organism.[74,138] Particles can also cause abrasion and irritation of the mucosa.[139] MP/NPs also provide a surface for microbes to colonize[140] which can cause alterations to the gut microbiome of animals as it has in mice,[77,96,97] springtails,[91]eathworms[73] and bees,[130] and negatively impact their health. MP and, even more so NPs, can interact with biological membranes, organelles and molecules to incite inflammation and oxidative stress. Such responses were seen in three *in vitro* studies on NP toxicity in human epithelial and cerebral cell lines.[45,141,142] In all cases, cell viability or mortality was unaffected while other sub-lethal effects were observed. Mahler et al.[141] saw significant transport of 50 nm (4.63×10^8 particles) and 200 nm (1.06×10^6 particle) PS particles in human epithelial cells at 37˚C, that disrupted iron transport proteins and cell permeability. Schirinzi et al.[142] exposed epithelial and cerebral cells to PE and PS NPs and found no change in cell viability but an increase in ROS production which could have been incited by an accumulation of plastics in endolysosomes.[45] These interactions are highly dependent on surface chemistry and size, which can be altered by weathering, a protein corona, or aggregation. [108]

Working outside individual organisms, the presence of MP and NP can alter soil properties such as microbial biodiversity, soil texture and structure.[138] They can also adsorb hydrophobic molecules or, if oxidized, charged molecules, which could disrupt molecules related to intra- and interspecies communication pathways or allow them to act as vector for toxic compounds. [143] Inversely, this adsorption capacity can also serve to reduce the bioavailability of other contaminants, like triphenyltin [143] and polyaromatic hydrocarbons (PAH), [144] reducing their effects

on organisms but also contributing to the persistence of contaminants in soil. One study observed that the biodegradation of PAHs, such as phenanthrene and anthracene, was reduced in the presence of PE MPs.[144] Plastics themselves can contain many plastic additives that can leach from the polymer matrix into the environment or within organisms.[138] Phthalates and BPA plasticizers have estrogenic activity that has been shown to affect a wide-range of vertebrates.[22,25] There are residual monomers that cause irritation to organs and skin, and some neurological disorders. [18] UV absorbers can accumulate in animal tissues, both marine and mammalian, and can cause liver damage.[18] However, most reported additives and monomer concentrations released often fall below guideline/legal limit values.[18] The hazard associated with the most widely produced plastics, PP, LDPE, PET, HDPE and PS, is low with the exception of PVC which is made from carcinogenic monomers.[145] Although, it should be noted that these guidelines often do not consider that low levels of endocrine disrupting chemicals cause an effect, and also the toxicity of mixtures.[18] Considering all these facets of MP/NP properties and interactions, a non-monotonic response, that is a non-linear response to exposure concentration, would be expected.[138]

Knowledge gaps

While studies evaluating the toxicity of MPs and NPs have vastly increased in number in the last decade and revealed the toxicity of these particles, inconsistency in reported endpoints and dose metrics make comparisons difficult. Studies on MPs consider only one or two exposure levels and so valuable dose-response relationships cannot be ascertained, making it difficult to build a clear picture of their risk.[61] While mounting evidence that environmentally relevant concentrations of plastics do not have lethal effects, there is data for sub-lethal effects in tissues and cellular activity that could lead to negative outcomes with chronic, long term exposure. As our study is one of the first using *D. melanogaster* as a model organism for MP and NP toxicity, our aim was to produce

basic toxicity information using the simplest and most common model of MP/NP particles (i.e. pristine commercial PS spheres) and measuring a variety of lethal and sub-lethal endpoints. This makes our study more comparable with existing research, and allows us to pinpoint useful exposure concentration and targets of MP/NP toxicity in *D. melanogaster* that can then become the focus in future experiments.

1.4 *Drosophila melanogaster* as *in vivo* model for toxicity

D. melanogaster, or the common fruit fly, has been used in research for more than 100 years. It is a small, highly fecund, rapid breeding insect with minimal demands for maintenance in laboratory. It is most widely used in genetics, being the basis for several major discoveries such as the chromosomal basis of heredity, the chemical nature of mutations, cell signaling, embryonic development and more.[146] These mechanisms have been found to be highly conserved across species. In fact, 75% of genes associated with a human disease have some homologue in *D. melanogaster*.[147] As such, its use has expanded in recent years to serve as a model for neurodegenerative diseases,[15] intestinal infection and pathology,[148] obesity and aging,[149] and sleep.[150] An ever expanding suite of tools is available for fly research including large well-curated databases like FlyBase, stock centers of well-documented mutants like the Bloomington Drosophila Stock Center, ready-made gene expression microarray chips for transcriptomic analysis, and automated activity monitors. Therefore, fruit flies show great potential as an alternative animal model in toxicology.[151,152]

Organism description

Wild-type fruit flies are approximately 3 mm long and 2 mm wide, with yellow-brown coloured bodies with black dorsal stripes on their abdomen, and bright red eyes (**Figure 1.4**). They have a chitinous exoskeleton covered with bristles that is segmented into three parts, the head, the thorax

and the abdomen, and three pairs of segmented legs. They also have two sets of wings: the larger main wings, and small altered hind wings, called halters, which serve to further balance flies during flight. *D. melanogaster* exhibits sexual dimorphism. Females are slightly larger and have less black pigmentation on their abdomen. Males have sexcombs and claspers, which are dark hairs on the tarsus of the first legs and around their reproductive organs, respectively, that assist males in attaching to females during mating.

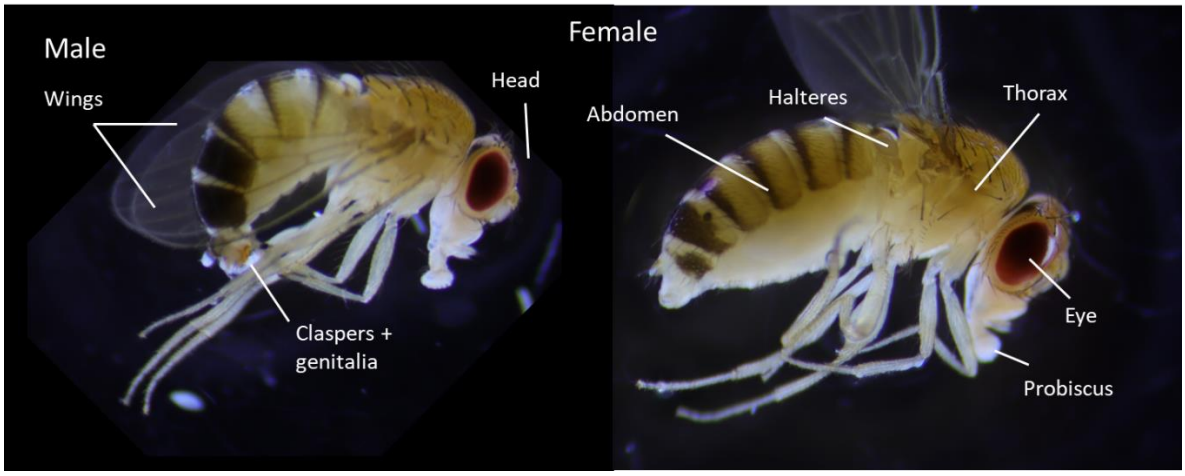


Figure 1.4. Adult male and female *D. melanogaster*.

Development and life cycle

The fruit fly life cycle consists of four distinct stages; egg, larva, pupa, and adult; with flies undergoing a complete metamorphosis. The rate of development is dependent on temperature, taking 20 days to complete at 18°C compared to only 10 days at 25 °C (**Figure 1.5**). Fruit flies live where they eat, being able to breed and feed on a variety of rotting vegetation, although having a preference for rotting fruit and yeast.[12] Individuals can live up to 40-60 days after emergence as adults.

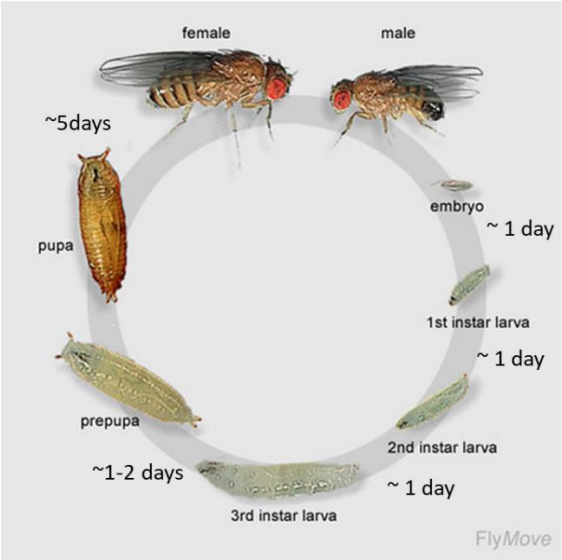


Figure 1.5. Life cycle of *D. melanogaster* at 25°C. Modified with permission from Weigmann et al.[153]

Eggs are 0.5 mm long ovoids that are covered with a thin but resilient protein envelope called the chorion. They also have two distinct dorsal appendages that are thought to facilitate gas exchange for the embryo by projecting out of the medium in which they were laid. [154] After approximately 24 hours, the first instar larvae will emerge from the egg leaving the chorion behind.

Larvae are white, segmented, worm-shaped burrowers with black mouth parts in the narrower head region. They feed continuously to sustain their growth and ingest solid food with their mouth hooks. The grow for about 4 days, completely shedding their outer layer of skin and teeth twice during molts. Once they attain the third and final larvae stage, they will begin to roam outside the food to find a dry place to pupate.

When pupating, larvae bodies shorten, and the exterior cuticle becomes hardened and pigmented. The hard, exterior shell is called the puparium and within it the fly will reconstruct itself into an adult fly. All adult structures originate from clusters of diploid cells of undifferentiated epithelium called imaginal discs. As time passes, adult features such as the eyes and wings become visible through the darkening case. Soon after, the flies will break through the case and emerge as adults.

Newly emerged adults have larger, pale bodies and a dark spot on their abdomen, called the meconium, which consists of the remains of their last meal before pupating. Flies will then mate as soon as 8 hours after eclosion, with females storing sperm to subsequently fertilize and lay eggs. Therefore, when the goal is to obtain specific cross of mutants, it is necessary to quickly collect virgin females and place them with their intended male mates. Once mated, females lay fertilized eggs as early as the next day, increasing the number of eggs layed per day until reaching a peak of about 80 eggs by day 7-10 before gradually decreasing with time.[155]

Genetics

Already established as an useful model for eukaryotic organisms, the genome of *D. melanogaster* was fully sequenced in 2000.[156] The genome comprises of 14,000 genes which when compared with mammalian proteins and expressed sequence tags, more than half had a similar counterpart in mammals. [146] These are distributed among 4 pairs of chromosomes: 1 pair of sex chromosomes, and 3 autosomes. Each chromosome has two arms, referred to as the left and the right. The chromosomal localities of individual genes are identified either by recombination units or numerical locations on each of these arms. This small number of chromosomes and well-defined chromosomal localities makes it easy to identify and track mutations. Heredity can be quickly determined thanks to the short reproductive cycle of flies.

At the cellular level, nanomaterials often induce oxidative stress, which plays an important role in both toxicity and genotoxicity.[157] Genotoxicity is of particular concern as it has serious implications for the health of individuals, resulting in severe developmental defects or carcinogenesis, and their subsequent offspring. The latter is especially concerning as it can have lasting effects on entire populations as seen by Vecchio et al. who created a new aberrant

phenotype in *Drosophila* that could be transmitted to descendants after exposure to 15 nm citrate-capped gold nanoparticles.[158]

Genotoxicity can be assessed at the tissue level with SMART (somatic mutation and recombination tests),[159] or in individual cells with the Comet assay. [160] The SMART assay is based on the principal that mutation in cells heterozygous for two recessive phenotypes will cause those cells to lose their heterozygosity, resulting in patches of mutant clones with mutant phenotypes.[159] For the SMART wing-spot assay, the multiple-wing-hairs *(mwh*) and the flare-3 (*flr*^{3}) strains, phenotype shown in **Figure 1.6**. The Comet assay extracts and separates intact and damaged DNA with electrophoresis and visualizes it with a fluorescent dye. [160] Damaged DNA will migrate further than intact DNA, producing a tail.

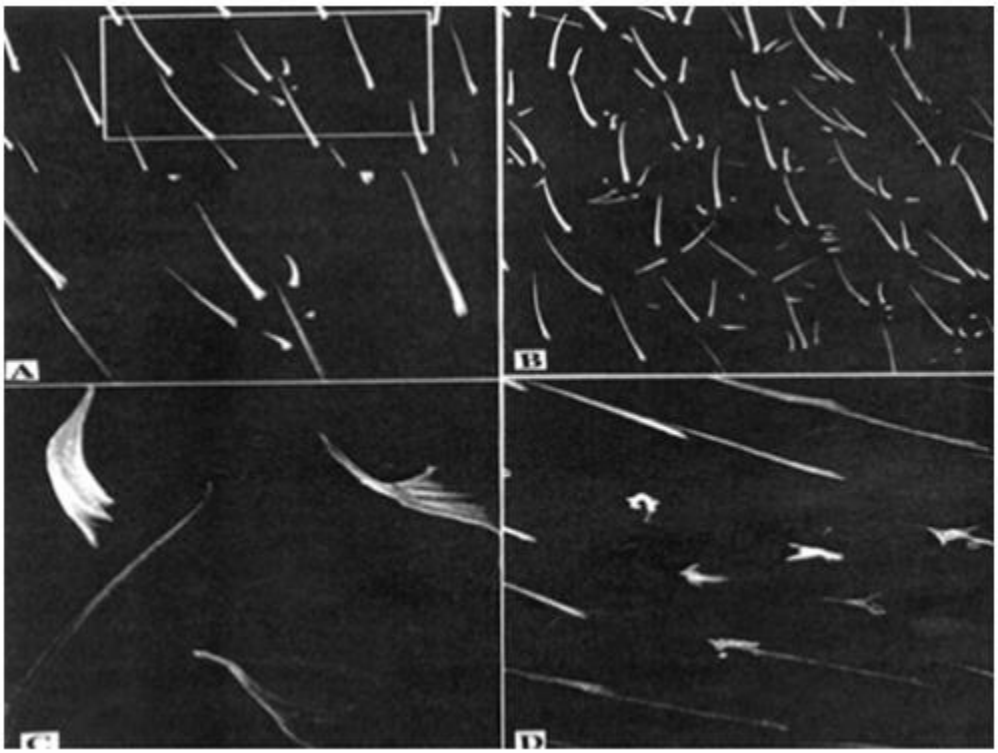


Figure 1.6. Electron microscopy images of (a-b) *mwh* phenotypes and (c-d) and *flr*^{3} phenotypes. Reprinted with permission from Marcos et al.[159] Copyright 2014 Springer Science+Business Media New York

Anatomy of digestive tract

When exposure occurs through ingestion, the first biological system toxicants will encounter is the digestive tract. In *Drosophila* the digestive tract consists of three general compartments that are distinguished based on their position and developmental origin: the foregut, the midgut and the hindgut, as shown in **Figure 1.7**.[16] The foregut consists of the proventriculus and the crop, the latter of which plays a similar role as the stomach in mammals. The midgut is divided into six distinct regions, each with specialized metabolic and digestive function, and transitions from an acidic to alkaline pH.[16] The hindgut stores waste for excretion.

All the compartments are surrounded by visceral muscles and are protected in the luminal side by mucous and a chitinous layer.[161] In the foregut and hindgut this chitinous layer is an impermeable cuticle; in the midgut it is a semi-permeable peritrophic matrix.[161] The peritrophic matrix is composed of chitin-fibrils and chitin-binding proteins that are assembled in the proventriculous and remodeled in the midgut.[161] The lumen also contains a unique gut microbiome that plays a role in digestion, growth and reproduction, and secretes a variety of ROS for defense against bacterial infections.[157,162]

While the arrangement and proportions will differ, all compartments contain three cells types: enterocytes, enteroendocrine cells (EECs) and stem cells. Enterocytes are large cells that secrete digestive enzymes and absorb nutrients. EECs secrete peptide-hormones and carry some neural-like functions in regulating intestinal physiology and relaying the state of nutrition to other organs.[161] As such, it is closely linked to energy metabolism (i.e. lipid storage) and contributes to the alteration of feeding, growth rate, sensory perception and olfactory behavior.[161] Stem cells constantly generate new enterocytes and EECs to regenerate and maintain the integrity of the gut.[16] MPs and NPs will likely cause alteration and damages to various aspects of this system and result

in changes in weight, behavior, microbiota and mortality. MPs and NPs will also need to first translocate through the intestinal wall to affect other systems.

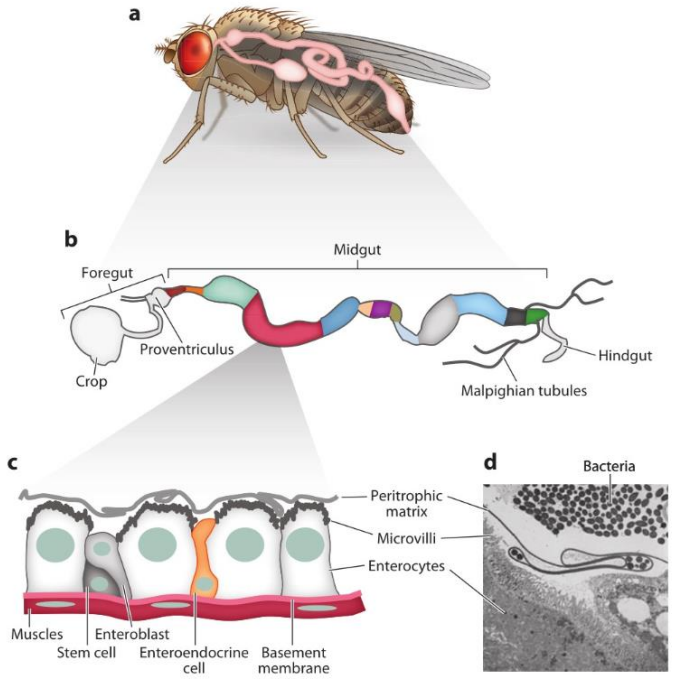


Figure 1.7. Schematic organization of the Drosophila digestive tract. (a) A 3D reconstruction of the digestive tract within the body cavity (b) The digestive tract is divided into three discrete domains of different developmental origin: foregut, midgut, and hindgut. Each of these domains is further subdivided into genetically distinct compartments (illustrated by different colors in the case of the midgut). (c) The midgut is composed of an epithelium surrounded by two layers of visceral muscles. The midgut epithelium consists of enterocytes, enteroendocrine cells (EEC), and progenitor cells. (d) Electron microscopy sections of a third-instar larval gut following infection with Erwinia carotovora 15. The peritrophic matrix establishes a physical barrier that prevents contact between bacteria and the epithelial cell. Reprinted with permission from LeMaitres et al. [16] Copyright 2020 Annu. Rev. Genet.

Anatomy of nervous system

While less complex than the mammalian brain, the central nervous systems of the fly is similarly composed of neurons and glia, is protected by a blood-brain barrier, and has many organizational similarities with vertebrate brains. [15] *Drosophila* neurons fire Na^+/K^+-based action potentials and use highly conserved mechanisms for synaptic vesicle release of conserved neurotransmitters, such

as γ-aminobutyric acid (GABA), glutamate, acetylcholine, and neuromodulators.[163] The central nervous system (CNS) is divided into distinct lobes including the lamina, the medulla, the lobula complex and the protocerebrum (**Figure 1.8**). [163,164] These are divided into two histological regions: the cortical cell cortex, where all CNS neuronal cell bodies reside; and the neuropil, to which axons and dendrites project and form neural circuits. [163] Some neurons branch out from the CNS to the periphery organs such as reproductive organs and muscles. [165] The peripheral nervous system consists of sensory neurons that relay information from sensory organs including the bristles and gut.[166]

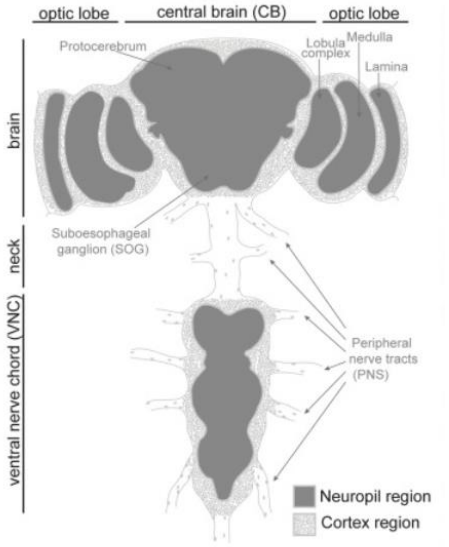


Figure 1.8. Schematic of the central nervous system (CNS). The cortical regions (dotted areas) contain all neuronal and most glial cell bodies, while the neuropile regions (grey areas) contain the synaptic connections. Reprinted with permission from Kremer et al. [164] Copyright 2017 John Wiley & Sons, Inc.

Together this system transmits signals between different parts of the body to control and coordinate behaviors like feeding, sleep and locomotion and regulate physiological processes. As a result, neurotoxicity can manifest itself in a wide variety of symptoms in flies. For example, a mutant named *drop dead* (*drd*) will suffer brain degeneration that produces abnormal phototaxis and circadian cycles.[15] While mutations in *swiss cheese* (*sws*) result in age-dependent loss in motor

activity and brain degeneration.[15] This type of neurotoxicity can be detected by monitoring synaptic connections at well-defined neuromuscular junction or through behavioral test.[167]

Behavior is the output of the nervous system. As such, there is a very strong connection between neurotoxicity and behavior. Behavioral tests assesses the overall functioning of the nervous system by observing reflexes, the autonomic system (ex. heartrate, breathing), innate behaviors (i.e. feeding, avoidance, response time, etc.) and learned behaviors.[168] These behaviors are important for survival and reproduction, and are often affected long before there are any dramatic changes in the organs.[78] Measuring behavior is challenging as most actions utilize multiple functions of the nervous system, such as memory, mechanosensory feedback, locomotion, response to olfactory cues, spatial learning and more. [169] In general, these functions can be separated in five domains; 1) autonomic , 2) neuromuscular 3) reactivity or excitability 4) sensory and 5) others (convulsions, tremors). [168] Each can contribute to the variability of a single behavior and make results more difficult to interpret. While proper controls can help isolate specific functions and reduce variability, it is recommended to perform a battery of tests.[168] Given its origin in the pharmaceutical industry, standardized tests have only been developed for mice.[168] However, the same general principles can be applied to experimental design for other models. Preliminary examinations should test neurological reflexes, motor and sensory function.[168] Focusing first on the effects at the lowest dose as the presence of more toxicant can expand effects to other systems. [168] Monotonic or inverted U-shaped curves are commons due to feedback regulations of the nervous system in mice.[168] Proper interpretation will require taking into account toxicity to other organs, the magnitude of the effects and dose-response.

Specialized equipment has been developed to automate behavioral assays with flies, such as the Drosophila Activity Monitor (DAM) in **Figure 1.9**. The DAM apparatus tracts the locomotor

activity level of individual flies by placing them in 5 mm x 60 mm transparent tubes with enough food to last for approximately 2 weeks. A single monitor holds 32 tubes, and an infrared beam crosses through the center of each tube. An activity is defined as a break in the infrared beam and measured as the number of beam-crossings per minute, while sleep is defined as a period of inactivity lasting at least 5 min.[170] A typical incubator can house dozens of these monitors, allowing the analysis of multiple variable at once including sex, exposure concentration, and timing of the exposure (i.e. egg, larval, pupal).

Figure 1.9. Drosophila Activity Monitor. Each glass tubes would contain one adult fly, food at the far end and a cotton plug.

Evaluating toxicity with Drosophila melanogaster

When evaluating the toxicity of a material in *D. melanogaster*, there are many endpoints that can be measured. The most direct, and most widely used endpoint, is survivorship. In this test, flies would be exposed to various concentrations of toxicant by oral, dermal and/or inhalation routes for a pre-determined period, and the number of deaths would be recorded regularly. This data can be used to generate dose-response curves and directly compare the toxicity of substances and the

sensitivity of developmental stages. For instance, exposure to 100 ppm of 20-30 nm silver nanoparticles during egg and larval stage not only reduced the emergence of adults,[171] but also affected the survivorship of subsequent generations who go without exposure.[172] In contrast, when exposure occurs at the adult stage, a higher concentration (200 ppm) of silver nanoparticle is required to cause mortality, demonstrating their lower sensitivity to silver nanoparticles.[173] Therefore, egg and larvae are more sensitive to silver nanoparticle toxicity. Dose-response curves also allow the comparison of exposure routes which have been shown to have significantly different impact on mortality for carbon nanotubes where dermal exposure was found to cause higher mortality than dietary uptake.[174]

Another common response to toxic substances is the increased production of ROS. Excess ROS can damage proteins, lipids and DNA in cells, eventually leading to a host of negative symptoms and disorders. As a result, it can be the primary mechanism of toxicity as it is for silver nanoparticles.[175,176] Due to the transient nature of ROS, their presence is often determined indirectly by assessing cellular damage or the expression of many antioxidant enzymes such as superoxide dismutase, catalase,[177] Hsp70,[178] and glutathione S transferase[179] among others. Protein levels can be directly measured with Western blots using the appropriate antibody, or by measuring enzyme activity within crude tissue extracts.[180] As these ROS biomarkers are found in most species, commercial kits to measure them are widely available. Protein expression can also be measured indirectly by quantifying the level of their respective mRNA with quantitative reverse transcription –polymerase chain reaction (qRT-PCR). [181,182] Finally, ROS reporter lines of fruit flies can monitor and identify tissues and organs *in viv*o that are susceptible to oxidative stress by attaching fluorescent tags to antioxidant proteins in their genome.[183,184]

Fertility and fecundity are a critical endpoint to measure, especially within the context of ecotoxicology, as effects here have implications for entire populations. Some nanomaterials have shown to have an effect on the fertility and fecundity of *Drosophila*[172] and MPs have been shown to affect reproduction of earthworms[70] and other insects.[34,74,76] The anatomical structures and cellular characteristics of germline stem cells in male and female reproductive organs has been well described, and detailed studies on the molecular and cellular mechanisms of toxicity in those cells lines can be carried out.[152] For a primary assessment, counting the number of eggs and/or offspring is sufficient.

When interpreting the results of any toxicity experiment, it is important to note that insects are very susceptible to stress,[185] different raising practices,[186] environmental conditions, and population density.[187] These factors can cause variation in biological responses and confound the toxic effects of substances. And so it is critical to maintain consistency in raising practices and healthy animal populations leading up to and during the entirety of these experiments.

Chapter 2: Polystyrene micro- and nanoplastics affect fertility and locomotion of *Drosophila melanogaster*

Sara Matthews, Elvis Genbo Xu, Eva Roubeau Dumont, Victoria Meola, Oluwadamilola Pikuda, Rachel S. Cheong, Mingrui Guo, Rui Tahara, Hans C. E. Larsson and Nathalie Tufenkji Manuscript under Review in Environ. Sci.: Nano as of Sept-10-2020. Contribution of authors outlined on page v.

2.1 Introduction

As a highly versatile material, plastic has found uses in a seemingly endless list of applications from construction, electronics, clothing, food packaging, and even cosmetics. As a result, demand is extremely high and yearly global production reached 360 million metric tonnes in 2018.[1] Of this, 70% ends up as waste with the majority (79%) going to landfills or directly into the environment.[2] The billions of metric tonnes of plastic pollution that has accumulated worldwide have developed into a problem that goes beyond the visible debris. Microplastics and nanoplastics are generally defined as plastic pieces with sizes between ~100 nm - 5 mm and < ~100 nm,[188] respectively, that can be introduced to the environment from weathering of bulk plastic or the release of primary plastic particles developed for commercial and industrial use.[30,189] These small fragments compose the vast majority (94%) of plastic pollution and their size allows them to be carried to even the most remote locations globally via ocean currents and winds.[5] Inevitably, a wide variety of species, including humans, has been exposed to these fragments.[189] Nanoplastics can pose additional risks as they have been shown to pass through biological barriers and accumulate in tissues and organs, triggering physiological distress, diminished reproductive fitness and early mortality.[7] These nanoparticles can also act as carriers for other contaminants that can enact toxic effects of their own.[27,190]

Although the toxicity of micro- and nanoplastics has been extensively studied in aquatic organisms with various effects on growth, development, behavior, reproduction, and mortality being observed

across a wide range of aquatic species,[60] studies in terrestrial systems are far less abundant despite soils being one of the largest sinks for plastics.[8] Plastics enter soils through aerial deposition,[191] littering, and the application of plastic films and sewage sludge to agricultural lands.[8,27,51,192] Sludges retain up to 99% of microplastics that pass through wastewater treatment plants (WWTPs), with 15385 particles kg^{-1} having been measured in samples from WWTPs in Ireland.[27,50] Sludge has been estimated to add up to 300 thousand tonnes of microplastics to North American farmlands per year.[51] Farmlands that use modern practices (e.g., plastic films and sludge) have reported higher microplastics concentrations (up to 42960 particles kg^{-1})[49] than those that do not (0.34 particles kg^{-1}).[47] In "hot spot" sites, concentrations can be extremely high with the soils outside an industrial site reporting concentrations as high as 67500 ppm[193], and 55.5 ppm in the Swiss floodplains soils.[48] Our understanding of the potential impacts of microplastics in soils is lacking, as studies in terrestrial organisms are scarce, representing only 10% of 45 peer-reviewed articles on microplastics in a recent review.[9] Few terrestrial models have been investigated, with earthworms predominating, and nanoplastics have been largely ignored.[9,34] Therefore, there is an urgent need to close this knowledge gap to gain a better understanding of the impacts of plastic pollution in terrestrial ecosystems. [194]

Drosophila is a diverse genus that is found in abundance worldwide in various environments, ranging from tropical to temperate climates.[12] In terrestrial ecosystems, it plays important roles as predator, prey, pollinator, and decomposer.[12–14] *Drosophila* lives and breeds in many different decaying vegetal tissues, with a strong preference for rotting fruits, [12] and thus environmental exposure is likely to occur. Fruits can contain plastics through the potential uptake of micro- and nanoplastics in plants,[195] the aerial deposition of airborne microplastics on their surface,[191] and, once fallen on the ground, from interactions with contaminated soils. Animals also interact with

soil directly as larvae can roam into the soil as they seek a dry place to pupate. Within this group, *Drosophila melanogaster*, known as the fruit fly, is a well-studied and highly tractable genetic model for understanding the molecular mechanisms of embryogenesis, signaling pathways, and various human diseases.[196] More recently, it has been growing in popularity as a model to assess the toxicity of nanomaterials[197–200] and insect toxicology[151] as it has many advantages including a short life-cycle (10-12 days), low cost, easy handling, a completely sequenced genome, and simpler homologues of the nervous[15] and digestive systems.[16] As one of first lines of defense during oral exposure, the digestive system will often suffer toxic effects from nanomaterials and *Drosophila* gut has proven a useful gut model for nanotoxicity.[201] The first study using *Drosophila* as a model for microplastic toxicity was published earlier this year.[202] In this study, flies were exposed to 200 µg/mL of 0.1 or 1 µm polystyrene particles for 7 days, alone or in combination with cadmium. Plastics alone caused no change in survival or epigenetics but decreased climbing ability and caused significant gut damage. Combined exposure with Cd aggravated the impact on these endpoints, suggesting additive or synergistic effects with metals like Cd. As only a single, high exposure concentration was tested, the effects of lower and more environmentally relevant concentrations, important chronic impacts on fly reproduction, as well as daily behaviors remain unknown.

The objective of this study was to assess the chronic effects of micro- and nanoplastics exposure in the fruit fly *D. melanogaster* by measuring various lethal and sublethal endpoints including mortality, development, locomotion, gene expression, daily activity rhythms, and fertility. This is one of the first studies to comprehensively assess the chronic dose-dependent impacts of both micro- and nanoplastics in the fruit fly. The toxicological data of this laboratory exposure

experiment is a prerequisite for understanding and predicting the potential ecological impacts in terrestrial environments.

2.2 Materials & Methods

Drosophila strains

The Oregon R strain of *D. melanogaster* was used for all experiments. Flies were cultured on Nutri-Fly Bloomington Formulation (Diamed) in a Panasonic MLR-352H-PA Versatile Environmental Test Chamber operating at 25˚C, 60% humidity and a 12/12 h day/night cycle. Egg collection and aging, and exposure experiments were also conducted under these conditions.

Polystyrene particles

Polystyrene particles have been found in soils.[47,48,193] In addition to its environmental importance, the commercial availability of polystyrene particles in a wide range of sizes, surface functionalities, fluorescent labelling and stable concentrations, make it the most widely adopted model plastic particle in toxicological studies.[60] To investigate the concentration-dependent effects, visualize the uptake *in situ*, avoid the use of surfactants, and compare to previous studies, carboxylated fluorescently labeled polystyrene particles in both micro- and nano size were also chosen for this study. Commercially prepared 1 µm red fluorescently labeled carboxylated polystyrene (580 nm excitation, 605 nm emission, catalog N° F8821) and 20 nm green-yellow polystyrene particles (505 nm excitation, 515 nm emission, catalog N° F8787) were purchased from ThermoFisher. To remove the confounding toxic effects of preservatives, surfactants as well as any free dye within the stock suspensions, particles were dialyzed for 7 days according to a method described by Xu *et al*.[64] This dialysis protocol was shown to remove excess fluorescent dye from these commercial particles, reducing the signal from leaked dye to negligible amounts.[64] Even in harsh simulated gastric conditions, no

detectable amount of fluorescent dye leached from similar polystyrene microspheres.[108] It should be noted that the fluorescent dye was not quantified and only used to confirm particle uptake in the present study. Fluorescent imaging of particles in suspension and in Ward's Instant Drosophila Media shows that both particles generally maintained their original size and dispersion (Figures S1-S2), except at high concentration (100 ppm) where some aggregates of nanoplastics formed when mixed into food media, with the largest aggregates being approximately 1 µm in diameter.

Developmental toxicity assay

Exposure conditions and workflow

Flies were exposed to plastic particles as described in Rand *et al*.[203] and the workflow is outlined in **Figure 2.1**. Briefly, populations of ~300-400 adult flies were housed in embryo collection cages on FlyStuff Grape Agar Premix (Diamed) with yeast paste (1 g/mL of Milli-Q water) to collect fresh eggs over an 8 h period. The eggs were aged for 24 h to 1st instar larvae stage and then exposed to a series of concentrations (0, 0.1, 1, 10, 50, 100 ppm) of micro- or nanoplastics and observed over 13 days. The 100 ppm concentration was equivalent to 2.27×10^{13} particles/mL of food and 1.8×10^{8} particles/mL of food for the 20 nm and 1 µm polystyrene particles, respectively, and falls within the concentration range of plastics in "hot spot" sites. Particle suspensions were prepared by diluting a 5000 or 5 ppm stock of dialyzed particles in Milli-Q water to desired exposure concentration. Then, 10 mL of the particle suspension was added to 2.1 g of Ward's Instant Drosophila Medium (VWR) in a 25 mm wide glass tube. The pH of the medium ranged from 4.9-5.0, which is comparable to that of fruit fly's natural foods such as pears and bananas (pH 5.2).[204] Fifty larvae were exposed in each vial, and 9 replicate vials were prepared for each condition. Of these, three vials were exposed for 3 days at which point 10 larvae from each vial were used in a larval crawling assay to assess locomotion, another 10 were observed by

fluorescence microscopy (Olympus SZX16 stereomicroscope; settings in supplementary information) to verify particle uptake, and 15 were placed on clean food for a 24 h depuration, being fluorescently imaged at 1, 4, 8, 12 and 24 h. The remaining vials were exposed for 13 days, with the number of pupae, adults, and mortality recorded daily, and the length of pupal casing measured on day 6 (**Figure S3**). On the final day, adult locomotion was assessed with an adult climbing assay before flies were anesthetized with carbon dioxide, counted, and sorted by sex. Nine mating pairs from each condition were randomly selected for a fertility assay. Thirty adult males and 30 females were preserved in 70% ethanol for imaging and body measurements (thorax length, **Figure S3**). Three females from control and 100 ppm conditions were prepared for nano-computed tomography x-ray scans. Six groups of 5 individuals per condition were placed in RNA*later*™ Stabilization Solution (Invitrogen) to preserve for gene expression analysis (details in SI). The remaining flies were cleared according to the ScaleS protocol developed by Hama *et al*. [205] for 10 days to reduce autofluorescence prior to fluorescence imaging with a stereomicroscope.

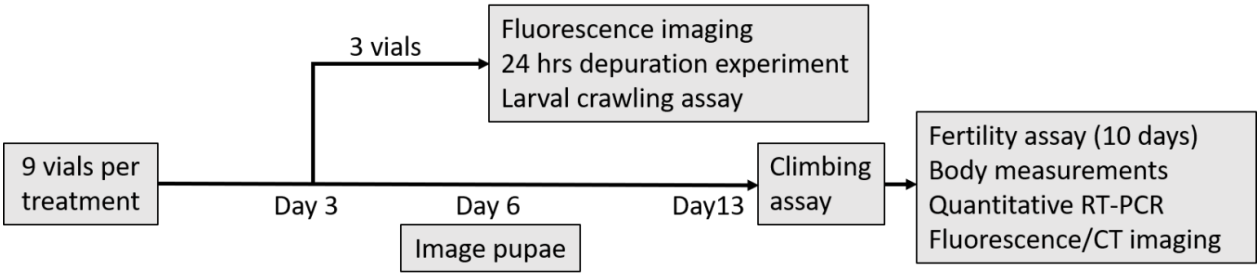


Figure 2.1. A workflow of the chronic toxicity assays. Each treatment had 9 vials with 50 individuals each. On day 3, 3 vials from each treatment were sacrificed to confirm particle uptake (by fluorescence imaging) and for depuration tests, as well as larval crawling assays. The remaining 6 vials were exposed until the emergence of adults (13 days). Their locomotion was assessed with climbing assay before being anesthetized and randomly sorted into fertility assay, euthanized for body measurements, preserved for quantitative real-time PCR expression analysis, or processed for fluorescence or X-ray CT imaging.

Larval crawling assay

Ten randomly selected larvae were placed in a glass-bottom culture dish (14 mm diameter, Matsunami Glass, VWR) filled with 100 µL diluted yeast paste (0.1 g/mL of Milli-Q water). The light source from the microscope was below the glass-bottom culture dish. The animals were allowed to adapt to the lighting environment for 3 min before being recorded for 60 s and the number of peristaltic contractions per min noted. As *Drosophila* larvae demonstrate social behavior,[206] locomotion was assessed individually and also in groups of ten.

Adult climbing assay

The locomotor performance of Drosophila adults was measured according to the method described by Pappus et al.[207] with minor modifications. Briefly, all emerged adult flies from each vial were transferred, without anesthesia, to a 100 mL glass graduated cylinder with a marking at 10 cm height and sealed in with a cotton plug. Flies were gently tapped down to the bottom of the vial and then allowed to ascend for 10 sec. The proportion of flies that passed the 10 cm mark, as well as those that remained at the bottom of the vial, was recorded. This experiment was repeated three times for each vial and the mean proportion of flies that passed the 10 cm mark or remained at the bottom was calculated.

Fertility assay

Nine mating pairs that had been previously exposed for 13 days to micro- or nanoplastics were placed in vials with food free of polystyrene particles for 10 days, being transferred to new vials every 48 h, and the number of progeny was recorded. Fertility was assessed on clean food to minimize the influence of oviposition preferences commonly observed in *Drosophila*, as females are seen to avoid laying eggs in unfavorable media.[208]

Gene expression

Stress-response genes (*HSP70, CAT, SOD2*) and genotoxic stress response gene (*P53*) were selected. A detailed description of the RT-PCR method can be found in supplementary information. Briefly, 15 males and 15 females from each condition were stored in groups of 5 in RNA*later* (Thermo Fisher) at -4 ˚C , giving a total of 6 biological replicates per condition. Total RNA was isolated using Purelink RNA Mini Kit (Thermo Fisher); the amount of RNA in each sample was determined using a spectrophotometer and the quality was analyzed using agarose gel electrophoresis. First-strand cDNA was obtained using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher). Real-time quantitative PCR (RT-PCR) was performed in triplicate for each sample with a 7900HT Fast Real-Time PCR System (Applied Biosystems) following manufacturer's instructions and using SYBR Green-based detection of PCR products. Melting curves were examined after amplification to exclude the presence of unspecific products. Relative expression was calculated through the $2^{-\Delta\Delta Ct}$ method and normalized to the transcript levels for alpha-tubulin.[209]

Nano-CT scan

Nano-computed tomography (CT) scans using Zeiss Xradia 520 Versa (Carl Zeiss Canada Limited) were performed on three randomly selected female fruit flies from control and 100 ppm treatments of the two particles for qualitative assessment of the integrity of the intestinal walls. This technique offers the advantage of preserving soft tissues close to their original state and can distinguish differences in organ size and tissue organization. The flies were fixed in FAE fixative (6:10:1 ratio of 40% formaldehyde, ethanol, and glacial acetic acid) for 24 h, decapitated with a razor, and then stained in 1% phosphotungstic acid solution in water for 2 weeks, changing to fresh reagent every 2-4 days. Flies were then placed in solutions of increasing ethanol concentration (25,

50, 70%) for 20 min increments before being mounted in 70% ethanol in a 20-200 µL pipette tip. All the scans were acquired at 1-3 µm resolution with 4× objective lens with 2×2 camera binning over a 360 degree-rotation. A total of 2401 and 201-501 projections were taken in high and low resolution scans respectively, at 60 kVp and 82 µA. High resolution scans of each specimen were taken with LE1 filter with 2.7 sec exposure, yielding 3 h scans in total whereas one of the 100 ppm with 20 nm plastic specimens was taken with LE2 filter with 3.5 sec exposure, resulting in 3.5 h scan. In low resolution scans, the same filters were used with 0.8-1.2 sec exposure, yielding 20-40 min scans. One control was scanned at low resolution with an LE2 filter and with additional projections (total 2401) yielding a 1.5 h scan time. The resulting images were qualitatively analyzed and 3D models were reconstructed with Dragonfly image analysis software (Object Research Systems Inc.). 3D models were generated based on a combination of the low and high resolution scan data.

Measuring daily activity and circadian rhythms

The daily activity and circadian rhythm of adult flies were assessed using a DAM2 Drosophila Activity Monitor (TriKinetics) following a protocol previously described by Chiu et al.[170] with some modifications. Two-day old adult males were loaded individually into glass tubes with food containing 0, 10, 50, and 100 ppm microplastics or nanoplastics (16 flies per treatment). Monitors were placed in an incubator at 25 ˚C, 60% humidity for 8 days. For the first 4 days, flies were subjected to 12 h/12 h light/dark cycle (LD) and then a 24 h dark cycle (DD) for the remaining 4 days. An activity event was defined as a break in the infrared beam that crosses the center of the activity tube and the number of events was recorded over 1-min intervals. Data were analyzed using Shiny-R software[210] to extract daily activity accounts, sleep events, bout lengths, and circadian rhythms. The first day of both cycles was a period of acclimatization and excluded from

the final statistical analysis along with data from dead flies. Further details can be found in supplementary information.

Statistical analyses

All statistical analyses were performed using R studio Software V3.3.1 [211] with agricolae and MASS packages.[212] Homoscedasticity was checked using Bartlett test and normality was checked on ANOVA residuals, with log-transformation when normality assumption was not met with raw data. Group comparisons were performed using one or two-way ANOVAs to assess the effect of concentration and, in the case of fertility, time. Considering the large difference in particle numbers of micro- and nano-sized particles at an equal mass concentration,[213] no comparison was made between particle sizes. Statistical significances were obtained using TukeyHSD test. Statistical significance was set to $p < 0.05$. Data in bar graphs and box plots are presented as mean ± standard error (SE) and mean ± standard deviation (SD), respectively.

2.3 Results and Discussion

Developmental exposure assay

Uptake and effects of micro- and nanoplastics in the gut

After 3 days of exposure to 1 µm or 20 nm particles, 3rd instar larvae were rinsed with de-ionized water and imaged with a fluorescence stereomicroscope. The images in **Figure 2.2A** show the accumulation of both types of particles in the gastrointestinal tract (GIT) of larvae. In contrast, only microplastics accumulated to levels that exceeded the background fluorescence in the GIT of adults in **Figure 2.2B**. Nanoplastic fluorescence was likely masked by the green background autofluorescence of adult bodies that could not be completely eliminated by the clearing protocol. Although the extent of fluorescence was not quantitatively analyzed, it can be noted that the intensity of the fluorescence signal generally increased with exposure concentration (**Figure S4**).

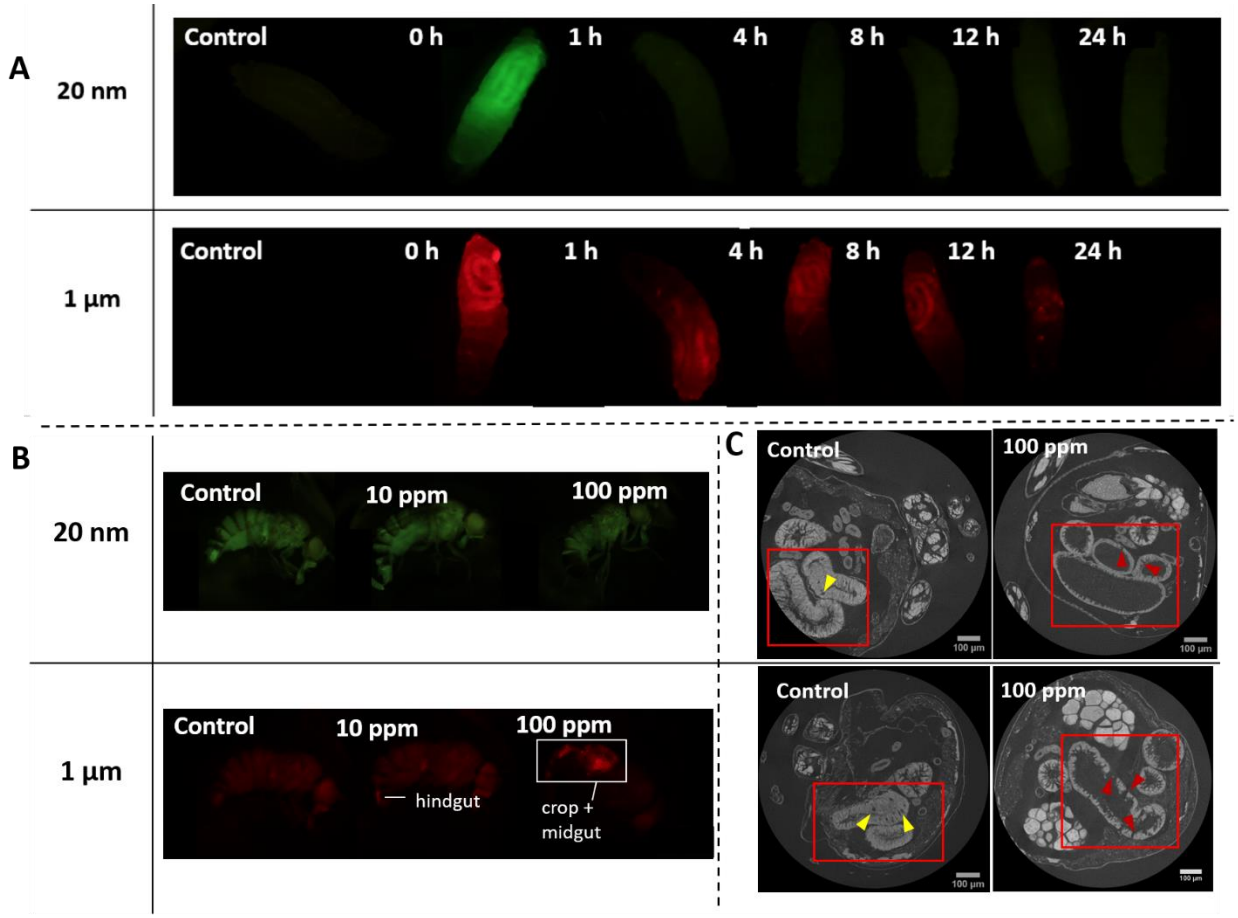


Figure 2.2. *D. melanogaster* larvae and adults exposed to various concentrations of 20 nm green-yellow or 1µm red fluorescent polystyrene in food. **A)** Larvae were exposed to 100 ppm concentration for 3-days and then underwent a 24 h depuration period. Nano- and microplastics mainly localize in the gastrointestinal tract of the larvae. Nanoplastics were largely excreted from the gastrointestinal tract after 1 h while it took the full 24 h for the microplastics to clear. **B)** Adults that emerged after 13-days of exposure were cleared according to the ScaleS protocol [205] for 10 days to reduce autofluorescence prior to fluorescence imaging with stereomicroscope. There is an accumulation of microplastics in the gastrointestinal tract that increases with exposure concentration. In contrast, there is no observable accumulation of nanoplastics. **C)** CT scans of gastrointestinal tracts are indicated in red squares. Normal secretory enteroendocrine cells are indicated by yellow arrows and signs of deterioration such as deep crypts or missing epithelia, are indicated by red arrows. See SI for larger images and 3D reconstructions.

Sequestration of micro- and nanoplastics in the GIT has been reported in many species including zebrafish,[214] daphnia,[215] fathead minnow,[215] and krill[216] as animals intake free-floating particles in the environment with their food. On the other hand, depuration is more varied depending on species and particle size. In general, microplastic depuration occurs relatively fast, usually from

hours to days. *Daphnia* and fathead minnows cleared 6 µm polystyrene spheres 72-96 h after exposure to 2×10^{-3} ppm,[215] and krill exposed to 20000 ppm of 27-32 µm polyethylene spheres cleared them in 24 h.[216] Nanoplastics tend to require longer depuration periods and a small portion often remains in the body. In scallops, 88% of ingested 24 nm polystyrene nanoparticles were excreted from the intestine after 3 days of depuration, but particles that had migrated to gills, hepatopancreas, gonad, and kidney remained.[217] In the 24 h depuration period of larvae in our experiment we observed the opposite trend for depuration times, with 1 µm microparticles requiring 24 h to clear compared to 1 h for 20 nm nanoparticles. After the nanoplastic depuration, there appeared to be some residual green fluorescence throughout the body of larvae suggesting some migration to other tissues as was seen in previous studies. More sensitive techniques such as fluorescence spectrophotometry of homogenized tissue[78] or histological analysis would be necessary to confirm this. The difference likely stems from the dispersion of particles in solid instead of liquid media, as well as the relative size of the particles to the animal gut. Microplastics are more comparable to the diameter of the gut (~160 µm),[218] which makes them more likely to form intestinal obstructions. Obstructions could lead to greater accumulation of microplastics, which in turn would require longer depuration times as we observed. Quantification of microplastics and nanoplastics within the GIT would be needed to confirm this hypothesis.

Intestinal blockages can also cause histopathological damages in the intestinal walls with such damage occurring from large (250-1000 µm),[72] intermediate (15 µm), [139] and nano-sized (100 nm) [202] polystyrene spheres in different species including *Drosophila*. Although the extent of the damage in the latter study was quantified using dye, it was not vizualized, leaving the nature of the tissue injury unknown. Here, a qualitative assessment of CT scans of female adults **(Figure 2C)** indicated tissue disorganization and damage after exposure to 100 ppm of either particle size.

Within the gut tube, the treatment flies appear to have little or no intact peritrophic matrix. These flies also appeared to have fewer or shrunken secretory enteroendocrine cells compared to controls. The enterocytes of control flies tightly filled the intestinal walls, whereas in plastic-treated flies, they were generally thinner and separated from each other, forming crypts that extended from the lumen to the basement membrane or, for 1 µm treated flies, gaps in the epithelia. However, there was a burst in the epithelia in one of the 3 controls, which suggests that larger gaps could have occurred due to processing. The 3D models constructed from the data show gross anatomical differences in the GIT (**Figure 2.3**). The midgut distal to the middle midgut (copper cell region) is lengthened in treatment flies leading to extra coils in the posterior midgut region. In controls, the anterior midgut anteroventrally covers the major loop of the posterior midgut for the exception of one replicate control (**Figure S12**). In all treatment individuals imaged, the hypertrophied posterior midgut extends anterior to the anterior midgut coil. Additionally, the descending portion of the hindgut leading to the ampulla is generally shorter in all treatment individuals. These length and coiling differences are expected to manifest during larval development and offer an insight into further research using larval life stages. In *Drosophila*, the gut is not only central to digestion and nutrient absorption, but has complex self-regulatory functions via neuronal and peptide signalling.[161] Damage to enteroendocrine cells has been shown to disrupt intestinal homeostasis and shorten adult life spans.[219] Immunohistochemistry in follow-up studies will be required to fully characterize these histological differences.

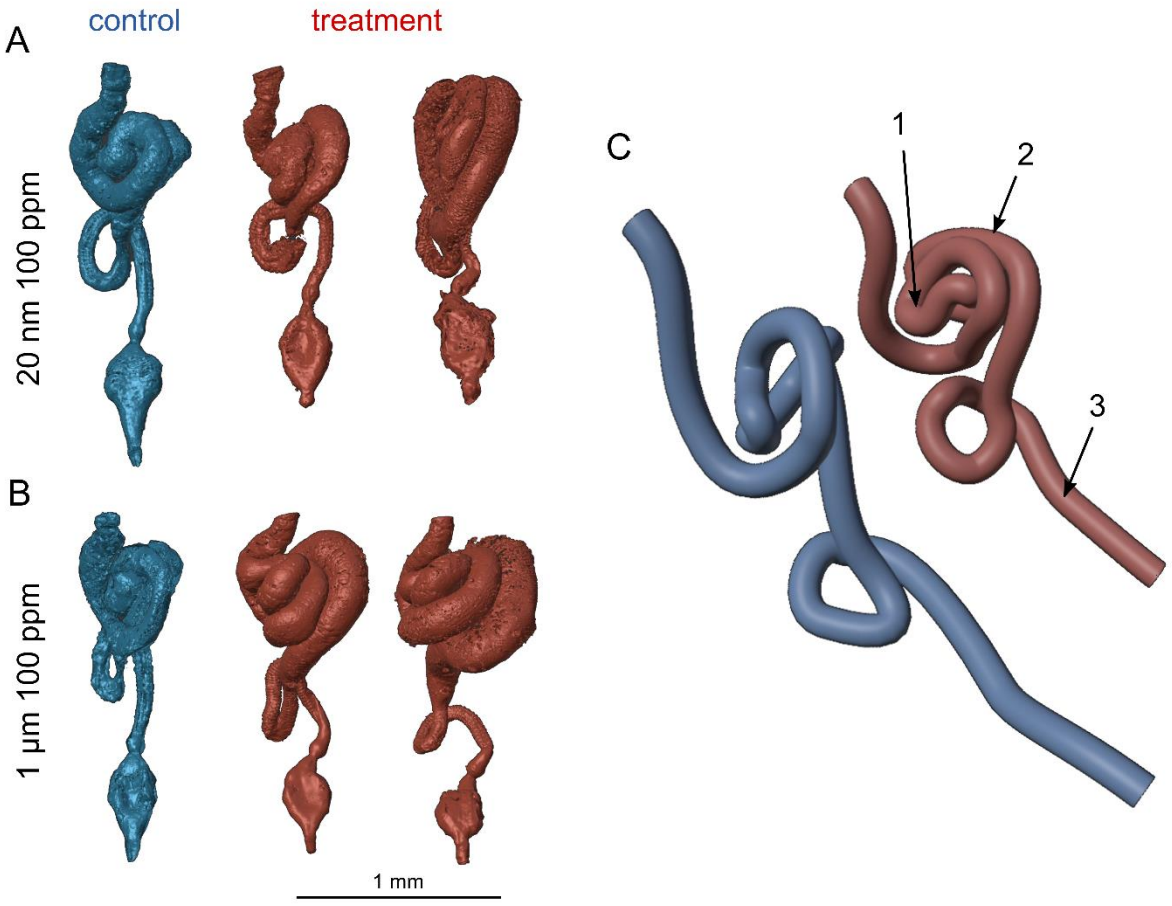


Figure 2.3. Reconstructed adult gastrointestinal tracts of *D. melanogaster* exposed to 100 ppm concentrations of **A)** 20 nm or **B)** 1 µm polystyrene particles. GITs are represented to the same scale from the anterior midgut to the ampulla and shown in ventral view with anterior toward the top. **C)** A right posteroventrolateral view of a simplified pipe representation of the GIT of the control and left treatment individual in (A). Labels: 1- middle midgut (copper cell region), 2 - hyptertrophied posterior midgut, and 3 - shortened descending hindgut.

No effect on development and mortality

The number of pupae, adults, and dead adults in each vial was recorded daily, and body measurements (length of the pupal case and adult thorax length, **Figure S3**) were taken to evaluate the development of fruit flies (**Table S2**). There was no significant delay in life-stage transitions or changes in pupae or thorax lengths in all treatments. While the previously discussed anatomical

changes in the GIT are likely due to a development change, they are not considered here as it is unknown whether it is a primary developmental change that is irreversible, or a secondary change that is plastic and reversible. The eclosion rate, defined as the transition from pupae to adult fly, was also unaffected with rates of 89% or more in all treatments. The mortality of flies was less than 10% for all treatments, which is contrary to studies that observed increased mortality and/or decrease in body weight in other models.[71,76] However, these studies used unwashed commercial particle suspensions that often contain toxic preservatives and surfactants like sodium azide.[220] When these chemicals are removed, as they were via dialysis in our study, mortality is significantly reduced in plastic exposures.[220] Similarly, low mortality for polystyrene spheres has been observed in *Drosophila*,[202] mice,[79] and different species of bacteria and algae[221,222] at similar or greater exposure concentrations of comparable polystyrene particles. Therefore, the developmental toxicity of clean spherical polystyrene micro- and nanoparticles in fruit flies is low as they are quickly moved through the digestive tract with minimal migration to other tissues, and in consequence, cause little to no lethal damages within this 13-day time frame.

Gene expression

The expression of stress-response genes (*HSP70, CAT, SOD2*) and genotoxic stress response gene (*P53)* of males and females was measured since reactive oxygen species (ROS) generation and/or genotoxicity have been observed with exposure to other metallic[158,180] and silica[201] nanoparticles. In our experiment, there was no significant difference in the expression of *CAT, SOD2,* and *P53* in all treatment groups (**Figure S5).** However, there was a significant upregulation of *HSP70* in flies exposed to microplastics ($F = 2.88$, $P = 0.033$) with a 4.5-fold increase compared to controls (**Figure 4B).** *HSP70* is a highly conserved gene that is induced in *Drosophila* by various physical, physiological, and chemical stressors. Its upregulation has been observed in response to gold

nanoparticles[158] and cadmium,[223] and is correlated with reduced lifespan.[224] The upregulation of *HSP70* might stem from potential dietary restriction, as the ingested plastics lack nutritional value and can also cause obstructions or physical damages. Given the longer depuration times observed for 1 µm spheres, the latter likely occurred to a greater extent for microplastics, hence consistently triggering the stress response and yielding a significant result.

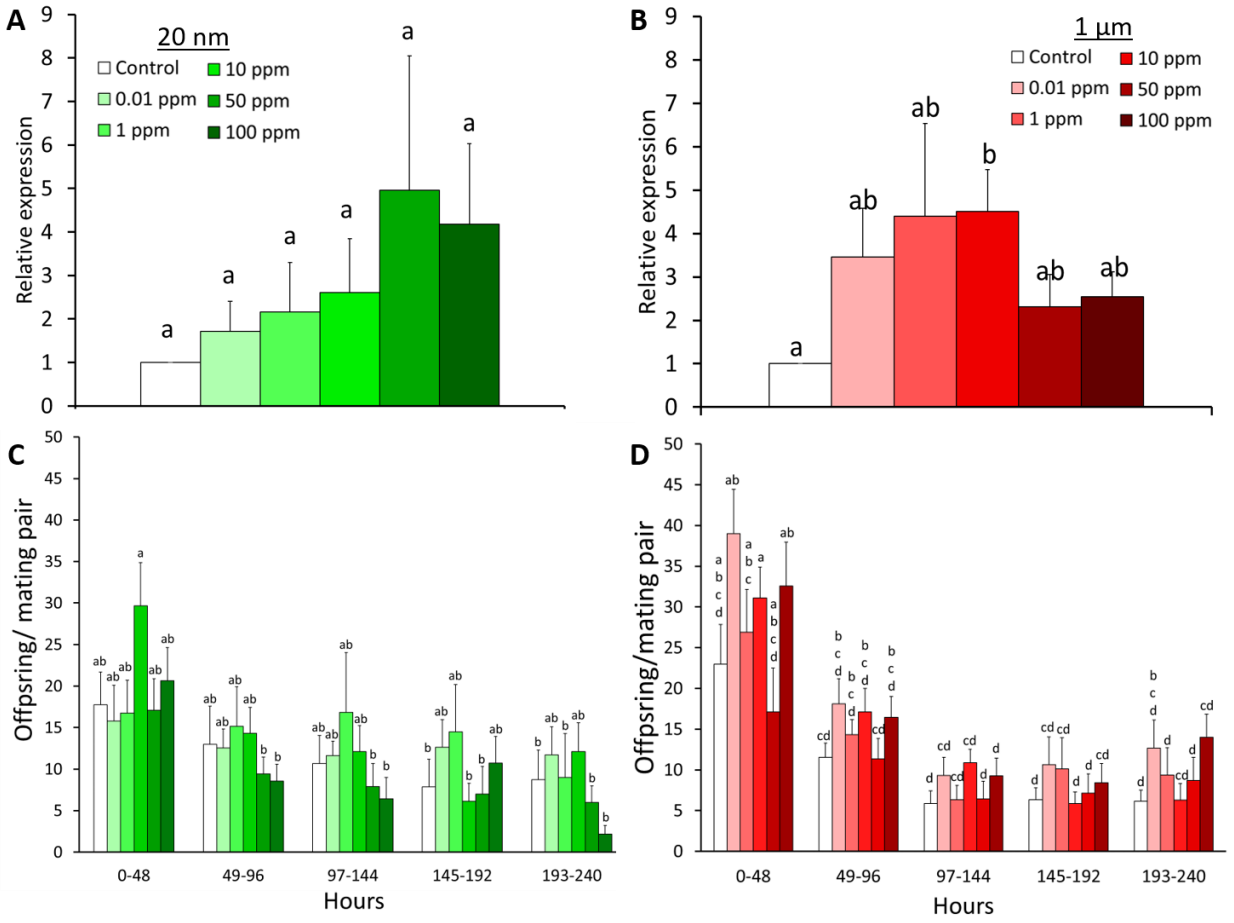


Figure 2.4. Average gene expression of *HSP70* (**A and B**) and fertility (**C and D**) of *D. melanogaster* post nano- or microplastic exposure with standard error. After exposure, mating pairs of flies were transferred to clean food for 240 h (or 10 days), with flies being transferred to new vials every 48 h (n = 9). In cases where flies were lost during transfer, n was reduced in subsequent days. The relative abundance of HSP70 was determined from total RNA extracts of groups of 5 individuals per replicate (n = 6), analyzed in three technical replicates. Tubulin served as the reference gene. Letters correspond to significant differences according to one or two-way ANOVA with Time and/or Concentration as factors. Combinations (Time×Concentration) with the same letter are not considered significantly different by Tukey-HSD test.

Effects on fertility

Fertility was measured by placing mating pairs on clean food for 10 days and counting the number of offspring (**Figure 2.4C and D**). Although any specific treatment group was not significantly different from the control within the same time frame, in general, there was a greater number of offspring in 1 µm treated groups than controls at all time points ($F = 4.75$, $P < 0.001$). However, this increase appears to have no dependence on concentration which, in addition to the high variability in offspring produced, suggests that these results fall within a normal range. Time was also a significant factor in offspring numbers in both particle exposures ($F = 8.44$ and 41.12 for 20 nm and 1 µm exposures, respectively, $P < 0.001$), with fewer offspring being produced in each subsequent day. This can be attributed to the natural decline of fertility with age in *Drosophila* as there is both a decrease in stem cell division and an increase in cell death in developing eggs in older females.[225] However, the rate of decline can vary from species to species, thus further studies on the aging and fertility of specific strains are required to distinguish the contribution of factors (i.e. aging, exposure) to the decline of fertility over time.[225]

Larval locomotion

Larval locomotion was defined as the average number of peristaltic contractions per minute. Locomotor and behavioral measurements are highly sensitive endpoints to toxicants.[226] The locomotor ability of flies was first assessed at the larval stage during the developmental toxicity assay. Nanoplastics showed no significant effect in individual tests, however, it impacted locomotion at concentrations ≥ 10 ppm when larvae were tested in a group ($F = 14.89$, $P < 0.001$) (**Figure 2.5B**). *D. melanogaster* larvae demonstrate social behavior and tend to spontaneously form social foraging groups[206] and can communicate via pheromone signaling.[227,228] These social interactions affect individual behavior such as burrowing[206] and localization.[228] Therefore, it is

likely that behavioral response is more sensitive to plastic exposure in a group; however, the potential mechanisms remain unknown. The non-monotonic response to nanoplastics we observed could result from competing effects from stress, nutrition and, potentially, neurotoxicity from translocated particles. Food scarcity can induce one of the two strategies depending on individual polymorphism: roving, when larvae actively forage for better feeding sites; or sitting, where energy is conserved by minimizing movement.[229,230] Nanoplastics have been observed to be neurotoxic in fish[65] and *C. elegans*[71] and adsorb to sensorial antenna and appendages of shrimp[63] and water fleas[64], all of which were associated with irregular motility in organisms. Depending on the nature of the damage, neurotoxicity can also alter behavior in either direction.[231,232] At 100 ppm concentration of nanoplastics, factors that increase crawling overcame those that suppress the movement. In contrast, microplastics consistently reduced the locomotion of larvae at concentrations ≥ 10 ppm (Individually: $F = 11.12$, $P < 0.001$; In groups: $F = 15.6$, $P < 0.001$) (**Figure 5A and B**). Given the lack of microplastic fluorescence outside the GIT, neurotoxicity is unlikely the major cause. Instead, occlusions within the intestine could also impede movement, as the same peristaltic movements are responsible for locomotion of the larvae and transport of food through the digestive tract.[233]

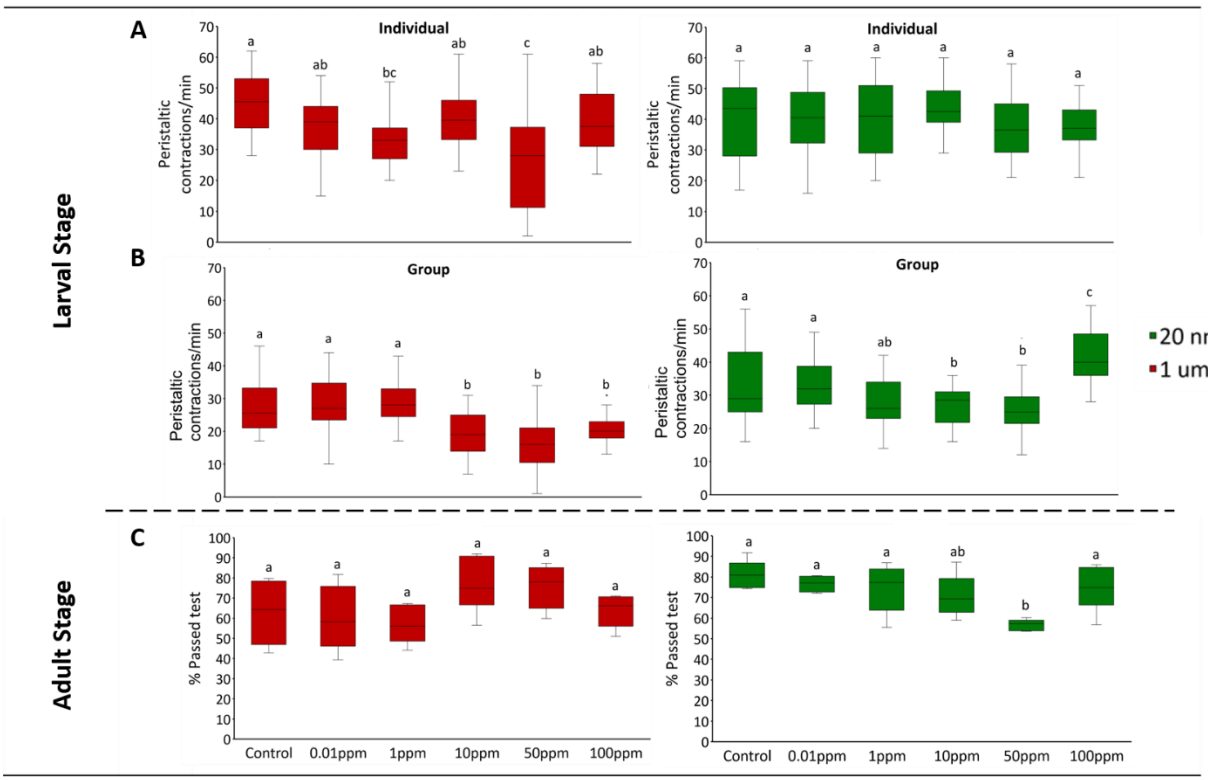


Figure 2.5. The locomotion of **A)** individual 3rd instar larvae, **B)** groups of ten 3rd instar larvae, **C)** adult *D. melanogaster*, was assessed via larval crawling and adult climbing assays after exposure to 20 nm green fluorescent or 1 µm red fluorescent polystyrene spheres for 3 days (larvae) or 13 days (adults). Bars represent standard deviation. Letters correspond to significant differences according to one-way ANOVA with concentration as the factor. Different letters indicate statistically significant differences by Tukey-HSD test; n = 10 in larval crawling assay; and n = 6 in adult climbing assay.

Adult locomotion

The locomotion of adult flies was assessed with a climbing assay. Adult locomotion was defined as the proportion of adults that passed 10 cm height after a 10-sec period. There was no difference in the proportion of flies that remained at the bottom in all conditions (**Figure S6**). Nanoplastics produced a similar but weaker response in adults as they did in larvae, while the effects of microplastics were absent in adults (**Figure 2.5C**). The disappearance of movement inhibition from exposure to 1 µm plastics in adulthood could be due to the larger size of adult digestive tracts and the decoupling of whole-body movement and digestion. In contrast, 50 ppm of 20 nm polystyrene significantly inhibited the climbing activity ($F = 4.49$, $P = 0.005$). This contrasts with

the results of Zhang et al.[202] who saw similar climbing inhibition from 1 µm and 100 nm polystyrene particles. However, this was at 200 µg/mL, twice the highest concentration in our study, which could explain the greater effect they observed. Like in larvae, the inhibited locomotion of adults could be attributed to the attachment of nanoplastics to mechanosensory organs such as bristles and/or neurotoxic effects. However, effects are dampened as any injury from particle exposure halts during the wandering phase of development, a period where larvae stop feeding and purge their gut prior to pupation. This may leave neurons a chance to regenerate and recover some locomotor ability.[234]

Daily activity and circadian rhythms

Adult male flies were exposed to 10, 50, and 100 ppm micro- and nanoplastics, and their daily activity was recorded. There were no significant effects on circadian rhythm, but daily activity counts generally increased with the concentrations of micro- ($F = 5.11$, $P = 0.003$) and nanoplastics ($F = 4.13$, $P = 0.012$) **(Figure 6A and 6C**, respectively). Total sleep was unaffected by nanoplastic exposure but decreased with 50 ppm of microplastics ($F = 9.84$, $P < 0.001$) (**Figure 2.6B**). The average sleep bout length was unaffected in all treatments (**Figure S7**), indicating that the number of sleep events was what diminished. The average activity bouts length increased at 50 ppm of micro- ($F = 5.71$, $P = 0.002$) and nanoplastics ($F = 3.23$, $P = 0.029$) (**Figure S7**). Overall, flies exposed to plastics were more active than controls, with higher activity counts, longer bouts of activity, and reduced total sleep. One possible reason is the effects of dietary restriction, that is defined as a decrease in nutrient intake without malnutrition, which has been shown to increase daily locomotor activity in flies.[235]

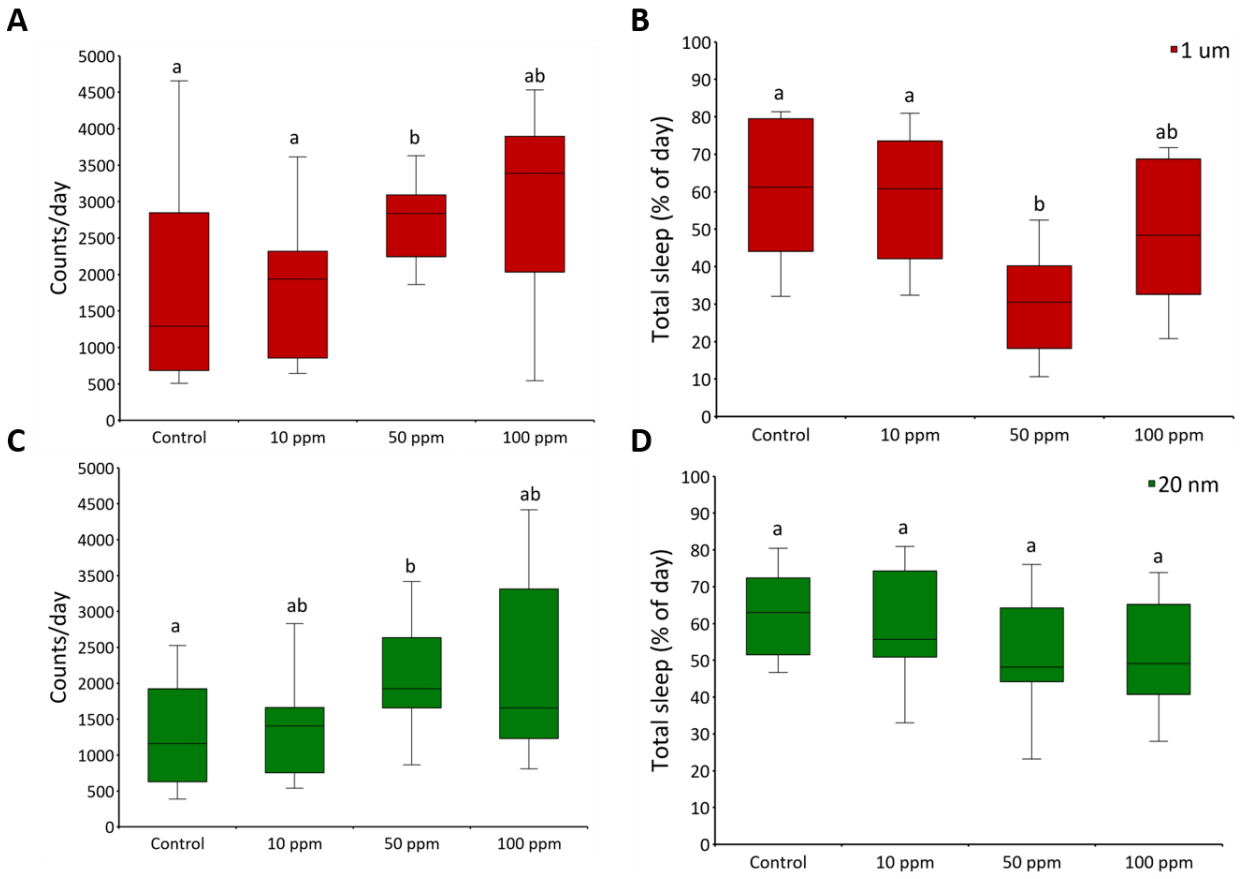


Figure 2.6. Daily activity and sleep. The daily activity of two-day-old adult male fruit flies was recorded by a Drosophila Activity Monitor 2 with activity counts being recorded in 1 min time intervals. The raw data were processed with Shiny-R software [210] to extract individual (**A and C**) daily activity counts, and (**B and D**) total sleep per day (≥ 5 min of inactivity). Letters correspond to significant differences according to one-way ANOVA with concentration as a factor; n = 16 for each concentration. There is a range of sample size as flies that died during the experiment were excluded from the analysis. Mortality was ≤ 12% in all controls.

2.4 Conclusions

We provided the first comprehensive data on the chronic effects of micro- and nanoplastics on *D. melanogaster* following exposures to a wide range of plastic concentrations. Overall, our results showed that clean spherical model polystyrene particles caused little to no toxicity at environmentally relevant concentrations up to 100 ppm in the fruit fly. In the absence of other toxicants, these particles had limited effect on severe endpoints (*i.e*. mortality, development, and

fertility), which suggests that the population of fruit flies would not be significantly impacted directly by exposure to up to 100 ppm of polystyrene micro- and nanospheres over one generation. However, there were several sub-lethal changes including upregulation of *HSP70* expression, intestinal damage, and significant changes in locomotion and daily activity after chronic exposures. Together, these may reduce the lifespan of individuals in the actual environment. For example, the reduced locomotion of larvae and adults may increase the likelihood of predation. Microplastics also induced upregulation of *HSP70* that is also associated with reduced lifespan,[224] and the intestinal damages could reduce nutrient absorption. Our results motivate the need for follow-up mechanistic studies using naturally weathered plastic particles to understand effects on sublethal endpoints in intestinal and neuronal tissues using biochemical and histochemical assays, as well as investigating the impact on the whole life-cycle of the organism.

Chapter 3: Conclusions and Future Work

While the toxicity of MPs has been extensively investigated in aquatic species, studies is terrestrial organisms have been limited. Data on NP toxicity is also rare. In this work, we used a new, environmentally relevant terrestrial model, *D. melanogaster* to investigate MP/NP toxicology. We identified the effects of MP/NPs by using chronic exposures to a wide range of environmentally relevant MP/NP concentrations. Although, NP concentrations in soil remains unknown due to the current methodological limitations in detection. The results of our study agreed with a growing body of literature that finds spherical micro and nano PS to have little to no effect on severe endpoints such as mortality, development and fertility. Instead, these particles induced sublethal changes in movement, behavior and intestinal damage at environmentally relevant concentrations of $\geq$ 10 ppm which can have an indirect impact on survival of fruit flies. This is likely to manifest as decrease in nutrient absorption and/or increased predation which, if either outpaces reproduction, could negatively impact fruit fly populations in natural environments. With the high intake of MP/NP in larvae and adult *Drosophila*, predation of exposed fruit flies could also result in trophic transfer of MP/NP previously observed in laboratory experiments with aquatic species.[61] However, if translocation from gastrointestinal system in predators is similar to what we observed in fruit flies, bioaccumulation is unlikely. Macrocosm experiments with *Drosophila* and predators, or field studies of natural population would be required to test these hypotheses.

To facilitate comparison with previous studies, we used spherical PS particles. However, there are many other types and shapes of plastics released into the environment, PE being even more abundant than PS, and fragments/fibers being the most common shapes. MPs are also rarely pristine and other chemicals are often present. Plastic surfaces are modified by ultraviolet light radiation and interact with other substances such as phthalates and heavy metals.[27] These

interactions have been shown to increase the toxicity and/or accumulation of pyrene, PCBs, PBDEs, chrome and benzo[a]pyrene when co-exposed with PE MPs.[60] Now that this work has provided a baseline for the toxicity of uniform micro- and nano PS spheres and identified sensitive endpoints, future experiments with *Drosophila* can focus on other particle shapes and contaminant interactions. Given the long lifetime of plastics in the environment and the significant sub-lethal effects observed in our study, more chronic exposure studies will also be needed to identify potential long-term effects of micro- and nanoplastics in terrestrial environments.

Reference

1 PlasticsEurope, *Plastics - the Facts 2019: An analysis of European plastics productions, demand and waste data*, PlasticsEurope, Belgium, Europe, 2019.
2 C. J. Rhodes, Plastic pollution and potential solutions, *Sci. Prog.*, 2018, **101**, 207–260.
3 L. Parker, *National Geographic*, 2019.
4 L. Parker, *National Geographic*, 2018.
5 W. J. Shim, S. H. Hong and S. Eo, *Marine Microplastics: Abundance, distribution, and composition*, Elsevier Inc., 2018.
6 F. Wang, C. S. Wong, D. Chen, X. Lu, F. Wang and E. Y. Zeng, Interaction of toxic chemicals with microplastics: A critical review, *Water Res.*, 2018, **139**, 208–219.
7 K. Mattsson, L.-A. Hansson and T. Cedervall, Nano-plastics in the aquatic environment, *Environ. Sci.: Process. Impacts*, 2015, **17**, 1712–1721.
8 L. Mai, L.-J. Bao, C. S. Wong and E. Y. Zeng, in *Microplastic contamination in aquatic environments*, Elsevier, 2018, pp. 365–378.
9 B. Nguyen, D. Claveau-Mallet, L. M. Hernandez, E. G. Xu, J. M. Farner and N. Tufenkji, Separation and analysis of microplastics and nanoplastics in complex environmental samples, *Acc. Chem. Res.*, 2019, **52**, 858–866.
10 J. Wang, X. Liu, Y. Li, T. Powell, X. Wang, G. Wang and P. Zhang, Microplastics as contaminants in the soil environment: A mini-review, *Sci. Total. Environ.*, 2019, **691**, 848–857.
11 B.-T. Lee, K.-H. Shin, J.-Y. Kim and K.-W. Kim, in *Advanced environmental monitoring*, eds. Y. J. Kim and U. Platt, Springer, Dordrecht, 2008.
12 J. H. Skevington and P. T. Dang, Exploring the diversity of flies (Diptera), *Biodiversity*, 2002, **3**, 3–27.
13 E. Galante and M. Marcos-Garcia, *Capinera JL (ed) Encyclopedia of Entomology*, 2008, 1158–1169.
14 J. Frouz, Use of soil dwelling Diptera (Insecta, Diptera) as bioindicators: a review of ecological requirements and response to disturbance, *Agric. Ecosyst. Environ.*, 1999, **74**, 167–186.
15 L. McGurk, A. Berson and N. M. Bonini, *Drosophila* as an *in vivo* model for human neurodegenerative disease, *Genetics*, 2015, **201**, 377–402.

16 B. Lemaitre and I. Miguel-Aliaga, The digestive tract of *Drosophila melanogaster*, *Annual Review of Genetics*, 2013, **47**, 377–404.
17 P. F. Mastro, in *Plastics product design*, ed. P. F. Mastro, Wiley, Hoboken, New Jerse, 2016, pp. 1–20.
18 J. N. Hahladakis, C. A. Velis, R. Weber, E. Iacovidou and P. Purnell, An overview of chemical additives present in plastics: Migration, release, fate and environmental impact during their use, disposal and recycling, *J. Hazard. Mater.*, 2018, **344**, 179–199.
19 E. Hansen, N. Nillson, D. Lithner and C. Lassen, *Hazardous substances in plastic materials*, COWI in cooperation with Danish Technological Institute, 2013.
20 Centers for Disease Control and Prevention, Agency for Toxic Substances & Disease Registry, https://www.atsdr.cdc.gov/.
21 M. V. Maffini, B. S. Rubin, C. Sonnenschein and A. M. Soto, Endocrine disruptors and reproductive health: The case of bisphenol-A, *Mol. Cell. Endocrinol.*, 2006, **254–255**, 179–186.
22 Y. Xu, S. H. Park, K. N. Yoon, S. J. Park and M. C. Gye, Effects of citrate ester plasticizers and bis (2-ethylhexyl) phthalate in the OECD 28-day repeated-dose toxicity test (OECD TG 407), *Environ. Res.*, 2019, **172**, 675–683.
23 C. Ducker, M. Kolodziejski, M. Jacques, J. Roark, A. Blakinger and T. Lehman, The risks of single-use bioprocess containers, *Am. Pharm. Rev.*, 2014.
24 A. Soares, B. Guieysse, B. Jefferson, E. Cartmell and J. N. Lester, Nonylphenol in the environment: A critical review on occurrence, fate, toxicity and treatment in wastewaters, *Environ. Int.*, 2008, **34**, 1033–1049.
25 S. C. Laws, Estrogenic activity of octylphenol, nonylphenol, bisphenol a and methoxychlor in rats, *Toxicol. Sci.*, 2000, **54**, 154–167.
26 Government of Canada, Draft screening assessment fatty amides group, https://www.canada.ca/en/environment-climate-change/services/evaluating-existing-substances/screening-assessment-fatty-amides.html#toc14, (accessed 24 November 2020).
27 O. S. Alimi, J. Farner Budarz, L. M. Hernandez and N. Tufenkji, Microplastics and Nanoplastics in aquatic environments: aggregation, deposition, and enhanced contaminant transport, *Environ. Sci. Technol.*, 2018, **52**, 1704–1724.
28 L. M. Hernandez, N. Yousefi and N. Tufenkji, Are there nanoplastics in your personal care products?, *Environ. Sci. Technol. Lett.*, 2017, **4**, 280–285.
29 M. Cole, P. Lindeque, C. Halsband and T. S. Galloway, Microplastics as contaminants in the marine environment: A review, *Mar. Pollut. Bull.*, 2011, **62**, 2588–2597.
30 A. L. Andrady, *Plastics and Environmental Sustainbility*, John Wiley & Sons, Inc, Hoboken, NJ, 2015.
31 P. J. Kole, A. J. Löhr, F. G. A. J. Van Belleghem and M. J. Ragas, Wear and tear of tires: A stealthy source of microplastics in the environment, *Int J Environ Res Public Health*, 2017, **14**, 1265.
32 H. Bouwmeester, P. C. H. Hollman and R. J. B. Peters, Potential health impact of environmentally released micro- and nanoplastics in the human food production chain: Experiences from nanotoxicology, *Environ. Sci. Technol.*, 2015, **49**, 8932–8947.
33 M. A. Browne, P. Crump, S. J. Niven, E. Teuten, A. Tonkin, T. Galloway and R. Thompson, Accumulation of microplastic on shorelines worldwide: Sources and sinks, *Environ. Sci. Technol.*, 2011, **45**, 9175–9179.

34 Y. Chae and Y.-J. An, Current research trends on plastic pollution and ecological impacts on the soil ecosystem: A review, *Environ. Pollut.*, 2018, **240**, 387–395.
35 A. Jahnke, H. P. H. Arp, B. I. Escher, B. Gewert, E. Gorokhova, D. Kühnel, M. Ogonowski, A. Potthoff, C. Rummel, M. Schmitt-Jansen, E. Toorman and M. MacLeod, Reducing uncertainty and confronting ignorance about the possible impacts of weathering plastic in the marine environment, *Environ. Sci. Technol. Lett.*, 2017, **4**, 85–90.
36 K. Mattsson, L. A. Hansson and T. Cedervall, *Nano-plastics in the aquatic environment*, Elsevier Inc., 2015, vol. 17.
37 K. N. Fotopoulou and H. K. Karapanagioti, Surface properties of beached plastics, *Environ Sci Pollut Res*, 2015, **22**, 11022–11032.
38 K. N. Fotopoulou and H. K. Karapanagioti, Surface properties of beached plastic pellets, *Mar. Environ. Res.*, 2012, **81**, 70–77.
39 J. R. Jambeck, R. Geyer, C. Wilcox, T. R. Siegler, M. Perryman, A. Andrady, R. Narayan and K. L. Law, Plastic waste inputs from land into the ocean, *ience*, 2015, **347**, 768–771.
40 Adventure Scientists, Global Microplastics Initiative, https://www.adventurescientists.org/microplastics.html.
41 S. L. Wright, R. C. Thompson and T. S. Galloway, The physical impacts of microplastics on marine organisms: A review, *Environ. Pollut.*, 2013, **178**, 483–492.
42 J. L. Conkle, C. D. Báez Del Valle and J. W. Turner, Are we underestimating microplastic contamination in aquatic environments?, *Environ. Manage.*, 2018, **61**, 1–8.
43 S. Lambert and M. Wagner, Characterisation of nanoplastics during the degradation of polystyrene, *Chemosphere*, 2016, **145**, 265–268.
44 L. M. Hernandez, E. G. Xu, H. C. E. Larsson, R. Tahara, V. B. Maisuria and N. Tufenkji, Plastic teabags release billions of microparticles and nanoparticles into tea, *Environ. Sci. Technol.*, 2019, **53**, 12300–12310.
45 D. Magrì, P. Sánchez-Moreno, G. Caputo, F. Gatto, M. Veronesi, G. Bardi, T. Catelani, D. Guarnieri, A. Athanassiou, P. P. Pompa and D. Fragouli, Laser ablation as a versatile tool to mimic polyethylene terephthalate nanoplastic pollutants: Characterization and toxicology assessment, *ACS Nano*, 2018, acsnano.8b01331.
46 S. Fuller and A. Gautam, A procedure for measuring microplastics using pressurized fluid extraction, *Environ. Sci. Technol.*, 2016, **50**, 5774–5780.
47 S. Piehl, A. Leibner, M. G. J. Löder, R. Dris, C. Bogner and C. Laforsch, Identification and quantification of macro- and microplastics on an agricultural farmland, *Sci. Rep.*, 2018, **8**, 17950.
48 M. Scheurer and M. Bigalke, Microplastics in swiss floodplain soils, *Environ. Sci. Technol.*, 2018, **52**, 3591–3598.
49 G. S. Zhang and Y. F. Liu, The distribution of microplastics in soil aggregate fractions in southwestern China, *Sci. Total. Environ.*, 2018, **642**, 12–20.
50 A. M. Mahon, B. O'Connell, M. G. Healy, I. O'Connor, R. Officer, R. Nash and L. Morrison, Microplastics in sewage sludge: Effects of Treatment, *Environ. Sci. Technol.*, 2017, **51**, 810–818.
51 E.-L. Ng, E. Huerta Lwanga, S. M. Eldridge, P. Johnston, H.-W. Hu, V. Geissen and D. Chen, An overview of microplastic and nanoplastic pollution in agroecosystems, *Sci. Total. Environ.*, 2018, **627**, 1377–1388.
52 K. A. V. Zubris and B. K. Richards, Synthetic fibers as an indicator of land application of sludge, *Environ. Pollut.*, 2005, **138**, 201–211.

53 S. A. Carr, J. Liu and A. G. Tesoro, Transport and fate of microplastic particles in wastewater treatment plants, *Water Res.*, 2016, **91**, 174–182.
54 L. Ramos, G. Berenstein, E. A. Hughes, A. Zalts and J. M. Montserrat, Polyethylene film incorporation into the horticultural soil of small periurban production units in Argentina, *Sci. Total. Environ.*, 2015, **523**, 74–81.
55 M. Liu, S. Lu, Y. Song, L. Lei, J. Hu, W. Lv, W. Zhou, C. Cao, H. Shi, X. Yang and D. He, Microplastic and mesoplastic pollution in farmland soils in suburbs of Shanghai, China, *Environ. Pollut.*, 2018, **242**, 855–862.
56 N. Weithmann, J. N. Möller, M. G. J. Löder, S. Piehl, C. Laforsch and R. Freitag, Organic fertilizer as a vehicle for the entry of microplastic into the environment, *Sci. Adv.*, 2018, **4**, eaap8060.
57 R. Lohmann, Microplastics are not important for the cycling and bioaccumulation of organic pollutants in the oceans—but should microplastics be considered POPs themselves?, *Integr. Environ. Asses.*, 2017, **13**, 460–465.
58 F. F. F. F. Wang, F. F. F. F. Wang and E. Y. Zeng, *Sorption of toxic chemicals on microplastics*, Elsevier Inc., 2018.
59 K. D. Cox, G. A. Covernton, H. L. Davies, J. F. Dower, F. Juanes and S. E. Dudas, Human consumption of microplastics, *Environ. Sci. Technol.*, 2019, **53**, 7068–7074.
60 Y. Chae and Y. J. An, Effects of micro- and nanoplastics on aquatic ecosystems: Current research trends and perspectives, *Mar. Pollut. Bull.*, 2017, **124**, 624–632.
61 E. E. Burns and A. B. A. Boxall, Microplastics in the aquatic environment: Evidence for or against adverse impacts and major knowledge gaps, *Environ. Toxicol. Chem.*, 2018, **9999**, 1–21.
62 A. Martins and L. Guilhermino, Transgenerational effects and recovery of microplastics exposure in model populations of the freshwater cladoceran *Daphnia magna* Straus, *Sci. Tot. Environ.*, 2018, **631–632**, 421–428.
63 E. Bergami, E. Bocci, M. L. Vannuccini, M. Monopoli, A. Salvati, K. A. Dawson and I. Corsi, Nano-sized polystyrene affects feeding, behavior and physiology of brine shrimp *Artemia franciscana* larvae, *Ecotox. Environ. Safe.*, 2016, **123**, 18–25.
64 E. G. Xu, R. S. Cheong, L. Liu, L. M. Hernandez, A. Azimzada, S. Bayen and N. Tufenkji, Primary and secondary plastic particles exhibit limited acute toxicity but chronic effects on *Daphnia magna*, *Environ. Sci. Technol.*, 2020, **54**, 6859–6868.
65 K. Mattsson, E. V. Johnson, A. Malmendal, S. Linse, L.-A. Hansson and T. Cedervall, Brain damage and behavioural disorders in fish induced by plastic nanoparticles delivered through the food chain, *Sci. Rep.*, 2017, **7**, 11452.
66 D. Guarnieri, A. Falanga, O. Muscetti, R. Tarallo, S. Fusco, M. Galdiero, S. Galdiero and P. a. Netti, Shuttle-mediated nanoparticle delivery to the blood-brain barrier, *Small*, 2013, **9**, 853–862.
67 P. Bhattacharya, S. Lin, J. P. Turner and P. C. Ke, Physical adsorption of charged plastic nanoparticles affects algal photosynthesis, *J. Phys. Chem. C*, 2010, **114**, 16556–16561.
68 C. Della Torre, E. Bergami, A. Salvati, C. Faleri, P. Cirino, K. A. Dawson and I. Corsi, Accumulation and embryotoxicity of polystyrene nanoparticles at early stage of development of sea urchin embryos *paracentrotus lividus*, *Environ. Sci. Technol.*, 2014, **48**, 12302–12311.
69 J. M. D'Souza, F. M. Windsor, D. Santillo and S. J. Ormerod, Food web transfer of plastics to an apex riverine predator, *Glob Change Biol*, 2020, **26**, 3846–3857.

70 E. Huerta Lwanga, H. Gertsen, H. Gooren, P. Peters, T. Salánki, M. van der Ploeg, E. Besseling, A. A. Koelmans and V. Geissen, Microplastics in the terrestrial ecosystem: implications for *Lumbricus terrestris* (Oligochaeta, Lumbricidae), *Environ. Sci. Technol.*, 2016, **50**, 2685–2691.
71 L. Lei, M. Liu, Y. Song, S. Lu, J. Hu, C. Cao, B. Xie, H. Shi and D. He, Polystyrene (nano)microplastics cause size-dependent neurotoxicity, oxidative damage and other adverse effects in *Caenorhabditis elegans*, *Environ. Sci.: Nano*, 2018, **5**, 2009–2020.
72 A. Rodriguez-Seijo, J. Lourenço, T. A. P. Rocha-Santos, J. da Costa, A. C. Duarte, H. Vala and R. Pereira, Histopathological and molecular effects of microplastics in *Eisenia andrei* Bouché, *Environ. Pollut.*, 2017, **220**, 495–503.
73 B. K. Zhu, Y. M. Fang, D. Zhu, P. Christie, X. Ke and Y. G. Zhu, Exposure to nanoplastics disturbs the gut microbiome in the soil oligochaete *Enchytraeus crypticus*, *Environ. Pollut.*, 2018, **239**, 408–415.
74 L. Tosetto, C. Brown and J. E. Williamson, Microplastics on beaches: ingestion and behavioural consequences for beachhoppers, *Marine Biology*, 2016, **163**, 199.
75 A. S. French and P. H. Torkkeli, in *Cell Physiology Source Book*, Elsevier, 2012, pp. 633–647.
76 B.-K. Zhu, Y.-M. Fang, D. Zhu, P. Christie, X. Ke and Y.-G. Zhu, Exposure to nanoplastics disturbs the gut microbiome in the soil oligochaete *Enchytraeus crypticus*, *Environ. Pollut.*, 2018, **239**, 408–415.
77 L. Lu, Z. Wan, T. Luo, Z. Fu and Y. Jin, Polystyrene microplastics induce gut microbiota dysbiosis and hepatic lipid metabolism disorder in mice, *Sci. Tot. Environ.*, 2018, **631–632**, 449–458.
78 Y. Deng, Y. Zhang, B. Lemos and H. Ren, Tissue accumulation of microplastics in mice and biomarker responses suggest widespread health risks of exposure, *Sci. Rep.*, 2017, **7**, 46687.
79 M. Rafiee, L. Dargahi, A. Eslami, E. Beirami, M. Jahangiri-rad, S. Sabour and F. Amereh, Neurobehavioral assessment of rats exposed to pristine polystyrene nanoplastics upon oral exposure, *Chemosphere*, 2018, **193**, 745–753.
80 M. E. Hodson, C. A. Duffus-Hodson, A. Clark, M. T. Prendergast-Miller and K. L. Thorpe, Plastic bag derived-microplastics as a vector for metal exposure in terrestrial invertebrates, *Environ. Sci. Technol.*, 2017, **51**, 4714–4721.
81 Y. Kim, J. Jeong, S. Lee, I. Choi and J. Choi, Identification of adverse outcome pathway related to high-density polyethylene microplastics exposure: Caenorhabditis elegans transcription factor RNAi screening and zebrafish study, *J. Hazard. Mater.*, 2020, **388**, 121725.
82 S. Pflugmacher, J. H. Huttunen, M.-A. von Wolff, O.-P. Penttinen, Y. J. Kim, S. Kim, S. M. Mitrovic and M. Esterhuizen-Londt, Enchytraeus crypticus avoid soil spiked with microplastic, *Toxics*, 2020, **8**, 10.
83 A. Rodríguez-Seijo, J. P. da Costa, T. Rocha-Santos, A. C. Duarte and R. Pereira, Oxidative stress, energy metabolism and molecular responses of earthworms (*Eisenia fetida*) exposed to low-density polyethylene microplastics, *Environ Sci Pollut Res*, 2018, **25**, 33599–33610.
84 A. Rodríguez-Seijo, B. Santos, E. Ferreira da Silva, A. Cachada and R. Pereira, Low-density polyethylene microplastics as a source and carriers of agrochemicals to soil and earthworms, *Environ. Chem.*, 2019, **16**, 8.

85 E. Huerta Lwanga, H. Gertsen, H. Gooren, P. Peters, T. Salánki, M. van der Ploeg, E. Besseling, A. A. Koelmans and V. Geissen, Incorporation of microplastics from litter into burrows of Lumbricus terrestris, *Environ. Pollut.*, 2017, **220**, 523–531.
86 X. Yang, E. H. Lwanga, A. Bemani, H. Gertsen, T. Salanki, X. Guo, H. Fu, S. Xue, C. Ritsema and V. Geissen, Biogenic transport of glyphosate in the presence of LDPE microplastics: A mesocosm experiment, *Environ. Pollut.*, 2019, **245**, 829–835.
87 Y. Chen, X. Liu, Y. Leng and J. Wang, Defense responses in earthworms (Eisenia fetida) exposed to low-density polyethylene microplastics in soils, *Ecotox. Environ. Safe.*, 2020, **187**, 109788.
88 Y. Cheng, L. Zhu, W. Song, C. Jiang, B. Li, Z. Du, J. Wang, J. Wang, D. Li and K. Zhang, Combined effects of mulch film-derived microplastics and atrazine on oxidative stress and gene expression in earthworm (*Eisenia fetida*), *Sci. Tot. Environ.*, 2020, **746**, 141280.
89 A. Jemec Kokalj, P. Horvat, T. Skalar and A. Kržan, Plastic bag and facial cleanser derived microplastic do not affect feeding behaviour and energy reserves of terrestrial isopods, *Sci. Total. Environ.*, 2018, **615**, 761–766.
90 C. J. M. Silva, A. L. P. Silva, C. Gravato and J. L. T. Pestana, Ingestion of small-sized and irregularly shaped polyethylene microplastics affect *Chironomus riparius* life-history traits, *Sci. Total. Environ.*, 2019, **672**, 862–868.
91 H. Ju, D. Zhu and M. Qiao, Effects of polyethylene microplastics on the gut microbial community, reproduction and avoidance behaviors of the soil springtail, *Folsomia candida*, *Environ. Pollut.*, 2019, **247**, 890–897.
92 C. J. Silva, A. L. Patrício Silva, D. Campos, A. M. Soares, J. L. Pestana and C. Gravato, *Lumbriculus variegatus* (oligochaeta) exposed to polyethylene microplastics: biochemical, physiological and reproductive responses, *Ecotox. Environ. Safe.*, 2021, **207**, 111375.
93 J. I. Kwak and Y.-J. An, Microplastic digestion generates fragmented nanoplastics in soils and damages earthworm spermatogenesis and coelomocyte viability, *J. Hazard. Mater.*, 2021, **402**, 124034.
94 A. P. da Costa Araújo and G. Malafaia, Microplastic ingestion induces behavioral disorders in mice: A preliminary study on the trophic transfer effects via tadpoles and fish, *J. Hazard. Mater.*, 2021, **401**, 123263.
95 C. Huang, Y. Ge, S. Yue, L. Zhao and Y. Qiao, Microplastics aggravate the joint toxicity to earthworm *Eisenia fetida* with cadmium by altering its availability, *Sci. Tot. Environ.*, 2021, **753**, 142042.
96 B. Li, Y. Ding, X. Cheng, D. Sheng, Z. Xu, Q. Rong, Y. Wu, H. Zhao, X. Ji and Y. Zhang, Polyethylene microplastics affect the distribution of gut microbiota and inflammation development in mice, *Chemosphere*, 2020, **244**, 125492.
97 Y. Deng, Z. Yan, R. Shen, M. Wang, Y. Huang, H. Ren, Y. Zhang and B. Lemos, Microplastics release phthalate esters and cause aggravated adverse effects in the mouse gut, *Environ. Int.*, 2020, **143**, 105916.
98 E.-J. Park, J.-S. Han, E.-J. Park, E. Seong, G.-H. Lee, D.-W. Kim, H.-Y. Son, H.-Y. Han and B.-S. Lee, Repeated-oral dose toxicity of polyethylene microplastics and the possible implications on reproduction and development of the next generation, *Toxicol. Lett.*, 2020, **324**, 75–85.
99 Y. Song, C. Cao, R. Qiu, J. Hu, M. Liu, S. Lu, H. Shi, K. M. Raley-Susman and D. He, Uptake and adverse effects of polyethylene terephthalate microplastics fibers on terrestrial snails (*Achatina fulica*) after soil exposure, *Environ. Pollut.*, 2019, **250**, 447–455.

100 J. D. Judy, M. Williams, A. Gregg, D. Oliver, A. Kumar, R. Kookana and J. K. Kirby, Microplastics in municipal mixed-waste organic outputs induce minimal short to long-term toxicity in key terrestrial biota, *Environ. Pollut.*, 2019, **252**, 522–531.
101 S. Maaß, D. Daphi, A. Lehmann and M. C. Rillig, Transport of microplastics by two collembolan species, *Environ. Pollut.*, 2017, **225**, 456–459.
102 M. T. Prendergast-Miller, A. Katsiamides, M. Abbass, S. R. Sturzenbaum, K. L. Thorpe and M. E. Hodson, Polyester-derived microfibre impacts on the soil-dwelling earthworm *Lumbricus terrestris*, *Environ. Pollut.*, 2019, **251**, 453–459.
103 S. Selonen, A. Dolar, A. Jemec Kokalj, T. Skalar, L. Parramon Dolcet, R. Hurley and C. A. M. van Gestel, Exploring the impacts of plastics in soil – The effects of polyester textile fibers on soil invertebrates, *Sci. Tot. Environ.*, 2020, **700**, 134451.
104 Y. Chae and Y.-J. An, Nanoplastic ingestion induces behavioral disorders in terrestrial snails: Trophic transfer effects: Via vascular plants, *Environ. Sci.: Nano*, 2020, **7**, 975–983.
105 R. Al-Jaibachi, R. N. Cuthbert and A. Callaghan, Up and away: ontogenic transference as a pathway for aerial dispersal of microplastics, *Biol. Lett.*, 2018, **14**, 20180479.
106 D. Cao, X. Wang, X. Luo, G. Liu and H. Zheng, Effects of polystyrene microplastics on the fitness of earthworms in an agricultural soil, *IOP Conf. Ser.: Earth Environ. Sci.*, 2017, **61**, 012148.
107 A. M. Brandon, S. H. El Abbadi, U. A. Ibekwe, Y.-M. Cho, W.-M. Wu and C. S. Criddle, fate of hexabromocyclododecane (HBCD), a common flame retardant, in polystyrene-degrading mealworms: Elevated HBCD levels in egested polymer but no bioaccumulation, *Environ. Sci. Technol.*, 2020, **54**, 364–371.
108 A. P. Walczak, P. J. M. Hendriksen, R. A. Woutersen, M. van der Zande, A. K. Undas, R. Helsdingen, H. H. J. van den Berg, I. M. C. M. Rietjens and H. Bouwmeester, Bioavailability and biodistribution of differently charged polystyrene nanoparticles upon oral exposure in rats, *J. Nanopart. Res.*, 2015, **17**, 231.
109 Y. Jin, L. Lu, W. Tu, T. Luo and Z. Fu, Impacts of polystyrene microplastic on the gut barrier, microbiota and metabolism of mice, *Sci. Total. Environ.*, 2019, **649**, 308–317.
110 Y. Qiu, Y. Liu, Y. Li, G. Li and D. Wang, Effect of chronic exposure to nanopolystyrene on nematode *Caenorhabditis elegans*, *Chemosphere*, 2020, **256**, 127172.
111 H. Fueser, M.-T. Mueller and W. Traunspurger, Rapid ingestion and egestion of spherical microplastics by bacteria-feeding nematodes, *Chemosphere*, 2020, **261**, 128162.
112 C. C. Parenti, A. Binelli, S. Caccia, C. Della Torre, S. Magni, G. Pirovano and M. Casartelli, Ingestion and effects of polystyrene nanoparticles in the silkworm *Bombyx mori*, *Chemosphere*, 2020, **257**, 127203.
113 M.-T. Mueller, H. Fueser, S. Höss and W. Traunspurger, Species-specific effects of long-term microplastic exposure on the population growth of nematodes, with a focus on microplastic ingestion, *Ecol. Indic.*, 2020, **118**, 106698.
114 D. Li, Y. Deng, S. Wang, H. Du, G. Xiao and D. Wang, Assessment of nanopolystyrene toxicity under fungal infection condition in *Caenorhabditis elegans*, *Ecotox. Environ. Safe.*, 2020, **197**, 110625.
115 X. Xie, T. Deng, J. Duan, J. Xie, J. Yuan and M. Chen, Exposure to polystyrene microplastics causes reproductive toxicity through oxidative stress and activation of the p38 MAPK signaling pathway, *Ecotox. Environ. Safe.*, 2020, **190**, 110133.
116 Y. Yang, H. Shao, Q. Wu and D. Wang, Lipid metabolic response to polystyrene particles in nematode *Caenorhabditis elegans*, *Environ. Pollut.*, 2020, **256**, 113439.

117 X. Jiang, Y. Chang, T. Zhang, Y. Qiao, G. Klobučar and M. Li, Toxicological effects of polystyrene microplastics on earthworm (*Eisenia fetida*), *Environ. Pollut.*, 2020, **259**, 113896.
118 Z. Li, S. Zhu, Q. Liu, J. Wei, Y. Jin, X. Wang and L. Zhang, Polystyrene microplastics cause cardiac fibrosis by activating Wnt/β-catenin signaling pathway and promoting cardiomyocyte apoptosis in rats, *Environ. Pollut.*, 2020, **265**, 115025.
119 S. W. Kim, D. Kim, S.-W. Jeong and Y.-J. An, Size-dependent effects of polystyrene plastic particles on the nematode *Caenorhabditis elegans* as related to soil physicochemical properties, *Environ. Pollut.*, 2020, **258**, 113740.
120 M. Qu, D. Li, Y. Zhao, Y. Yuan and D. Wang, Exposure to low-dose nanopolystyrene induces the response of neuronal JNK MAPK signaling pathway in nematode *Caenorhabditis elegans*, *Environ. Sci. Eur.*, 2020, **32**, 58.
121 Y. Yang, H. Du, G. Xiao, Q. Wu and D. Wang, Response of intestinal Gα subunits to nanopolystyrene in nematode: *Caenorhabditis elegans*, *Environ. Sci.: Nano*, 2020, **7**, 2351–2359.
122 M.-T. Mueller, H. Fueser, L. N. Trac, P. Mayer, W. Traunspurger and S. Höss, Surface-related toxicity of polystyrene beads to nematodes and the role of food availability, *Environ. Sci. Technol.*, 2020, **54**, 1790–1798.
123 H. M. Kim, N. P. Long, J. E. Min, N. H. Anh, S. J. Kim, S. J. Yoon and S. W. Kwon, Comprehensive phenotyping and multi-omic profiling in the toxicity assessment of nanopolystyrene with different surface properties, *J. Hazard. Mater.*, 2020, **399**, 123005.
124 H. Jin, T. Ma, X. Sha, Z. Liu, Y. Zhou, X. Meng, Y. Chen, X. Han and J. Ding, Polystyrene microplastics induced male reproductive toxicity in mice, *J. Hazard. Mater.*, 2021, **401**, 123430.
125 S. Wang, H. Liu, Y. Zhao, Q. Rui and D. Wang, Dysregulated mir-354 enhanced the protective response to nanopolystyrene by affecting the activity of TGF-β signaling pathway in nematode Caenorhabditis elegans, *NanoImpact*, 2020, **20**, 100256.
126 Y. Yu, H. Chen, X. Hua, Y. Dang, Y. Han, Z. Yu, X. Chen, P. Ding and H. Li, Polystyrene microplastics (PS-MPs) toxicity induced oxidative stress and intestinal injury in nematode *Caenorhabditis elegans*, *Sci. Tot. Environ.*, 2020, **726**, 138679.
127 X. Shang, J. Lu, C. Feng, Y. Ying, Y. He, S. Fang, Y. Lin, R. Dahlgren and J. Ju, Microplastic (1 and 5 μm) exposure disturbs lifespan and intestine function in the nematode *Caenorhabditis elegans*, *Sci. Tot. Environ.*, 2020, **705**, 135837.
128 M. Qu, D. Li, Y. Qiu and D. Wang, Neuronal ERK MAPK signaling in response to low-dose nanopolystyrene exposure by suppressing insulin peptide expression in *Caenorhabditis elegans*, *Sci. Tot. Environ.*, 2020, **724**, 138378.
129 Y. Zhao, D. Li, Q. Rui and D. Wang, Toxicity induction of nanopolystyrene under microgravity stress condition in *Caenorhabditis elegans*, *Sci. Tot. Environ.*, 2020, **703**, 135623.
130 K. Wang, J. Li, L. Zhao, X. Mu, C. Wang, M. Wang, X. Xue, S. Qi and L. Wu, Gut microbiota protects honey bees (*Apis mellifera L.*) against polystyrene microplastics exposure risks, *J. Hazard. Mater.*, 2021, **402**, 123828.
131 S. W. Kim and Y.-J. An, Soil microplastics inhibit the movement of springtail species, *Environ. Int.*, 2019, **126**, 699–706.

132 J. Wang, S. Coffin, C. Sun, D. Schlenk and J. Gan, Negligible effects of microplastics on animal fitness and HOC bioaccumulation in earthworm *Eisenia fetida* in soil, *Environ. Pollut.*, 2019, **249**, 776–784.
133 Y. Zhou, X. Liu and J. Wang, Ecotoxicological effects of microplastics and cadmium on the earthworm *Eisenia fetida*, *J. Hazard. Mater.*, 2020, **392**, 122273.
134 D. Zhu, Q.-F. Bi, Q. Xiang, Q.-L. Chen, P. Christie, X. Ke, L.-H. Wu and Y.-G. Zhu, Trophic predator-prey relationships promote transport of microplastics compared with the single *Hypoaspis aculeifer* and *Folsomia candida*, *Environ. Pollut.*, 2018, **235**, 150–154.
135 E. Bergami, E. Rota, T. Caruso, G. Birarda, L. Vaccari and I. Corsi, Plastics everywhere: first evidence of polystyrene fragments inside the common Antarctic collembolan *Cryptopygus antarcticus*: Microplastics in Antarctic collembola, *Biol. Lett.*, 2020, **16**, 20200093.
136 J. Carlin, C. Craig, S. Little, M. Donnelly, D. Fox, L. Zhai and L. Walters, Microplastic accumulation in the gastrointestinal tracts in birds of prey in central Florida, USA, *Environ. Pollut.*, 2020, **264**, 114633.
137 S. L. Wright and F. J. Kelly, Plastic and Human Health: A Micro Issue?, *Environ. Sci. Technol.*, 2017, **51**, 6634–6647.
138 A. A. de Souza Machado, W. Kloas, C. Zarfl, S. Hempel and M. C. Rillig, Microplastics as an emerging threat to terrestrial ecosystems, *Glob Change Biol*, 2018, **24**, 1405–1416.
139 R. Qiao, Y. Deng, S. Zhang, M. B. Wolosker, Q. Zhu, H. Ren and Y. Zhang, Accumulation of different shapes of microplastics initiates intestinal injury and gut microbiota dysbiosis in the gut of zebrafish, *Chemosphere*, 2019, **236**, 124334.
140 H. Saygin and A. Baysal, Biofilm Formation of Clinically Important Bacteria on Bio-Based and Conventional Micro/Submicron-Sized Plastics, *B. Environ. Contam. Tox.*, 2020, **105**, 18–25.
141 G. J. Mahler, M. B. Esch, E. Tako, T. L. Southard, S. D. Archer, R. P. Glahn and M. L. Shuler, Oral exposure to polystyrene nanoparticles affects iron absorption, *Nat. Nanotechnol.*, 2012, **7**, 264–271.
142 G. F. Schirinzi, I. Pérez-Pomeda, J. Sanchís, C. Rossini, M. Farré and D. Barceló, Cytotoxic effects of commonly used nanomaterials and microplastics on cerebral and epithelial human cells, *Environ. Res.*, 2017, **159**, 579–587.
143 X. Yi, J. Wang, Z. Li, Z. Zhang, T. Chi, M. Guo, W. Li and H. Zhou, The effect of polystyrene plastics on the toxicity of triphenyltin to the marine diatom Skeletonema costatum—influence of plastic particle size, *Environ Sci Pollut Res*, 2019, **26**, 25445–25451.
144 J. Kleinteich, S. Seidensticker, N. Marggrander and C. Zarfl, Microplastics Reduce Short-Term Effects of Environmental Contaminants. Part II: Polyethylene Particles Decrease the Effect of Polycyclic Aromatic Hydrocarbons on Microorganisms, *IJERPH*, 2018, **15**, 287.
145 D. Lithner, A. Larsson and G. Dave, Environmental and health hazard ranking and assessment of plastic polymers based on chemical composition, *Sci. Tot. Environ.*, 2011, **409**, 3309–3324.
146 A. Letsou and D. Bohmann, Small flies - Big discoveries: Nearly a century of *Drosophila* genetics and development, *Dev. Dyn.*, 2005, **232**, 526–528.
147 T. E. Lloyd and J. P. Taylor, Flightless flies: *Drosophila* models of neuromuscular disease: Fly models of neuromuscular diseases, *Ann. NY Acad. Sci.*, 2010, **1184**, E1–E20.
148 Y. Apidianakis and L. G. Rahme, *Drosophila melanogaster* as a model for human intestinal infection and pathology, *Dis. Models Mech*, 2011, **4**, 21–30.

149 M. Gáliková and P. Klepsatel, Obesity and aging in the *Drosophila* model, *IJMS*, 2018, **19**, 1896.
150 C. Cirelli and D. Bushey, Sleep and wakefulness in *Drosophila melanogaster*, *Ann. NY Acad. Sci.*, 2008, **1129**, 323–329.
151 J. G. Scott and N. Buchon, *Drosophila melanogaster* as a powerful tool for studying insect toxicology, *Pestic. Biochem. Phys.*, 2019, **161**, 95–103.
152 C. Ong, L.-Y. L. Yung, Y. Cai, B.-H. Bay and G.-H. Baeg, *Drosophila melanogaster* as a model organism to study nanotoxicity, *Nanotoxicol.*, 2015, **9**, 396–403.
153 K. Weigmann, R. Klapper, T. Strasser, C. Rickert, G. Technau, H. Jäckle, W. Janning and C. Klämbt, FlyMove - a new way to look at development of *drosophila*, *Trends Genet*, 2003, **19**, 310.
154 M. Osterfield, C. A. Berg and S. Y. Shvartsman, Epithelial patterning, morphogenesis, and evolution: *Drosophila* eggshell as a model, *Dev. Cell.*, 2017, **41**, 337–348.
155 J. David, Y. Cohet and P. Fouillet, The variability between individuals as a measure of senescence: a study of the number of eggs laid and the percentage of hatched eggs in the case of *Drosophila melanogaster*, *Exp. Geront.*, 1975, **10**, 17–25.
156 M. D. Adams, S. E. Celniker, R. A. Holt, C. A. Evans, J. D. Gocayne, P. G. Amanatides, S. E. Scherer, P. W. Li, R. A. Hoskins, R. F. Galle, R. A. George, S. E. Lewis, S. Richards, M. Ashburner, S. N. Henderson, G. G. Sutton, J. R. Wortman, M. D. Yandell, Q. Zhang, L. X. Chen, R. C. Brandon, Y.-H. C. Rogers, R. G. Blazej, M. Champe, B. D. Pfeiffer, K. H. Wan, C. Doyle, E. G. Baxter, G. Helt, C. R. Nelson, G. L. Gabor, Miklos, J. F. Abril, A. Agbayani, H.-J. An, C. Andrews-Pfannkoch, D. Baldwin, R. M. Ballew, A. Basu, J. Baxendale, L. Bayraktaroglu, E. M. Beasley, K. Y. Beeson, P. V. Benos, B. P. Berman, D. Bhandari, S. Bolshakov, D. Borkova, M. R. Botchan, J. Bouck, P. Brokstein, P. Brottier, K. C. Burtis, D. A. Busam, H. Butler, E. Cadieu, A. Center, I. Chandra, J. M. Cherry, S. Cawley, C. Dahlke, L. B. Davenport, P. Davies, B. de Pablos, A. Delcher, Z. Deng, A. D. Mays, I. Dew, S. M. Dietz, K. Dodson, L. E. Doup, M. Downes, S. Dugan-Rocha, B. C. Dunkov, P. Dunn, K. J. Durbin, C. C. Evangelista, C. Ferraz, S. Ferriera, W. Fleischmann, C. Fosler, A. E. Gabrielian, N. S. Garg, W. M. Gelbart, K. Glasser, A. Glodek, F. Gong, J. H. Gorrell, Z. Gu, P. Guan, M. Harris, N. L. Harris, D. Harvey, T. J. Heiman, J. R. Hernandez, J. Houck, D. Hostin, K. A. Houston, T. J. Howland, M.-H. Wei, C. Ibegwam, M. Jalali, F. Kalush, G. H. Karpen, Z. Ke, J. A. Kennison, K. A. Ketchum, B. E. Kimmel, C. D. Kodira, C. Kraft, S. Kravitz, D. Kulp, Z. Lai, P. Lasko, Y. Lei, A. A. Levitsky, J. Li, Z. Li, Y. Liang, X. Lin, X. Liu, B. Mattei, T. C. McIntosh, M. P. McLeod, D. McPherson, G. Merkulov, N. V. Milshina, C. Mobarry, J. Morris, A. Moshrefi, S. M. Mount, M. Moy, B. Murphy, L. Murphy, D. M. Muzny, D. L. Nelson, D. R. Nelson, K. A. Nelson, K. Nixon, D. R. Nusskern, J. M. Pacleb, M. Palazzolo, G. S. Pittman, S. Pan, J. Pollard, V. Puri, M. G. Reese, K. Reinert, K. Remington, R. D. C. Saunders, F. Scheeler, H. Shen, B. C. Shue, I. Sidén-Kiamos, M. Simpson, M. P. Skupski, T. Smith, E. Spier, A. C. Spradling, M. Stapleton, R. Strong, E. Sun, R. Svirskas, C. Tector, R. Turner, E. Venter, A. H. Wang, X. Wang, Z.-Y. Wang, D. A. Wassarman, G. M. Weinstock, J. Weissenbach, S. M. Williams, T. Woodage, K. C. Worley, D. Wu, S. Yang, Q. A. Yao, J. Ye, R.-F. Yeh, J. S. Zaveri, M. Zhan, G. Zhang, Q. Zhao, L. Zheng, X. H. Zheng, F. N. Zhong, W. Zhong, X. Zhou, S. Zhu, X. Zhu, H. O. Smith, R. A. Gibbs, E. W. Myers, G. M. Rubin and J. C. Venter, The genome sequence of *Drosophila melanogaster*, *Science*, 2000, **287**, 2185.

157 M. Alaraby, B. Annangi, R. Marcos and A. Hernández, *Drosophila melanogaster* as a suitable *in vivo* model to determine potential side effects of nanomaterials: A review, *J Toxicol Environ Health B Crit Rev*, 2016, **19**, 65–104.
158 G. Vecchio, A. Galeone, V. Brunetti, G. Maiorano, S. Sabella, R. Cingolani and P. P. Pompa, Concentration-Dependent, Size-Independent Toxicity of Citrate Capped AuNPs in Drosophila melanogaster, *PLoS ONE*, 2012, **7**, e29980.
159 R. Marcos, L. M. Sierra and I. Gaivão, in *Genotoxicity and DNA Repair*, eds. L. M. Sierra and I. Gaivão, Humana Press, 2014, pp. 283–295.
160 H. R. Siddique, D. K. Chowdhuri, D. K. Saxena and A. Dhawan, Validation of *Drosophila melanogaster* as an *in vivo* model for genotoxicity assessment using modified alkaline Comet assay, *Mutagenesis*, 2005, **20**, 285–290.
161 I. Miguel-Aliaga, H. Jasper and B. Lemaitre, Anatomy and physiology of the digestive tract of *Drosophila melanogaster*, *Genetics*, 2018, **210**, 357–396.
162 E.-M. Ha, C.-T. Oh, Y. S. Bae and W.-J. Lee, A direct role for dual oxidase in *drosophila* gut immunity, *Science*, 2005, **310**, 5.
163 M. R. Freeman, *Drosophila* central nervous system glia, *Cold Spring Harb Perspect Biol*, 2015, **7**, a020552.
164 M. C. Kremer, C. Jung, S. Batelli, G. M. Rubin and U. Gaul, The glia of the adult *Drosophila* nervous system: Glia anatomy in adult *Drosophila* nervous system, *Glia*, 2017, **65**, 606–638.
165 D. Pauls, C. Blechschmidt, F. Frantzmann, B. el Jundi and M. Selcho, A comprehensive anatomical map of the peripheral octopaminergic/tyraminergic system of *Drosophila melanogaster*, *Sci Rep*, 2018, **8**, 15314.
166 J. Modolell, Patterning of the adult peripheral nervous system of *Drosophila*, *Perspect. Dev. Neurobiol.*, 1997, **4**, 285–296.
167 H. V. B. Hirsch, G. Lnenicka, D. Possidente, B. Possidente, M. D. Garfinkel, L. Wang, X. Lu and D. M. Ruden, *Drosophila melanogaster* as a model for lead neurotoxicology and toxicogenomics research, *Front. Gene.*, 2012, **3**, 68.
168 V. C. Moser, in *Comprehensive Toxicology*, Elsevier, 2010, pp. 337–350.
169 H. L. Needleman, Behavioral toxicology., *Environ Health Perspect.*, 1995, **103**, 77–79.
170 J. C. Chiu, K. H. Low, D. H. Pike, E. Yildirim and I. Edery, Assaying locomotor activity to study circadian rhythms and sleep parameters in *Drosophila*, *J. Vis. Exp.*, 2010, 2157.
171 T. J. Webster, D. Gorth and D. Rand, Silver nanoparticle toxicity in *Drosophila*: size does matter, *IJN*, 2011, 343.
172 A. Raj, P. Shah and N. Agrawal, Dose-dependent effect of silver nanoparticles (AgNPs) on fertility and survival of *Drosophila*: An *in-vivo* study, *PLoS ONE*, 2017, **12**, e0178051.
173 S.-E. A. Araj, N. M. Salem, I. H. Ghabeish and A. M. Awwad, Toxicity of nanoparticles against *Drosophila melanogaster* (Diptera: Drosophilidae), *J. Nanomater.*, 2015, **2015**, 1–9.
174 X. Liu, D. Vinson, D. Abt, R. H. Hurt and D. M. Rand, Differential toxicity of carbon nanomaterials in *Drosophila* : Larval dietary uptake is benign, but adult exposure causes locomotor impairment and mortality, *Environ. Sci. Technol.*, 2009, **43**, 6357–6363.
175 R. Posgai, C. B. Cipolla-McCulloch, K. R. Murphy, S. M. Hussain, J. J. Rowe and M. G. Nielsen, Differential toxicity of silver and titanium dioxide nanoparticles on *Drosophila melanogaster* development, reproductive effort, and viability: Size, coatings and antioxidants matter, *Chemosphere*, 2011, **85**, 34–42.

176 A. Panacek, R. Prucek, D. Safarova, M. Dittrich, J. Richtrova, K. Benickova, R. Zboril and L. Kvitek, Acute and chronic toxicity effects of silver nanoparticles (NPs) on *Drosophila melanogaster*, *Environ. Sci. Technol.*, 2011, **45**, 4974–4979.
177 T. Liu, L. Li, F. Zhang and Y. Wang, Transcriptional inhibition of the Catalase gene in phosphine-induced oxidative stress in *Drosophila melanogaster*, *Pestic. Biochem. Phys.*, 2015, **124**, 1–7.
178 R. Morimoto, Cells in stress: transcriptional activation of heat shock genes, *Science*, 1993, **259**, 1409–1410.
179 T. L. Parkes, A. J. Hilliker and J. P. Phillips, Genetic and biochemical analysis of glutathione- *S* -transferase in the oxygen defense system of *Drosophila melanogaster*, *Genome*, 1993, **36**, 1007–1014.
180 M. Ahamed, R. Posgai, T. J. Gorey, M. Nielsen, S. M. Hussain and J. J. Rowe, Silver nanoparticles induced heat shock protein 70, oxidative stress and apoptosis in *Drosophila melanogaster*, *Toxicol. Appl. Pharm.*, 2010, **242**, 263–269.
181 M. Alaraby, A. Hernández and R. Marcos, New insights in the acute toxic/genotoxic effects of CuO nanoparticles in the *in vivo Drosophila* model, *Nanotoxicology*, 2016, **10**, 749–760.
182 B. Wang, N. Chen, Y. Wei, J. Li, L. Sun, J. Wu, Q. Huang, C. Liu, C. Fan and H. Song, Akt signaling-associated metabolic effects of dietary gold nanoparticles in *Drosophila*, *Sci. Rep.*, 2012, **2**, 1–7.
183 G. P. Sykiotis and D. Bohmann, Keap1/Nrf2 Signaling regulates oxidative stress tolerance and lifespan in *Drosophila*, *Dev. Cell.*, 2008, **14**, 76–85.
184 K. Nilangekar, N. Murmu, G. Sahu and B. V. Shravage, Generation and characterization of germline-specific autophagy and mitochondrial reactive oxygen species reporters in *Drosophila*, *Front. Cell Dev. Biol.*, 2019, **7**, 47.
185 H. Khaira, T. Trinidad, J. Luu, S. Khan and N. Crockenberg, Measuring the effects of larval life stress on adult anxiety behavior in *Drosophila melanogaster*, *J. Biol. Sci.*, 2019, **5**, 6.
186 N. R. Bartholomew, J. M. Burdett, J. M. VandenBrooks, M. C. Quinlan and G. B. Call, Impaired climbing and flight behaviour in *Drosophila melanogaster* following carbon dioxide anaesthesia, *Sci Rep*, 2015, **5**, 15298.
187 M. N. Günther, G. Nettesheim and G. T. Shubeita, Quantifying and predicting *Drosophila* larvae crawling phenotypes, *Sci Rep*, 2016, **6**, 27972.
188 M. Miernicki, T. Hofmann, I. Eisenberger, F. von der Kammer and A. Praetorius, Legal and practical challenges in classifying nanomaterials according to regulatory definitions, *Nat. Nanotechnol.*, 2019, **14**, 208–216.
189 S. Karbalaei, P. Hanachi, T. R. Walker and M. Cole, Occurrence, sources, human health impacts and mitigation of microplastic pollution, *Environ. Sci. Pollut. R.*, 2018, **25**, 36046–36063.
190 F. Wang, F. Wang and E. Y. Zeng, in *Microplastic contamination in aquatic environments: An emerging matter of environmental urgency*, Elsevier, 2018, pp. 225–247.
191 J. Brahney, M. Hallerud, E. Heim, M. Hahnenberger and S. Sukumaran, Plastic rain in protected areas of the United States, *Science*, 2020, **368**, 1257–1260.
192 D. Briassoulis, M. Hiskakis, G. Scarascia, P. Picuno, C. Delgado and C. Dejean, Labeling scheme for agricultural plastic wastes in Europe: Labeling scheme for APW in Europe, *Qual. Assur. Saf. Crop.*, 2010, **2**, 93–104.
193 S. Fuller and A. Gautam, A Procedure for Measuring Microplastics using Pressurized Fluid Extraction, *Environ. Sci. Technol.*, 2016, **50**, 5774–5780.

194 M. C. Rillig and A. Lehmann, Microplastic in terrestrial ecosystems Research shifts from ecotoxicology to ecosystem effects and Earth system feedbacks, *Science*, 2020, **368**, 1430–1431.
195 M. C. Rillig, Plastic and plants, *Nat Sustain*, , DOI:10.1038/s41893-020-0583-9.
196 U. B. Pandey and C. D. Nichols, Human Disease Models in *Drosophila melanogaster* and the role of the fly in therapeutic drug discovery, *Pharmacol. Rev.*, 2011, **63**, 411–436.
197 M. Alaraby, B. Annangi, R. Marcos and A. Hernández, *Drosophila melanogaster* as a suitable *in vivo* model to determine potential side effects of nanomaterials: A review, *J. Toxicol. Env. Heal. B*, 2016, **19**, 65–104.
198 N. Agrawal and P. Shah, *Toxicology of nanoparticles: Insights from drosophila*, Springer, Singapore, 2020.
199 A. S. Anand, U. Gahlot, D. N. Prasad, Amitabh and E. Kohli, Aluminum oxide nanoparticles mediated toxicity, loss of appendages in progeny of *Drosophila melanogaster* on chronic exposure, *Nanotoxicology*, 2019, **13**, 977–989.
200 M. Alaraby, E. Demir, J. Domenech, A. Velázquez, A. Hernández and R. Marcos, *In vivo* evaluation of the toxic and genotoxic effects of exposure to cobalt nanoparticles using *Drosophila melanogaster*, *Environ. Sci.: Nano*, 2020, **7**, 610–622.
201 A. Pandey, S. Chandra, L. K. S. Chauhan, G. Narayan and D. K. Chowdhuri, Cellular internalization and stress response of ingested amorphous silica nanoparticles in the midgut of *Drosophila melanogaster*, *BBA-Gen. Subjects.*, 2013, **1830**, 2256–2266.
202 Y. Zhang, M. B. Wolosker, Y. Zhao, H. Ren and B. Lemos, Exposure to microplastics cause gut damage, locomotor dysfunction, epigenetic silencing, and aggravate cadmium (Cd) toxicity in *Drosophila*, *Sci. Tot. Environ.*, 2020, **744**, 140979.
203 M. D. Rand, S. L. Montgomery, L. Prince and D. Vorojeikina, in *Current Protocols in Toxicology*, eds. L. G. Costa, J. C. Davila, D. A. Lawrence and D. J. Reed, John Wiley & Sons, Inc., Hoboken, NJ, USA, 2014, p. 1.12.1-1.12.20.
204 W. Seifriz, Breakdown of fruit and vegetables tissues due to an electric current., *Plant Physiol.*, 1936, **11**, 195–200.
205 H. Hama, H. Hioki, K. Namiki, T. Hoshida, H. Kurokawa, F. Ishidate, T. Kaneko, T. Akagi, T. Saito, T. Saido and A. Miyawaki, ScaleS: an optical clearing palette for biological imaging, *Nat. Neurosci.*, 2015, **18**, 1518–1529.
206 Z. Durisko, R. Kemp, R. Mubasher and R. Dukas, Dynamics of social behavior in fruit fly larvae, *PLoS ONE*, 2014, **9**, e95495.
207 S. A. Pappus, B. Ekka, S. Sahu, D. Sabat, P. Dash and M. Mishra, A toxicity assessment of hydroxyapatite nanoparticles on development and behaviour of *Drosophila melanogaster*, *J. Nanopart. Res.*, 2017, **19**, 136.
208 B. Moreteau, S. R'Kha and J. R. David, Genetics of a nonoptimal behavior: Oviposition preference of *Drosophila mauritiana* for a toxic resource, *Behav. Genet.*, 1994, **24**, 433–441.
209 F. Ponton, M.-P. Chapuis, M. Pernice, G. A. Sword and S. J. Simpson, Evaluation of potential reference genes for reverse transcription-qPCR studies of physiological responses in *Drosophila melanogaster*, *J. Insect Physiol.*, 2011, **57**, 840–850.
210 K. Cichewicz and J. Hirsh, ShinyR-DAM: a program analyzing *Drosophila* activity, sleep and circadian rhythms, *Commun. Biol.*, 2018, **1**, 25.
211 R Core Team, *R: A language and environment for statistical computing.*, R Foundation for Statistical Computing, Vienna, Austria, 2016.

212 W. Venables and B. Ripley, *Modern Applied Statistics with S*, Springer, New York, NY, 4th edn., 2002.
213 G. OberdÃ¶rster, Safety assessment for nanotechnology and nanomedicine: concepts of nanotoxicology: Symposium: Nanotoxicological concepts, *J. Intern. Med.*, 2010, **267**, 89–105.
214 Y. Lu, Y. Zhang, Y. Deng, W. Jiang, Y. Zhao, J. Geng, L. Ding and H. Ren, Uptake and accumulation of polystyrene microplastics in zebrafish (*Danio rerio*) and toxic effects in liver, *Environ. Sci. Technol.*, 2016, **50**, 4054–4060.
215 A. Elizalde-Velázquez, A. M. Carcano, J. Crago, M. J. Green, S. A. Shah and J. E. Cañas-Carrell, Translocation, trophic transfer, accumulation and depuration of polystyrene microplastics in *Daphnia magna* and *Pimephales promelas*, *Environ. Pollut.*, 2020, **259**, 113937.
216 A. Dawson, W. Huston, S. Kawaguchi, C. King, R. Cropp, S. Wild, P. Eisenmann, K. Townsend and S. Bengtson Nash, Uptake and depuration kinetics influence microplastic bioaccumulation and toxicity in antarctic krill (*Euphausia superba*), *Environ. Sci. Technol.*, 2018, **52**, 3195–3201.
217 M. Al-Sid-Cheikh, S. J. Rowland, K. Stevenson, C. Rouleau, T. B. Henry and R. C. Thompson, Uptake, whole-body distribution, and depuration of nanoplastics by the scallop *Pecten maximus* at environmentally realistic concentrations, *Environ. Sci. Technol.*, 2018, **52**, 14480–14486.
218 N. Buchon, D. Osman, F. P. A. David, H. Yu Fang, J.-P. Boquete, B. Deplancke and B. Lemaitre, Morphological and molecular characterization of adult midgut compartmentalization in *Drosophila*, *Cell. Rep.*, 2013, **3**, 1725–1738.
219 A. Amcheslavsky, W. Song, Q. Li, Y. Nie, I. Bragatto, D. Ferrandon, N. Perrimon and Y. T. Ip, Enteroendocrine cells support intestinal stem-cell-mediated homeostasis in *Drosophila*, *Cell Rep.*, 2014, **9**, 32–39.
220 O. Pikuda, E. G. Xu, D. Berk and N. Tufenkji, Toxicity assessments of micro- and nanoplastics can be confounded by preservatives in commercial formulations, *Environ. Sci. Technol. Lett.*, 2019, **6**, 21–25.
221 M. Heinlaan, K. Kasemets, V. Aruoja, I. Blinova, O. Bondarenko, A. Lukjanova, A. Khosrovyan, I. Kurvet, M. Pullerits, M. Sihtmäe, G. Vasiliev, H. Vija and A. Kahru, Hazard evaluation of polystyrene nanoplastic with nine bioassays did not show particle-specific acute toxicity, *Sci. Total. Environ.*, 2020, **707**, 136073.
222 M. Okshevsky, E. Gautier, J. M. Farner, L. Schreiber and N. Tufenkji, Biofilm formation by marine bacteria is impacted by concentration and surface functionalization of polystyrene nanoparticles in a species-specific manner, *Env. Microbiol. Rep.*, 2020, **12**, 203–213.
223 X. Hu, W. Fu, X. Yang, Y. Mu, W. Gu and M. Zhang, Effects of cadmium on fecundity and defence ability of *Drosophila melanogaster*, *Ecotox. Environ. Safe.*, 2019, **171**, 871–877.
224 J. Yang and J. Tower, Expression of hsp22 and hsp70 transgenes is partially predictive of *Drosophila* survival under normal and stress conditions, *J. Gerontol. A. Biol.*, 2009, **64A**, 828–838.
225 P. B. Miller, O. T. Obrik-Uloho, M. H. Phan, C. L. Medrano, J. S. Renier, J. L. Thayer, G. Wiessner and M. C. Bloch Qazi, The song of the old mother: Reproductive senescence in female drosophila, *Fly*, 2014, **8**, 127–139.

226 T. D. Algarve, C. E. Assmann, T. Aigaki and I. B. M. da Cruz, Parental and preimaginal exposure to methylmercury disrupts locomotor activity and circadian rhythm of adult *Drosophila melanogaster*, *Drug Chem. Toxicol.*, 2020, **43**, 255–265.
227 J. Mast, C. Moraes, H. Alborn, L. Lavis and D. Stern, Evolved differences in larval social behavior mediated by novel pheromones, *eLife*, 2014, **3**, e.04205.
228 Z. Durisko and R. Dukas, Attraction to and learning from social cues in fruit fly larvae, *Proc. R. Soc. B*, 2013, **280**, 20131398.
229 M. B. Sokolowski, H. S. Pereira and K. Hughes, Evolution of foraging behavior in *Drosophila* by density-dependent selection, *Proc. Natl. Acad. Sci. USA*, 1997, **94**, 7373–7377.
230 R. K. Vijendravarma, S. Narasimha and T. J. Kawecki, Evolution of foraging behaviour in response to chronic malnutrition in *Drosophila melanogaster*, *Proc. R. Soc. B*, 2012, **279**, 3540–3546.
231 S. D. Mhatre, V. Satyasi, M. Killen, B. E. Paddock, R. D. Moir, A. Saunders and D. Marenda, Synaptic abnormalities in a *Drosophila* model of Alzheimer's disease, *Dis. Model. Mech.*, 2014, **7**, 373–385.
232 A. B. Pizzo, C. S. Karam, Y. Zhang, H. Yano, R. J. Freyberg, D. S. Karam, Z. Freyberg, A. Yamamoto, B. D. McCabe and J. A. Javitch, The membrane raft protein Flotillin-1 is essential in dopamine neurons for amphetamine-induced behavior in *Drosophila*, *Mol. Psychiatry.*, 2013, **18**, 824–833.
233 D. R. LaJeunesse, B. Johnson, J. S. Presnell, K. Catignas and G. Zapotoczny, Peristalsis in the junction region of the *Drosophila* larval midgut is modulated by DH31 expressing enteroendocrine cells, *BMC. Physiol.*, 2010, **10**, 14.
234 L. DeVault, T. Li, S. Izabel, K. L. Thompson-Peer, L. Y. Jan and Y. N. Jan, Dendrite regeneration of adult *Drosophila* sensory neurons diminishes with aging and is inhibited by epidermal-derived matrix metalloproteinase 2, *Genes Dev.*, 2018, **32**, 402–414.
235 S. Ghimire and M. S. Kim, Enhanced locomotor activity is required to exert dietary restriction-dependent increase of stress resistance in *Drosophila*, *Oxid. Med. Cell. Longev.*, 2015, **2015**, 1–8.
236 K. J. Livak, Q. F. Wills, A. J. Tipping, K. Datta, R. Mittal, A. J. Goldson, D. W. Sexton and C. C. Holmes, Methods for qPCR gene expression profiling applied to 1440 lymphoblastoid single cells, *Methods*, 2013, **59**, 71–79.
237 A. J. Rocha, R. de S. Miranda, A. J. S. Sousa and A. L. C. da Silva, in *Polymerase Chain Reaction for Biomedical Applications*, ed. A. Samadikuchaksaraei, InTech, 2016.

Appendix A: Supplementary Materials

Fluorescence imaging of plastic spheres in situ

Particle suspensions and spiked food media were prepared as described in the method section. Slides were prepared by spreading 2 µL aliquots of suspension or food media between a clean glass slide and coverslip. Slides were mounted on the stage of a fluorescence microscope (Olympus IX71) and monitored using a 40x objective (LUCPlanFI, numerical aperture 0.6, working distance 4 mm). Exposure time was 30 ms and 200 ms for red and green fluorescent images, respectively.

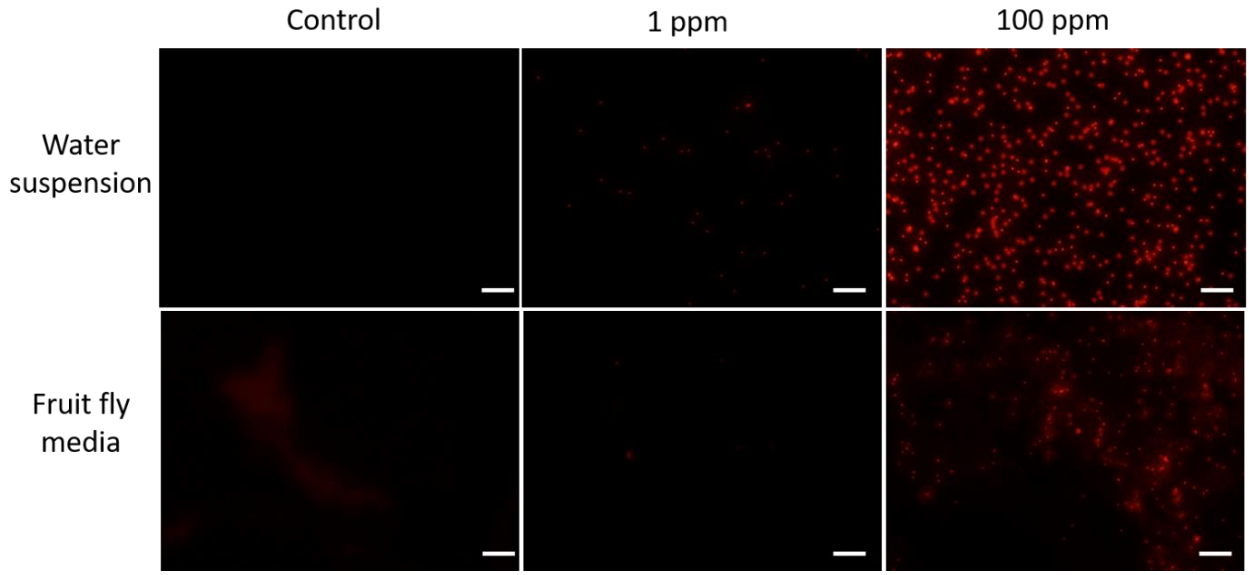


Figure S1. Fluorescence imaging of 1 µm red fluorescent carboxylated polystyrene spheres (ThermoFisher) in water suspension and mixed into Ward's Instant Drosophila Medium. The white scale bar represents 20 µm. Particles show strong fluorescence compared to fluorescence from food (seen in control) and are clearly visible. There is no significant change in particle shape or size when incorporated into food.

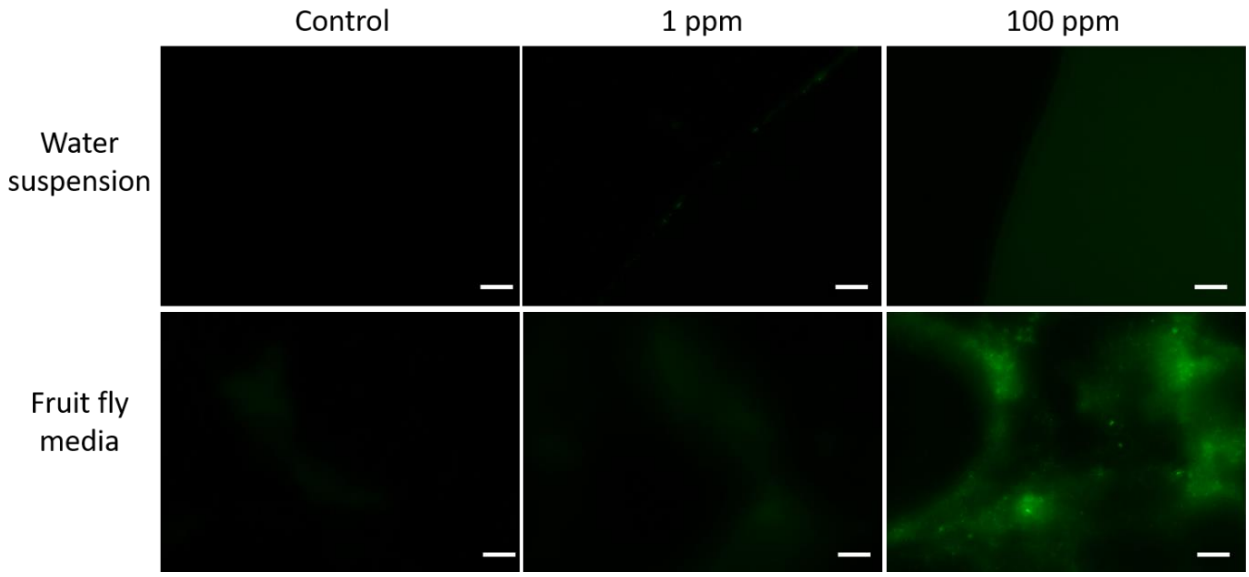


Figure S2. Fluorescence imaging of 20 nm green-yellow fluorescent carboxylated polystyrene spheres (ThermoFisher) in water suspension and mixed into Ward's Instant Drosophila Medium. The white scale bar represents 20 µm. Some aggregates of nanoplastics were observed at high concentrations when mixed into food media, with the largest aggregates being approximately 1µm.

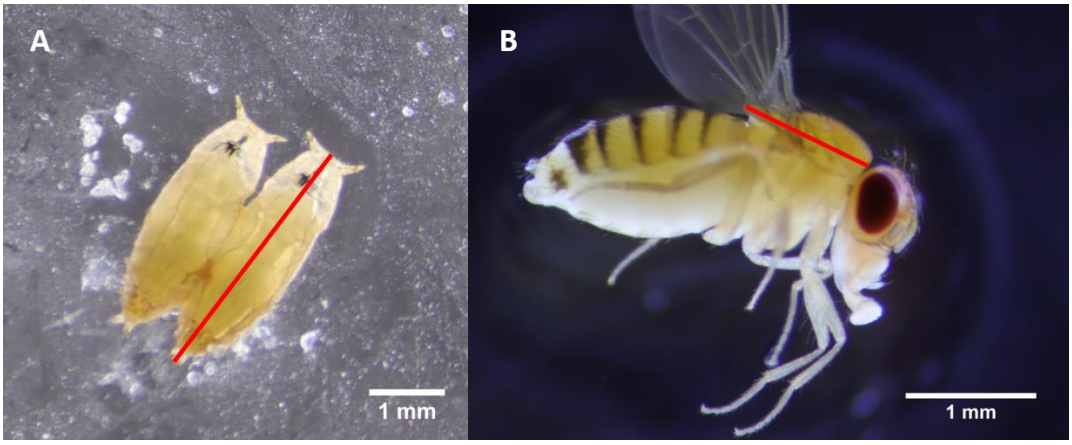


Figure S3. Body measurements were taken to compare the growth and development of treatment groups. **A)** The length of the pupal case was measured on day 6 of exposure, and **B)** the thorax length of adults on the final day.

Quantitative Real-Time PCR

Stress-response genes (*HSP70, CAT, SOD2*) and genotoxic stress response gene (*P53*) were selected, and primers were designed with Benchling (https://benchling.com) (**Table S1**) and targets were verified by NCBI Primer-Blast. Fifteen males and 15 females from each condition were stored in groups of 5 in RNA*later* (Thermo Fisher) at -4 °C, giving a total of 6 biological replicates per condition. Total RNA was isolated from flies using Purelink RNA Mini Kit (Thermo Fisher); the amount of RNA in each sample was determined using a spectrophotometer ($OD_{260/280}$ 1.8; concentration > 5 ng/uL), and the RNA quality was analyzed using agarose gel electrophoresis (1%) in TAE buffer prepared in-house. First-strand cDNA was prepared from 300-500 ng of total RNA using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher) in 20 µL reactions and then diluted to a concentration of 2 ng/µL with nuclease-free, RNAse free water. Real-time quantitative PCR (RT-PCR) was performed in triplicate for each sample with a 7900HT Fast Real-Time PCR System (Applied Biosystems) following manufacturer's instructions and using SYBR Green-based detection of PCR products. Melting curves were examined after amplification to exclude the presence of unspecific products. For each gene, 10 ng of cDNA was mixed with 10 µL of Power SYBR Green PCR Master Mix (Thermo Fisher), 1 µL of 1 mM of gene-specific primers, and 3 µL of water in a 394 well-plate. The RT-PCR was performed with the following protocol: 1 cycle of denaturation at 95 °C for 10 min; 40 cycles at 95 °C for 15 sec and 60 °C for 1 min. Following the amplification reaction, a melting curve program (60-95 °C) was carried out and fluorescence data were collected at 0.5 °C intervals. Relative expression was calculated through the $2^{-\Delta\Delta Ct}$ [236]method and normalized to the transcript levels for alpha-tubulin.

Table S1. Primers for quantitative real-time PCR.

Gene	**GeneBank Acc. No**	**F**	**R**	**Size**	**Average gene efficiency** [237]
HSP70	NM_169441.2	AGGGTCAGATCC ACGACATC	CGTCTGGGTTGATG GATAGG	117	97
CAT	NM_080483.3	GATGCGGCTTCC AATCAGTTG	GCAGCAGGATAGGT CCTCG	139	78
P53	NM_206544.2	TGCGGACACAAA TCGCAACTGCT	ACGACGCGGACTTG TGAAGACTC	79	105
SOD2	NM_057577.3	CTCCTGCCCTGC GTTTCA	GTCAGCGTGGTCAG CTCCTT	160	98
TUB	NM_057424	TGTCGCGTGTGA AACACTTC	AGCAGGCGTTTCCA ATCTG	96	86

Measuring daily activity and circadian rhythm

The daily activity and circadian rhythm of adult flies were assessed using a DAM2 Drosophila Activity Monitor (TriKinetics) following a protocol previously described by Chiu et al.[38] with some modifications. Food with 0, 10, 50, and 100 ppm microplastics or nanoplastics were prepared as previously described and approximately 0.6 mL was injected into glass tubes (5 mm × 65 mm, TriKinetics) with a small syringe before being sealed with parafilm. Two-day old adult male fruit flies were then anesthetized with carbon dioxide, placed in tubes individually, and capped with

100% cotton string to allow air circulation in the tube. Two monitors were loaded with 32 tubes (16 flies per treatment, 64 in total) and placed in an incubator at 25 ˚C, 60% humidity for 8 days. For the first 4 days, flies were subjected to 12 h/12 h light/dark cycle (LD) and then a 24 h dark cycle (DD) for the remaining 4 days. The dark conditions were created within the incubator by using a cardboard box with small ventilation holes. The light intensity in the incubator and in the dark box was 30 and 0 μmol/m^2s, respectively, when measured using a UV detector (Apogee, MU-200). An activity event was defined as a break in the infrared beam that crosses the center of the activity tube and the number of events was recorded over 1-min intervals. Data were analyzed using Shiny-R software[39] to extract daily activity accounts, sleep events, bout lengths, and circadian rhythms. The software used the standard definition of sleep as a continuous period of inactivity lasting at least 5 min and dead flies were defined as those with < 50 counts per day. The first day of both cycles was considered to be a period of acclimatization and excluded from the final statistical analysis along with data from dead flies.

Fluorescence imaging of live larvae. Fluorescent images were taken using an Olympus SZX16 stereomicrope at 2X magnification and GFP (5-SX810) or FRFP2 (5-SX822) filter. Images were captured with an EOS Rebel SL2 camera with ISO 800 and exposure time of 5 and 4 seconds for GFP and FRFP2 filters, respectively.

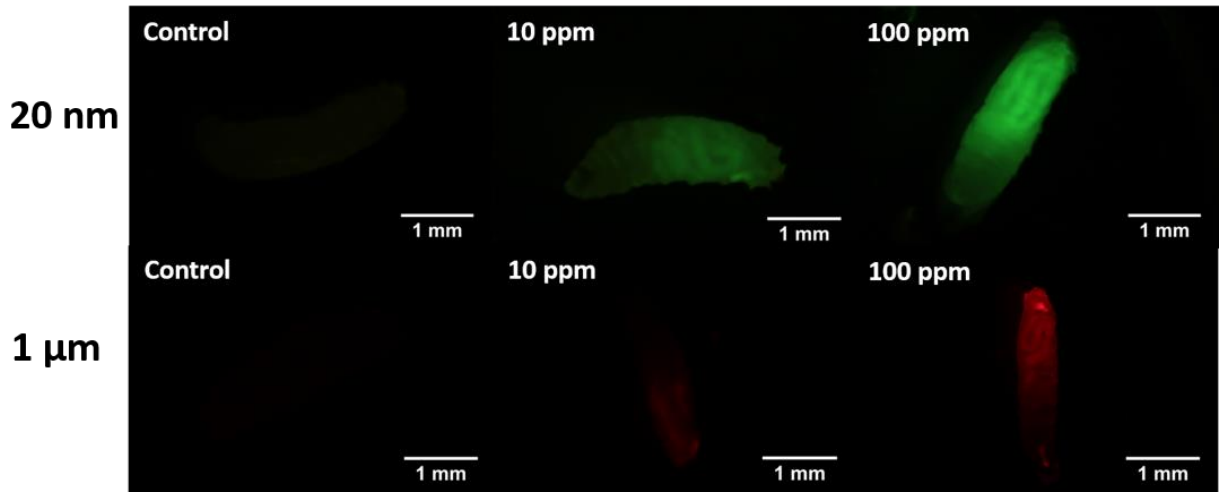


Figure S4. Fluorescence microscopy images of fruit fly larvae exposed to various concentrations of 20 nm yellow-green or 1 µm red fluorescent polystyrene spheres for 3 days.

Table S2. Mortality and development of *D. melanogaster*. The development and mortality of fruit flies exposed to nano- and microplastics were tracked by marking the timing and success of life-stage transitions, body size, and mortality. The average and standard deviations of the endpoints are shown in this table. No statistically significant difference was found between control and experimental groups in all conditions (ANOVA with post-hoc turkey test, significance p-value < 0.05).

	20 nm polystyrene						**1 µm polystyrene**					
Concentration (ppm)	**Control**	**0.01**	**1**	**10**	**50**	**100**	**Control**	**0.01**	**1**	**10**	**50**	**100**
Day of first pupae	4	4	4	5	4	4	4	5	5	4	4	4
Day of first adult emergence	9	9	9	9	8	8	7	8	9	8	9	9
Pupae length (mm) n=30	3.12 ±0.13	3.03 ±0.19	3.19 ±0.22	3.15 ±0.24	3.00 ±0.21	3.24 ±0.13	3.06 ±0.022	2.92 ±0.21	2.97 ±0.23	3.05 ±0.22	2.88 ±0.15	3.09 ±0.17
Female thorax length (mm) n=30	1.03 ±0.04	1.04 ±0.05	1.04 ±0.05	1.04 ±0.05	1.01 ±0.05	1.10 ±0.16	1.03 ±0.07	1.03 ±0.06	099 ±0.06	1.06 ±0.06	1.01 ±0.05	1.05 ±0.06
Male thorax length (mm) n=30	0.91 ±0.04	0.90 ±0.05	0.92 ±0.03	0.92 ±0.04	0.91 ±0.04	0.92 ±0.04	0.92 ±0.04	0.90 ±0.08	0.89 ±0.05	0.901 ±0.05	0.91 ±0.05	0.91 ±0.04
Eclosion rate (%) n=6	89 ± 7	93 ± 3	93 ± 5	96 ±2	97 ±2	97 ±2	97 ±4	97 ±5	93 ±3	97 ±1	98 ±3	98 ±2
Mortality (%) n=6	4 ± 3	4 ±3	5 ± 6	2 ±2	4 ± 4	4 ± 2	6 ±3	6 ±5	4 ±1	3 ±2	6 ±4	5 ±4

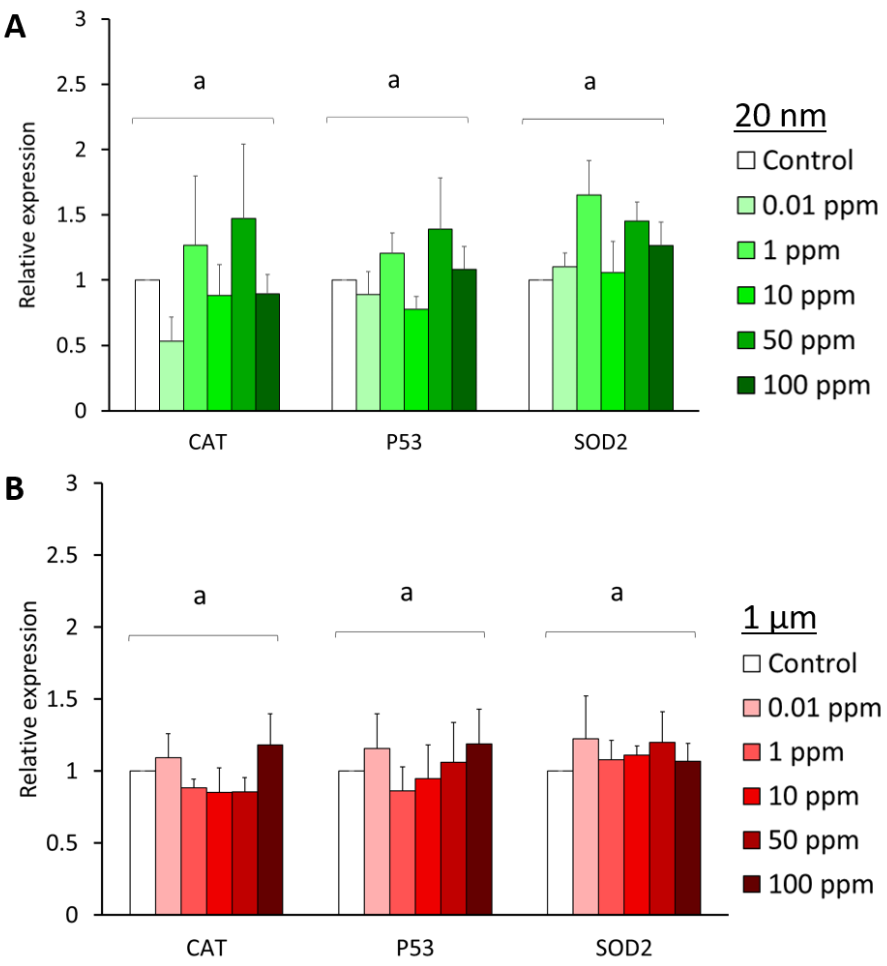


Figure S5. Gene expression in fruit flies. The relative abundance of stress-response genes (*CAT*, *SOD2*) and genotoxicity (*P53*) was determined from total RNA extracts of groups of 5 individuals per replicate (n = 6). Tubulin served as the reference gene. There were no significant differences in one-way ANOVA ($P > 0.05$).

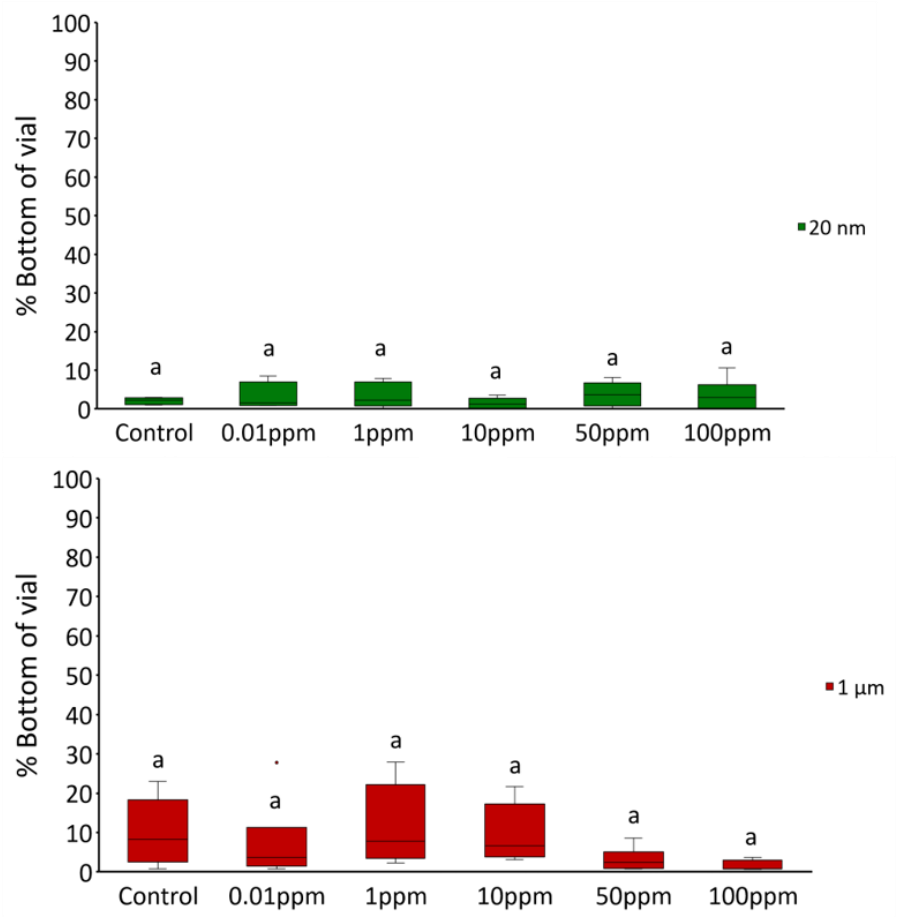


Figure S6. The locomotion of adult *D. melanogaster* was assessed via climbing assays after exposure to 20 nm green or 1 µm red fluorescent polystyrene spheres for 13 days (larvae to adult). There were no significant differences in one-way ANOVA ($P > 0.05$).

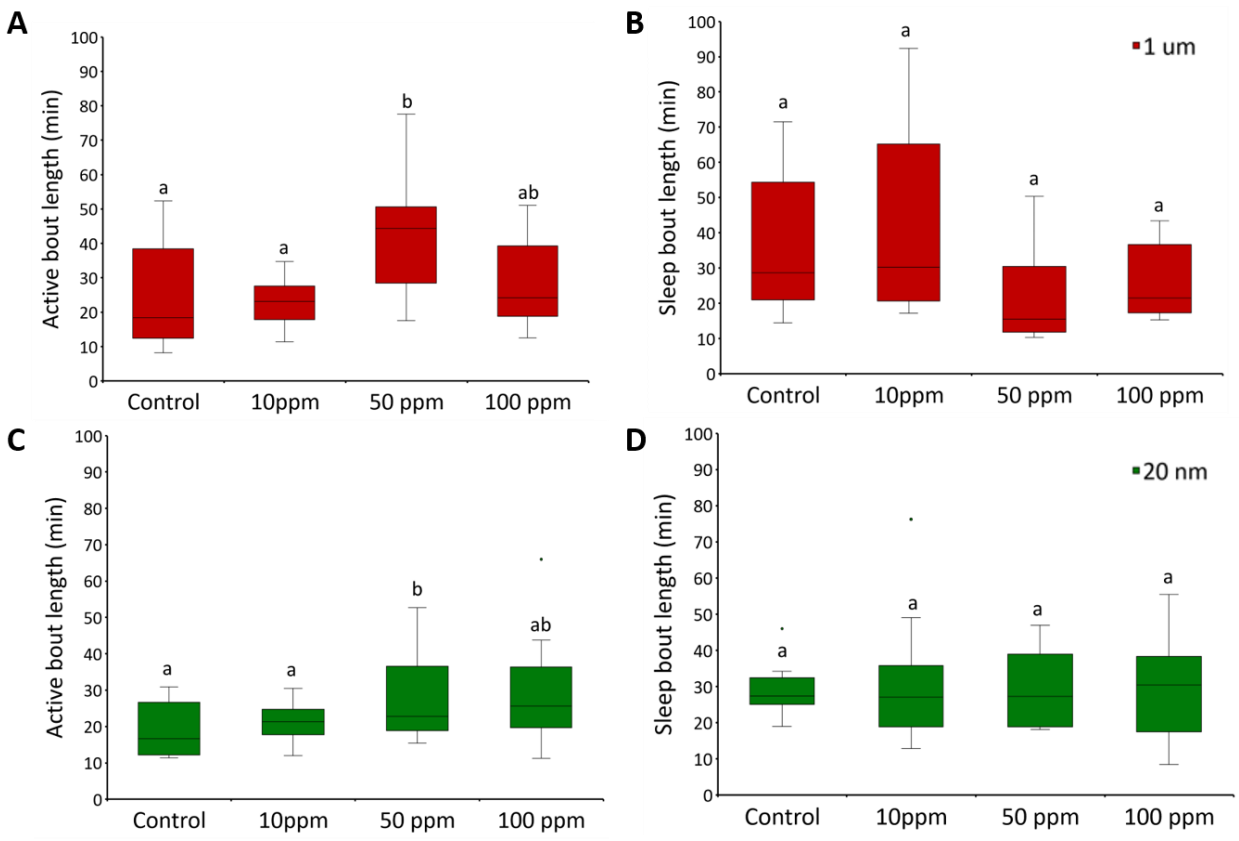


Figure S7. Activity and sleep bout lengths. The daily activity of two-day-old adult male fruit flies was recorded by a Drosophila Activity Monitor 2 with activity counts being recorded in 1 min time intervals. The raw data were processed with Shiny-R software[39] to extract individual (**A and C**) daily active bout lengths and (**B and D**) sleep bout lengths (≥ 5 min of inactivity). Letters correspond to significant differences according to one-way ANOVA with concentration as factors, n=16 for each concentration. There is a range of sample size as flies that died during the experiment were excluded from the analysis. Mortality was ≤ 12% in all controls.

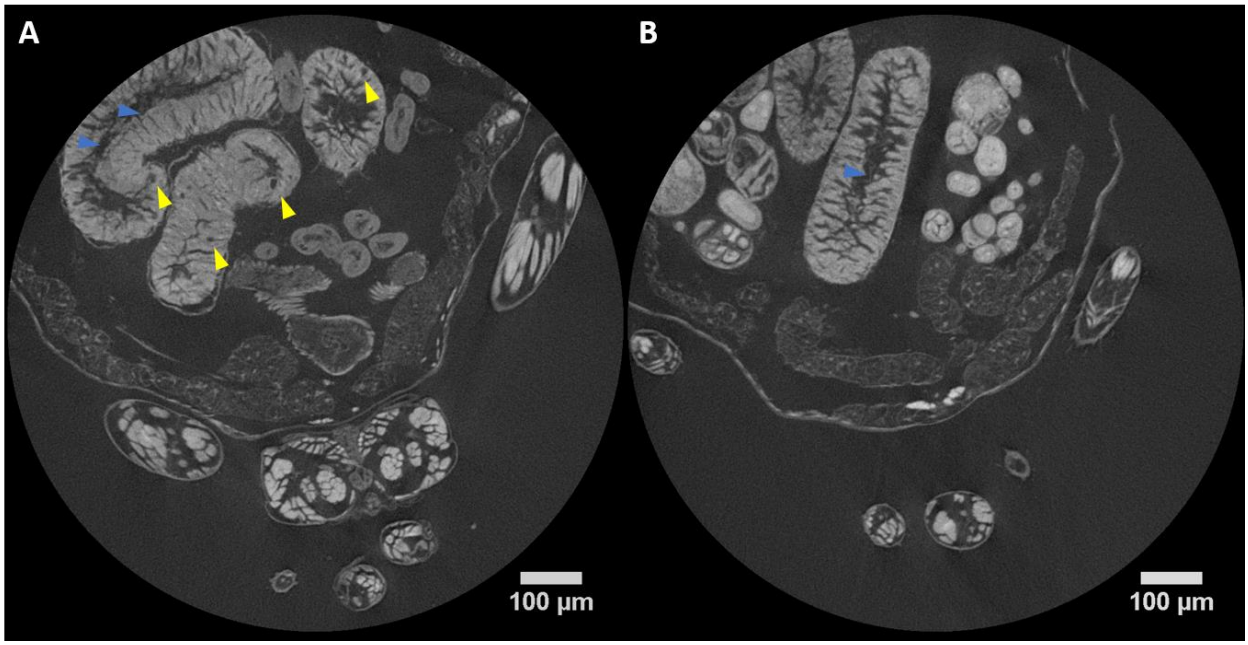


Figure S8. Computed tomography (CT) scanned images of **A)** midgut and **B**) hindgut adult female fruit flies from the control of developmental assay, which consisted of a 13-day exposure to 20 nm dialyzed yellow-green fluorescent polystyrene particles from larval to adult stage. Yellow arrows indicated secretory enteroendocrine cells and blue, the peritrophic matrix. Scan was acquired at 1 µm resolution with 4x objective lens with 2x2 camera binning, LE1 filter with 2.7 sec exposure. 2401 projections were taken over a 360 degree-rotation scan, at 60 kVp and 82 µA.

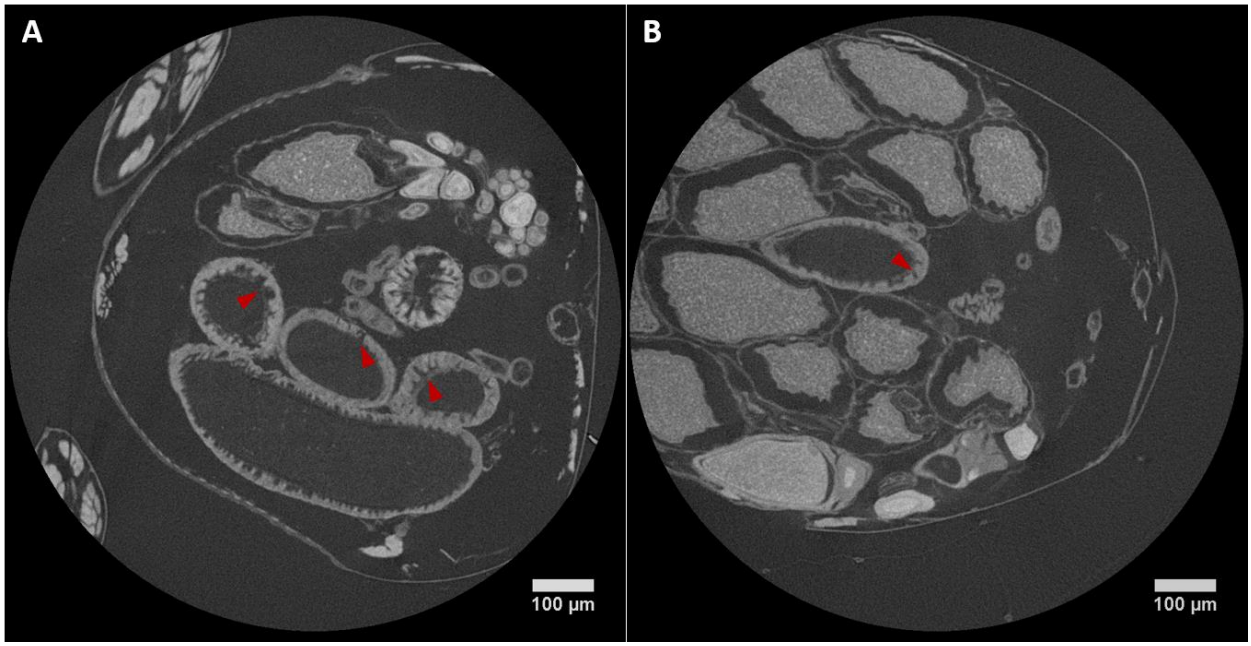


Figure S9. Computed tomography (CT) scanned images of **A)** midgut and **B**) hindgut adult female fruit flies from 100 ppm treatment of developmental assay, which consisted of a 13-day exposure to 20 nm dialyzed yellow-green fluorescent polystyrene particles from larval to adult stage. There are signs of intestinal damage such as a thinner epithelial layer, a lack of secretory enteroendocrine cells and peritrophic matrix, and deep crypts which are indicated by red arrows. Scan was acquired at 1 µm resolution with 4x objective lens with 2x2 camera binning, LE2 filter with 3.5 sec exposure. 2401 projections were taken over a 360 degree-rotation scan, at 60 kVp and 82 µA.

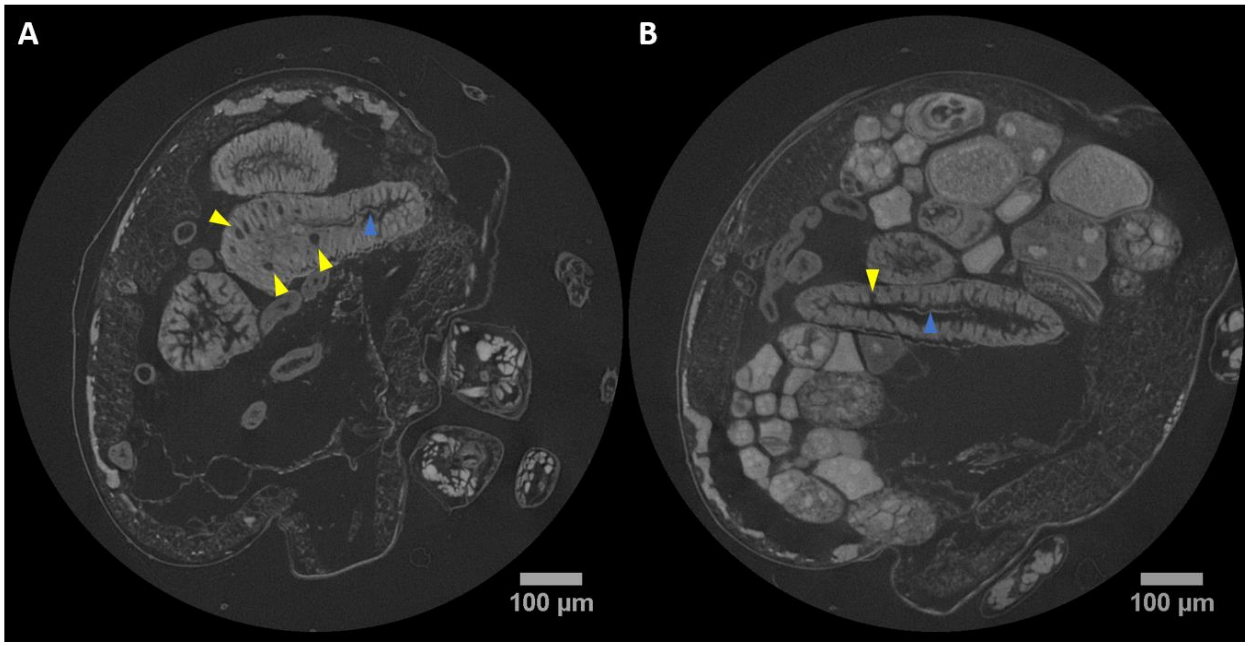


Figure S10. Computed tomography (CT) scanned images of **A)** midgut and **B**) hindgut adult female fruit flies from the control of developmental assay, which consisted of a 13-day exposure to 1 µm dialyzed red fluorescent polystyrene particles from larval to adult stage. Yellow arrows indicated secretory enteroendocrine cells and blue, the peritrophic matrix. Scan was acquired at 1 um resolution with 4x objective lens with 2x2 camera binning, LE1 filter with 2.7 sec exposure. 2401 projections were taken over a 360 degree-rotation scan, at 60 kVp and 82 µA.

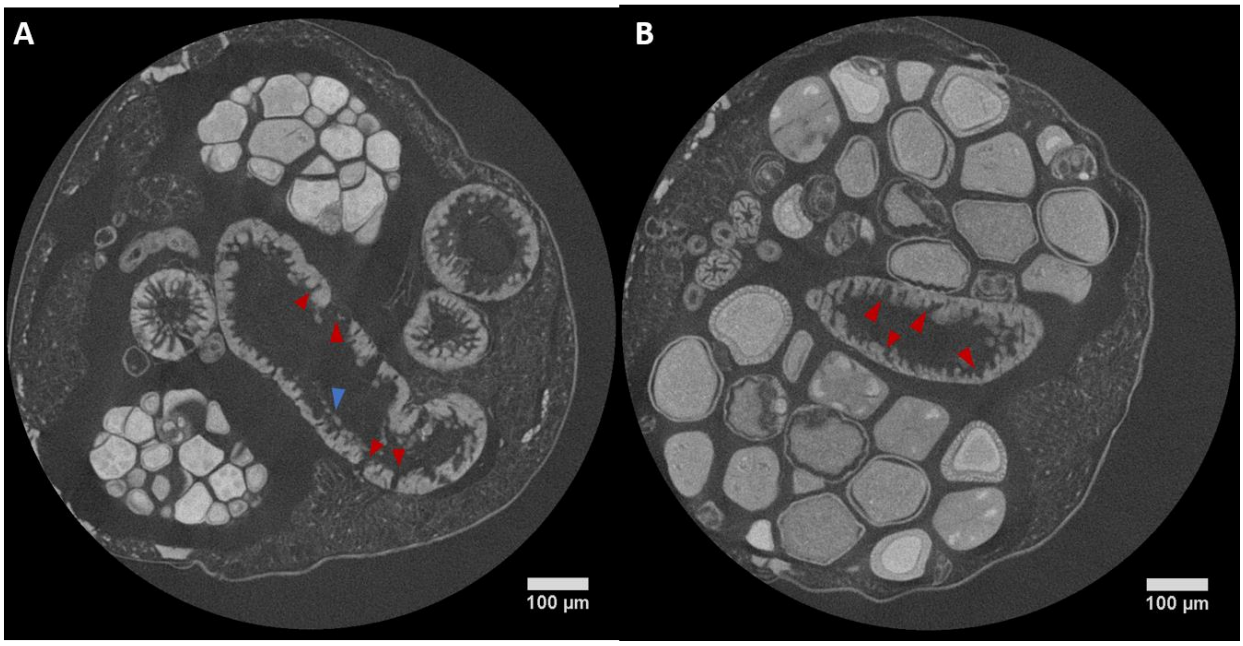


Figure S11. Computed tomography (CT) scanned images of **A)** midgut and **B**) hindgut adult female fruit flies from 100 ppm of developmental assay, which consisted of a 13-day exposure to 1 µm dialyzed red fluorescent polystyrene particles from larval to adult stage. Blue arrows indicated the peritrophic matrix. There are signs of intestinal damage such as a thinner or missing epithelial layer, shrunken enteroendocrine cells and peritrophic matrix, and deep crypts which are indicated by red arrows. Scan was acquired at 1 um resolution with 4 x objective lens with 2 x 2 camera binning, LE1 filter with 2.7 sec exposure. 2401 projections were taken over a 360 degree-rotation scan, at 60 kVp and 82 µA.

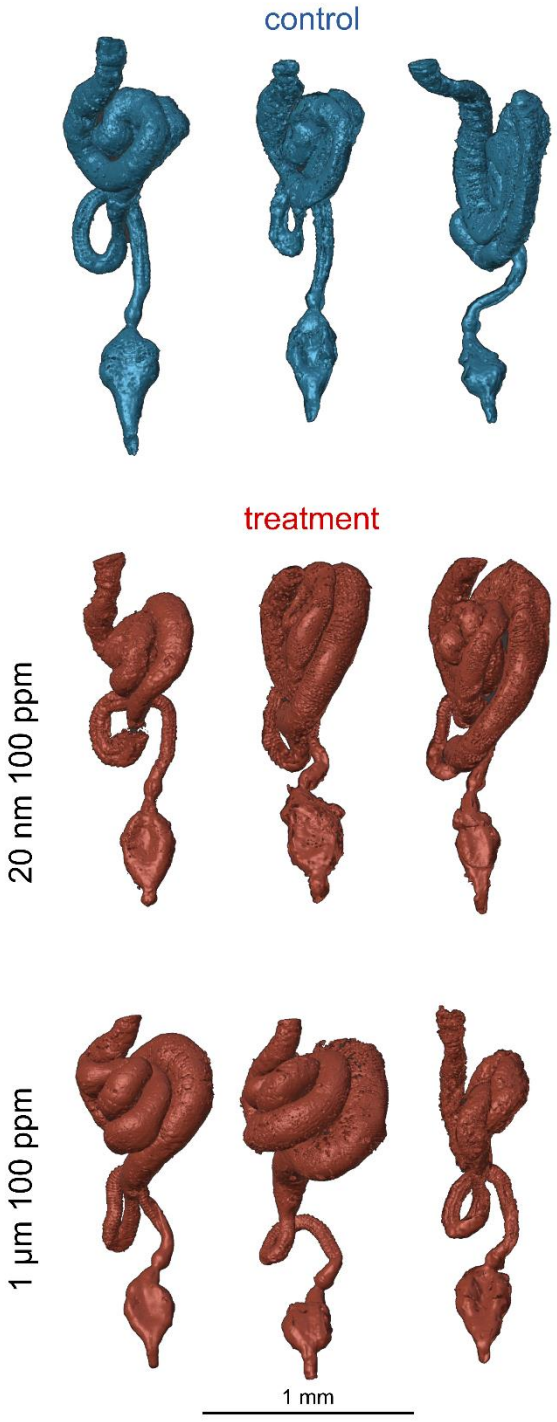


Figure S12. Reconstructed adult gastrointestinal tracts of *D. melanogaster* exposed to 100 ppm concentrations of 20 nm or 1 µm polystyrene particles. GITs are represented to the same scale from the anterior midgut to the ampulla and shown in ventral view with anterior toward the top.

Table S3. Summary table of the P-values and significance levels (*,**, ***) for factorial ANOVAs testing the effects of the variables Treatment (*i.e.* particle concentrations, 1-way) and Time (*i.e.* duration of exposure, 2-ways) for two particle sizes, 20 and 1000nm. *F* = Fischer calculated values, followed by degree of freedom and residual degree of freedom. Significance was set at $P < 0.05$.

Endpoint	Factor	20nm	1000nm
Fertility - Offspring	Treatment Time	$F_{5,201} = 9.48$, $P = 0.136$ $F_{4,\ 201} = 0.842$, $P = 0.660$	$F_{5,\ 219} = 4.754$, $P = 0.000382$ *** $F_{4,\ 219} = 41.122$, $P <$ 2e-16 ***
Ind.Larv.Crawl	Treatment	$F_{5,\ 160} = 1.777$, $P = 0.12$	$F_{5,\ 165} = 11.12$, $P =$ 3.01e-09 ***
Group.Larv.Crawl	Treatment	$F_{5,\ 160} = 14.89$, $P =$ 5.47e-12 ***	$F_{5,\ 167} = 15.6$, $P =$ 1.38e-12 ***
Climbing - Pass	Treatment	$F_{5,\ 25} = 4.458$, $P = 0.00485$ **	$F_{5,\ 28} = 2.268$, $P = 0.075$
Climbing - Fail	Treatment	$F_{5,\ 25} = 0.53$, $P = 0.752$	$F_{5,\ 28} = 2.868$, $P = 0.0326$ *
Daily activity count	Treatment	$F_{3,\ 43} = 4.128$, $P = 0.0117$ *	$F_{3,\ 55} = 5.108$, $P = 0.00344$ **
Total sleep average	Treatment	$F_{3,\ 55} = 2.506$, $P = 0.0685$	$F_{3,\ 41} = 9.845$, $P =$ 5.13e-05 ***
Sleep bout length	Treatment	$F_{3,\ 43} = 1.835$, $P = 0.155$	$F_{3,\ 55} = 0.363$, $P = 0.78$
Activity bout length	Treatment	$F_{3,\ 55} = 3.23$, $P = 0.0292$ *	$F_{3,\ 43} = 5.71$, $P = 0.0221$ **
HSP70	Treatment	$F_{5,\ 22} = 0.383$, $P = 0.855$	$F_{5,\ 26} = 2.883$, $P = 0.0335$ *
CAT	Treatment	$F_{5,\ 24} = 2.131$, $P = 0.0962$	$F_{5,\ 21} = 0.701$, $P = 0.629$
P53	Treatment	$F_{5,\ 23} = 0.220$, $P = 1.353$	$F_{5.\ 21} = 0.363$, $P = 0.868$
SOD	Treatment	$F_{5,\ 23} = 1.926$, $P = 0.129$	$F_{5,\ 21} = 0.09$, $P = 0.993$

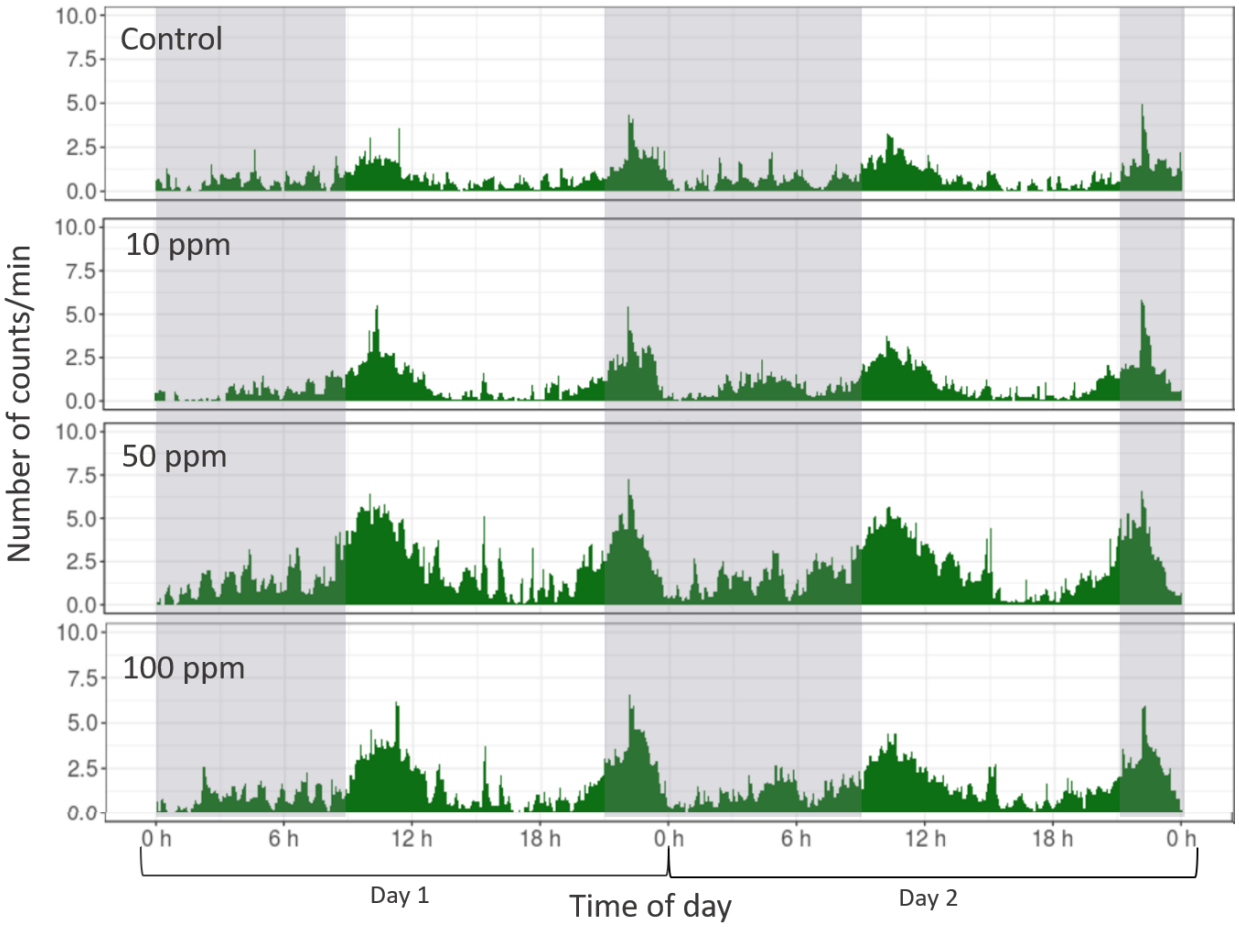


Figure S13. Actograms of two-day-old adult male fruit flies exposed to various concentration of 20 nm yellow-green fluorescent polystyrene spheres. The activity was recorded by a Drosophila Activity Monitor 2 in 1 min time intervals. Light periods are indicated by a white background and dark periods by grey. There were 16 flies in each treatment but there is a range of sample size as flies that died during the experiment were excluded from the analysis.

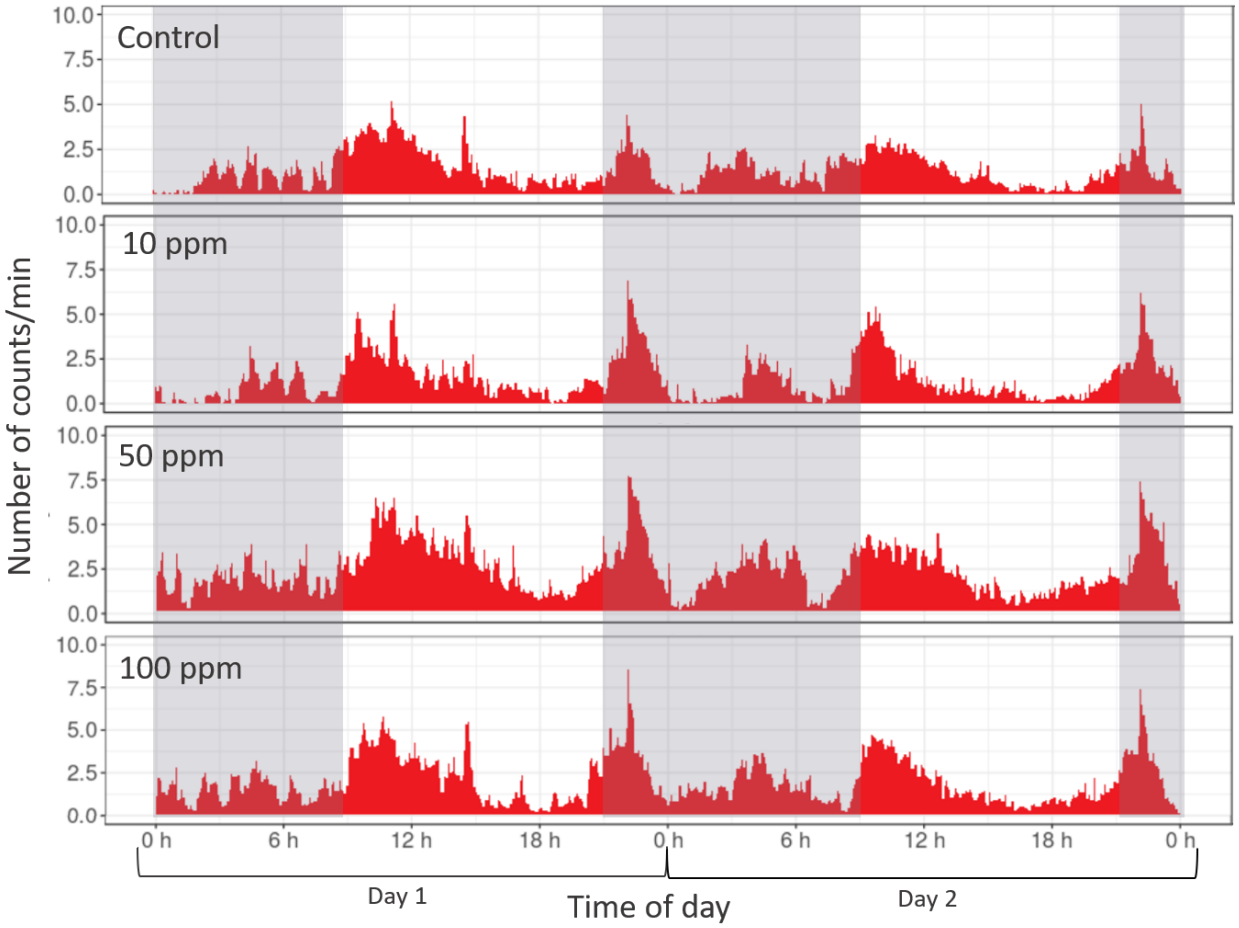


Figure S14. Actograms of two-day-old adult male fruit flies exposed to various concentration of 1 µm red fluorescent polystyrene spheres. The activity was recorded by a Drosophila Activity Monitor 2 in 1 min time intervals. Light periods are indicated by a white background and dark periods by grey. There were 16 flies in each treatment but there is a range of sample size as flies that died during the experiment were excluded from the analysis.

Appendix B – Particle Number Concentration Calculation

The commercial spherical particles were delivered in a 2% (w/v), or 20 000 ppm (mg/L), solutions. These particles suspensions are highly monodisperse and so the particle concentrations were calculated based on the density of polystyrene (1050 kg/m^3) and the volume of single spherical particle as follows:

$$Particle\ number\ concentration\ \left(\frac{particle}{mL}\right) = \frac{mass\ concentgration\ (\frac{kg}{mL})}{volume\ \frac{1}{6}\pi d^3\ (m^3) \times particle\ density\ (\frac{kg}{m^3})}$$

An example calculation using 1 µm polystyrene particles at 100 ppm (100 mg/L) concentration would be as follows:

$$Particle\ number\ concentration\ \left(\frac{particle}{mL}\right) = \frac{100\ (\frac{mg}{L})(\frac{1L}{1000\ mL})}{(\frac{1}{6}\pi\ (1 \times 10^{-6}m)^3) \times 1050\ (\frac{kg}{m^3})}$$

$$Particle\ number\ concentration\ \left(\frac{particle}{mL}\right) = \ 1.8\ \times 10^8 \frac{particles}{mL}$$

For 20 nm polystyrene particles at 100 ppm (100 mg/L) concentration would be as follows:

$$Particle\ number\ concentration\ \left(\frac{particle}{mL}\right) = \frac{100\ (\frac{mg}{L})(\frac{1L}{1000\ mL})}{(\frac{1}{6}\pi\ (20 \times 10^{-9}m)^3) \times 1050\ (\frac{kg}{m^3})}$$

$$Particle\ number\ concentration\ \left(\frac{particle}{mL}\right) = \ 2.27\ \times 10^{13} \frac{particles}{mL}$$