Title

Microplastic (1 and 5 μm) exposure disturbs lifespan and intestine function in the nematode Caenorhabditis elegans

Authors

Shang, Xu
Lu, Jiawei
Feng, Cheng
et al.

Publication Date

2020-02-01

DOI

10.1016/j.scitotenv.2019.135837

Peer reviewed

Science of the Total Environment 705 (2020) 135837

Contents lists available at ScienceDirect

Science of the Total Environment

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

Microplastic (1 and 5 μm) exposure disturbs lifespan and intestine function in the nematode *Caenorhabditis elegans*

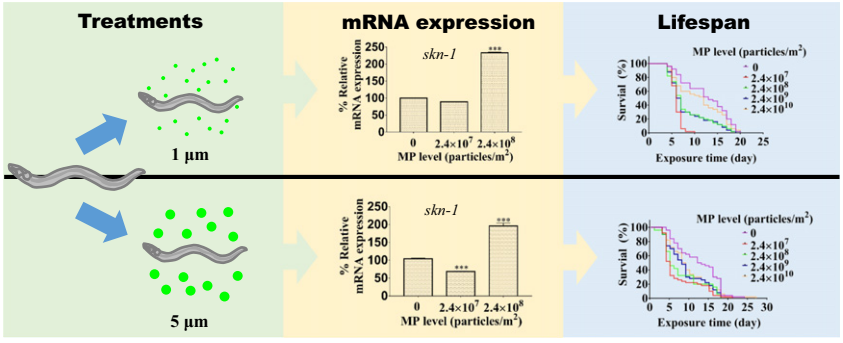
Xu Shang [a,b], Jiawei Lu [a], Cheng Feng [a], Yimeng Ying [a], Yuanchen He [a], Sheng Fang [a], Ying Lin [a], Randy Dahlgren [b,c], Jingjuan Ju [a,b,*]

[a] Department of Preventive Medicine, School of Public Health and Management, Wenzhou Medical University, Wenzhou, Zhejiang Province 325035, China
[b] Key Laboratory of Watershed Sciences and Health of Zhejiang Province, School of Public Health and Management, Wenzhou Medical University, Wenzhou 325035, China
[c] Department of Land, Air and Water Resources, University of California Davis, CA 95616, USA

HIGHLIGHTS

- Toxicological effects of 1 and 5 μm microplastics (MPs) (10^7–10^{10} particles/m^2) were studied on *Caenorhabditis elegans*.
- Intake of 1 and 5 μm MPs increased as exposure concentrations and duration increased.
- The lifespan of nematodes at lower concentration decreased faster than those at higher concentration after exposure to MPs.
- Down-regulated expression of *skn-1* at the lowest exposure concentration may be responsible for the shortened lifespan.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 22 July 2019
Received in revised form 27 November 2019
Accepted 27 November 2019
Available online 30 November 2019

Editor: Damia Barcelo

Keywords:
C. elegans
Microplastics
Lifespan
Defecation
Toxicity

ABSTRACT

As an emerging environmental pollutant, microplastics (MPs) are increasingly viewed as a serious health concern to terrestrial and aquatic ecosystems. However, previous toxicological studies examining MPs on freshwater and terrestrial organisms provide contradictory results, possibly due to few investigations at environmentally relevant concentrations. Here, the nematode *Caenorhabditis elegans* (*C. elegans*), a model organisms with both aquatic and terrestrial free-living forms, was employed to investigate the effects of 1 and 5 μm MPs (10^7–10^{10} particles/m^2) on the intake, lifespan, defecation rhythm, defecation-related neurons and transcriptional expression of related genes (*skn-1*, *mkk-4*, *pmk-1*, *cpr-1* and *itr-1*). We demonstrated that the percentage of MP-contaminated nematodes increased with increasing exposure concentrations and duration. The lifespan of nematodes in the lower concentration exposure groups (2.4×10^7 and 2.4×10^8 particles/m^2) decreased more prominently than that of higher concentration groups (2.4×10^9 and 2.4×10^{10} particles/m^2) after a 72-h exposure period. Concomitantly, expression of the *skn-1* gene, involved in detoxification and lifespan regulation, was significantly altered at lower MP concentrations. Physiologically, the defecation rhythm after a 72-h exposure period was most strongly affected by 1 μm MPs at 2.4×10^8 particles/m^2. The significant up-regulation of related genes by 1 μm MPs appears responsible for the shortened defecation interval. Results of this study identified a potential toxicity threat to *C. elegans* from exposure to MPs at environmentally relevant concentrations and provide novel evidence for MP risks to freshwater and terrestrial organisms.
Capsule.

* Corresponding author at: Department of Preventive Medicine, School of Public Health and Management, Wenzhou Medical University, Wenzhou, Zhejiang Province 325035, China.
E-mail address: jjj0810@wmu.edu.cn (J. Ju).

https://doi.org/10.1016/j.scitotenv.2019.135837

After exposure to 1 and 5 μm MPs (10^7–10^{10} particles/m^2), the lifespan of *C. elegans* decreased more rapidly at lower concentrations.

1. Introduction

Approximately 8.3 billion metric tons of plastics haves been manufactured since plastic production was introduced in the 1950s (Geyer et al., 2017). Because of its low recyclability (<10% of plastic products are recycled), ubiquitous use in society, poor waste management and persistence in the environment, plastic pollution has been designated as "one of the biggest environmental challenges of this lifetime" (UNEP, 2018). Plastic polymer debris with a ≤5-mm diameter is termed microplastics (MPs) and has received considerable attention in recent years as they are easily ingested by organisms, thus presenting the possibility for toxicity-related exposure (David et al., 2009; Foley et al., 2018). The common plastics productions include polyethylene (PE), polypropylene (PP), polystyrene (PS), polyvinylchloride (PVC) and so on (Geyer et al., 2017). Plastic debris is often categorized as primary, in which the particle was manufactured as a MP, or as secondary, in which the particles formed from the degradation of macroplastics (>5 mm) (Andrady, 2017; Cole et al., 2011). Primary MPs are typically manufactured in the micron size range in form of acrylic or polyester beads and fibers, plastic pre-production pellets ('nurdles'), polyethylene 'microbeads', etc., and are commonly used as abrasives in sandblasting, personal care products, medical products, etc. (Browne et al., 2011; Horton et al., 2017; Rochman et al., 2013). Secondary MPs vary in form and origin, and often result from the breakdown of macro- and larger microplastics through ultraviolet irradiation, microbial, and/or physical degradation processes (Andrady, 2011; David et al., 2009).

Although the majority of research to date has examined plastics in marine systems, an increasing number of studies show a prevalence of MPs in freshwater systems as well. So far, knowledge about the toxicity of MPs can be diverse in varying size, shape, composition, exposure concentrations, surfactants and contaminants sorbed to their surfaces, etc. MPs may accumulate in the digestive system of various organisms, while smaller particles (nanoplastics less than ~100 nm) may enter the blood or lymph circulation through the digestive system (da Costa et al., 2016; Triebskorn et al., 2019). MP exposure results in a wide variety of toxic insults from feeding disruption to reproductive performance, disturbances to energy metabolism, changes in liver physiology, and synergistic/antagonistic actions from hydrophobic organic contaminants sorbed to their surfaces (Horton et al., 2017; Rochman et al., 2013; Anbumani and Kakkar, 2018). However, some studies found minimal or no hazardous effects from MP exposure to isopods, fishes and earthworms (Giedrė et al., 2018; Kokalj et al., 2017; Wang et al., 2019). Although many environmental and biological variables can cause discrepancies among MP toxicity studies, some of these discrepancies may result from the wide range of exposure concentrations found in the natural environment, as many studies utilized very high MP exposure concentrations in laboratory simulation studies (Horton et al., 2017).

Environmental pollutants are usually most toxic at high doses, and the toxic effects tend to increase progressively with increasing concentration. However, some environmental pollutants exhibit elevated toxicity at lower exposure concentrations, which has raised considerable concern for chronic ecotoxicology of these compounds within the food web (Vandenberg et al., 2012), especially for endocrine-disrupting chemicals such as BPA (vom Saal and Hughes, 2005). Most previous studies of MP toxicity on animals documented higher toxicities at higher MP concentrations. At high MP concentrations (10^8–10^{18} particles/m^3), generally higher than ambient environmental conditions (0–10^6 particles/m^3), several studies found that feeding, development and reproduction of copepods were negatively affected (Cole et al., 2015; Lee et al., 2013), but other studies found minimal effects (Beiras et al., 2018). Other studies using low MP exposure concentrations (10^5–10^7 particles/m^3) showed little effect on aquatic animals (Guven et al., 2018; Kaposi et al., 2014). However, the ingestion of plastic debris at environmentally relevant concentrations (10^4–10^5 particles/m^3) was shown to alter endocrine system function in adult fish raising concerns for low-level exposure (Rochman et al., 2014). In total, these studies indicate some discrepancies in the dose effect - toxicity response of MPs highlighting the need for further research.

Nematodes are a diverse animal phylum inhabiting a broad range of terrestrial and aquatic environments. Their numerical dominance, diversity of lifecycles, and presence at various trophic levels make them an important component in many food webs and ecosystems (Lorenzen, 1994). This is especially true for benthic communities in marine and freshwater systems where MPs tend to accumulate by sedimentation (Wang et al., 2018). Among nematodes, *C. elegans* is a free-living, transparent nematode having several advantages as a model organism owing to its small size (~1 mm), rapid development (3–4 days), moderate lifespan (2–3 weeks), ease of cultivation and observation, and complete sequencing of its genome. Its transparent body is highly conducive for observing the ingestion of MPs, the genetic background is well-described, and mutant and transgenic nematodes are readily available and highly homologous to humans facilitating elucidation of molecular mechanisms (Brenner, 1974; Leung et al., 2008; Michaelson, 2001). *C. elegans* has been used to assess the toxicity of many environmental pollutants, and shows a low-dose response to several chemicals (Huang et al., 2016; Jingjuan et al., 2013; Ju et al., 2014; Zhou et al., 2016). *C. elegans* was used to assess the toxicity of nano- and microplastics from 0.1 to 5 μm in size in a few previous studies. Nanoplastics ingestion by *C. elegans* induced a functional deficit in the intestinal barrier, toxicity effects in combination with titanium dioxide, and transgenerational toxicity at environmental concentrations (Dong et al., 2018; Li et al., 2017; Qu et al., 2018). Although 1 and 5 μm MPs resulted in higher toxicity with respect to most endpoints of survival, development, and motor-related neurons than nanoplastics (≤100 nm) in nematodes (Lei et al., 2018a; Lei et al., 2018b), information concerning low-dose effects of MPs with contrasting size on *C. elegans* is very limited.

To address this knowledge gap, this study employed *C. elegans* to investigate the effects of 1 and 5 μm polystyrene spheres over a wide concentration range (2.4×10^7, $\times 10^8$, $\times 10^9$, $\times 10^{10}$ particles/m^2) for 72 h (chronic exposure duration for *C. elegans*). Polystyrene (PS) microplastics were one of most commonly used and detected plastics in the environment (Geyer et al., 2017; Andrady, 2011). Lifespan analysis and defecation rhythm were assessed to evaluate toxicity at the individual level. Motor neurons response and transcriptional expression of five genes associated with lifespan and defecation in the intestinal tract of *C. elegans* were determined to gain a mechanistic understanding of the response to MP exposure.

2. Materials and methods

2.1. Strains

Wild *C. elegans* type N2, EG1285 [unc-47:: GFP + lin-15(+)] and *E. coli* OP50 were purchased from the Caenorhabditis Genetics Center (University of Minnesota, USA). Nematodes were maintained on nematode growth medium (NGM) plates seeded with OP50 at 20 °C (Brenner, 1974). Age-synchronized eggs were obtained as described

by Ju et al. (2013). L3 larvae were acquired by further incubation on NGM for 36 h and used as the starting stage for exposure studies.

2.2. Preparation of exposure plates with MPs

Stock solutions of 1 and 5 μm fluorescent, polystyrene spheres were purchased from Da'e Technology Co. (Tianjin, China). A scanning electron microscope (SEM) (SURPRA 55, ZEISS, Germany) was used to confirm the size and morphologies of the 1 and 5 μm MPs (Fig. S1A~B). Fourier-transform infrared spectroscopy (FTIR) (Nicolet iN10 MX, Thermo Scientific, USA) was used to analyze and confirm the polymeric composition of the MPs (Fig. S1C).

E. coli OP50 solution was used to prepare dilutions of four MP exposure concentrations (0.01, 0.1, 1 and 10% (v/v)), corresponding to MP surface area concentrations of ~2.4×10^7, 2.4×10^8, 2.4×10^9, and 2.4×10^{10} particles/m^2. The lower MP concentrations (10^7 particles/m^2) used in this study fall within the upper concentration limit for MPs (10^7 particles/m^2) reported for riverine sediment samples providing environmental relevance to our study design (Rowshyra et al., 2014; Wang et al., 2018). In addition, as MPs reported in these studies only quantified particles sizes >20 μm and size-frequency spectra were skewed towards small particles, the total MP concentrations in these studies may be under-estimated due to the prevalence of smaller MPs in riverine sediment samples (Goldstein et al., 2013).

All MP stock and exposure solutions were sonicated for 30 min immediately prior to use. No new particles were detected by SEM after sonication. A 100-μL volume of exposure solution was placed on a 3.5 cm NGM plate, dried and sterilized by ultraviolet irradiation for 20 min in a vertical clean bench (DL-CJ-2NDI, Beijing HDL Apparatus Co., China). MP distribution was observed using a fluorescent microscope (Leica DM4000M, Germany) and quantified by Leica LAS Image Analysis (Leica Microsystems, Germany). A 100-μL sonicated *E. coli* OP50 solution was used as a blank control. The MP distribution on the exposure plates showed a relatively uniform dispersal of MP particles.

2.3. Intake of MPs

The MPs showed a green fluorescence when ingested within *C. elegans* and when present in excreted fecal materials allowing quantification using a fluorescent stereo microscope (M165 FC, Leica, Germany). Ingestion of MPs by nematodes was observed 24, 48, 72 and 96 h after exposure. *C. elegans* containing any number of MPs were regarded as contaminated nematodes, and results were reported as the percentage of contaminated nematodes to the total number of nematodes in each treatment. A total of 20 nematodes were used in each treatment group and each treatment contained three replicates.

2.4. Lifespan analysis

C. elegans lifespan was assessed after exposing L3 larvae to 1 and 5 μm MPs at concentrations of 0, 2.4×10^7, 2.4×10^8, 2.4×10^9 and 2.4×10^{10} particles/m^2 for 72 h. A total of 50 nematodes were used in each treatment group and each treatment contained three replicates. The date of L3 larvae transfer was recorded as Day 0, and the number of living nematodes was recorded each day. The experiment was terminated when all nematodes within a given treatment had died. The date of the last death was recorded as maximum survival length and the number of living nematodes was recorded each day to calculate an average daily survival metric for each treatment group.

2.5. Analysis of defecation rhythm

Defecation rhythm of nematodes was recorded as previously described by Ju et al. (2014). The defecation behavior was divided into four stages (Fig. S2); the contraction of the tail muscles can be clearly seen in the pBoc stage. Using a 50× magnification stereo microscope, the interval between two pBocs was recorded as the defecation interval. After exposing to 0, 2.4×10^7 and 2.4×10^8 particles/m^2 of 1 and 5 μm MPs for 72 h, 10 nematodes were used for each treatment group and each treatment contained two replicates.

2.6. Neuronal analysis

The motor neurons in AVL (in the head) and DVB (in the tail) stimulate enteric muscles during the defecation cycle by releasing γ-aminobutyric acid (GABA) (Walker et al., 2017). Green fluorescent protein (GFP) in the AVL and DVB was imaged using the EG1285 mutant (Fig. S3). The EG1285 mutant was synchronized and cultivated to L3 larvae and then exposed to 1 μm MPs at concentrations of 2.4×10^7 and 2.4×10^8 particles/m^2. After a 72-h exposure, 20 nematodes were photographed in each exposure and control group, and the average fluorescence density of the two neurons was analyzed separately by Image Pro Plus (Media Cybernetics, Rockville, MD, USA).

2.7. Quantitative real-time PCR

The transcriptional expression was analyzed for five genes associated with lifespan and defecation in the intestinal tract of *C. elegans*: *skn-1*, *mkk-4*, *pmk-1*, *cpr-1* and *itr-1*. *Skn-1* is involved in detoxification and lifespan regulation in nematodes (Blackwell et al., 2015). *Mkk-4* and *pmk-1* are involved in the intestinal intercellular MAPK signaling pathway (Altun and Hall, 2009a), while *cpr-1* and *itr-1* regulate the function of the foregut and hindgut, respectively (Altun and Hall, 2009a; Baylis and Vázquez-Manrique, 2012). L3 larvae of N2 were exposed to 1 and 5 μm MPs at concentrations of 2.4×10^7 and 2.4×10^8 particles/m^2 for 72 h. Total RNA for each treatment and control group was extracted according to standard protocols (Invitrogen Trizol reagent, Carlsbad, CA, USA). The quantity and quality of RNA were measured with a Nanodrop-200 spectrophotometer (Nanodrop, Thermo Scientific, Waltham, MA, USA). One to 2 μg of total RNA were used for reverse transcription to synthesize cDNA using Takara RR037A reverse transcriptase (Takara Bio, Shiga, Japan). Real-time PCR was performed on the reverse transcription products using a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and Toyobo SYBR® Green Realtime PCR Master Mix Code No. QPK-201 (Toyobo, Osaka, Japan). The primers used in this study were synthesized by BGI (Shenzhen, China) and are shown in Table S1. Relative RNA expression levels were determined using the $2^{-\Delta\Delta Ct}$ method (Pereira et al., 2012). Three replicates were conducted for each control and treatment group and the *act-1* gene was used as a reference control.

2.8. Statistics

All statistical analyses were performed using SPSS 20.0 (IBM, Armonk, NY, USA), with the exception of the intake modeling. Data are presented as mean ± SE or percentages (if not otherwise indicated). Differences among groups were assessed using ANOVA. A two-way ANOVA was used to assess treatment combinations of particle size and exposure concentrations. Mean differences among exposure and control groups, or different particle sizes, were determined by LSD. If particle size × exposure concentration interactions were statistically significant, the independent effect was shown. Normality and equal variance assumptions were tested before statistical analysis. If assumptions were not met, the non-parametric Kruskal-Wallis H test and Kruskal-Wallis pairwise comparison tests were used to assess differences between treatment groups. Statistical significance was considered as $p \leq .05$.

The intake modeling was conducted with SPSS 23.0 (IBM, Armonk, NY, USA), Empower(R) (www.empowerstats.com, X&Y Solutions, Boston MA, USA) and R software. The primary hypothesis that the proportion of MP-containing nematodes changes as the exposure concentration, particle size and duration increase was tested by fitting

a generalized additive mixed-effects model via maximum likelihood with the percentage of MP-containing nematodes as the outcome.

3. Results

3.1. MP intake by C. elegans

Intake of MPs by *C. elegans* was assessed by examining the percentage of MP-containing nematodes at different exposure concentrations, MP size and times. The proportion of nematodes containing 1 and 5 μm MPs increased with increasing exposure concentration and duration (Fig. 1). After a 72-h exposure to 1 μm MPs, the percentage of nematodes containing MPs increased with increasing MP concentrations (2.4×10^7, $\times 10^8$, $\times 10^9$, $\times 10^{10}$ particles/m^2) reaching 48.3, 73.3, 96.7 and 100.0%, respectively, while the control group was 0%. A similar dose-response was found for 5 μm MPs with the percentage of nematodes containing MPs for the low to high MP levels attaining 47.5, 67.5, 87.5 and 97.5%, respectively (Fig. 1).

The generalized additive mixed-effects model described the data well indicating increased MP-containing nematode percentages of 36.9, 53.1, 79.2, and 90.6%, with increasing MP exposure concentrations (2.4×10^7, $\times 10^8$, $\times 10^9$, $\times 10^{10}$ particles/m^2), respectively ($P < .0001$). Increasing exposure time from 24 h to 48, 72 and 96 h increased the percentage of MP-containing nematodes from 30.8% to 48.0, 62.0 and 67.0%, respectively ($P < .0001$). There was no significant difference in percentage of MP-containing nematodes between the 1 and 5 μm MP size fractions ($P = .58$) (Table 1).

3.2. MP effect on C. elegans lifespan

Compared with the control group, *C. elegans* lifespan was shortened in both 1 and 5 μm MP exposure groups, except for the highest MP concentration (2.4×10^{10} particles/m^2) (Fig. 2A and B). The life expectancy of nematodes in the 2.4×10^7 particles/m^2 exposure group with 1 μm MPs was the shortest among 1 μm treatments with an average lifespan of 6.0 ± 0.2 d, which was 52.4% lower than the control group 12.6 ± 0.7 d ($P < .001$) (Fig. 2C). The lifespan of nematodes in the group with 2.4×10^7 particles/m^2 of 5 μm MP was the shortest among 5 μm treatments with average lifespans of 7.0 ± 0.7 d (Fig. 2C). When comparing between 1 and 5 μm MP treatments, no statistical difference was found (Fig. 2C).

3.3. MP effects on defecation rhythm

Exposure to 1 and 5 μm MPs at different concentrations showed some effects on defecation rhythm of nematodes. After a 72-h exposure to 1 μm MPs, the defecation rhythm was affected at 2.4×10^8 particles/m^2, with a shorter defecation interval (48.0 ± 1.2 s) compared to the control (53.3 ± 2.2 s) ($P < .05$). In contrast, the 72-h exposure to 5 μm MPs at 2.4×10^8 particles/m^2 showed an increased defecation interval (57.3 ± 1.4 s) relative to the 1 μm treatment, but the defecation interval was not significantly different from the control (Fig. 3).

Table 1
Intake ratio analysis using a generalized additive mixed-effects model.

		Difference (95% CI)	*P* value
(Intercept)		−0.201 (−0.301, −0.100)	0.0002
Exposure concentration (particles/m^2)	0	-	-
	2.4×10^7	0.369 (0.253, 0.485)[a]	<0.0001
	2.4×10^8	0.531 (0.415, 0.647)[a]	<0.0001
	2.4×10^9	0.792 (0.676, 0.908)[a]	<0.0001
	2.4×10^{10}	0.906 (0.790, 1.022)[a]	<0.0001
Exposure duration (h)	24	-	-
	48	0.172 (0.098, 0.246)[b]	<0.0001
	72	0.312 (0.238, 0.386)[b]	<0.0001
	96	0.362 (0.288, 0.436)[b]	<0.0001
Particle size (μm)	1	-	-
	5	−0.021 (−0.094, 0.053)[c]	0.58

[a] Compared to the control group.
[b] Compared to 24 h group.
[c] Compared with 1 μm MP group.

3.4. MP effects on fluorescence expression of defecation-related neurons

After exposure to 1 μm MPs for 72 h, mean fluorescence intensities of the AVL at concentrations of 2.4×10^7 and 2.4×10^8 particles/m^2 showed an increasing tendency, and the difference between the 2.4×10^7 particles/m^2 group and the control group was statistically significant ($P < .05$). However, mean fluorescence intensities of the DVB were not significantly different between the exposure and control groups (Fig. 4).

3.5. MP effects on expression of lifespan and defecation-related mRNA in the intestinal tract of C. elegans

L3 larvae exposed to 1 and 5 μm MPs at concentrations of 2.4×10^7 and 2.4×10^8 particles/m^2 for 72 h showed differences in gene expression for all 5 genes with respect to both MP size and concentration (Fig. 5). For the 1-μm exposure treatment, the expression of *skn-1* showed a nearly significant decrease at 2.4×10^7 particles/m^2 ($P = .051$ vs control) and a large increase at 2.4×10^8 particles/m^2. The 5-μm treatments showed a similar response as the 1-μm treatment with the expression level for *skn-1* decreasing at 2.4×10^7 particles/m^2 and

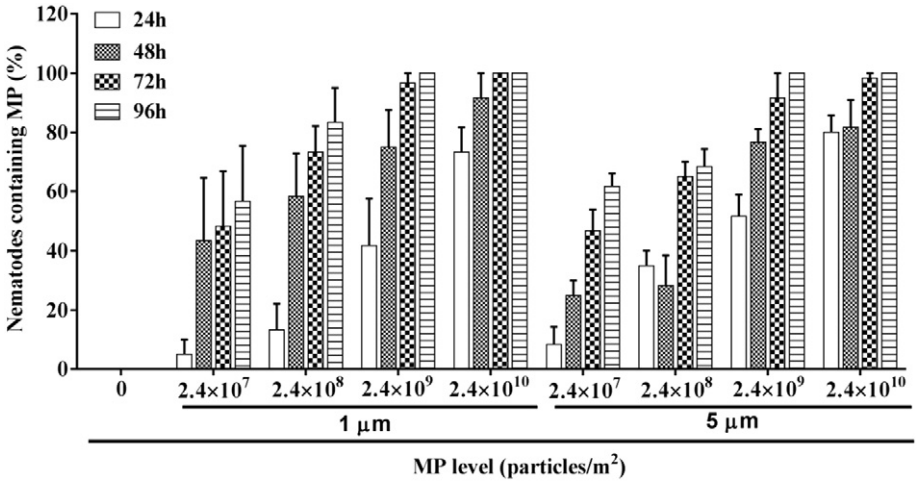


Fig. 1. Intake of 1 and 5 μm MPs after a 24, 48, 72 and 96 h exposure period.

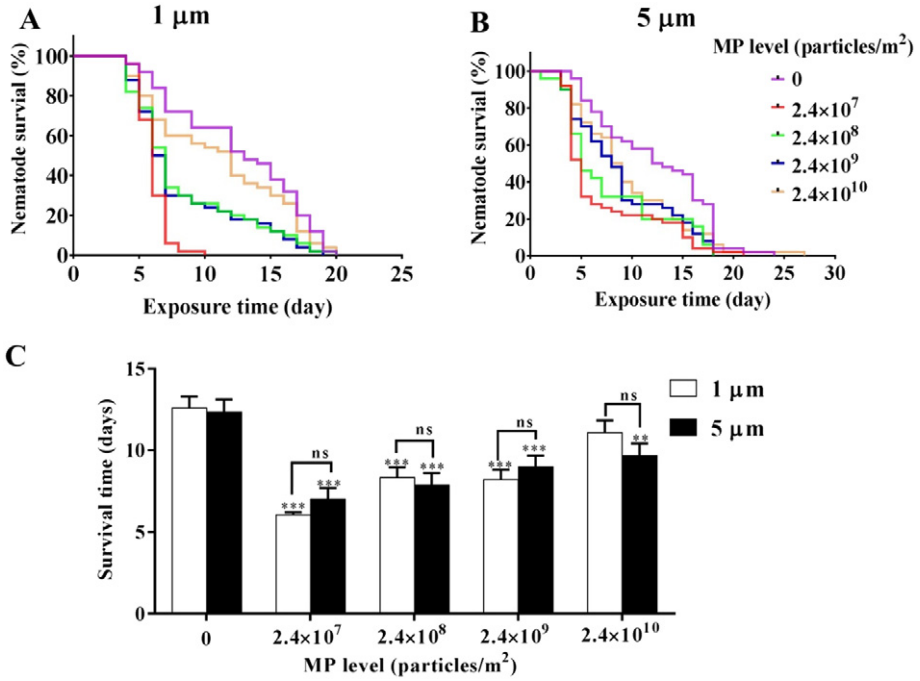


Fig. 2. Changes in lifespan due to exposure to 1 and 5 μm MPs. A: lifespan curves for 1 μm MPs after 72 h exposure; B: lifespan curves for 5 μm MPs after 72 h exposure; C: nematode survival after 72 h exposure to 1 and 5 μm MPs. **: $P < .01$, ***: $P < .001$; ns: no significant difference, vs the control (if not specifically indicated).

increasing at 2.4×10^8 particles/m^2. Expression levels for *skn-1* were significantly lower for the 5-μm vs 1-μm treatment at both MP concentrations.

The expression levels for *pmk-1* showed similar trends for 1 and 5 μm MPs with decreased expression levels at 2.4×10^7 particles/m^2 and increased expression levels at 2.4×10^8 particles/m^2 relative to the control. For both MP concentrations, the *pmk-1* expression was higher for the 5-μm treatment relative to the 1-μm treatment. Expression levels of *mkk-4* increased for both exposure concentrations relative to the control with the 5-μm treatment higher than the 1-μm treatment for the 2.4×10^7 particles/m^2 concentration and the 1-μm treatment higher than the 5-μm treatment for the 2.4×10^8 particles/m^2 treatment. The expression levels for *cpr-1* and *itr-1* increased after exposure to 1 and 5 μm MP at 2.4×10^8 particles/m^2, but showed a differential response at 2.4×10^7 particles/m^2 where expression levels decreased for the 1-μm treatment, but increased for the 5-μm treatment. Moreover, the expression levels for all five genes changed significantly between 1 and 5-μm MP treatments, but not always in the same direction (Fig. 5).

4. Discussion

Previous studies showed that MPs can be ingested into the digestive system where they can be excreted, remain in the digestive tract, or be absorbed into the body tissue (Browne et al., 2008; Wright et al., 2013). In this study, the ingested MPs were found exclusively in the intestine (Fig. S4). The intake ratio for 1 and 5 μm MPs showed no significant difference. The intestine is the primary digestive organ of *C. elegans*, which secretes digestive enzymes into the lumen and takes up processed nutritional materials along with potential toxicants (Altun and Hall, 2009a). Our results are consistent with those of Julia et al. (2014) who found MPs to mainly accumulate in the digestive tract, and the amount of MPs increased as the exposure concentration increased.

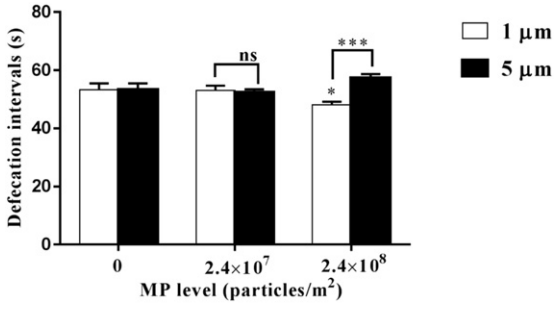


Fig. 3. Effect of MPs on defecation rhythm after 72 h exposure to 1 and 5 μm MPs. *: $P < .05$; ***: $P < .001$; ns: no significant difference, vs the control (if not specifically indicated).

Our results showed accumulation of MPs was only in the intestine and the MPs were largely expelled by the muscle contraction of defecation. Exposure to 1 μm MPs at 2.4×10^8 particles/m^2 induced a significant decrease on defecation interval, while there was no change for the 5-μm MP treatment at the same concentration (Fig. 3). Lei et al. (2018b) also found that 1 μm particles resulted in a larger toxicity to *C. elegans* than larger particles. The 1 μm MPs may be more actively taken up and accumulated in *C. elegans* as their size and shape are closer to that of *E. coli* cells, their primary food source in these studies (Yuya et al., 2012). The *C. elegans* pharynx, in combination with the buccal cavity, is adapted to entrap and transport particles of a specific size range dictated by their bacteria-dominated food source (Christopher et al., 2009). The *E. coli* OP50 has an approximate size range of 0.5×2 μm making them similar in size to the 1 μm MPs used in this study. Therefore, uptake of 1 μm MPs could hinder *E. coli* uptake and result in changes in *C. elegans* physiological and biochemical levels. This observation is consistent with MP exposure to earthworms (*Lumbricus terrestris*), *P. nana* and zebrafish (*Danio rerio*), which showed that a greater proportion of smaller plastics were retained, as opposed to larger plastics (Batel et al., 2018; Fackelmann and Sommer, 2019; Huerta Lwanga et al., 2016; Jeong et al., 2017). This could result from preferential uptake of smaller sized MPs contributing to a greater retention potential.

In addition to MP excretion by muscle contraction and expulsion within the intestine, excretion may occur by other means, such as the nematode excretory cells, whose role includes osmotic/ionic regulation and waste elimination, analogous to the renal system of higher animals (Altun and Hall, 2009b). Further information concerning the excretion dynamics of nematodes in response to MP exposure is an important

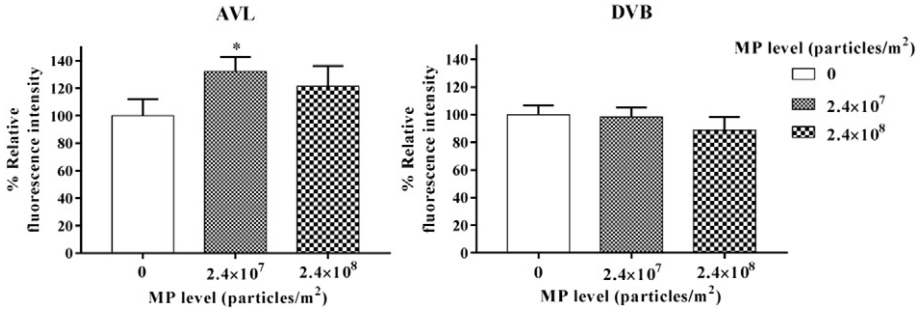


Fig. 4. Effect of 1 μm MPs on the fluorescence expression of AVL and DVB. *: $P < .05$ vs the control.

future research topic given the possibility of nematodes introducing MPs into the food web.

Defecation rhythm is regulated not only by physiological factors, but also by genetic and molecular responses in the intestine of *C. elegans* (Espelt et al., 2005). AVL and DVB neurons were reported to be involved in the control of defecation behavior (Sun et al., 2015; Wu et al., 2014). Exposure to 1 μm MPs increased the fluorescence intensity of the AVL motor neurons, which suggests that damage of the AVL and DVB neurons resulted in an abnormal defecation behavior.

After exposure to 1 and 5 μm MPs, nematode lifespan decreased by about 50% for the most severely impacted treatments. Unexpectedly, the lifespan for the lower concentration exposure groups decreased more than for the higher concentration exposure groups. Commonly, dose–response relationships in toxicological studies show a threshold or linear non-threshold response. However, Calabrese and Baldwin (2003) reported that a biphasic dose-response, or hormesis-like dose–response, characterized by a low dose stimulation or beneficial effect versus a high dose inhibitory or toxic effect, was also common. Some common environmental pollutants like endocrine-disrupting compounds have been reported to have more severe low dose responses (Vandenberg et al., 2012).

The lifespan of *C. elegans* seems to be an appropriate metric to reflect the effects of chemicals on a spectrum of changes over time, and sensitive enough to assess the low-dose effect. As previously reported, acrylamide induced a significant decrease in lifespan even at an extremely low dose of 0.5 μg/L, while such adverse effects were not found at 5 mg/L (Hasegawa et al., 2004). The more adverse effect of MPs on *C. elegans* lifespan at lower concentrations may result from prolonged retention of MPs at lower concentrations (data not shown). The intestinal mucus secretion level and protein levels of the cystic fibrosis transmembrane conductance regulator (CFTR), which play a critical role in regulation of epithelial secretions in the intestinal canal, were shown to decrease more significantly at lower exposure concentrations to 5 μm MPs (Jin et al., 2019). However, other experimental studies found that MPs did not significantly reduce the lifespan of marine worms (Kaposi et al., 2014).

The changes in lifespan were further supported by the gene expression results in this study. *Skn-1* is mainly involved in detoxification and lifespan regulation in nematodes (Blackwell et al., 2015). Loss of *skn-1* shortens lifespan and *skn-1*over-expression or gain-of-function usually increases the lifespan (Tullet et al., 2017). Thus, the shortened lifespan may be attributed to down-regulated expression of *skn-1* at the 2.4×10^{-7} particles/m^2 concentration for both particle size groups.

We further investigated the expression level of genes known to regulate the development and function of the intestine (Altun and Hall, 2009a; Maduro, 2017). In this study, we found that exposure to 1 and 5 μm MPs at a concentration of 2.4×10^8 particles/m^2 for 72 h increased the transcriptional expressions of *mkk-4, pmk-1, cpr-1* and *itr-1* (Fig. 5), which may induce a protection response to MP exposure. Qu et al. (2018) found that exposure of acs-22 mutant nematodes to nanopolystyrene particles (1 μg/L) enhanced intestinal permeability by triggering a protection mechanism through enhanced transcriptional expression of genes in the SOD, catalases, and Nrf signaling pathways. These possible protection responses in our study seem to counteract the toxicity induced by MPs to varying degrees, which may explain why the defecation rhythm response and the expression of

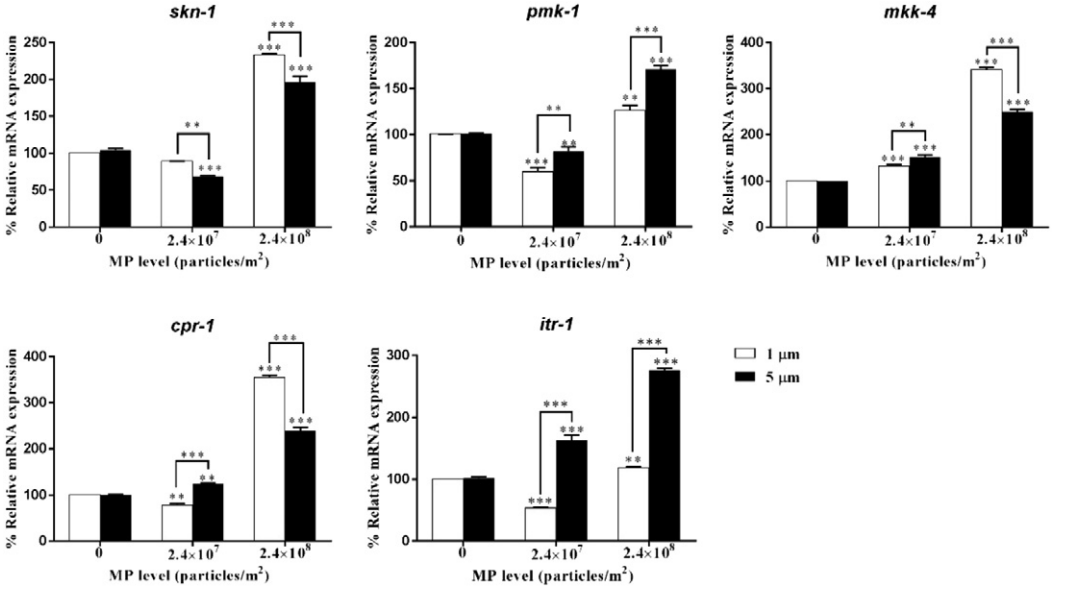


Fig. 5. Gene expressions related with lifespan and defecation in the intestinal tract. **: $P < .01$, ***: $P < .001$, vs the control (if not specifically indicated).

corresponding neurons were differentially mediated by 1 and 5 μm MPs at the 2.4×10^8 particles/m^2 concentration (Figs. 3 and 4). In sum, the gene expression results provide primary evidence that the lifespan and intestine function of nematodes responded to MP exposure and demonstrated enhanced sensitivity to lower MP concentrations.

5. Conclusions

In this study, *C. elegans* was exposed to 1 and 5-μm MPs across a concentration range of 10^7–10^{10} particles/m^2 for 72 h. Results demonstrated that 1 and 5 μm MPs were readily ingested. Ingestion increased at higher exposure concentrations, and induced toxic effects on lifespan and the intestine at the physiological, genetic, and molecular levels. The 1 μm MPs, especially at the lowest concentration evaluated (2.4×10^7 particles/m^2), produced more adverse effects than the 5 μm MPs. Unexpectedly, lower MP concentrations resulted in more severe impacts on *C. elegans* lifespan than higher MP concentrations suggesting a biphasic dose-response to MP exposure.

Declaration of competing interest

There are no conflicts to declare.

Acknowledgements

We thank Dr. Hongying Shi (Wenzhou Medical University) for analysis of the mixed-effects intake modeling and Dr. Hongmei Wu (Wenzhou Medical University) for her comments to improve the manuscript.

Funding

The project was supported by Zhejiang Provincial Natural Science Foundation of China [LQ16H260001] and the National Natural Science Foundation of China [81602829].

Data availability

Data are available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.135837.

References

Altun, Z.F., Hall, D.H., 2009a. Alimentary System, Intestine. WormAtlas https://10.3908/wormatlas.1.4.
Altun, Z.F., Hall, D.H., 2009b. Excretory system. WormAtlas https://10.3908/wormatlas.1.17.
Anbumani, S., Kakkar, P., 2018. Ecotoxicological effects of microplastics on biota: a review. Environ. Sci. Pollut. Res. 25, 14373–14396. https://10.1007/s11356-018-1999-x.
Andrady, A.L., 2011. Microplastics in the marine environment. Mar. Pollut. Bull. 62, 1596–1605. https://doi.org/10.1016/j.marpolbul.2011.05.030.
Andrady, A.L., 2017. The plastic in microplastics: a review. Mar. Pollut. Bull. 119, 12–22. https://doi.org/10.1016/j.marpolbul.2017.01.082.
Batel, A., Borchert, F., Reinwald, H., Erdinger, L., Braunbeck, T., 2018. Microplastic accumulation patterns and transfer of benzo[a]pyrene to adult zebrafish (Danio rerio) gills and zebrafish embryos. Environ. Pollut. 235, 918–930. https://10.1016/j.envpol.2018.01.028.
Baylis, H.A., Vázquez-Manrique, R.P., 2012. Genetic analysis of IP 3 and calcium signalling pathways in *C. elegans*. Biochim. Biophys. Acta 1820, 1253–1268. https://10.1016/j.bbagen.2011.11.009.
Beiras, R., Bellas, J., Cachot, J., Cormier, B., Cousin, X., Engwall, M., Gambardella, C., Garaventa, F., Keiter, S., Le Bihanic, F., Lopez-Ibanez, S., Piazza, V., Rial, D., Tato, T., Vidal-Linan, L., 2018. Ingestion and contact with polyethylene microplastics does not cause acute toxicity on marine zooplankton. J. Hazard. Mater. 360, 452–460. https://10.1016/j.jhazmat.2018.07.101.
Blackwell, T.K., Steinbaugh, M.J., Hourihan, J.M., Ewald, C.Y., Meltem, Isik M., 2015. SKN-1/Nrf, stress responses, and aging in *Caenorhabditis elegans*. Free Radic. Biol. Med. 88, 290–301. https://https://doi.org/10.1016/j.freeradbiomed.2015.06.008.
Brenner, S., 1974. The genetics of *Caenorhabditis elegans*. Genetics 77, 71–94.
Browne, M.A., Dissanayake, A., Galloway, T.S., Lowe, D.M., Thompson, R.C., 2008. Ingested microscopic plastic translocates to the circulatory system of the mussel, *Mytilus edulis* (L). Environmental Science & Technology 42, 5026–5031. https://https://doi.org/10.1021/es800249a.
Browne, M.A., Crump, P., Niven, S.J., Teuten, E., Tonkin, A., Galloway, T., Thompson, R., 2011. Accumulation of microplastic on shorelines woldwide: sources and sinks. Environmental Science & Technology 45, 9175–9179. https://10.1021/es201811s.
Calabrese, E.J., Baldwin, L.A., 2003. Toxicology rethinks its central belief. Nature 421, 691–692. https://10.1038/421691a.
Christopher, F.Y., Leon, A., Samuel, A.D.T., 2009. Two size-selective mechanisms specifically trap bacteria-sized food particles in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. U. S. A. 106, 20093–20096. https://https://doi.org/10.1038/421691a.
Cole, M., Lindeque, P., Halsband, C., Galloway, T.S., 2011. Microplastics as contaminants in the marine environment: a review. Mar. Pollut. Bull. 62, 2588–2597. https://10.1016/j.marpolbul.2011.09.025.
Cole, M., Lindeque, P., Fileman, E., Halsband, C., Galloway, T.S., 2015. The impact of polystyrene microplastics on feeding, function and fecundity in the marine copepod Calanus helgolandicus. Environmental Science & Technology 49, 1130–1137. https://10.1021/es504525u.
da Costa, J.P., Santos, P.S.M., Duarte, A.C., Rocha-Santos, T., 2016. (Nano) plastics in the environment - sources, fates and effects. Science of theTotal Environment 566-567, 15–26. https://doi.org/10.1016/j.scitotenv.2016.05.04.
David, K.A., Barnes, F.G., Richard, C., Thompson, M.B., 2009. Accumulation and fragmentation of plastic debris in global environments. Philos. Trans. R. Soc. B 364, 1985–1998. https://10.1098/rstb.2008.0205.
Dong, S., Qu, M., Rui, Q., Wang, D., 2018. Combinational effect of titanium dioxide nanoparticles and nanopolystyrene particles at environmentally relevant concentrations on nematode *Caenorhabditis elegans*. Ecotoxicology & Environmental Safety 161, 444–450. https://10.1016/j.ecoenv.2018.06.021.
Espelt, M.V., Estevez, A.Y., Yin, X., Strange, K., 2005. Oscillatory Ca^{2+} signaling in the isolated *Caenorhabditis elegans* intestine: role of the inositol-1,4,5-trisphosphate receptor and phospholipases C beta and gamma. J. Gen. Physiol. 126, 379–392. https://https://doi.org/10.1085/jgp.200509355.
Fackelmann, G., Sommer, S., 2019. Microplastics and the gut microbiome: how chronically exposed species may suffer from gut dysbiosis. Mar. Pollut. Bull. 143, 193–203. https://doi.org/10.1016/j.marpolbul.2019.04.030.
Foley, C.J., Feiner, Z.S., Malinich, T.D., Höök, T.O., 2018. A meta-analysis of the effects of exposure to microplastics on fish and aquatic invertebrates. Sci. Total Environ. 631–632, 550–559. https://10.1016/j.scitotenv.2018.03.046.
Geyer, R., Jambeck, J.R., Law, K.L., 2017. Production, use, and fate of all plastics ever made. Sci. Adv. 3, 7. https://10.1126/sciadv.1700782 e1700782.
Giedrė, A.K.L., Ingrid, U., Joachim, S., Bethanie, C.A., 2018. Size matters: ingestion of relatively large microplastics contaminated with environmental pollutants posed little risk for fish health and fillet quality. Environmental Science & Technology 52, 14381–14391. https://doi.org/10.1021/acs.est.8b04849.
Goldstein, M.C., Titmus, A.J., Ford, M., 2013. Scales of spatial heterogeneity of plastic marine debris in the northeast Pacific Ocean. PLoS One 8, e80020. https://10.1371/journal.pone.0080020.
Guven, O., Bach, L., Munk, P., Dinh, K.V., Mariani, P., Nielsen, T.G., 2018. Microplastic does not magnify the acute effect of PAH pyrene on predatory performance of a tropical fish (*Lates calcarifer*). Aquat. Toxicol. 198, 287–293. https://doi.org/10.1016/j.aquatox.2018.03.011.
Hasegawa, K., Miwa, S., Tsutsumiuchi, K., Taniguchi, H., Miwa, J., 2004. Extremely low dose of acrylamide decreases lifespan in *Caenorhabditis elegans*. Toxicol. Lett. 152, 183–189. https://doi.org/10.1016/j.toxlet.2004.04.037.
Horton, A.A., Walton, A., Spurgeon, D.J., Lahive, E., Svendsen, C., 2017. Microplastics in freshwater and terrestrial environments: evaluating the current understanding to identify the knowledge gaps and future research priorities. Sci. Total Environ. 586, 127–141. https://10.1016/j.scitotenv.2017.01.190.
Huang, C.W., Li, S.W., Liao, H.C., 2016. Chronic ZnO-NPs exposure at environmentally relevant concentrations results in metabolic and locomotive toxicities in *Caenorhabditis elegans*. Environ. Pollut. 220, 1456–1464. https://https://doi.org/10.1016/j.envpol.2016.10.086.
Huerta Lwanga, E., Gertsen, H., Gooren, H., Peters, P., Salánki, T., van der Ploeg, M., Besseling, E., Koelmans, A.A., Geissen, V., 2016. Microplastics in the terrestrial ecosystem: implications for Lumbricus terrestris (Oligochaeta, Lumbricidae). Environmental Science & Technology 50, 2685–2691. https://10.1021/acs.est.5b05478.
Jeong, C.B., Kang, H.M., Lee, M.C., Kim, D.H., Han, J., Hwang, D.S., Souissi, S., Lee, S.J., Shin, K.H., Park, H.G., Lee, J.S., 2017. Adverse effects of microplastics and oxidative stress-induced MAPK/Nrf2 pathway-mediated defense mechanisms in the marine copepod Paracyclopina nana. Sci. Rep. 7, 41323. https://10.1038/srep41323.
Jin, Y., Lu, L., Tu, W., Luo, T., Fu, Z., 2019. Impacts of polystyrene microplastic on the gut barrier, microbiota and metabolism of mice. Sci. Total Environ. 649, 308–317. https://10.1016/j.scitotenv.2018.08.353.
Jingjuan, J., Qinli, R., Xiaobo, L., Ran, L., Yunhui, L., Yuepu, P., Lihong, Y., Dayong, W., 2013. Neurotoxicological evaluation of microcystin-LR exposure at environmental relevant concentrations on nematode *Caenorhabditis elegans*. Environ. Sci. Pollut. Res. 20, 1823–1830. https://https://doi.org/10.1007/s11356-012-1151-2.
Ju, J., Saul, N., Kochan, C., Putschew, A., Pu, Y., Yin, L., Steinberg, C.E., 2014. Cyanobacterial xenobiotics as evaluated by a *Caenorhabditis elegans* neurotoxicity screening test. Int. J. Environ. Res. Public Health 11, 4589–4606. https://10.3390/ijerph110504589.
Julia, H., Lar,s, G., Angela, K., Saborowski, R., 2014. Fate of microplastics in the marine isopod Idotea emarginata. Environmental Science & Technology 48, 13451–13458. https://doi.org/10.1021/es501385y.
Kaposi, K.L., Benjamin, M., Kelaher, B.P., Dworjanyn, S.A., 2014. Ingestion of microplastic has limited impact on a marine larva. Environmental Science & Technology 48, 1638–1645. https://https://doi.org/10.1021/es404295e.

Kokalj, A.J., Horvat, P., Skalar, T., Kržan, A., 2017. Plastic bag and facial cleanser derived microplastic do not affect feeding behaviour and energy reserves of terrestrial isopods. Sci. Total Environ. 615, 761–766. https://https://doi.org/10.1016/j.scitotenv.2017.10.020.

Lee, K.W., Shim, W.J., Kwon, O.Y., Kang, J.H., 2013. Size-dependent effects of micro polystyrene particles in the marine copepod Tigriopus japonicus. Environmental Science & Technology 47, 11278–11283. https://10.1021/es401932b.

Lei, L., Liu, M., Yang, S., Lu, S., Hu, J., Cao, C., Bing, X., Shi, H., He, D., 2018a. Polystyrene (nano)microplastics cause size-dependent neurotoxicity, oxidative damages and other adverse effects in *Caenorhabditis elegans*. Environ. Sci.: Nano 5, 2009–2020. https://10.1039.C1038EN00412A.

Lei, L., Wu, S., Lu, S., Liu, M., Song, Y., Fu, Z., Shi, H., Raley-Susman, K.M., He, D., 2018b. Microplastic particles cause intestinal damage and other adverse effects in zebrafish Danio rerio and nematode *Caenorhabditis elegans*. Sci. Total Environ. 619–620, 1–8. https://10.1016/j.scitotenv.2017.11.103.

Leung, M.C., Williams, P.L., Benedetto, A., Au, C., Helmcke, K.J., Aschner, M., Meyer, J.N., 2008. *Caenorhabditis elegans*: an emerging model in biomedical and environmental toxicology. Toxicol. Sci. 106, 5–28. https://10.1093/toxsci/kfn121.

Li, Z., Man, Q., Wang, D., Wong, G., 2017. Transgenerational toxicity of nanopolystyrene particles in the range of μg/L in nematode *Caenorhabditis elegans*. Environmental Science Nano 4, 2356–2366. https://https://doi.org/10.1039/c7en00707h.

Lorenzen, S., 1994. The Phylogenetic Systematics of Free Living Nematodes. The Ray Society, London, UK.

Maduro, M.F., 2017. Gut development in *C. elegans*. Semin. Cell Dev. Biol. 66, 3–11. https://10.1016/j.semcdb.2017.01.001.

Michaelson, L., 2001. *C. elegans*: A Practical Approach. Oxford University Press, Oxford.

Pereira, V.M., Bortolotto, J.W., Kist, L.W., Azevedo, M.B.D., Fritsch, R.S., Oliveira, R.D.L., Pereira, T.C.B., Bonan, C.D., Vianna, M.R., Bogo, M.R., 2012. Endosulfan exposure inhibits brain AChE activity and impairs swimming performance in adult zebrafish (Danio rerio). Neurotoxicology 33, 469–475. https://10.1016/j.neuro.2012.03.005.

Qu, M., Xu, K., Li, Y., Wong, G., Wang, D., 2018. Using acs-22 mutant *Caenorhabditis elegans* to detect the toxicity of nanopolystyrene particles. Sci. Total Environ. 643, 119–126. https://10.1016/j.scitotenv.2018.06.173.

Rochman, C.M., Browne, M.A., Halpern, B.S., Hentschel, B.T., Hoh, E., Karapanagioti, H.K., Rios-Mendoza, L.M., Takada, H., Teh, S., Thompson, R.C., 2013. Classify plastic waste as hazardous. Nature 494, 169–171. https://10.1038/494169a.

Rochman, C.M., Kurobe, T., Flores, I., Teh, S.J., 2014. Early warning signs of endocrine disruption in adult fish from the ingestion of polyethylene with and without sorbed chemical pollutants from the marine environment. Sci. Total Environ. 493, 656–661. https://10.1016/j.scitotenv.2014.06.051.

Rowshyra, A.C., Suncica, A., M. Anouk, S., Anthony, R., 2014. Microplastic pollution discovered in St. Lawrence River sediments. Can. J. Fish. Aquat. Sci. 88, 5–6. http://https://doi.org/10.1139/cjfas-2014-0281.

Sun, L., Lin, Z., Liao, K., Xi, Z., Wang, D., 2015. Adverse effects of coal combustion related fine particulate matter (PM2.5) on nematode *Caenorhabditis elegans*. Sci. Total Environ. 512–513, 251–260. https://doi.org/10.1016/j.scitotenv.2015.01.058.

Triebskorn, R., Braunbeck, T., Grummt, T., Hanslik, L., Huppertsberg, S., Jekel, M., Knepper, T.P., Krais, S., Müller, Y.K., Pittroff, M., Ruhl, A.S., Schmieg, H., Schür, C., Strobel, C., Wagner, M., Zumbülte, N., Köhler, H.R., 2019. Relevance of nano- and microplastics for freshwater ecosystems: a critical review. Trends Anal. Chem. 110, 375–392. https://doi.org/10.1016/j.trac.2018.11.023.

Tullet, J.M.A., Green, J.W., Au, C., Benedetto, A., Thompson, M.A., Clark, E., Gilliat, A.F., Young, A., Schmeisser, K., Gems, D., 2017. The SKN-1/Nrf2 transcription factor can protect against oxidative stress and increase lifespan in *C. elegans* by distinct mechanisms. Aging Cell 16, 1191–1194. http://10.1111/acel.12627.

UN Environment Programme (UNEP), 2018. World Environment Day Overview (WEF). https://www.environment.gov.za/event/worldenvironmentday2018.

Vandenberg, L.N., Colborn, T., Hayes, T.B., Heindel, J.J., Jacobs Jr., D.R., Lee, D.H., Shioda, T., Soto, A.M., vom Saal, F.S., Welshons, W.V., Zoeller, R.T., Myers, J.P., 2012. Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. Endocr. Rev. 33, 378–455. https://10.1210/er.2011-1050.

vom Saal, F.S., Hughes, C., 2005. An extensive new literature concerning low-dose effects of bisphenol a shows the need for a new risk assessment. Environ. Health Perspect. 113, 926–933. http://10.1289/ehp.7713.

Walker, D.S., Chew, Y.L., Schafer, W.R., 2017. Genetics of behavior in *C. elegans*. The Oxford Handbook of Invertebrate Neurobiology.

Wang, Z., Su, B., Xu, X., Di, D., Huang, H., Mei, K., Dahlgren, R.A., Zhang, M., Shang, X., 2018. Preferential accumulation of small (<300 μm) microplastics in the sediments of a coastal plain river network in eastern China. Water Res. 144, 393–401. http://10.1016/j.watres.2018.07.050.

Wang, J., Coffin, S., Sun, C., Schlenk, D., Gan, J., 2019. Negligible effects of microplastics on animal fitness and HOC bioaccumulation in earthworm Eisenia fetida in soil. Environ. Pollut. 249, 776–784. https://doi.org/10.1016/j.envpol.2019.03.102.

Wright, S.L., Rowe, D., Thompson, R.C., Galloway, T.S., 2013. Microplastic ingestion decreases energy reserves in marine worms. Curr. Biol. 23, 1031–1033. http://https://doi.org/10.1016/j.cub.2013.10.068.

Wu, Q., Zhao, Y., Fang, J., Wang, D., 2014. Immune response is required for the control of in vivo translocation and chronic toxicity of graphene oxide. Nanoscale 6, 5894–5906. https://10.1039/c4nr00699b.

Yuya, K., Kohji, M., Yasumi, O., 2012. Active uptake of artificial particles in the nematode *Caenorhabditis elegans*. J. Exp. Biol. 215, 1178–1183. http://https://doi.org/10.1242/jeb.067199.

Zhou, D., Yang, J., Li, H., Cui, C., Yu, Y., Liu, Y., Lin, K., 2016. The chronic toxicity of bisphenol a to *Caenorhabditis elegans* after long-term exposure at environmentally relevant concentrations. Chemosphere 154, 546–551. https://10.1242/jeb.067199.