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Ingestion of microplastics by nematodes depends on feeding strategy and buccal cavity size☆

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ABSTRACT

Microplastics are hardly biodegradable and thus accumulate rather than decompose in the environment. Due to sedimentation processes, meiobenthic fauna is exposed to microplastics. Within the meiofauna, nematodes are a very abundant taxon and occupy an important position in benthic food webs by connecting lower and higher trophic levels. However, the key determinants of the uptake of microplastics by freshwater nematodes are still unknown. To investigate the bioaccessibility of microplastics for nematodes, we performed single- and multi-species ingestion experiments in which the ability of seven nematode species (six bacterial and one fungal feeder), diverse in their buccal cavity morphology (1.3 −10.5 μm), to ingest fluorescence-labelled polystyrene (PS) beads along with their natural diet was examined. Applied beads sizes (0.5, 1.0, 3.0 and 6.0 μm), exposure time (4, 24 and 72 h) and concentration (3×10^6 PS beads ml^{-1} and 10^7 PS beads ml^{-1}) were varied. Ingested beads were localized and quantified via fluorescence microscopy in the nematodes. In contrast to fungal-feeding nematode species with a stylet, bacterial-feeding species ingested 0.5- and 1.0-μm PS beads with up to 249 and 255 beads after 24 h, respectively. Microplastics ≥0.5 μm could only be ingested and transported into the gastrointestinal tract, if the buccal cavities were considerably (>1.3 times) larger than the beads. At concentrations of 10^7 PS beads ml^{-1} ingestion rates were influenced by exposure time and PS bead concentration. In case of a known microplastic size distribution in the environment, predictions on the potential ingestion for nematode communities can be made based on the feeding type composition and the size of their buccal cavities.

1. Introduction

Because microplastics (polymer particles <5 mm; Arthur et al., 2009; European Commission, 2013) are hardly biodegradable, they rather accumulate than decompose in the environment (Barnes et al., 2009) and therefore have become a major environmental concern (Haegerbaeumer et al., 2019; Thompson et al., 2009). Microplastics enter the environment either as primary microplastics (e.g. manufactured for cosmetics) or emerge as secondary microplastics result from the breakdown of larger plastic items via the mechanical action that occurs during weathering, oxidative processes, and biological degradation (Andrady, 2011; Browne et al., 2007). Microplastics originate from land-based sources (Andrady, 2011; Browne et al., 2007; Browne et al., 2011), however accumulate in freshwater streams (Eerkes-Medrano et al., 2015). Many polymers are transported to the sediments of water bodies (van Cauwenberghe et al., 2015), because they either show higher specific densities than water or biofouling processes enhance their sedimentation (e.g. Harrison et al., 2018; Kaiser et al., 2017; Kooi et al., 2017), leading to up to 10,000-fold higher microplastic densities in sediments compared to the water surface (Wendt-Potthoff et al., 2017). Sediment-inhabiting fauna (e.g. benthic invertebrates) may be therefore continuously exposed to higher concentrations of microplastics than pelagic organisms (e.g. Haegerbaeumer et al., 2019; Zhang et al., 2015). Due to the sampling of European freshwaters with cut-off sizes >80 μm, exposure concentrations of microplastics might be underestimated (Adam et al., 2019) and no exposure data for particles <5 μm is available (Triebskorn et al., 2019). However, the smallest particles are

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potentially those that are most easily ingested (Dris et al., 2015) and the risk for freshwater organisms seems to increase with decreasing particle size (Triebskorn et al., 2019).

Nematodes are one of the most abundant and species-rich metazoan phyla: in fine sediments, they account for up to 90% of meiobenthic organisms (e.g. Bergtold and Traunspurger, 2005; Majdi et al., 2017; Strayer, 1985; Traunspurger, 2000; Traunspurger et al., 2012). Moreover, by connecting lower (bacteria) and higher (e.g. macrozoobenthic organisms, fish) trophic levels, nematodes occupy a key position in benthic food webs (Majdi and Traunspurger, 2015; Schmid-Araya and Schmid, 2000; Weber and Traunspurger, 2015). A trophic transfer of microplastics was already demonstrated from the mussel *Mytilus edulis* to the crab *Carcinus maenas* (Farrell and Nelson, 2013) and from mesozooplankton to mysid shrimps (Setälä et al., 2014). Hence, ingestion by nematodes may be an important bottom-up route by which microplastics enter the aquatic food web and investigations of the bioaccessibility of microplastics for nematodes are therefore essential.

Most research on the uptake of microplastics by organisms has focused on the marine environment. Although the number of studies in freshwater is increasing (Adam et al., 2019; Triebskorn et al., 2019), freshwater biota are still underrepresented (Scherer et al., 2018). A few studies have shown that microplastics are ingested by freshwater invertebrates, irrespective of the feeding type (e.g. Canniff and Hoang, 2018; Imhof et al., 2013; Jeong et al., 2016; Rehse et al., 2016). Among the limited studies of microplastic particle ingestion by nematodes, *Caenorhabditis elegans* has been the only species investigated (e.g. Zhao et al., 2017; Lei et al., 2018). Kiyama et al. (2012) examined food/non-food discrimination by *C. elegans* exposed to fluorescent polystyrene microspheres differing in size that actively ingested 0.5- and 1.0-μm microspheres mainly into the intestine while those with diameters <0.5 μm or >3.0 μm were rarely ingested (Fang-Yen et al., 2009). These findings were supported by other studies showing that *C. elegans* only ingested latex beads with a maximum diameter of 3.4 μm (Boyd et al., 2003) and 4.5−μm beads could not enter the buccal cavity (Fang-Yen et al., 2009). The ingestion depends upon microplastics particle size among other factors (Phuong et al., 2016) and according to Lehtiniemi et al. (2018) the size more than the shape is an important determining factor for influencing the numbers of ingested microplastics.

Buccal cavities of nematodes show a great morphological diversity in form and size, which results in a specific feeding strategy for each morphological form (Yeates et al., 1993). For freshwater nematodes, there are feeding-types defined based on morphological characteristics: deposit feeder, epistrate feeder, chewer and suction feeder (Traunspurger, 1997). Deposit-feeding nematodes are characterized by their unarmed (small tooth is absent) buccal cavity. The objective of this study was to quantitatively investigate the ingestion of microplastics by bacterial-feeding nematodes, differing in the morphology of their buccal cavities, along with their natural bacterial diet. Therefore, the nematodes were exposed to beads of polystyrene (PS), a common polymer form of microplastics in river shore sediments (Klein et al., 2015). Fluorescent-labelled PS beads were applied in various nematode-relevant diameters (0.5, 1.0, 3.0 and 6.0 μm) in single- and multi-species exposure experiments to exclude inter- or intraspecific competition and to relate any differences in PS bead uptake to individual ingestion rates of the nematode species and ingested PS beads were localized in the nematode's body after 4, 24 and 72 h via fluorescence microscopy. Selected PS beads (1.0 μm diameter) were tested at two different exposure concentrations. Moreover, the PS bead ingestion of the bacterial feeding species was compared to that of the fungal-feeding species *Aphelenchoides parietinus* that mainly feeds on dissolved food using a stylet (Abebe et al., 2006). We hypothesized that (1) the uptake of the PS beads occurs only via a buccal cavity, (2) the ingestion of PS beads is related to the size of beads and that of the nematode buccal cavities and (3) the number of ingested PS beads is positively correlated with exposure time and bead concentration.

2. Material and methods

2.1. Test organisms, feeding strategy and stock cultures

The used nematode species *Caenorhabditis elegans*, *Panagrolaimus thienemanni*, *Plectus acuminatus, Poikilolaimus regenfussi* and *Acrobeloides nanus*, belong to the group of deposit-feeding nematodes, which can engulf the whole prey in the bacterial-size range. *Caenorhabditis elegans* (N2 strain; bacterial feeder) stock cultures were obtained from the Caenorhabditis Genetics Centre (University of Minnesota, Minneapolis, MN, USA). They were grown on nematode growth medium [17 g agar l^{-1}, 2.5 g peptone l^{-1}, and 3 g NaCl l^{-1}, with 1 ml 1 M $CaCl_2$, 1 ml 1 M $MgSO_4$, 25 ml 1 M KPO_4 buffer pH 6 (108.3 g KH_2PO_4 l^{-1}, 35.6 g K_2HPO_4 l^{-1}), and 1 ml cholesterol solution (5 mg ml^{-1} in ethanol) added after autoclaving (Brenner, 1974)] like *P. pacificus* (wild isolate; bacterial feeder) and *A. nanus* (bacterial feeder). As food, the agar plates were spotted with OP50, an uracil-requiring mutant of *Escherichia coli* that avoids overgrowth of the bacterial lawn (Brenner, 1974), following standard procedures (Stiernagle, 2006). *Pristionchus pacificus* is commonly described as a bacterial feeder, but this species shows a mouth dimorphism (Ragsdale, 2015) induced by low dietary densities (Bento et al., 2010) allowing them to live predacious and to specialize on alternative, larger food sources such as nematode prey (Lieven and Sudhaus, 2000). Stock cultures of *P. acuminatus* (wild isolate from Lake Constance, Germany; bacterial feeder) were maintained on "pond water agar" plates (1.7% agar and filtered pond water without additional salts) and *E. coli* OP50 as the food source. Monoxenic stock cultures of *P. thienemanni* and *P. regenfussi* (wild isolates from Movile Cave, Romania; bacterial feeders) were maintained on semi-fluid nematode growth gelrite (Muschiol and Traunspurger, 2007) devoid of peptone to prevent additional bacterial growth. *Aphelenchoides parietinus* as a suction feeder has a sharp, tapered and retractable stylet in the buccal cavity for piercing inner contents of a variety of prey (e.g. fungal hyphae). Stock cultures of *A. parietinus* (fungal suction feeder with stylet) were maintained on malt-extract agar (1.7% agar, 0.5% malt, Volvic water). All stock culture plates were stored at 20 °C in the dark and buccal cavities were microscopically photographed (Progres Gryphax Subra, Jenoptik, Jena, Germany) and measured at 1000 × magnification (Zeiss Axio Scope.A1, Jena, Germany) using fluorescence-free immersion oil (PanReac AppliChem, AppliChem GmbH, Darmstadt, Germany).

2.2. Food source

An *E. coli* OP50 culture grown overnight at 37 °C in Luria-Bertani (LB) medium (1% peptone, 0.5% yeast extract, 1% NaCl) served as the nematode food source. A bacterial suspension with a final density of 1×10^9 *E. coli* cells ml^{-1} was used in the ingestion experiment, which was shown to be optimal for nematode larvae (Schiemer, 1982). The inoculated LB medium was centrifuged for 20 min at 2000 g and cells were washed with K-medium (3.1 g NaCl l^{-1}, 2.4 g KCl l^{-1}) or, in the case of OP50 used for *P. acuminatus*, Volvic water. The bacterial density was spectrophotometrically determined for five subsamples (1:20 dilution) at an optical density of OD_{600} (Varian Cary 50 Bio UV−Visible). The aim was to achieve a bacterial

density twice as high as the intended test concentration. Cholesterol solution (5 mg ml^{-1} in ethanol) was then added to a concentration of 4 µl ml^{-1}.

2.3. Polystyrene bead suspension

The microplastic suspensions were prepared by diluting stock suspensions of 0.5-µm (0.47 ± 0.01 µm), 1.0-µm (0.91 ± 0.01 µm), 3.0-µm (3.0 ± 0.15 µm) and 6.0-µm (6.1 ± 0.24 µm) Fluoresbrite® Yellow Green microspheres (excitation maxima at 441 nm, emission maxima at 485 nm; Polysciences Europe GmbH, Baden-Wuerttemberg, Germany) with K-medium (or Volvic water for the *P. acuminatus* single-species experiment) to achieve a concentration twice as high as the intended test concentrations of 10^7 (0.5 µm: 0.69 mg l^{-1}; 1.0 µm: 5.49 mg l^{-1}; 3.0 µm: 148.81 mg l^{-1}; 6.0 µm: 1190.00 mg l^{-1}) and 3×10^6 PS beads ml^{-1} (1.0 µm: 1.65 mg l^{-1}). According to the results of preliminary ingestion experiments (Fig. S1 in supplementary material), the final test concentrations ensured the uptake and precise quantification of the beads. The surface charge of the used PS beads showed to be negative (zeta potential of 1.0–µm PS bead: −82.3 ± 2.2 mV; measured in 1% M9-medium at 10^7 PS beads ml^{-1}; Zetasizer Nano ZS, Malvern Panalytical GmbH, Kassel, Germany).

2.4. Experimental setup

The bioaccessibility experiments (schematic illustration Fig. S2 in supplementary material), in which the nematodes were exposed to the PS beads, were carried out in standard polystyrene cell culture plates (cell growth area per well: 3.85 cm^2; VWR International GmbH, Darmstadt, Germany). For the single-species ingestion experiment each well contained five adult nematodes of the same species and for the multi-species experiment ten adult nematodes per species (50 individuals per well in total). Twenty-four hours prior to the start of the experiments, 0.5 ml of PS bead suspension and 0.5 ml of bacterial suspension were mixed in each well. The plates were then incubated at 4 °C in the dark. Nematodes were starved for 30 min prior to the experiment start. A food density of 10^9 cells ml^{-1} and a PS bead concentration of 10^7 beads ml^{-1} and 3×10^6 PS beads ml^{-1} (2.6×10^6 and 7.8×10^5 PS beads cm^{-2}) respectively were applied. Six nematode species (the five bacterial feeders, *C. elegans*, *P. pacificus*, *P. acuminatus*, *P. thienemanni*, *P. regenfussi*, and the fungal feeder *A. parietinus*; five adult nematodes per species per well) were exposed to three treatments of the microplastic-bacteria suspension, containing PS beads of 0.5, 1.0, and 6.0 µm in diameter at exposure concentrations of 10^7 beads ml^{-1}, for 24 h in a single-species ingestion experiment. Five nematode species (*C. elegans*, *P. pacificus*, *P. thienemanni*, *A. nanus* and *A. parietinus*; 10 adult nematodes per species and thus 50 individuals per well in total) were exposed to 1.0, 3.0 and 6.0 µm-beads at concentrations of 10^7 beads ml^{-1} for 4 h, 24 h and 72 h in a multi-species ingestion experiment. The most ingested PS bead size (1.0 µm) was additionally applied at a lower concentration of 3×10^6 PS beads ml^{-1} (30% of the highest concentration applied) in a separate experiment. The ingestion of PS beads was stopped by heat-killing the nematodes at 80 °C for 20 min, which is a reasonable procedure to avoid the egestion of the gut content and fluorescent properties and shape of the PS beads remained unaffected. The nematodes were then washed with K-medium to remove beads adhered to their cuticle and transferred onto a microscopic slide. Ingested PS beads were localized and quantified at 400 × magnification using a fluorescence microscope (Zeiss Axio Scope.A1, Jena, Germany). The beads in several nematode gastrointestinal tract regions (buccal cavity, pro- and metacorpus, isthmus, terminal bulb, pre-intestine, intestine, post-intestine, and rectum; Fig. S3 in supplementary material) were quantified separately. However, the egestion of PS beads was not evaluated in this study.

2.5. Statistical analysis

All data were checked for normality (Shapiro-Wilk test) and for homoscedasticity (Levene's test) but were not transformed to improve normality. A significance level of $p < 0.05$ was used for all comparisons. A Kruskal-Wallis test was used to test for significant differences of non-paired and not normally distributed data e.g. the buccal cavity sizes between the nematode species (post hoc test: Dunn). Two way ANOVA on ranks were performed for the PS bead ingestion after 24 h in the single-species and in the multi-species ingestion experiment after exposure times of 4, 24, 72 h as well as for the PS bead ingestion between the single-vs. multi-species treatments of the species *C. elegans* and *P. pacificus*. The PS bead ingestion as a factor of exposure time for 1.0, 3.0 and 6.0 µm PS beads separately and the ingestion of 1.0 µm PS beads as a factor of exposure time at two exposure concentrations for each nematode species were also checked with a two way ANOVA on ranks. In addition, a PERMANOVA was performed for the factors exposure time, exposure concentration of PS beads and nematode species to include their interactions in the statistical analysis. All pairwise multiple comparison procedures regarding the number of ingested PS beads were checked for significant differences with the post hoc Holm-Sidak's method. Statistical analyses were performed using SigmaPlot 12.0 (Systat Software Inc.).

3. Results

3.1. Buccal cavity sizes of various nematode species

Measurements of all seven nematode species showed species-specific differences in the size of their buccal cavities ($H = 126.767$; $p < 0.001$; Fig. 1). *Aphelenchoides parietinus*, as a suction-feeding species, had the smallest buccal cavity (1.3 (± 0.1 standard deviation) µm). Among the six bacterial feeders, the buccal cavity of *P. thienemanni* was the smallest (1.5 ± 0.2 µm) followed by that of *A. nanus* (1.8 ± 0.3 µm). The buccal cavities of *P. regenfussi* (3.7 ± 0.7 µm), *P. acuminatus* (4.3 ± 0.7 µm) and *C. elegans* (4.4 ± 0.5 µm) were comparable in size whereas that of *P. pacificus* (10.5 ± 1.3 µm) was the largest (Table S1 in supplementary material). Based on the measured buccal cavity sizes, all bacterial-feeding nematode species were expected to take up PS beads of 0.5 and 1.0 µm, *P. regenfussi*, *P. acuminatus* and *C. elegans* up to 3.0 µm and *P. pacificus* beads up to 6.0 µm in diameter.

3.2. Influence of bead size, buccal cavity size and exposure time on PS bead ingestion

In the single-species ingestion experiment, all bacterial-feeding nematode species except the suction-feeding *A. parietinus* ingested PS beads. About 53% of *P. thienemanni*, 88% of *P. regenfussi*, 100% of *P. acuminatus*, 100% of *C. elegans*, and 100% of *P. pacificus* ingested PS beads of 0.5 µm in size (Fig. 2).

The largest number of 0.5-µm PS beads was detected in the body of *P. acuminatus* (153 (± 15 standard error) beads; $n = 17$) and the smallest number in that of *P. thienemanni* (6 ± 2 beads; $n = 18$). 1.0-µm PS beads were ingested by four of the five bacterial-feeding species (Fig. 2; 75% of the individuals; 0% of *P. thienemanni*, 100% of *P. regenfussi*, 73% of *P. acuminatus*, 100% of *C. elegans*, and 100% of *P. pacificus*) but not by *P. thienemanni* although its buccal cavity, ranging from 1.3 to 1.9 µm ($n = 18$), should have been large enough to do so. After 24 h *P. pacificus* contained the fewest 1.0-µm beads

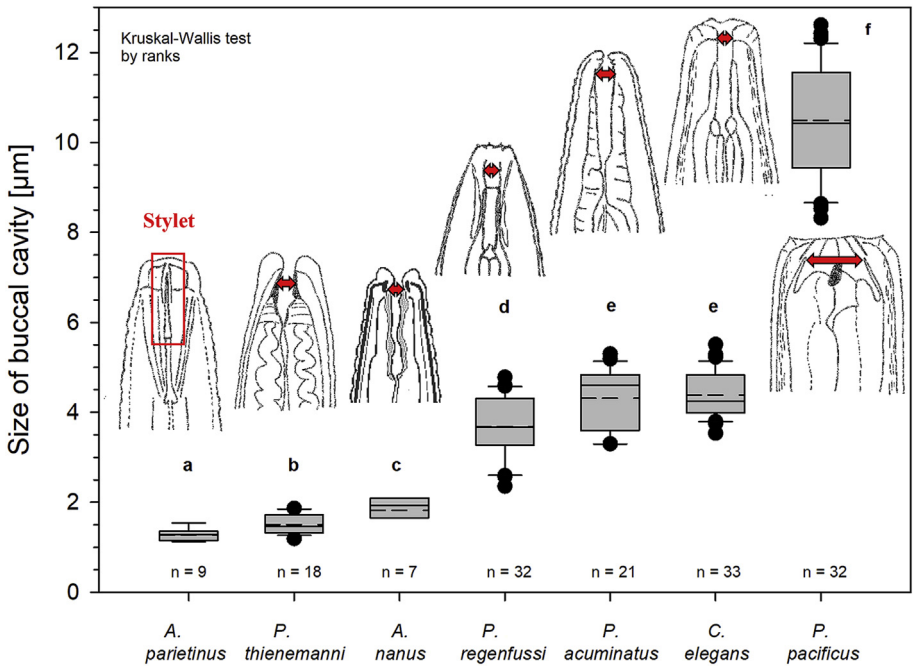

Fig. 1. Sizes and drawings of the buccal cavities of the seven tested nematode species. Different letters indicate significant differences (Kruskal-Wallis test; Dunn's method; $p < 0.05$). Median (solid line) and mean (dashed line); boxes represent 50% (interquartile range) and each whisker 25% of the data. SigmaPlot 12.0 (Systat Software Inc.).

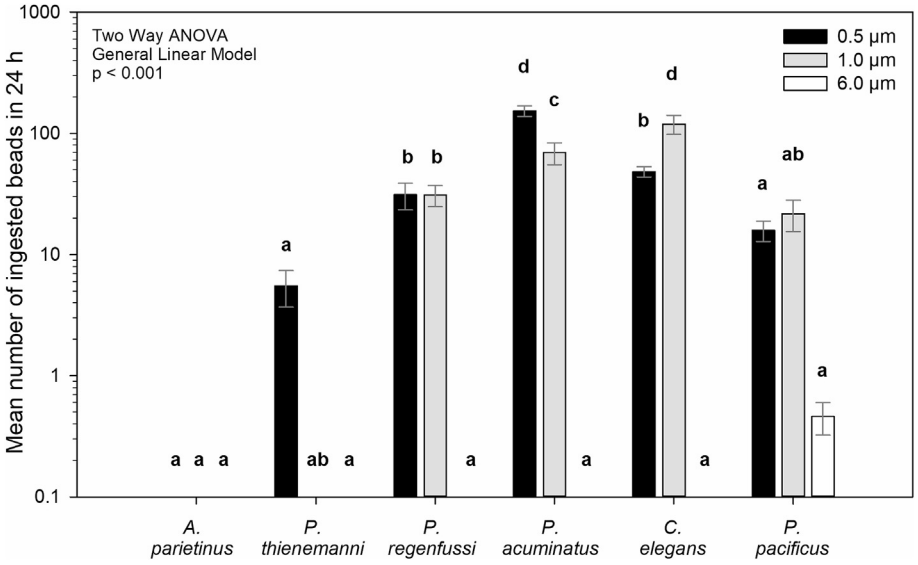

Fig. 2. Ingestion of 0.5-, 1.0-, and 6.0-µm polystyrene beads by the nematode species in the single-species ingestion experiment. Different letters indicate significant differences (Two Way ANOVA; Holm Sidak's method; $p < 0.05$). $n = 15$ per bead size class. The mean ± standard error is shown. SigmaPlot 12.0 (Systat Software Inc.).

(24 ± 6 beads; $n = 12$) and *C. elegans* with 102 ± 20 beads ($n = 15$) the most. However, significant species-specific differences in the number of ingested 0.5- and 1.0-µm PS beads were found for two species: *P. acuminatus* had ingested significantly more 0.5-µm than 1.0-µm beads ($n = 15$; $t = 7.430$, $p < 0.001$), while *C. elegans* significantly more 1.0-µm than 0.5-µm beads ($n = 15$; $t = 0.6.375$, $p < 0.001$). Only *P. pacificus* ingested 6.0-µm PS beads and in 46% of the evaluated individuals of this species in the single-species ingestion experiment, a single 6.0-µm bead located in the buccal cavity was observed after 24 h, but none was found in the intestine.

In the multi-species ingestion experiment with exposure times of 4, 24 and 72 h, the number of ingested beads differed between species ($F = 44.192$, $p < 0.001$) and PS bead diameter ($F = 24.534$, $p < 0.001$). Three of the four bacterial-feeding nematodes were able to ingest 1.0-µm. 3.0-µm beads were ingested by *C. elegans* and *P. pacificus* exclusively (Fig. 3).

The ingestion differed between the two species ($F = 13.832$, $p < 0.001$), but not between the single- and multi-species treatments of each species ($t = 0.366$, $p < 0.715$ and $t = 0.811$, $p < 0.420$; Table S2 in supplementary material). The ingestion potential of various PS bead sizes by nematodes was therefore compared regardless of the single- and multi-species treatment.

About 11% of *P. thienemanni*, 95% of *C. elegans*, 13% of *A. nanus* and 87% of *P. pacificus* ingested PS beads of 1.0 µm in size after 24 h

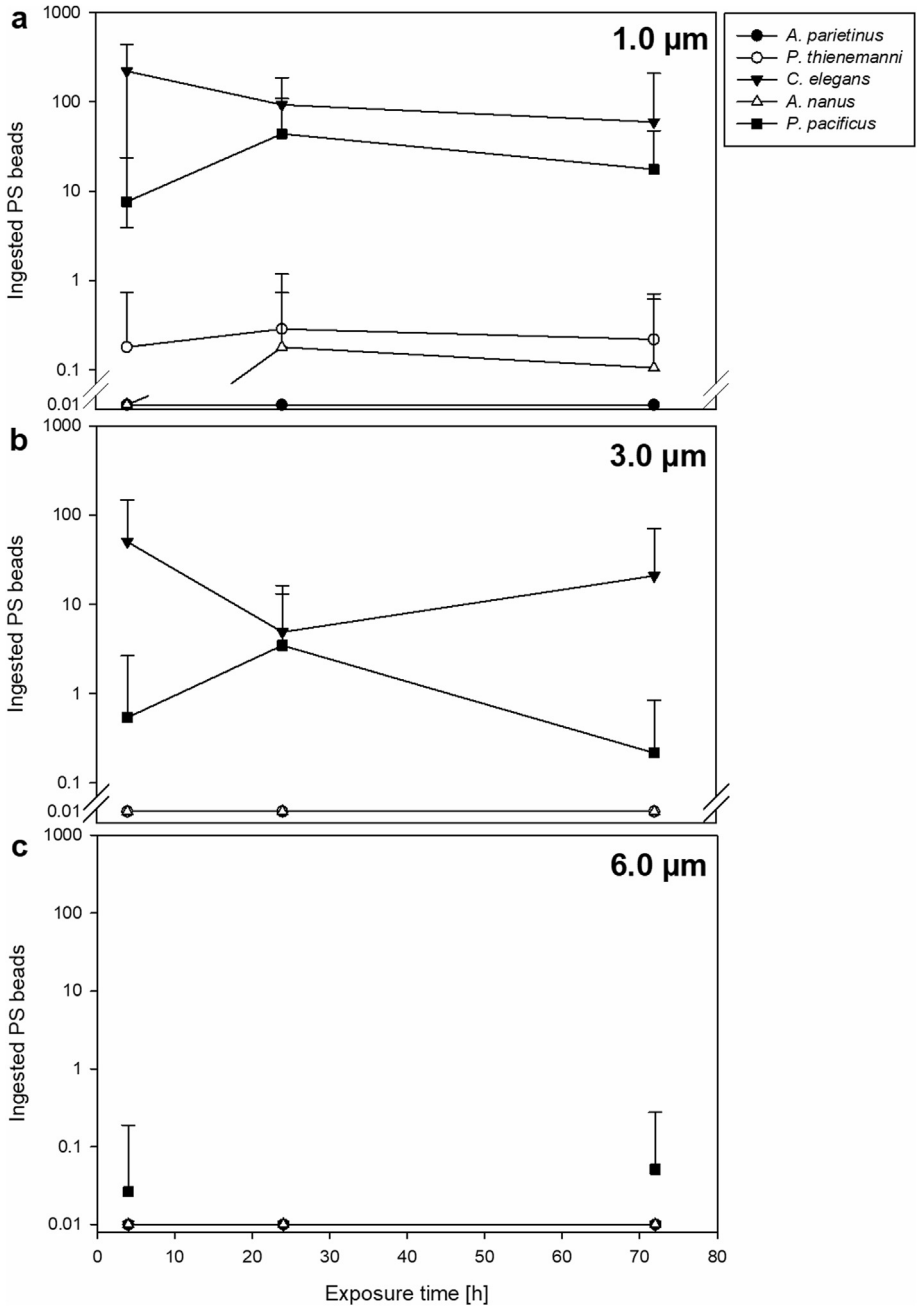

Fig. 3. Ingestion of 1.0- (a), 3.0- (b) and 6.0-µm polystyrene beads (c) by the nematode in the multi-species ingestion experiment after 4 h, 24 h and 72 h of direct exposure. n = 21–40 per species and bead size class. The mean ± standard error is shown. SigmaPlot 12.0 (Systat Software Inc.).

(Fig. 3a) and two of the four bacterial-feeding species ingested 3.0-µm beads after 24 h (0% of *P. thienemanni*, 60% of *C. elegans*, 0% of *A. nanus* and 39% of *P. pacificus*; Fig. 3b). Beads of 6.0 µm could only be detected in the buccal cavity of a few individuals of *P. pacificus* after 4 h and 72 h (Fig. 3c). The largest number of PS beads was detected in the body of *C. elegans* (222 ± 34 beads; 1.0 µm; 4 h; n = 40) and significantly fell to 60 ± 24 beads (72 h; n = 38; t = 6.266; $p < 0.001$) with increasing exposure time. After 72 h of exposure to 3.0-µm PS beads, *C. elegans* significantly ingested less beads than after 4 h (t = 2.665; p = 0.017). Just a few individuals of *A. nanus* (up to 5 of 39; 24 h) and *P. thienemanni* (up to 6 of 32; 72 h) had ingested 1.0-µm beads, but no beads of 3.0 or 6.0 µm in size. *Caenorhabditis elegans* at any exposure time (n = 38–40; t = 11.749, $p < 0.001$) and *P. pacificus* after 24 h (n = 37–39; t = 5.706, $p < 0.001$) significantly ingested more beads of 1.0 than 3.0 µm.

3.3. Influence of exposure concentration on PS bead ingestion

Since the overall ingestion of PS beads of *A. nanus* and *P. thienemanni* was poor, those species were excluded from the comparison of both exposure concentrations (Fig. 4; Table S3 in supplementary material).

The ingestion of 1.0-µm beads by *C. elegans* and *P. pacificus* was significantly higher at exposure concentrations of 10^7 PS beads ml^{-1} than at 3×10^6 PS beads ml^{-1}; F = 17.221, $p < 0.001$) regardless of exposure time. Significant species-specific differences were

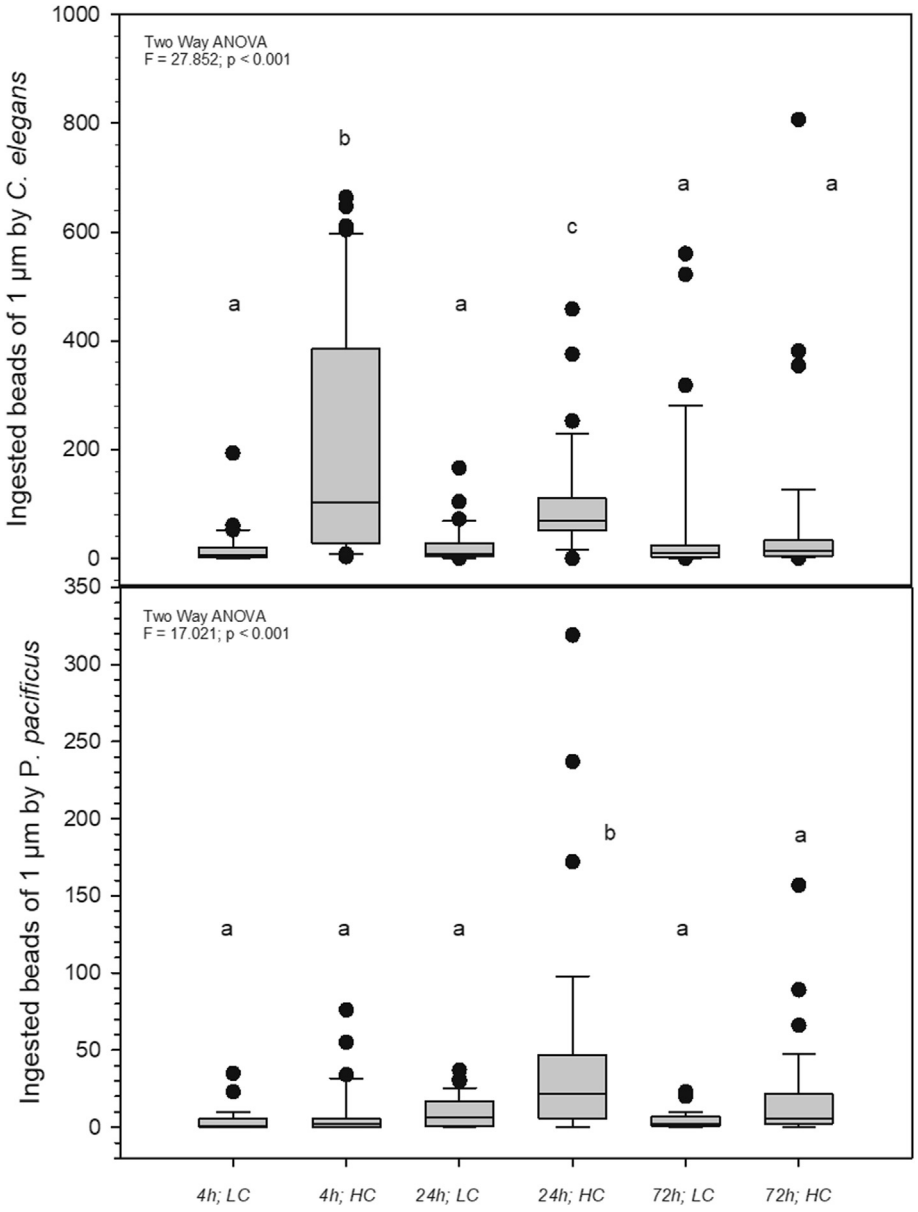

Fig. 4. Comparison of the ingestion of 1.0-µm polystyrene beads by *C. elegans* and *P. pacificus* at different exposure concentrations and times. Mean numbers of ingested 1.0−µm PS beads were plotted for the nematode species that had ingested the most PS beads after 4, 24 and 72 h at two exposure concentrations (high concentration HC = 10^7 PS beads ml^{-1} and low concentration LC = 3×10^6 PS beads ml^{-1}). Different letters indicate significant differences (Two Way ANOVA; Holm Sidak's method; $p < 0.05$). Median (solid line); boxes represent 50% (interquartile range) and each whisker 25% of the data. SigmaPlot 12.0 (Systat Software Inc.).

recorded after 72 h at 3×10^6 PS beads ml^{-1} ($t = 2.858$; $p = 0.004$) and within the highest exposure concentration of 10^7 PS beads ml^{-1} ($t = 8.185$; $t < 0.001$).

3.4. Translocation of PS beads in the intestine

We observed that PS beads tend to cluster at narrow passages in the front and rear part of the intestine potentially impeding the intestinal flow. More than 80% of the ingested PS beads regardless of the size were found in the oesophagus after 24 h of exposure (Table 1). With increasing PS bead size, the percentage of ingested PS beads in the oesophagus increased for *P. pacificus* that had the largest buccal cavity of tested nematode species, however, the vast majority was still located in the intestine.

4. Discussion

In addition to the microplastic size and shape (e.g. Gray and Weinstein, 2017; Lehtiniemi et al., 2018), surface properties (Bråte et al., 2018), exposure concentration and time, morphological factors (e.g. buccal cavity size, intestine dimensions, presence/absence of a valve apparatus in the oesophageal bulb) or species-specific feeding habits probably constrain the microplastic ingestion and uptake in nematodes. Since *A. parietinus*, as a representative for suction-feeding nematode species (plant and fungal feeders), was unable to ingest PS beads ≥0.5 µm, the uptake of microplastics must exclusively occur through the buccal cavity, which confirms hypothesis (1). The stylet of *Aphelenchoides* species is about 10−12 µm in length with an opening of <0.2 µm (e.g. Fortuner, 1970; Franklin, 1955) implying that nematodes with a stylet are

Table 1
Localization of ingested polystyrene beads within the gastrointestinal tract of the nematodes: Exposure time: 24 h; exposure concentration: 10^7 PS beads ml^{-1}. Nematode body regions: Oes = oesophagus (including buccal cavity), Int = intestine, Rec = rectum. nt = not tested.

PS bead diameter	0.5 μm			1.0 μm			3.0 μm			6.0 μm		
Species	Oes	Int	Rec	Oes	Int	Rec	Oes	Int	Rec	Oes	Int	Rec
A. parietinus	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
P. thienemanni	3%	97%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
A. nanus	nt	nt	nt	82%	18%	0%	0%	0%	0%	0%	0%	0%
P. regenfussi	15%	83%	2%	8%	91%	1%	nt	nt	nt	0%	0%	0%
P. acuminatus	15%	84%	1%	11%	88%	1%	nt	nt	nt	0%	0%	0%
C. elegans	13%	80%	7%	4%	86%	10%	7%	91%	2%	0%	0%	0%
P. pacificus	10%	82%	8%	12%	84%	4%	29%	67%	4%	100%	0%	0%

not able to ingest microplastics ≥0.5 μm. In accordance with our findings based on buccal cavity measurements the results confirm that the ingestion of PS beads is related to the size ratio of beads and the nematode buccal cavities (Fig. 5). Whenever the size of the buccal cavity is at least 1.3 times larger than the PS beads, the beads can be readily taken up. At a ratio between 1.3 and 3.6 the buccal cavity strongly limits the ingestion of PS beads. At ratios exceeding 3.6, the ingestion is no longer limited by the buccal cavity but by species-specific feeding differences, exposure time and exposure concentration. For example, 1.0-μm PS beads were the most effectively ingested bead size in the ingestion experiments and were ingested by all studied bacterial-feeding nematode species with buccal cavity and PS beads size ratios of more than 1.3: *P. thienemanni* (ratio: 1.5), *A. nanus* (ratio: 1.8), *P. regenfussi* (ratio: 3.7), *P. acuminatus* (ratio: 4.3), *C. elegans* (ratio: 4.4), *P. pacificus* (ratio: 10.5). In case of a known size distribution of microplastics in the environment, predictions on the potential ingestion for nematode communities can be made based on the feeding type composition and the size of their buccal cavities.

In the case of *P. pacificus*, only one 6.0-μm bead could enter the buccal cavity and, however, was wedged in the buccal cavity, which probably prevented the uptake of other particles, including bacterial cells. The present data confirm the threshold value of 1.3 obtained by Boyd et al. (2003) that showed that *C. elegans* only ingested latex beads with a maximum of 3.4 μm in diameter.

There were no significant differences in the ingestion rate of PS beads between the single- and the multi-species treatment within each nematode species that generally showed an efficient uptake of PS beads. Due to the sufficiently high concentrations of PS beads, nematodes found non-depletive conditions in single- and multi-species set ups, so that any inter- or intraspecific competition could be excluded and any differences in PS bead uptake could be related to individual ingestion rates of the nematode species, the exposure time and exposure concentration.

Differences in the ingestion of 0.5-μm and 1.0-μm PS beads by *C. elegans* and *P. acuminatus* could have been caused by distinct feeding and size-selective feeding behaviours between rhabditid and plectid nematodes. While Rhabditidae feed continuously when enough food is available, Plectidae feed more intermittently with active sweeping motions during feeding and being more specialized in collecting bacteria associated with substrate (Moens et al., 2004). *Caenorhabditis elegans* showed a rapid uptake of PS beads

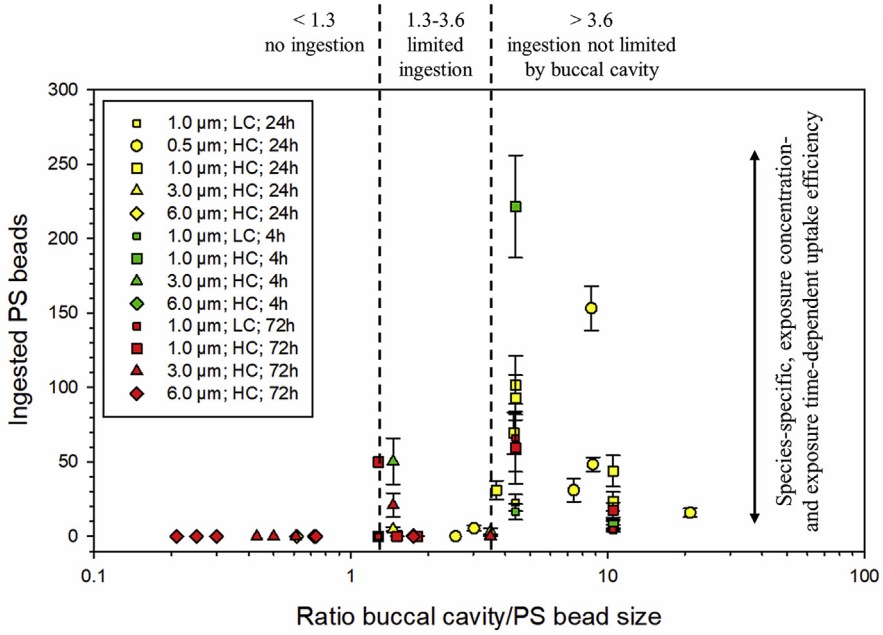

Fig. 5. Relation of mean number of ingested polystyrene (PS) **beads to the size ratio of nematode buccal cavity and PS beads.** Mean numbers of ingested PS beads with sizes of 0.5, 1.0, 3.0 and 6.0 μm were plotted for each species, exposure time (4, 24 and 72 h) and exposure concentration (high concentration HC = 10^7 PS beads ml^{-1} and low concentration LC = 3×10^6 PS beads ml^{-1}). Nematode species with a size ratio of more than 1.3 were able to ingest PS beads along with the bacterial diet. At ratios >3.6 the uptake efficiency was not influenced by the buccal cavity size anymore but by the exposure concentration, exposure time and species-specific feeding differences. The mean ± standard error is shown. SigmaPlot 12.0 (Systat Software Inc.).

into the gastrointestinal tract within 4 h in the multi-species experiment (exposure times 4, 24 and 72 h), while *P. pacificus* needed longer. Although both rhabditid nematodes have a similar morphology (terminal bulb), they show a different feeding behaviour. In general, bacterial-feeding nematodes feed by drawing suspended bacteria into the pharynx by rhythmic pharyngeal pumping followed by a posterior moving isthmus peristaltic contraction in about one out of four pharyngeal pumps that transports the bacteria to the intestine (Fang-Yen et al., 2009; Scholz et al., 2016; Trojanowski et al., 2016). The rate of pumping depends on feeding history, quality and quantity of food, and the familiarity of food (Hobson et al., 2006; Nicholas et al., 1973; Shtonda and Avery, 2006; Song et al., 2013). In *C. elegans*, in addition to the muscular corpus also the anterior part of the isthmus has a pumping function (Chiang et al., 2006), but in *P. pacificus* the pumping behaviour is restricted to the corpus (Riebesell and Sommer, 2017). A declining number of PS beads with exposure time in the body of *C. elegans* could hint that egestion remained constant or increased while the ingestion decreased simultaneously. A full intestine of PS beads seemed to affect the feeding activity. Whether it was due to a learning effect of the nematode or a blockade of the terminal bulbus has to be tackled in pumping rate experiments separately, since the rate of pumping is the primary indicator of food intake (Avery, 1993).

Besides feeding behaviour differences, the egestion of 1.0-μm beads may occur more slowly than smaller PS beads and the retention of 1.0-μm beads in the nematode's gastrointestinal tract may be longer, due to their doubled size and an eightfold higher mass than that of single 0.5-μm beads. In general, 1.0-μm beads were found to be the most ingested microplastics co-ingested with bacterial cells for nematodes. *Escherichia coli* OP50 cells have a nearly spherical shape and sizes between that of 0.5-μm and 1.0-μm beads (Kiyama et al., 2012), which may explain this microplastic size to be most relevant for bacterial-feeding nematodes. Due to the negative zeta potential of the used PS beads, they did not hetero-agglomerate with negatively charged bacterial cells (Hanna et al., 2018). However, in the static test system, the PS beads as well as the bacterial cells settled to the bottom of the wells since PS beads are denser than water, which might benefit the nematode's encounter rate of a particle and then the probability of being ingested since nematodes seek out for bacterial assemblages.

The findings of the ingestion experiments are in good agreement with Kiyama et al. (2012) since (1) *C. elegans* was able to ingest bead sizes of 0.5, 1.0 and 3.0 μm; (2) most of the ingested beads were localized in the intestine, and some in the pharynx and (3) beads of 1.0 μm were the most ingested bead size by *C. elegans*. The predominant ingestion by adult *C. elegans* of PS bead sizes between 0.5 μm and 3.0 μm (Fang-Yen et al., 2009) was confirmed in the ingestion experiments, in which *C. elegans* ingested 0.5-μm, 1.0-μm and 3.0-μm beads but not 6.0-μm beads. Fang-Yen et al. (2009) demonstrated that beads with a diameter of 4.5 μm could not enter the buccal cavity of *C. elegans* due to relaxation of the valve-like metastomal flaps, which prevent the entry of particles >3.0 μm. In *P. pacificus*, the flint-shaped dorsal tooth in the buccal cavity (stenostomatous hermaphrodite; Ragsdale, 2015) might have hindered the uptake of 6.0-μm beads into the intestine. Some non-rhabditid aquatic nematodes are even able to widen their buccal cavity to ingest prey that is considerably larger than the buccal cavity size (Boucher, 1973). The size ratio of buccal cavity and PS bead may therefore give an underestimation of the maximum particle size for some prey items that the nematodes can ingest. Since the ingestion of different PS bead sizes is also dependent upon the size of the nematode buccal cavities and thus particularly bacterial-feeding nematodes are expected to ingest most microplastics, hypothesis (2) can be verified.

In addition to microplastic size, shape might be a very relevant factor also for nematodes. For instance, fibers were more toxic to *Hyalella azteca* than spheres (10-day lethal concentrations LC50: 71 fibers ml^{-1} and 4.5×10^4 spheres ml^{-1}), maybe as a result of internal structures becoming damaged, and needed longer egestion times than spheres (Au et al., 2015). Moreover, they might not be fully egested and retained fibers may become entangled within the intestinal tract over time (Gray and Weinstein, 2017).

Higher ingestion rates for 1.0-μm beads were observed when higher concentrations of PS beads were offered in the medium. Thus, the higher the concentration of exposed microplastics is, the more likely they will be ingested by nematodes due to higher encounter rates. This finding suggests that the ingestion of microplastics seems to be accidental rather than intended. To summarize, hypothesis (3) can only be partially verified since ingestion rates were higher when more PS beads were offered in the ingestion experiment.

Exposure concentrations of microplastics used in our ingestion experiment (3×10^6 and 10^7 PS beads ml^{-1}) may rarely be reached in natural aquatic ecosystems (Klein et al., 2015; Peng et al., 2018). The number of microplastic particles reported for aquatic environments strongly depends on the detected particle size, sampling processing and identification methods (e.g. Enders et al., 2015; Imhof et al., 2013; Ivleva et al., 2017). However, present environmental data do not provide enough information on the environmental concentrations of microplastics <20 μm (Adam et al., 2019; Ivleva et al., 2017; Phuong et al., 2016) since smaller size fractions can still not be quantified properly in sediment samples due to technical limitations (Lenz et al., 2016; Triebskorn et al., 2019). So far, natural concentrations for smaller particles can only be extrapolated by stating that the number of microplastic particles will scale inversely with the particle radius to the power of 2.7 (Lenz et al., 2016). Therefore, when we extrapolate measured environmental concentrations of microplastics >20 μm to the smallest PS bead size (0.5 μm) used in our laboratory experiments, the estimated environmental concentrations for 0.5-μm PS beads might reach 10^8 beads l^{-1} (10^5 beads ml^{-1}), which is 30–100 times lower than the actual concentration used in the present study. However, we chose elevated concentrations to ensure an optimal uptake of PS beads to evaluate the microplastic ingestion potential of nematodes properly and to allow a proof-of-concept demonstration of PS bead uptake (Huvet et al., 2016). Therefore, the demonstrated uptake of small PS beads by nematodes in this study may be an important basis for estimating their availability to organisms that feed on nematodes since fragments of plastics are expected to end up in the food web (Lehtiniemi et al., 2018).

5. Conclusion

The uptake of PS beads in nematodes is predominantly governed by their feeding habit and can be predicted by the morphology of the buccal cavity: buccal cavities must be considerably (>1.3 times) larger than the beads, before they can be taken up. If the buccal cavity is >3.6 times larger than the beads other factors, such as species-specific feeding differences, exposure time and exposure concentration become important for controlling the quantity of microplastic uptake. Therefore, it can be assumed that for predatory organisms, feeding on nematodes (e.g. planarians, chironomids, young fishes), besides overall microplastic concentrations, size and feeding type composition of the nematode community will be crucial factors for the dietary uptake of microplastics. As nematodes are the most abundant taxon in benthic habitats and occupy an important basal position in benthic food webs, future studies should examine whether microplastics that had been ingested by nematodes can be transferred to higher-trophic levels and whether

the nematode species composition is relevant for the trophic transfer.

Author contributions

H.F., M-T.M., L.W., S.H. and W.T. designed the direct ingestion experiments of nematode species in single- and multi-species designs. H.F., M-T.M. and L.W. counted and localized the ingested PS beads via fluorescence microscopy. H.F. surveyed the nematode buccal cavity sizes. H.F. and M-T.M. wrote the manuscript. W.T. and S.H. supervised the work. All authors reviewed and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2019.113227.

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