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Uptake of microplastics by marine worms depends on feeding mode and particle shape but not exposure time

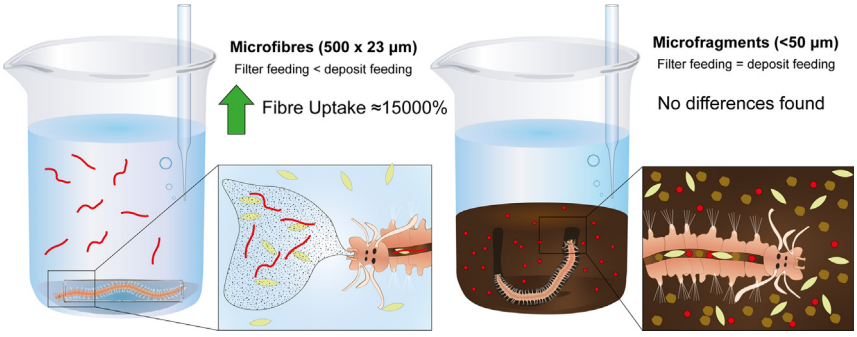
Adam Porter [a,*], Dan Barber [a], Catherine Hobbs [a], John Love [a], Ann L. Power [a], Adil Bakir [b], Tamara S. Galloway [a], Ceri Lewis [a]

[a] College of Life and Environmental Sciences, Biosciences, Geoffrey Pope Building, University of Exeter, Stocker Road, Exeter EX4 4QD, United Kingdom
[b] Centre for Environment Fisheries and Aquaculture Science (CEFAS), Pakefield Road, Lowestoft NR33 0HT, United Kingdom

HIGHLIGHTS

- Microfibres and -fragments ingested by filter and deposit feeding *H. diversicolor*.
- Worms reached a body burden in equilibrium with their exposure media rapidly.
- Filter feeders took up ≈15,000 % more microfibres than deposit feeders.
- No difference between filter and deposit feeders feeding on microfragments.
- Findings shed insight onto environmental uptake of microplastics.

GRAPHICAL ABSTRACT



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ABSTRACT

The uptake of microplastics into marine species has been widely documented across trophic levels. Feeding mode is suggested as playing an important role in determining different contamination loads across species, but this theory is poorly supported with empirical evidence. Here we use the two distinct feeding modes of the benthic polychaete, *Hediste diversicolor* (The Harbour Ragworm) (O.F. Müller, 1776), to test the hypothesis that filter feeding will lead to a greater uptake of microplastic particles than deposit feeding. Worms were exposed to both polyamide microfragments and microfibres in either water (as filter feeders) or sediment (as deposit feeders) for 1 week. No effect of exposure time was found between 1 day and 1 week ($p > 0.19$) but feeding mode was found to significantly affect the number of microfibres recovered from each worm ($p < 0.001$). When exposed to microfibers, filter feeding worms took up ≈15,000 % more fibres than deposit feeding worms ($p < 0.001$), whereas when feeding on microfragments there was no difference between feeding modes. Our data demonstrate that both feeding mode and particle characteristics significantly influence the uptake of microplastics by *H. diversicolor*. Using imaging flow cytometry, filter feeders were found to take up a broader size range of particles, with significantly more smaller and larger particles than deposit feeders ($p < 0.05$), commensurate with the range of plastics isolated from the guts of ragworms recovered from the environment. These results demonstrate that biological traits are useful in understanding the uptake of plastics into marine worms and warrant further exploration as a tool for understanding the bioaccessibility of plastics to marine organisms.

* Corresponding author.
E-mail address: a.porter@exeter.ac.uk (A. Porter).

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1. Introduction

Contamination of the environment by microplastics is a widespread and growing problem (Wong et al., 2020). The presence of microplastics has been reported in marine, freshwater, atmospheric, and terrestrial ecosystems whilst adverse effects of varying severities have been described in many organisms, from small, sediment dwelling invertebrates to humans (Wright and Kelly, 2017a; Wright and Kelly, 2017b; Wong et al., 2020; Cai et al., 2017; Carr et al., 2016; Leslie et al., 2022). An enormous variety of organisms can ingest plastics in situ in their natural habitats as shown from numerous studies on field collected organisms (Lusher, 2015), but very little is known about the actual ingestion and depuration rates of microplastics in many species and across trophic levels, which is vital to understanding the true exposure rates and cumulative impacts of plastic ingestion (Miller et al., 2020; Foley et al., 2018). Further, the ecotoxicological consequences of those species ingesting plastic are not fully understood; although the emerging picture is negative (Foley et al., 2018). Finally, it is difficult to demonstrate causality between microplastic ingestion and negative effects in wild-caught organisms due to the myriad of stressors organisms face in the Anthropocene (Miller et al., 2020). It is imperative then, that attention is focused on understanding the risk plastics play in marine ecosystems (Koelmans et al., 2022), which functional groups may be most at risk, and to study those organisms whose distributions overlap in time and space with the distribution of microplastic (Lusher, 2015; Burns and Boxall, 2018).

The extent and rates of microplastic uptake into marine organisms is likely to be dictated by the interactions of a range of organismal factors including; habitat, geographic location, environmental position within the habitat (epifaunal, infaunal etc.), feeding mode, size, lifespan, environmental contamination levels or the distance from the source or sources of plastic pollution. The organismal factors are often referred to as traits and can give insights into a species ecological function and therefore its resilience (Bremner et al., 2006).

A key trait in determining ingestion rates is organismal feeding mode (Setälä et al., 2016; Bour et al., 2018), yet to date there is little empirical evidence to support this widely held paradigm in relation to microplastics. To address this knowledge gap, here we utilise the plasticity in feeding mode of the sediment dwelling marine worm *Hediste diversicolor* (O.F. Müller, 1776) to compare the microplastic ingestion for this species when it is filter feeding to when it is deposit feeding (Harley, 1950; Hird et al., 2016). *H. diversicolor* is a polychaete worm that inhabits the shallow sediment strata in an interconnected network of burrows with several connections to the sediment surface, known as the gallery (Gebhardt, 2019; Davey, 1994) in estuaries across the North Atlantic, The Mediterranean, and The Black Sea (OBIS, 2022). *H. diversicolor* are omnivorous with several distinct feeding modes including scavenging, carnivory, filter feeding of suspended particulate matter in the water column and deposit feeding from organic matter and detritus in and around the surface layers of sediment (Harley, 1953; Olivier et al., 1997).

When deposit feeding, the ragworm will emerge partially from its burrow and consume sediment, macroalgae, carrion, detritus and plant material using its eversible pharynx (Aberson et al., 2016) or browse the upper layers of subsurface sediment for food. In comparison, when filter feeding *H. diversicolor* secretes a mucus net across the opening of the burrow, bodily undulations then create a current to draw down water through the net and the net is subsequently consumed. *H. diversicolor* have been reported to ingest microplastics in situ, with between 0.8 and 3.7 particles per individual reported for field collected worms (Bour et al., 2018; Lourenço et al., 2017; Missawi et al., 2020). The phenotypic plasticity of feeding modes exhibited by *H. diversicolor* in their capacity to switch between filter feeding and deposit feeding, makes them an ideal species to test the role of feeding mode on microplastic uptake.

Here, we exposed worms for varying times to the presence of two test microplastic particles and then quantified particle uptake using an imaging flow cytometry (IFC), to test the hypotheses that: (H1) the number of particles in the worms will increase with exposure time, (H2) filter feeding worms will have a greater body burden of plastics than deposit feeding worms, and (H3) fragments will be found in higher quantities than fibres in the worms due to their shape making them easier to ingest. Lastly, we confirmed the presence of microplastics and investigated the range of shape, polymer, and size classes found in wild caught worms and sediments from local estuarine study sites.

2. Methods

2.1. Animal husbandry for H diversicolor used in experiments

Hediste diversicolor (mean ± S.E.: wet weight: 0.66 ± 0.03 g) were collected from Exton (Devon, UK), and transported to the University of Exeter along with 20 L of sediment from the same location. Worms were transferred to a single large covered holding tank for acclimation (3 days) filled with 0.2 μm filtered artificial seawater (ASW) (12 °C, 22 ppt) and fucoid seaweeds to add habitat complexity. Worms were fed ad libitum with crushed trout pellets. Worms were also collected at the same location to use as control worms to ensure that the flow cytometry method did not misidentify our exposure plastics. These worms were returned to the lab and immediately killed by placing in the freezer in individual 15 mL falcon tubes.

2.2. Sediment preparation

Sediment was sieved to 500 μm to remove large particulates, organisms and organic matter and mixed in with 0.2 μm filtered 22 ppt ASW to allow organic detritus to float off. The sediment was then allowed to settle and the overlying water poured off and topped up twice a day for 3 days.

2.3. Microplastic preparation

The microfragments used in both sediment and water exposures were polyamide fragments generated by cryo-milling 3 mm polyamide (Nylon 6,6) granules (purchased from Goodfellow Cambridge Ltd.) to a size of <50 μm (See Supplementary Section 1.1 and Supplementary Fig. 1. for details and histogram of size ranges). The microfragments were stained using Nile Red (as per Maes et al. (2017)), rinsed three times with acetone to remove excess Nile Red, rinsed in 0.2 μm Milli-Q water and then suspended in filtered ASW. Microfibres were purchased from Barnet Europe as precision cut fibres of 23 × 500 μm and subsequently dyed with Nile Red using the same protocol as the microfragments. These particles were chosen as they are easy to disperse in experimental systems, and have an affinity to Nile Red dye which made their visualisation and recovery easier.

2.4. Pre-exposure depuration and feeding initiation

The worms (n = 180) were transferred into 400 mL glass beakers, and the beakers placed inside an opaque plastic box with lid to achieve total darkness, with one individual worm per beaker and 30 beakers per box. The beakers were filled with ASW and gently aerated through the lid of the exposure boxes and a 6 cm long glass tube (diameter 8 mm) was added to act as an artificial burrow. Worms were kept in these conditions for a total pre-exposure period of 72 h and then split into two cohorts: deposit and filter feeding with 90 individuals in either cohort. The worms were left for 48 h to allow them to depurate, then transferred into clean beakers of ASW and given food, and allowed to feed for 24 h in two separate manners. To induce filter feeding, 400 μL of algae paste was added to each beaker (Reed Mariculture Shellfish Diet 1800, ~2 billion cells mL^{-1}) and feeding was characterised by the production of mucus nets at burrow entrances (Riisgård, 1991). To maintain deposit feeding in the deposit feeding cohort, each worm was fed ~200 dead *Artemia* sp., cultured at the University of Exeter and frozen to euthanise, 24 h before feeding. They were enumerated and subsequently pipetted into the beakers.

2.5. Experimental design

To test between filter and deposit feeding modes and across the two plastic types, four treatments were set up. (1) Deposit feeding on microfragments, (2) filter feeding on microfragments, (3) deposit feeding on microfibres, and (4) filter feeding on microfibres. Each treatment comprised 45 worms, each in individual exposure beakers, totalling 180 worms. After the 72 h pre-exposure period (48 h depuration + 24 h feeding) worms were split into their four treatments, and each treatment ran for 168 h. Nine worms from each treatment were removed from the exposure at specific time points; at 24, 48, 72, 96, and 168 h.

2.6. Microfragment exposures

Microfragments were pre-mixed into the sediment collected from Exton using a bottle roller. 1.2 L of sediment was added to 6 × 2 L duran bottles and microfragments added to the bottles at a nominal concentration of 1000 particles per mL of sediment. 1000 particles per mL were selected based on preliminary work and concentrations below this resulted in a lack of uptake of fibres by worms and therefore constituted our limit of uptake for *H. diversicolor*. The same concentration of microfragments was maintained in the sediment and water exposures so at to provide both feeding modes with the same number of microplastics per unit volume of exposure matrix. This equates to half the total number of plastics being available to the worms per beaker in the sediment exposures than in the seawater only exposures as the volume of exposure matrix was 170 mL of sediment compared to 340 mL of water.

Once mixed, 170 mL of plastic contaminated sediment was transferred to 45 beakers for the deposit feeding worms. Subsequently, 170 mL of ASW was carefully poured over the sediment down the side of the beaker so as to avoid any resuspension. For the filter feeding worms, 45 beakers were filled with 340 mL of ASW and a clean glass burrow placed in the beaker. Microfragments were then pipetted into the water and an individual worm was added to each beaker giving 45 replicates for each treatment, again at a nominal concentration of 1000 particles per mL. Twenty-five microliters of algae paste was also added to continue to stimulate filter feeding in the filter feeding worms. Worms were left to feed in their respective treatments and at the specific time points, removed from their beaker, rinsed, placed in a 15 mL falcon tube, and frozen to euthanise them.

2.7. Microfibre exposures

The same methodology was undertaken to expose both feeding modes to microfibers. In brief, mixing microfibers into the sediment, and then exposing 45 worms to microfibers at a nominal concentration of 1000 fibres per mL in 170 mL of sediment for the worms to feed by deposit feeding and 45 worms at a nominal concentration of 1000 fibres per mL in 340 mL of ASW to feed via filter feeding. As with the microfragments, the concentrations were maintained so as to provide each worm access to the same number of particles per unit volume of the media they were feeding from. Worms were left for the same time periods (9 worms removed at 24/48/72/96/168 h exposure time) and euthanised in the same manner.

2.8. Measuring microplastic uptake in exposed H. diversicolor

2.8.1. Sample preparation

Microplastics were recovered and enumerated from each individual *H. diversicolor* in two ways. For the microfragments, worms were initially digested using potassium hydroxide (KOH) at a concentration of 20 %. Eleven millilitres of KOH was added to each individual falcon tube and placed in an oven for 48 h at 60 °C. The digestate contained a relatively high organic matter content and needed further digesting. Samples were homogenised by shaking, 1 mL of homogenate was then removed into a 2.5 mL reaction tube and 1 mL of 30 % hydrogen peroxide (H_2O_2) added to the same tube and leaving at room temperature for 24 h. This left ~2 mL of dilute worm homogenate that was analysed via IFC to determine microfragment concentrations. Light and fluorescent microscopy was used to manually count larger particulate.

2.8.2. Quantification of microfragments using Imaging Flow Cytometry (IFC)

Imaging flow cytometry allowed the rapid (thousands of objects per second) analysis, quantification, fluorescence detection and morphological characterisation of particulate within the worm homogenate. Samples were hydrodynamically focused, allowing the individual interrogation of each particulate by a series of lasers and brightfield illumination. This allowed a visual separation of fluorescent, Nile Red stained microplastics from the non-fluorescent worm homogenate, for quantification and morphological characterisation. The application of IFC in this way, as an alternative to time-consuming manual counting and measuring of microplastics via optical fluorescence microscopy, allowed rapid, high-throughput analysis with technical replications, removed human bias and recovered microplastics that may otherwise be obscured by worm homogenate or difficult to detect and measure due to their small size.

To count the particles using IFC, first samples of the stock, ground microfragments were scanned and scans refined to capture true fluorescent signals (Fig. 1A and B). A selection of objects was also visually verified as fragments (Fig. 1C). Control worm homogenate samples, not exposed to plastic, were scanned to ensure that worm homogenate did not fluoresce in the plastic "gate" (Fig. 1D and E). Finally, the gates used to 'accept' a plastic particle was refined to ensure only plastic particles were captured (Fig. 1F) and a bespoke mask applied automatically to all particles scanned to allow quantification and morphological measurements to be made (Fig. 1G). For more details, please see section 2 of the Supplementary Materials.

2.8.3. Manual counting of microfibres via light and fluorescent microscopy

To recover the microfibers, an initial digestion step using KOH was performed as with the microfragments. However, due to their larger size these could not be easily detected via IFC and, as they were easily visible by light and fluorescent microscopy, they were counted manually. Each worm digestate was filtered on to a 47 mm, 5 μm polycarbonate membrane filter paper, placed in a 50 mm petri dish, and the microfibers were counted to give a number of fibres per worm.

2.8.4. Assessing microplastics in natural H. diversicolor populations and sediments

To describe the characteristics and abundance of MPs taken up in-situ by natural populations of *H. diversicolor*, three estuaries were sampled in the South West of the United Kingdom. The estuaries and sites were located on the Plym, Plymouth (50°22′22.3″ N 4°06′10.6″ W), at Bowcombe Creek on the Kingsbridge Estuary (50°16′36.9″ N 3°45′37.8″W), and at Exton on the Exe Estuary (50°40′03.1″ N 3°26′39.2″ W).

Using a garden fork, 30 *H. diversicolor* individuals were collected from estuarine sediments at each of the three sites on the mid shore at low tide during January and February of 2019. Each worm was washed in situ using 0.2 μm filtered Milli-Q water and transferred to individual 15 mL falcon tubes. In the laboratory the worms were individually weighed, frozen in liquid nitrogen, and stored at −20 °C. Twenty-five millilitres of a 0.2 μm filtered, 10 % solution of potassium hydroxide (KOH) was added to each falcon tube containing an individual worm which was then placed in an oven for 48 h at 60 °C until all soft tissues had been digested. The digestate was then vacuum filtered through a 10 μm Cyclopore polycarbonate membrane filter.

Sediment samples were also collected using a 50 mL falcon tube as a push core, to collect ten 50 mL sediment samples in the same area the worms were collected and were frozen on return to the lab. Defrosted sediment from each falcon tube was then placed into individual 500 mL beakers, mixed using a metal spatula and left to dry in an oven at 60 °C for 12 h. From each of these samples (ten per site), 50 g of dry sediment was isolated for the identification of potential microplastics. Density floatation using Sediment-Microplastic Isolation (SMI) units (see Coppock et al. (2017)) were then used to separate plastic-like particles from the sediment.

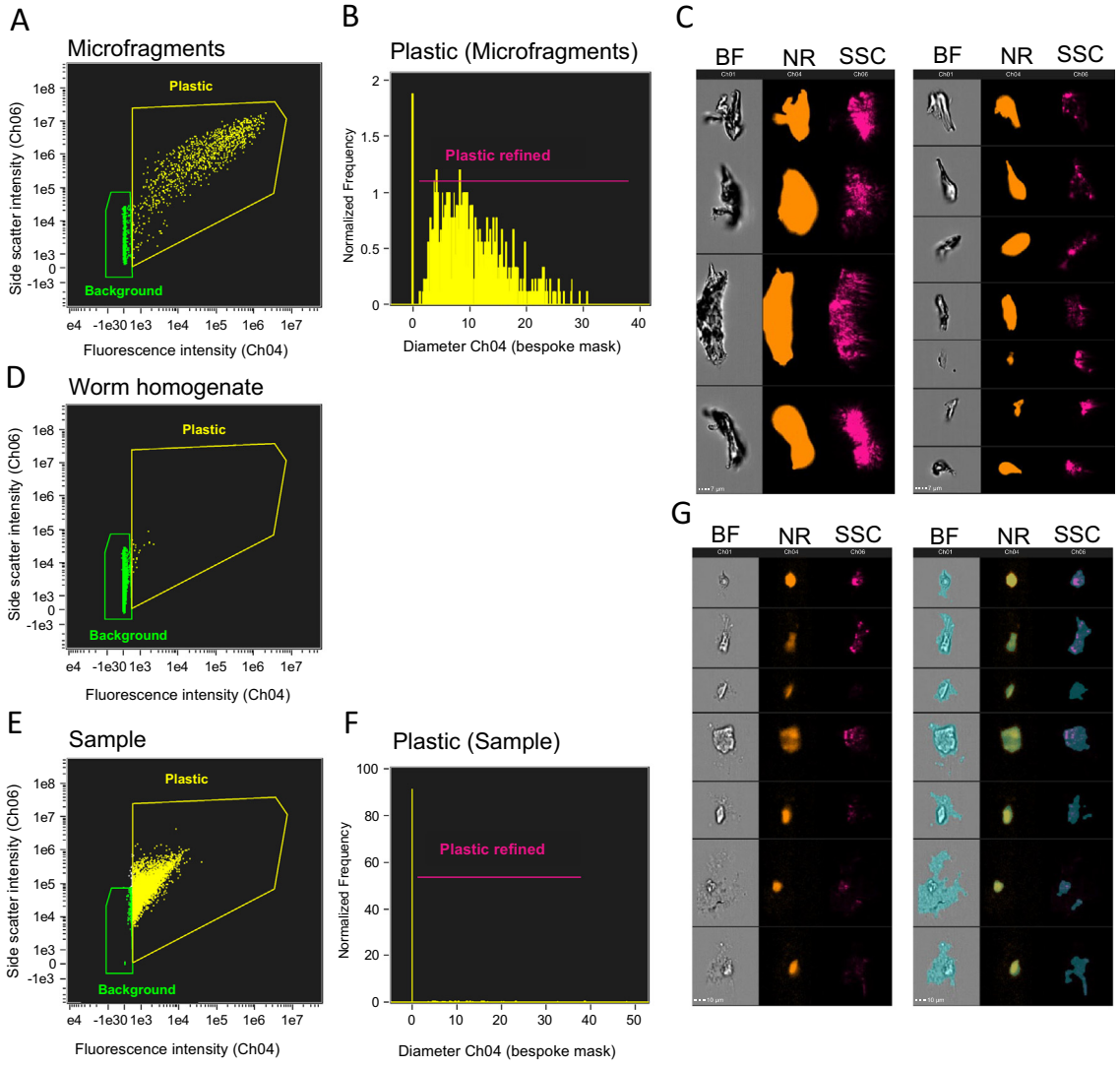

Fig. 1. Imaging flow cytometry allowed the identification and quantification of Nile Red dyed plastic fragments within worm homogenate. To distinguish plastic fragments from background worm material, a positive, microfragment control was analysed (A) and characterised as 'plastic' based on fluorescence (Ch04) and side scatter (Ch06) intensities. Microfragments were further refined to exclude objects not exhibiting a true fluorescence (B). Exemplar images from the 'plastic refined' population are presented (C) showing Brightfield, (BF) Nile Red fluorescence (NR) and side scatter (SSC). Control worms, containing no microfragments were also analysed to ensure 'background' worm homogenate did not fluoresce in the 'plastic' region (D). For plastic fed worm samples, background worm homogenate was not collected during acquisition (E). To ensure the exclusion of any background worm homogenate and non-fluorescence objects from ingested microfragments, a 'plastic refined' gate was applied. Microfragments from within the 'plastic refined' gates (G) were quantified and morphologically characterised using a bespoke mask fitted to the Nile Red fluorescence of objects (blue overlay) to avoid any background worm homogenate contributing to the measurements.

A filtered solution of zinc chloride ($ZnCl_2$) at a density of 1.5 gcm^{-2} was mixed with each 50 g of sediment in a separate SMI, placed in a fume hood and after settling, was vacuum filtered through 10 μm Cyclopore polycarbonate membrane filters.

The filters from the worm digestions and sediment flotations were subsequently visually inspected for likely microplastic particles. These were particles that looked of anthropogenic origin based on shape and colour. Using an Olympus SZX16 microscope each filter paper was scanned at magnifications 1.6 and 3.2× for 5 min per filter paper. Any particle identified as a likely microplastic was then photographed using the attached Olympus XC10 camera, classified by shape (fibre, fragment or film) and colour and then transferred to a clean Sterlitech 5 μm silver membrane filter paper for subsequent Fourier-Transformed Infrared (FTIR) analysis. Images were loaded into ImageJ (Rasband, 1997-2017) version 1.47 and the maximum and minimum lengths were measured.

Polymer identification was performed according to Jones et al. (2021) using a Perkin Elmer Spotlight 400 FTIR spectrometer using the attenuated total reflection (ATR) attachment for particles >1 mm and the μFTIR Imaging System (MCT detector, KBr window) for particles <1 mm. Spectral matches >60 % were accepted with characteristic peaks compared with standards to help confirm matches. Highly-modified cellulose and biopolymers, in addition to traditional plastics are included in our definition of plastic particles in this study as they are a significant aspect of marine micro-litter, found in the same locations as microplastics, generated and formed in similar ways, and they exert similar mechanisms of toxicity (Hartmann et al., 2019; Slaughter et al., 2011).

2.9. QA/QC

All laboratory processes were conducted in a laminar flow hood and with the researchers wearing cotton lab coats. Atmospheric contamination blanks consisted of a wetted Whatman GF/C filter paper in a petri dish open on the bench, in the fume hood or oven, whenever a sample was open and being analysed. Procedural blanks were also carried out, placing KOH in an empty falcon tube and $ZnCl_2$ in an isolation unit without sediment and filtered to account for any contamination arising from our method.

Atmospheric and procedural blanks underwent microscopy and FTIR analysis. The procedural blanks taken from the worm digestion protocol contained an average of 0.56 ± 0.22 black fibres and 1.12 ± 0.32 clear fibres. The procedural blanks collected by running the sediment processing protocol without a sediment sample contained an average of 1.42 ± 0.15 black, 0.32 ± 0.05 clear and 0.22 ± 0.06 blue fibres. The atmospheric contamination controls contained an average of 0.21 ± 0.13 black fibres and 0.34 ± 0.09 blue fibres. The mean number of particles for each category (colour × shape) was subtracted from its respective matrix data (worm or sediment) prior to further analysis and therefore is not included in any data presented.

2.10. Statistical analyses

All data were initially tested for normality using a Shapiro-Wilk normality test and subsequently tested for homogeneity of variances using the Levene's in R (R CORE TEAM, 2019). To investigate the influence of time or feeding mode on microplastic uptake, a Two-Way ANOVA was undertaken with time as an independent factor with 5 levels (24, 48, 72, 96, & 168 h) and feeding mode as an independent factor with three levels (control, deposit feeding and filter feeding) using an additive interaction term. These were performed separately on the microfragment data and the microfibre data and the level of significance set at $p < 0.05$. To further investigate the influence of feeding mode on microplastic uptake the data found to be not normal ($p < 0.05$). Subsequently a nonparametric Kruskall-Wallis test was undertaken to test whether each feeding mode (filter feeding or deposit feeding) populations has significantly different levels of microplastic ingested. A Dunn's Post-hoc test was then used to identify differences between groups with p values adjusted using The Benjamini-Hochberg method. To test whether worms had ingested similar amounts of different sized microfragments the differences between the mean size ingested, largest size ingested, and smallest size ingested by each worm were tested with the feeding mode use as the independent variable in each case. Again, Kruskall-Wallis test was performed to test for differences between the feeding modes. Finally, to test for differences in the uptake of microplastics by worms at the three sample sites, a Kruskall-Wallis test followed by Dunn's Test was used. To test for differences between the microplastic numbers found in the sediments at each of the three sample sites, the data were normally distributed and so a One-Way ANOVA followed by Tukey's Post-Hoc test was applied. Data visualisations were made using the "ggplot2" (Wickham, 2016) and "ggsci" packages (Xiao, 2021).

3. Results and discussion

3.1. The influence of time on uptake of microfragments and microfibres

Microplastics were ingested by worms in each of the 4 treatments but no effect of time on the number of particles ingested per worm was found for any of the 4 treatments between 24 and 168 h (Fig. 2) and so Hypothesis 1 was rejected. A two-way ANOVA identified feeding mode as a significant driver in uptake for the microfibers ($F(1, 81) = 22.06$, $p \leq 0.001$, Fig. 2A) but not microfragments ($p = 0.18$, Fig. 2B). Time was also found to not be a significant factor in the ingestion of microfragments or microfibres by either filter or deposit feeding worms. Simple main effects analysis found no difference between feeding modes ($p = 0.70$) or time ($p = 0.67$) for microfragments, and time similarly was not a significant factor for microfibers ($F(5,81) = 1.54$, $p = 0.19$).

Worms filter feeding on fibres ingested on average between 20.8 and 140.2 fibres (±SE 8 and 41 fibres respectively) and when deposit feeding on fibres, ingested on average between 0.2 and 1.2 fibres (±SE 0.1 and 0.4 fibres respectively) (Fig. 2A). In contrast, worms filter feeding on fragments ingested on average between 3983 and 12,205 fragments (±SE 938 and 7323 fragments respectively) and when deposit feeding on fragments, ingested on average between 4468 and 8549 fragments (±SE 853 and 2219 respectively) (Fig. 2B). An equilibrium state where the internal concentration of plastic within a worm (gut) becomes stable seems to occur here over a period of hours rather than days for both feeding modes, ingesting both shapes of plastic.

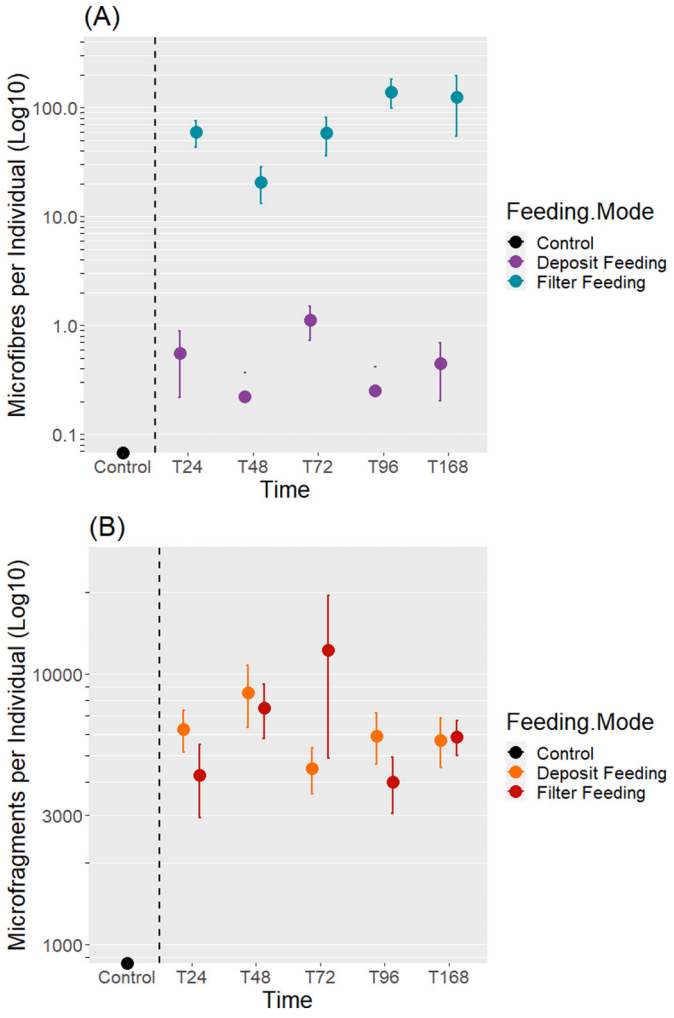

Fig. 2. (A) The ingestion of microfibres over the investigated time points: 24, 48, 72, 96, and 168 h by *Hediste diversicolor* and (B) the ingestion of microfragments over the same time points by *Hediste diversicolor*. Microplastic uptake is Log10 transformed for data visualisation. Control worms contained no microfragments or microfibres and nine worms were used at each time point totalling 45 worms per feeding mode for the microfibre and microfragment exposures respectively.

A similar finding is reported by Missawi et al. (2021) who demonstrated that at a high microplastic concentration (100 mg of microplastics per kilo of sediment) exposed worms reached an equilibrium state after 3 days. Further, as *H. diversicolor* is capable of eliminating 89 % of microplastics from its gut when placed in clean artificial seawater after exposure to microplastics in sediment after 24 h (Revel et al., 2020), it seems likely that the equilibrium state the worms have reached is due to a constant ingestion and equivalent egestion of microplastics over time.

3.2. The influence of feeding mode on uptake of microfragments and microfibres

As time was not a controlling factor determining the uptake of microplastics by *H. diversicolor*, and feeding mode seemingly important, all time points were pooled to test hypothesis 2; that feeding mode will influence uptake (measured as number of particles per worm) and hypothesis 3; that uptake of microfibres and microfragments will differ. We hypothesised that filter feeders to contain more microplastics than deposit feeders as this has been shown in previous work by Setälä et al. (2016), however, this was experimentally tested between six

different organisms of differing body sizes and each with a different feeding mode. Our data suggest that the ingestion of microplastics is not uniform between feeding modes based on the particle shape the worms are exposed to. When exposed to microfibers, filter feeding worms contained ≈15,000 % more fibres than deposit feeding worms (Kruskall-Wallis Test, H(1) = 56.09, $p \leq 0.001$), containing an average of 0.52 (±0.13 SE) fibres per individual when deposit feeding and 80.28 (±17.37 SE) when filter feeding (Fig. 3A) at the time of sampling. When exposed to microfragments there was no significant difference between feeding modes with deposit feeders containing an average of 6167.2 (±638.3 SE) microfragments per individual and filter feeders containing 6933.9 (±1694.2 SE) microfragments per individual (Fig. 3B).

The differing feeding responses of the two feeding modes to the different plastic shapes is of interest as there is an emerging picture in the literature that organismal ingestion of microplastics can be different based on the characteristics of the microplastics encountered. For example, The predatory gastropod snail *Reishia clavigera* has been found to take up significantly more polypropylene microfibers (63 and 250 μm) than polypropylene (PP) microfragments (63 and 125 μm) following exposure to plastic contaminated mussel tissues (Xu et al., 2022). Conversely, the mainly detritivorous daggerblade glass shrimp *Palaemonetes pugio*, took up significantly more

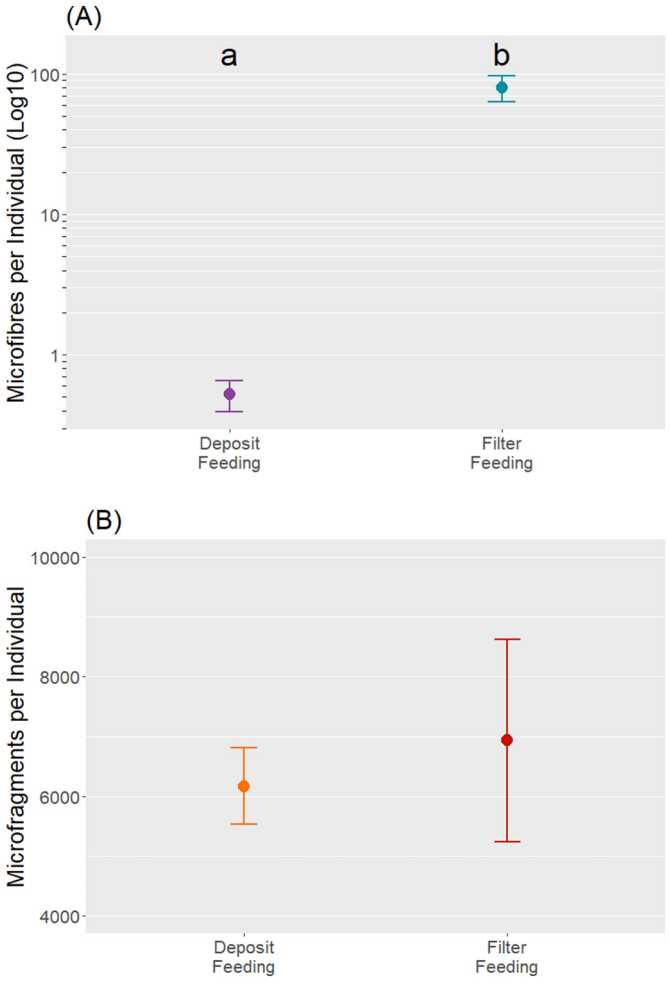

Fig. 3. (A) A plot of the microfibre uptake by deposit and filter feeding *Hediste diversicolor*. Points that do not share the same letter were statistically different from each other. (B) The microfragment uptake of deposit feeding and filter feeding *Hediste diversicolor*, which were not significantly different from each other. Data were Log10 transformed where indicated for visualisation only, and data for both A and B constitute a combination of data across all time points sampled and therefore each point represents the mean of 45 worms.

fragments (PP; 34 & 93 μm) than spheres (PS; 30 & 75 μm, PE; 35, 59, 83, 116 & 165 μm) and fibres (PP; 34 & 93 μm) (Gray and Weinstein, 2017) in a high concentration, acute (3 h) exposure study. Here, we show within a single species that the ingestion of particles may change based on the shape (and size) of the particle, but also this will differ based on how an organism feeds or encounters the particles.

H. diversicolor is, perhaps surprisingly, a highly efficient facultative filter feeder and as such should be considered a useful indicator species for microplastic contamination. The harbour ragworm has a particle capture efficiency of 100 % for particles >7.5 μm and has a clearance rate of around 0.54 L per hour (Riisgård, 1991) and, as it can be present in extremely high densities in estuaries (densities up to 20,000 individuals per m^2 found by Ólafsson and Persson (1986)), is considered a key organism in the control of phytoplankton abundance (Riisgård, 1991).

The efficiency of filter feeding in *H. diversicolor* may explain why ≈15,000 % more microfibres were taken up when worms were filter feeding compared to when deposit feeding (Fig. 3A) in our exposures. Mucus nets effectively immobilise microplastics (Costa et al., 2006) creating a concentrated agglomeration of microplastics to be ingested, which may also mask the plastic surface characteristics of these microfibres and encourage the ingestion of these plastic particles (Porter et al., 2019; Savoca et al., 2016; Hodgson et al., 2018). Deposit feeding worms may be actively rejecting fibres in the sediment due to their shape and orientation on encountering them. A study by Delefosse and Kristensen (2012) observed that *H. diversicolor* could not ingest seeds of *Zostera marina* (eelgrass); 3.5 × 1.5 mm in size, and perhaps this demonstrates the need for *H. diversicolor* to encounter our longer fibres "end-on" to ingest them leading to lower ingestion rates in the deposit feeding worms due to a mouth size restriction. Individuals of *H. diversicolor* collected from contaminated natural populations have been found to have ingested microplastics up to 3 and 4 mm in length (Hodgson, 2019; Delefosse and Kristensen, 2012), but as these were wild caught animals it is not possible to tell under which feeding mode these particles were captured. The much lower proportion of available microfibres taken up (number ingested compared to total added to the exposure beaker) compared to microfragments, regardless of feeding mode, further suggests that fibres are less bioaccessible to *H. diversicolor* than microfragments. Based on average uptakes reported in Fig. 3 roughly 0–0.2 % of fibres and roughly 2–3 % of fragments were ingested.

Hediste diversicolor is well adapted to the ingestion of particulate material including sediment grains when deposit feeding and polychaetes in general are able to discriminate between different sized particles for digestion by size-selective feeding mechanisms (Self and Jumars, 1988). In our exposures, the concentration of microplastics in each treatment was the same, however, since the volume of sediment per beaker was half that of the water only exposures (to allow for overlying seawater), the total number of particles available to deposit feeders within the beaker was 50 % less than those available to the filter feeders. Despite this the uptake of microfragments by *H. diversicolor* was not significantly different between feeding modes (Fig. 3B). *H. diversicolor* may therefore, even be actively selecting for the polyamide fragments in the search for nutritional particles in the sediment, as deposit feeders have demonstrated some selection for particles of lower specific gravities (Taghon, 1982) such as plastic. *Owenia fusiformis*, a tube dwelling worm, has been shown to have an increased selection for particles of a lower specific gravity; selecting for plastic over glass regardless of size (size range 3–648 μm) (Self and Jumars, 1988). This is made more likely in *H. diversicolor* as sediment only accounts for a minor part of ingested material in the species as it does not conduct sediment feeding in a strict sense (Gebhardt, 2019).

3.3. Size selection from the microfragments between deposit and filter feeding worms

Due to the nature of cryomilling and fractional sieving of the virgin polyamide granules into microfragments, a size range of fragments was produced ranging from 2.3 μm to 79.6 μm (Fig. 4). It was therefore possible to further compare the selectivity of particle sizes between the two feeding

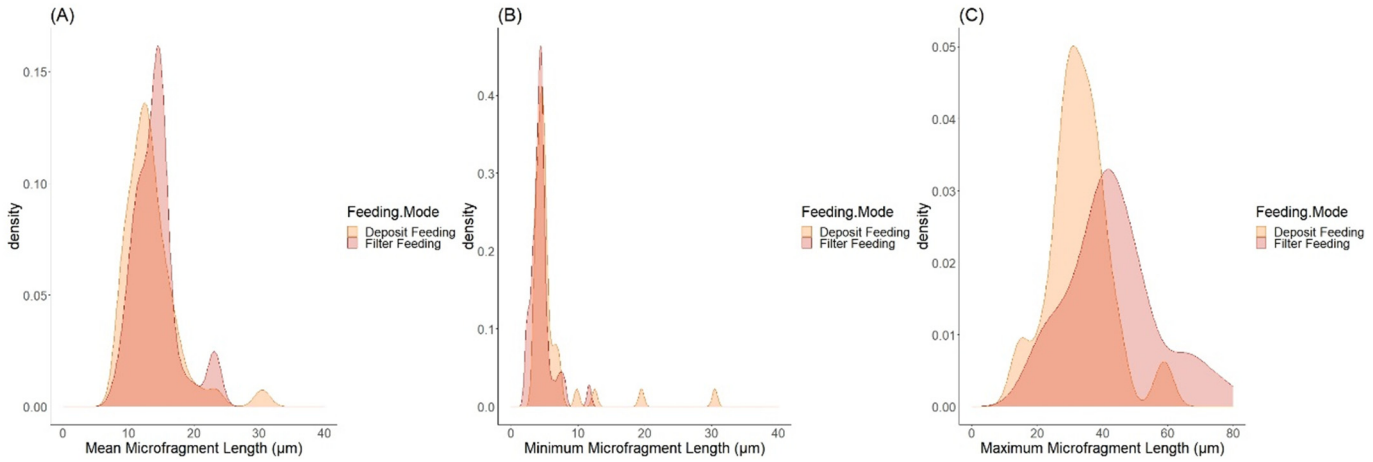

Fig. 4. (A) A kernel density estimation (density) plot of the mean microfragment lengths taken up by each individual *Hediste diversicolor* by either deposit or filter feeding, (B) a density plot of the minimum microfragment length taken up by each individual worm by either deposit or filter feeding, and (C) a density plot of the maximum microfragment length taken up by each individual worm by either deposit or filter feeding. These data were generated from the microfragments recovered from all of the 90 fragment feeding worms (filter feeding and deposit feeding modes had 45 replicate worms each).

modes exposed to microfragments specifically. A Kruskall-Wallis test showed that there was no significant difference between the mean microfragment length ingested by filter and deposit feeders ($H(1) = 0.7$, $p = 0.38$) (Fig. 4A). Filter feeders did however take up significantly more smaller, and larger fragments than deposit feeders (Fig. 4B & C) (Min. Frag. Length: $H(1) = 15.2$, $p < 0.001$, Max. Frag. Length: $H(1) = 6.27$, $p = 0.012$). These differences are interesting as they reflect the *H. diversicolor*'s ability to sense particle size and therefore the selectivity of each feeding mode.

When given the choice *H. diversicolor* is more likely to feed by predation or deposit feeding than filter feeding as the available energy per prey item is likely to be much higher (Barnes et al., 2009). Optimal foraging theory (Schoener, 1969; Hughes, 1980) dictates the choices an organism makes with regards to food selectivity, based on the available food supply. In the filter feeding exposures there was an available supply of algae which initiated facultative filter feeding in the worms; a more indiscriminate feeding practice than predation or deposit feeding in *H. diversicolor*. Optimal foraging theory dictates that suspension feeders should preferentially ingest larger particles because the calorific value of suspended particulates is directly related to their volume (Shimeta and Koehl, 1997). This may explain the significant consumption of larger microfragments in the facultative filter feeding worms compared to those deposit feeding (Fig. 4C), and additionally contribute to the higher ingestion of fibres in the filter feeding worms compared to deposit feeders.

Whilst most inorganic material will pass through a *H. diversicolor* when deposit feeding particle size is considered by the species and explains the tighter size distribution in deposit feeders feeding on microfragments (Fig. 4). Revel et al. (2020) exposed *H. diversicolor* to 0.4–400 μm polypropylene microfragments and they were found to contain only those in the 25–70 μm range when sampled, which aligns with our data. Size selectivity therefore seems dependent on feeding mode when feeding on both fibres and fragments. In the microfragment exposures, particle morphology seems to dictate whether the particles are bioaccessible (i.e. ingestible) and perhaps selected for or not. Beyond feeding mode, particle size and specific gravity, the amount of mucus production, mouth size, water flow rate, food availability and food type seem to be factors in the feeding selectivity in marine worms (Taghon, 1982; Self and Jumars, 1988; Gebhardt, 2019; Hentschel, 1996; Shimeta and Koehl, 1997; Leppänen, 1995).

3.4. Imaging flow cytometry

Imaging flow cytometry (IFC) proved to be an extremely versatile tool for our microplastic exposure study as it combines high throughput flow cytometry with digital microscopy generating quantitative data such as counts but also morphometric data on the recovered particles (Park et al., 2020) (Fig. 4). This could be of great benefit for studies using a range of particle shapes and/or sizes as data can be collected for what particles, from a suite of those exposed, an organism took up for example. Furthermore, these can be fluorescently labelled so as to be identified as different even if their shapes and size ranges overlap. This could allow high throughput *co*-exposures of various plastic property combinations (shape × size × polymer) allowing us to better understand the dynamics of particle type uptake by organisms.

3.5. Microplastic ingestion by wild caught H. diversicolor

Finally, we confirmed the presence of microplastic particles in both worms and the sediment at all three sites that were sampled. Wild populations of *Hediste diversicolor* were found to have ingested plastic at every site investigated, with an average of 0.71 (±0.09 SE) anthropogenic particles per individual across all sites. A Kruskall-Wallis test identified that significantly more plastics were found in worms from The Plym and Exton (0.83 ± 0.14 and 0.96 ± 0.20 SE particles per individual respectively) than those from Kingsbridge (0.33 ± 0.10 SE particles per individual) ($H(2) = 9.34$, $p = 0.01$) (Fig. 5Ai), likely due to the greater population densities in the Plym and Exe catchments. These data followed broadly the trend of contamination level in the sediment (Fig. 5Aii). A One-Way ANOVA identified the Plym Estuary as the most contaminated with an average of 5.8 (±0.90 SE) particles per 50 g of sediment, followed by Exton with an average contamination of 4.9 (±0.50 SE) and Kingsbridge with an average contamination of 3.1 (±0.65) particles per 50 g sediment ($H(2) = 37.8$, $p = 0.036$). It would seem therefore that the sediment contamination level is a major driver of microplastic ingestion in *H. diversicolor*.

Of the particles the worms had ingested, fibres comprised the majority of particle shapes recovered ranging from 44 to 60 % of the total particles in worms at each site, with fragments 32–40 %, and films 0–24 % making up the rest (Fig. 5Bi). In the sediment fibres and fragments were more evenly distributed with 35–56 % of the total being fibres, 37.5–50 % being fragments, and with films again being the smallest group of particle shape found with 6.25–16.1 % of the total (Fig. 5Bii). In our experimental work, fibres were less bioaccessible than fragments. In the environment, it would seem that the picture is not so clear-cut. This is possibly because our exposure fibres were monofilament polyamide nylon, which may be much more obvious as a non-nutritious particle than those found in the sediment may. Of the polymers recovered from the worms 74 % of the fibres were anthropogenically modified cellulose (Supplementary Fig. 2) which are more similar to natural fibres such a cotton or sisal (Cai et al., 2019) and therefore may be more readily ingested. Their flexibility, state of

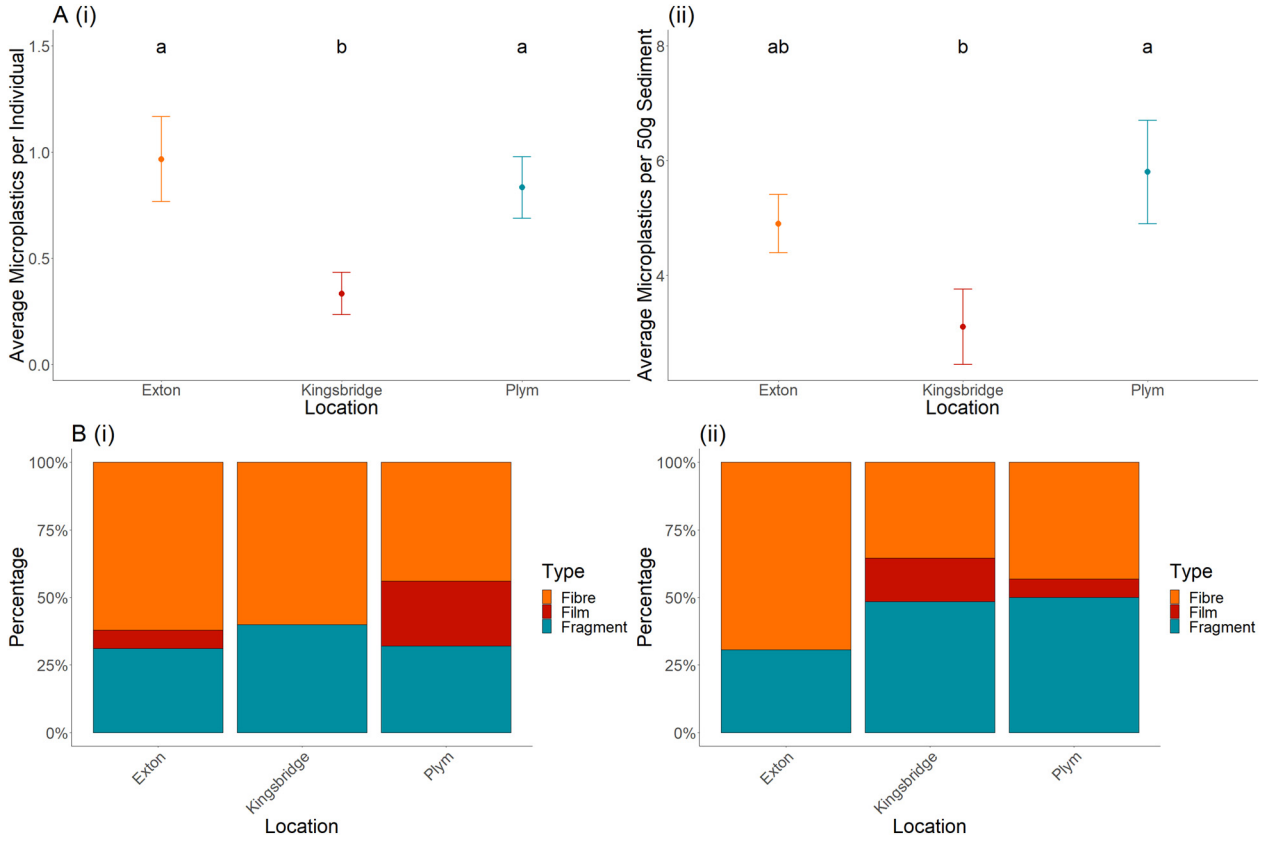

Fig. 5. (Ai) The average number of microplastics per individual recovered from *Hediste diversicolor* collected from each sample site: 30 worms at Exton on the Exe estuary, 30 worms at Bowcombe Creek on the Kingsbridge Estuary, and 30 worms on the Plym Estuary. (Aii) The average number of microplastics recovered from 50 g of sediment collected at the same sites with 10 replicates at each site totalling 500 g of sediment analysed. Points that do not share a letter were significantly different from each other. (Bi) The percentage contribution of fibres, films and fragments recovered from the 30 worms at each site and (Bii) the percentage distribution of fibres, films and fragments recovered from 500 g of sediment at each site.

degradation (cellulose will degrade more rapidly than microplastics making it less recalcitrant (Macieira et al., 2021)), and biofouling may also play a role in their bioaccessibility as each individual worm assesses particles for ingestion.

3.6. Biological traits: a general discussion

The benthos is expected to accumulate large quantities of plastic over time and this will in turn lead to increased body burdens of plastic in marine benthic organisms (Berlino et al., 2021; Everaert et al., 2018). To date, many organismal responses to microplastic ingestion have been recorded through exposure experiments using benthic invertebrates, demonstrating physical and chemical impacts on mortality, development, behaviour, reproduction, and cellular responses (for a good review of these see Haegerbaeumer et al. (2019)). However, many of these studies have exposed organisms to microplastics in an aqueous phase and 72 % of benthic microplastic exposure studies reviewed by Haegerbaeumer et al. (2019) used arthropods and molluscs highlighting our relative lack of investigation of the benthos as a whole and the delinking of species and their environment. Here we demonstrate that organisms should be exposed in scenarios similar to their environmental exposure routes and that particle type significantly influences uptake with our experimental worms taking up more fibres in aqueous phase when filter feeding, and possibly actively selecting for microfragments in the sediment when deposit feeding. We would therefore encourage the investigation of multiple particle (co)exposures to look at relative rates of uptake, perhaps using IFC as highlighted here to expedite the process. Similarly, we would encourage the investigation of other benthic fauna; particularly abundant organisms that live within the sediment to help increase our knowledge of uptake into benthic invertebrate species. Further, there remains a paucity of data on retention of microplastics within the benthos and limited understanding of the dynamics of plastic particles as they move from the aqueous to sedimentary realm and so investigations that include depuration phases should be encouraged. Benthic invertebrates contribute up to 90 % of prey biomass to freshwater and marine fishes (Haegerbaeumer et al., 2019) and so the trophic implications for benthic invertebrate particle uptake are as yet unknown.

Biological traits are a well-established framework for understanding the response of organisms and ecosystems to anthropogenic stress. Here we used the trait of feeding mode to investigate aspects of microplastics uptake in the model organism *Hediste diversicolor* and found that whilst feeding mode affected the uptake of microplastics the particle shape did so too. It is clear that biological traits are useful tools in the understanding of microplastic uptake but that other traits (such as mouth size) and environmental factors will play an important role also. Further research, comparing feeding modes, and other traits will be important in elucidating their use in risk modelling. Of particular importance will be in understanding how strongly individual traits may dictate particle uptake to fully understand their usefulness in such risk modelling. Other ecological theories can help in part explain the likelihood of ingestion also, such as optimal foraging theory, however they must always be used in the context of considering the whole animal and ecosystem also as an organisms feeding biology cannot be adequately described by one parameter (Taghon, 1982). Understanding the threat a collection of microplastic properties (polymer, shape size etc.) pose to any organism (Koelmans et al., 2022; Bucci and Rochman, 2022), combined with the bioaccessibility of plastic particles (using biological traits), may allow us to expedite the investigation of the risk microplastics

play to fauna in the benthos, where high species turnover and patchiness (Chapman, 1999; Fromentin et al., 1997), and methodological complexity have constrained our investigations thus far.

CRediT authorship contribution statement

Adam Porter: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Dan Barber:** Methodology, Validation, Data curation. **Catherine Hobbs:** Methodology, Formal analysis, Investigation, Data curation. **John Love:** Resources, Project administration. **Ann Power:** Resources, Methodology, Formal analysis, Validation, Data curation, Writing – review & editing, Visualization. **Adil Bakir:** Conceptualization, Validation, Resources. **Tamara Galloway:** Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Ceri Lewis:** Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Data availability

The research data supporting this publication are openly available from NERC EDS British Oceanographic Data Centre NOC at: https://doi.org/10.5285/e7a3050e-9c4f-6119-e053-6c86abc0f558.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.159287.

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