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Towards the understanding of the uptake and depuration of microplastic in the ragworm *Hediste diversicolor*: Field and laboratory study

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Abstract :

An important number of studies have evaluated the presence of microplastics, particles with a size below 5 mm, in aquatic organisms. Studies have shown that these fragments are widely present in the marine environment, but research on the estuarine ecosystem is still scarce. In this study, two different approaches were used to evaluate the presence and ingestion of plastic particles in the ragworm Hediste diversicolor: a field study for the environmental assessment and a laboratory experiment in controlled condition. For the environmental evaluation, ingestion of microplastics was evaluated in the ragworm H. diversicolor sampled from the mudflats of the Seine estuary (France) during March and June 2017 and 2018, on two locations: S1 and S2, both characterized by high anthropogenic pressures, and for S2 a more influential hydrodynamic component. Ingestion of microplastics was measured in ragworms tissues and in gut content (sediment) after depuration. The number of particles as well as their size, shape and color were reported and compared between sampling period and locations. Results showed the presence of a low number of particles in both worms and gut content. In gut content, 45.6% and 87.58% of samples from site S1 and S2 respectively contained plastic like particles. In worms, 41.7% (S1) and 75.8% (S2) of analysed samples contained plastic like items. The lowest mean number of particles was 0.21 ± 0.31 (S1 in June 2017) in worms' tissues, but 0.80 ± 0.90 (S1 in June 2017) in the gut content and the highest was 1.47 ± 1.41 (S2 in April 2017) while the highest number was 2.55 ± 2.06 (S2 in June 2017) in worms and gut content respectively. The majority of suspected microplastics observed were fibers (66%) and fragments (27%), but films (3.7%) foam (2.1%), and granules (0.2%) were also identified. In addition, the most polymer type observed by Raman spectroscopy was polypropylene. Furthermore, a preliminary study of the ingestion and egestion of fluorescent polyethylene (PE) microbeads in the digestive tract of ragworms was conducted after exposure through water, during 1h at 1.2 × 106 MP/mL. Results showed a rapid turnover of PE microbeads throughout the digestive tract of worms especially after exposure through water. This study revealed that microplastics are ingested by the ragworm H. diversicolor but do not seem to bioaccumulate. More research is needed to measure potential chronic effects of microplastics on physiological parameters of H. diversicolor and potential trophic transfer of microplastics.

Highlights

► Very high variability in the number of plastic-like particles detected in ragworm *Hediste diversicolor*. ► More particles are found in the depurated sediment from the gut. ► Model polyethylene microbeads ingested by *Hediste diversicolor* from water or sediment area rapidely egested.

Keywords : Hediste diversicolor, ragworm, ingestion, egestion, microplastics, estuary, sediment

1. Introduction

Microplastics (MPs) have been observed in every ecosystem such as rivers, coastal region, oceans or even the arctic. Estuaries are essential ecosystems as they constitute an intermediate region between the ocean and rivers. Valuable for cities to develop tourism, industrial and agricultural activities, they represent ecologically significant habitats. The

pressure of anthropogenic activities can be challenging for estuarine species but also for marine organisms using the estuary as a shelter or for feeding (Chapman et Wang 2001). Previous studies have evaluated the ingestion of microplastics (MPs) by marine organisms from phytoplankton to large mammals (Phuong et al. 2016) and studies on MPs in estuaries are increasing (Wu et al., 2022; Malli et al. 2022). Because of their small size, MPs can be ingested, through water or sediment, and induce several injuries such as intestinal lesions, inflammation or even blockages (Lei et al. 2018; Rezania et al. 2018; Ahrendt et al. 2020).

The ragworm *Hediste diversicolor* is an annelid polychaete which is an excellent biological indicator to assess the health of estuarine ecosystems. Individuals are not very mobile, and mainly live in the sediment but can go on the surface during the tide. The ragworm *H. diversicolor* is also an important food resource for fish and birds. Few studies have evaluated ingestion of MPs in the ragworm located in the coast of Tunisia (Missawi, Bousserrhine, Belbekhouche, et al. 2020) and toxic effects related to oxidative stress or immunotoxicity were measured after field or laboratory exposures (Missawi, Bousserrhine, Zitouni, et al. 2020; Revel et al. 2018; Silva et al. 2020; Urban-Malinga et al. 2022). While knowledge on the effects of MP ingestion is increasing, mechanisms of MPs ingestion and egestion in estuarine species was recently investigated (Porter et al. 2023) but the retention and egestion process has not been included. In the study of Porter et al. (2023), authors exposed H. diversicolor to microfibres and microfragments for 1 day or 1 week through water or sediment and observed that the ingestion of MPs was different between feeding modes (filter feeders or deposit feeders) with microfibers ingested by filter feeders.

The objectives of this study were to (i) estimate the occurrence, shapes, colors, sizes and types of polymers of particles observed in worms living in the mudflats of the Seine estuary near Le Havre (France) and (ii) to investigate the ingestion and egestion of a model MP: fluorescent polyethylene (PE) microbeads. Among European rivers, the Seine is

considered to be one of the most polluted since it is subjected to serious anthropogenic pressure due to the important number of people living in Paris metropole (Burgeot et al. 2017; Dévier et al. 2013), but also two large agglomerations and harbors in Rouen and Le Havre (Normandie region), and many industrial and agricultural areas bordering the Seine River (Tavakoly et al. 2019; Gardes et al. 2020). Many contaminants from different activities end up in the Seine river such as hydrocarbons, metals, pesticides, but also plastic debris (Gasperi et al. 2014). To study ingestion and egestion of MPs, worms were exposed under controlled laboratory conditions to red fluorescent polyethylene microbeads 45-53µm in size.

2. Material and methods

2.1 Sampling of ragworms

Ragworms and their burrows were collected in March and June 2017 and 2018 in the intertidal mudflat located on the French Atlantic coast (Le Havre, France) in two sites characterized by high anthropogenic pressures (S1 and S2) and one of them with an important hydrodynamism (S2) (8 different sampling in total) (Fig.1).

Fig.1. Ragworms sampling sites in (A) le Havre, France, in (B) the intertidal mudflats (S1 and S2) of the Seine estuary (map data with GPS coordinates: google, 2020).

Sampling was operated in 5 quadrats ($1m^2$) every 100 m with for each quadrat, 12 worms similar size were gently collected by hand as well as 1 pool of burrows (5 per site and 40 pools in total) were collected. In total, per site, 60 worms (480 for all site and sampling period) and 5 pools of burrows (40 pools for all site and sampling period) were collected and transported to the laboratory. Worms were then depurated individually for 24 h, to eliminate their gut content, in glass beakers (previously rinsed with ultra-pure water) containing aerated artificial seawater (ASW) prepared with reconstituted salt (Tropic Marin Neu, Tropicarium Buchshlag, Dreieich, Germany) at the salinity corresponding to the site (14-30 psu, measured with a multiprobe) and at 15°C in the dark. After 24 h, worms were individually frozen in

aluminium foil at -20°C and gut content, corresponding to the depurated sediment, were sampled with glass pipettes and stored individually in clean glass tubes at -20°C.

2.2 Identification of MPs in worm bodies and gut content

Before digestion of ragworms, the total fresh weight and size frequency of worms were measured using the length L3 indicator (prostomium, peristomium and first chaetiger) (Gillet et Torresani 2003).

Analyses of MPs were performed on individual ragworms and depurated sediments. Total worm tissues were placed into a 50 mL beaker with 15 mL of 10% KOH (m/v) (Revel et al. 2018) and the mix was then heated at 60°C with a 24 h agitation. For depurated sediments, samples were dried at 60°C, weighted and placed into a 50 mL beaker following the same digestion protocol as worm tissues. The solutions were then filtered using a Büchner filter and a fiberglass microfiber filter with a porosity of 1.6 µm (GE Healthcare, Whatman™) and the filters were dried at room temperature in a glass Petri dish until analysis.

2.3 MPs analysis through microscopy and spectroscopy

After filtration, first identification of particles suspected to be MP and called suspected microplastics (foam, pellet, granule, film, fiber or fragments) was conducted under a stereomicroscope. The whole filter was observed, and each particle was described in shape and color, and measured with the micrometric ocular (10 mm, Pierron).

Filters with at least 2 particles, representative of each type of suspected microplastics particles observed, were selected for spectroscopic analysis with a total of 41 samples analysed (fibers could not be analysed) representing 40% of the samples. Raman analyses were conducted according to (Frère et al. 2016). Briefly, a mapping of the integrity of the

sample was conducted using a LabRAM HR800 Raman micro-spectrometer (Horiba Scientific), equipped with a Horiba Scientific ParticleFinder module for LabSpec6. This equipment provided easy and quick localization, counting and morphological characterization (size, area, perimeter, shape) of the particles. After particle localization and morphological characterization, all particles were analyzed using laser wavelength set at 785 nm (Laser diode, Oxxius, Lannion, France). Experimental conditions for Raman analyses – integration time, accumulation and laser power were set to limit fluorescence and increase the spectral quality of the analysed particles. Particle's identifications were performed by comparing acquired spectra to home-made database including environmental polymers spectra and the following reference polymers spectra: Low Density Polyethylene (LDPE), High Density Polyethylene (HDPE), Polypropylene (PP), Polystyrene (PS), Unplasticized Polyvinyl Chloride (uPVC), Polyethylene Terephthalate (PET), Polyamide-6 (PA-6), Polyamide-12 (PA-12), Polytetrafluoroethylene (PTFE), Polymethylmethacrylate (PMMA), Acrylonitrile-Butadiene-Styrene.

2.4 Quality control

Several precautions were taken to avoid any aerial contamination. All equipment used, such as glass beakers and tubes, glass cover lid, dissecting tools, were previously rinsed with 70% ethanol and ultra-pure water. They were dried in a dedicated fume hood and kept under aluminium foil to prevent any contact with the ambient air. During the biometric measurements, laboratory coats made of cotton were worn (but no gloves) and samples were kept in aluminium foil of clean glass material. Digestion of tissues or sediment (gut content or burrows) was operated under a fume hood, beakers were covered with glass lids and a blank, consisting of 10% KOH (m/v), was included at each session of digestion as an experimental control. This control (digestion without tissues or depurated sediment) was conducted each

time a series of digestion was performed (48 replicates in total for ragworms). For the filtration, Büchner filter was rinsed before and in between samples with 70% ethanol and once the filtration was performed, the filters were kept in glass Petri dishes until microscopic and spectroscopy analysis.

Particles (mostly fibers) observed in control blank filters were noted and compared to the final result to evaluate external contamination and avoid overestimation of suspected microplastics in analysed samples.

2.5 Study of the ingestion polyethylene microbeads in ragworms

2.5.1 Sample collection

Organisms *H. diversicolor* and sediments were collected in June 2019 on an intertidal mudflat located on the French Atlantic coast (1° 59′ 02′ 80″ W, 47° 01′ 49.20″ N, Port du Collet, Bay of Bourgneuf, France). This site was considered as a reference site due to the absence of industrial activity and reduced anthropogenic pressures. Worms with similar size were carefully collected by hand and sediment was sampled before being transported to the laboratory. Worms were then put in artificial seawater (salinity of 30 psu) for an acclimatation period of 72h.

2.5.2 MPs solution preparation

Fluorescent red polyethylene microspheres (UVPMS-BR-0.995 45-53µm, Cospheric LLC) were used to directly observe the ingestion/egestion by worms through fluorescent microscopic analysis. The solution used for water contamination was prepared as 7.5 mg of MP dissolved in 5 mL of a solution containing 0.05 % of Tween and MQ water. This solution that has a concentration of 1.2 $x10^6$ particles of MPs/mL, was then sonicated 30 min and stored at 4°C. As it is a preliminary study on this model organism, a high concentration was selected to enable the observation and analysis of plastic particles.

2.5.3 Exposure to MP through water or sediment

Worms of approximately 200 mg wet weight were selected to avoid a potential influence of weight. They were then exposed to MPs either through contaminated sediment or contaminated artificial seawater. The exposure concentration used corresponds to an extremely high concentration of MPs and is much higher than the one which can be found in the aquatic environment, but it was chosen in order to be able to visualize as well as possible the ingestion of the beads in the digestive tract of the worms.

For water contamination, worms were exposed to MPs for 1 h, as suggested by the American Society for Testing and Materials for initial studies on a new contaminant (ASTM 2013). Worms were exposed in a glass tank filled with 500 mL of 30 psu aerated artificial seawater (ASW) without MP (control, 15 worms) or contaminated with MPs at 1.2 x 10^6 MPs/mL (15worms). Three worms from each tank were removed, immediately fixed just after the exposure without depuration time; 12 worms were removed from the contaminated tank, placed in glass beakers (filled with 50 mL of ASW) in triplicate and subjected to different depuration times as follow: 15 minutes (T15), 30 minutes (T30), 60 minutes (T60) or 120 minutes (T120) then fixed in ethanol to study the ingestion and egestion of PE microbeads.

For sediment contamination, 54 worms of the same weight were used and exposed to either clean sediment or sediment with 67 mg of MPs particles/kg for 96 h under the same conditions, according to ASTM (2013) recommendations with minor adaptations. Glass tanks of 5 L were filled with 3 kg of control (clean without MPs, 21 worms) or contaminated sediment (21 worms) and covered with 100 mL of clean ASW (Mouneyrac et al. 2014). For each condition, 3 worms were immediately removed, fixed in ethanol just after the exposure without depuration time (T0) and the others were placed in glass beakers (filled with 50 mL of ASW) in triplicate and subjected to different depuration times: 15 minutes (T15), 30 minutes (T30), 60 minutes (T60) or 120 minutes (T120) (n=15). The total number of worms

used for the analysis of ingestion and excretion was 30 worms (15 worms from water-exposure and 15 worms from sediment-exposure). The difference in exposure time duration between water (1h) and sediment (96h) is explained in previous results from our laboratory showing that the ingestion of MPs by worms exposed through water was faster than through sediment (Revel et al. 2018).

For both types of exposure, glass tanks were covered with aluminum foil to prevent contamination by MPs from the air. The organisms were not fed during exposure and were left in a room at a controlled temperature of 16°C ± 1°C, in the dark.

2.5.4 Evaluation of ingestion and egestion of plastic microbeads

After 1 h exposure through water or 96 h exposure through sediment, 3 worms were analyzed at T0 and per depuration time (T15, T30, T60 and T120) to follow MPs ingestion and excretion. Each worm was individually placed in a closed Petri dish in the presence of absolute ethanol to avoid the expulsion of fluids (coeloma), then fixed in 70% ethanol and finally dissected under a binocular magnifier (Leica Mz 7.5). The digestive tract was separated in two parts (anterior part called A and posterior part called B) and delicately crushed on a slide in order to facilitate observation under epifluorescence microscopy (Olympus BX2, 100× objective) of whole part A and B. For each worm, the presence of MPs was recorded for both part of the digestive tract by a direct visual counting of fluorescent MPs and from photos taken during observation.

2.6 Statistical analysis

Suspected microplastics found in worms and gut content were reported per gram of sample. After a Shapiro-Wilk test, worms related data did not follow a normal distribution and non-parametric tests (Kruskal-Wallis and Mann-Whitney U tests) were used to test for difference

of ingested suspected microplastics between all sites Statistical analyses were conducted using the software R (version 3.3.0, R Core Team, 2016) and data were considered significant when the p-value was < 0.05.

3. Results

3.1 Field study

3.1.1 Biometric measurements

In the first site S1, 2017, the mean size of L3 and weight (± Standard deviation) of ragworms was 1.9 ± 0.5 mm and 0.5 ± 0.3 g in 2017, and 2.6 ± 1.1 mm and 0.98 ± 0.48 g in 2018. For the second site S2, the mean size of L3 and weight of ragworms was 1.7 ± 0.5 mm and 0.27 ± 0.13 g in 2017 and 1.8 ± 0,4 mm and 0.32 ± 0.08 g in 2018.

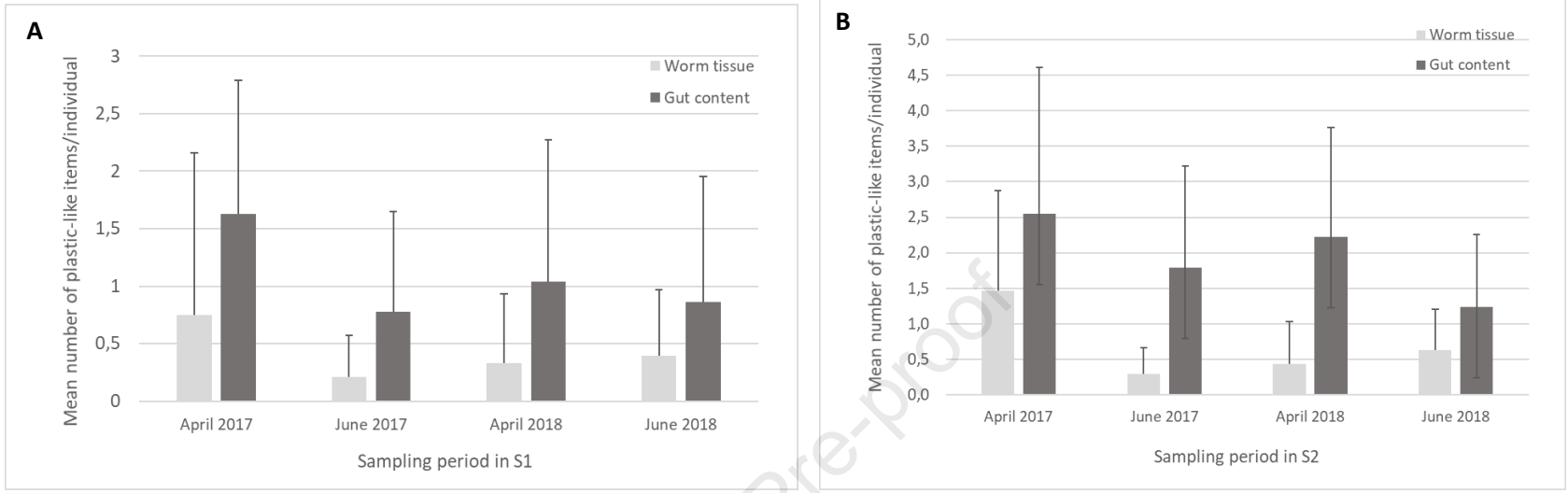
3.1.2 Mean number and proportion of suspected microplastics *in tissues and gut content of worms*

The mean number and percentage of suspected microplastics identified (with a stereomicroscope) in worms and gut content from each site and sampling campaign is describe in Fig.2. For worms, 41.7% and 75.8 % of samples contained plastic like items for S1 and S2 respectively; For the gut content of worms, 45.6 % (S1) and 87.5 % (S2) of samples contained plastic like items. A total of 214 suspected microplastics including 122 particles (90 fragments, 21 films, 2 granules and 9 foams) and 155 fibers were observed in the 237 filters from ragworms digestion, with a number of 147 and 67 particles observed in all pools of worms for 2017 and 2018 respectively. In the gut content of worms, 531 suspected microplastics including 163 particles (144 fragments, 11 films and 8 foams), and 403 fibers

were identified in the 209 filters, from the depurated sediment corresponding to the gut content of worms with 324 and 207 particles observed in all pools from 2017 and 2018.

In worms from S1, the presence of suspected microplastics (Fig.2 A) varied from 0.75 ± 0.70 to 0.21 ± 0.31 in April and June 2017 respectively, and from 0.33 ± 0.66 to 0.40 ± 0.43 particles/individual in April and June 2018. In gut content of worms, the presence of suspected microplastics varied from 1.63 ± 1.16 to 0.80 ± 0.90 in April and June 2017 respectively, and from 1.04 ± 1.23 to 0.86 ± 1.09 particles/individual in April and June 2018. In worms from S2 (Fig.2 B), the presence of suspected microplastics varied from 1.47 ± 1.41 to 0.3 ± 0.36 in April and June 2017 respectively, and from 0.43 ± 0.60 to 0.63 ± 0.57 particles/individual in April and June 2018. However, no statistical differences were observed between the number of suspected microplastics/individual in worm's tissues between sampling period in S1 or S2 ($p>0.05$). In gut content of worms, the presence of suspected microplastics varied from 2.55 ± 2.06 to 1.79 ± 1.42 in April and June 2017 respectively, and from 2.23 ± 1.53 to 1.24 ± 1.01 particles/individual in April and June 2018. However, for both sites (or between them), no statistical differences were observed between the number of suspected microplastics in worm's tissues or gut content ($p>0.05$).

The mean number of suspected microplastics seemed higher in the gut content (depurated sediment) than in worm tissues especially in samples from S1 in June 2017 with 0.002 ± 0.003 particles/mg of worm tissues and 0.360 ± 0.932 particles/mg of gut content for S1 and 0.003 ± 0.005 particles/mg of worm tissues and 0.309 ± 0.442 particles/mg of gut content for S2 (Fig.2 C). However, no statistical differences were observed between the number of suspected microplastics/mg of worm's tissues or gut content between sampling period and S1 and S2. This is probably due to the wide difference of suspected microplastics content between samples.



C

	S1							
	April 2017		June 2017		April 2018		June 2018	
	Worm	Gut content	Worm	Gut content	Worm	Gut content	Worm	Gut content
Mean number of plastic-like particles ± SD/mg of worm tissue or gut conte	0,005 ± 0,007	-	0,002 ± 0,003	0,342 ± 0,932	0,0006 ± 0,0012	0,013 ± 0,022	0,0006 ± 0,0007	0,067 ± 0,113
Mean number of plastic-like particles/ filter* (blank)	0	0	0,75 ± 0,51	0,30 ± 1,00	0,33 ± 0,53	2,00 ± 1,00	0,83 ± 0,75	1,33 ± 1,21

	S2							
	April 2017		June 2017		April 2018		June 2018	
	Worm	Gut content	Worm	Gut content	Worm	Gut content	Worm	Gut content
Mean number of plastic-like particles ± SD/mg of worm tissue or gut conte	0,011 ± 0,138	0,279 ± 0,298	0,003 ± 0,005	0,309 ± 0,442	0,003 ± 0,004	0,065 ± 0,050	0,003 ± 0,003	0,123 ± 0,173
Mean number of plastic-like particles/ filter* (blank)	1,00 ± 0,89	1,00 ± 0,63	1,00 ± 1,26	1,50 ± 1,05	0,33 ± 0,52	2,00 ± 2,17	2,17 ± 1,17	2,33 ± 1,51

5 to 8 filters per sampling analysis (one site+one period+one matrice)

Fig2. Presence of suspected microplastics in worms *Hediste diversicolor* sampled at the Seine estuary (le Havre). Mean number of plastic-like particles in worm's tissue and gut content/individual from S1 (A) or S2 (B). Percentage of fibers and mean number of suspected microplastics in worm tissues or gut content (depurated sediment)/mg, and mean number of plastic-like particles in blanks.

3.1.3 Analysis of shapes, sizes, colors and polymer types of the suspected microplastics

Among plastic-like items, fibers and fragments were predominantly blue (51.3% and 42%). For fibers, 25.4% were black and 12.6 % red and below 10 % were in another color. Fragments observed were also green (29.6%), red (7.6%), black (7.6%) or transparent (5%) and below 8 % were in another color. If we compare between sites or worms and gut content, results show that between 44 and 54.4% of suspected microplastics items observed in tissues or gut content are blue. If we analyse in detail the differences between sites, months of sampling and type of tissue, we see a greater diversity in terms of suspected microplastics (fibers, fragments, granules, films, pellet or foams) in samples from 2017 which seems more pronounce in worm tissues and S1 (Fig.3). For S1, more fibers were observed in worms in April 2017 compared to June (52.4 % versus 15.8 %) and in 2018 and 2017 with 70 % in April and and 88.9 % in June. A majority of fibers was observed in gut content of worms with 63.5 and 100 % for April and June 2017 and 95.5 and 77.8% for April and June 2018. For S2, the same tendency was observed with 52.4 and 52.2 % of fibers observed in worms sampled in April and June 2017, and 100 and 86.67 % in April and June 2018 (Fig 4). A majority of fibers was also observed in gut content of worms sampled in April and June 2017 with 79.6 and 87.1 % of fibers respectively. For 2018, fibers were only observed in gut content from June sampling campaign and were predominant (91.1%).

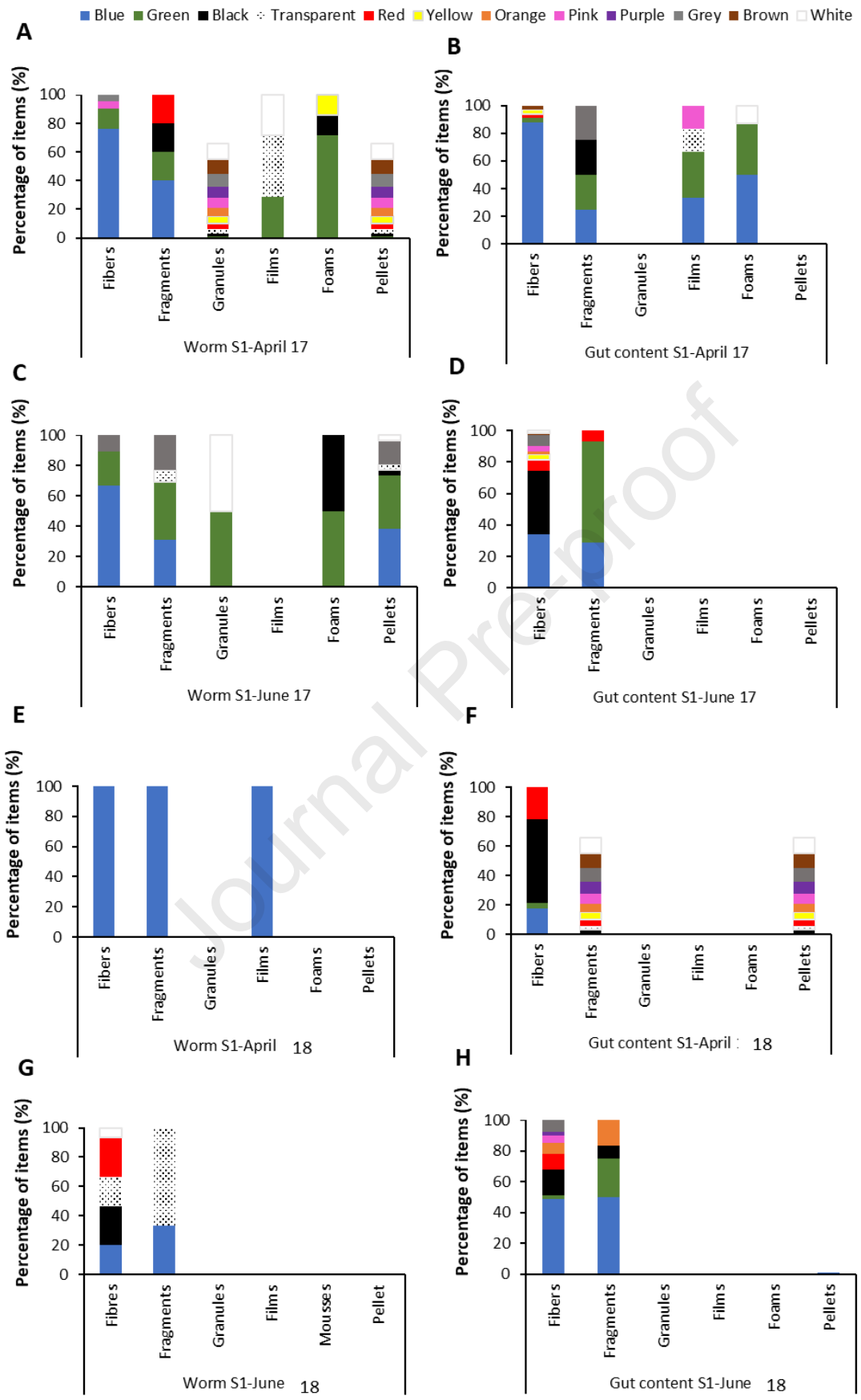


Fig 3. Proportion of suspected microplastics according to colors in worms (A, C, E, G) or their gut content (B, D, F, H) sampled in April or June 2017 and 2018 in site 1 (S1)

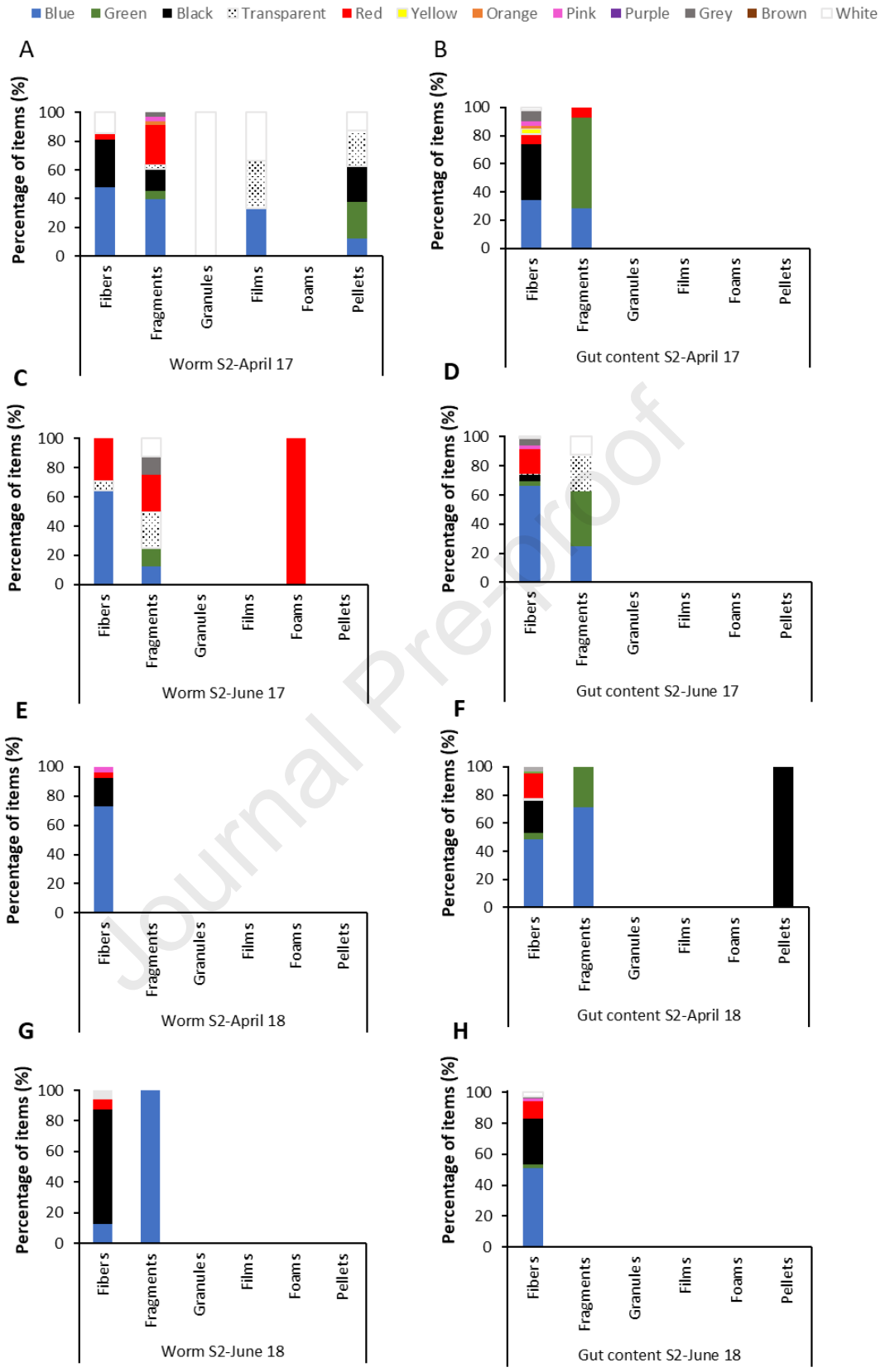


Fig 4. Proportion of suspected microplastics according to colours in worms (A, C, E, G) or their gut content (B, D, F, H) sampled in April or June 2017 and 2018 in site 2 (S2)

Suspected microplastics with the size comprised between 12 and 5000 µm were observed in both worms and gut content with different proportions per size classes (Fig.5.). Overall, the size of the majority of suspected microplastics (for S1 and S2) observed in both worms and gut content was higher than 500 µm (between 43% and 56% of the total items). In worm tissues from S1, more items between 12 and 100 µm were observed compared to the gut content, with 44% in June 2017 and 40% in April 2017 for S1 and S2 respectively. In 2017, between 75 and 90 % of the items observed were below 1000 µm for both sites. In April 2018, 30 and 40 % of the items were above 1000 µm when in June 2018 it was 50 and 60 % of the items for S1 and S2 respectively.

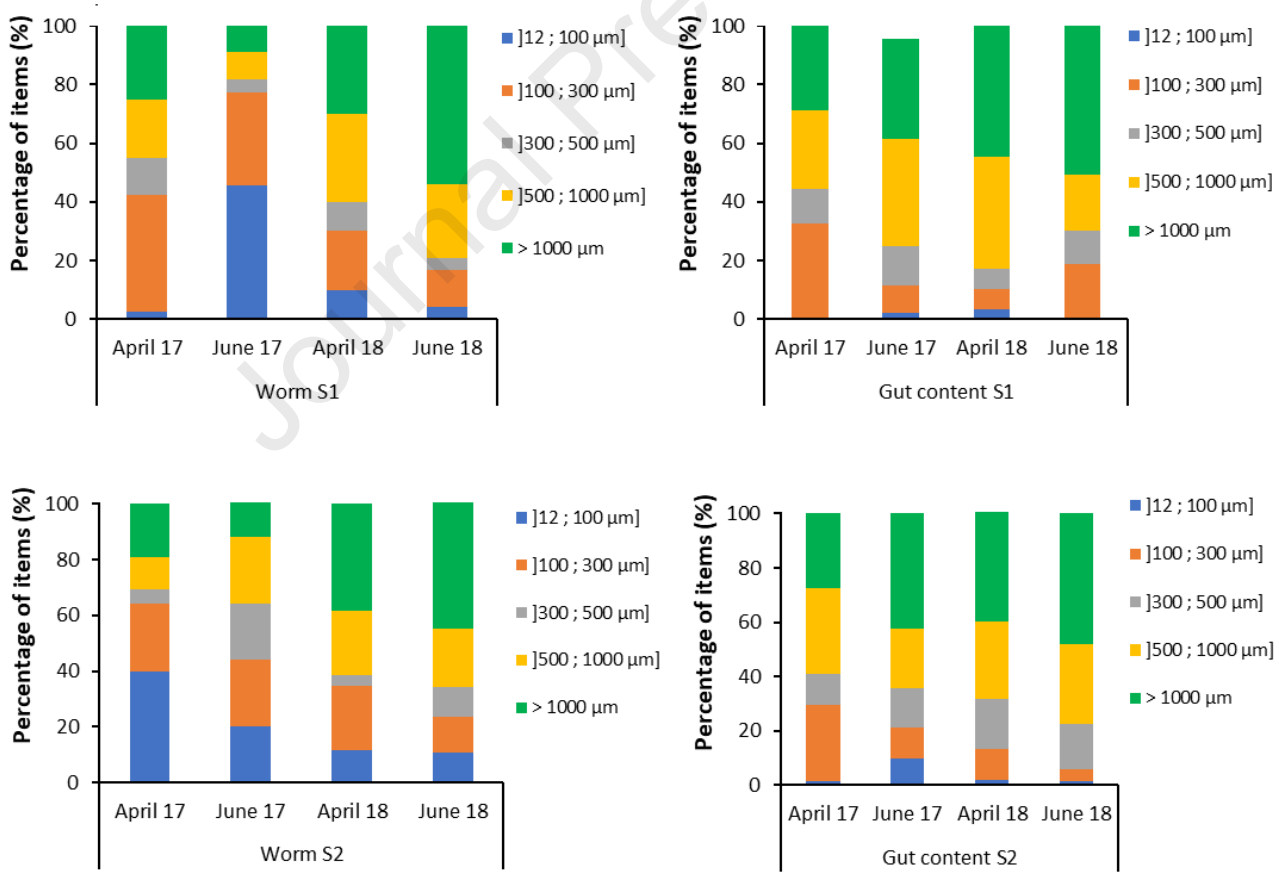


Fig 5. Proportion of suspected microplastics according to size classes found in worms and their gut content sampled at the Seine estuary site 1 (S1) and site 2 (S2)

In our study, approximately between 6 and 12% of all filters were identified with Raman spectroscopy with 23 worm filters and 18 gut content filters, and all items found on each filter were analysed. Unfortunately, we were only able to analyse the particles and not the fibers due to the Raman technique used. For worm filters, 69 suspected microplastics were identified in total including 23 particles (fragments, foams, granules or films). For the gut content, 91 suspected microplastics were identified in total including 25 particles. In worms, the polymer PE was identified in S2 (13.3%), but in the gut content, PP associated with TiO_2 was identified in S1 (9.1%) and polystyrene (6.2%) and PP (31.2%) in S2 (Fig.6). Three blue pigments known as the phthalocyanine blue 15 (PB15) were also observed in worms from S2 (June 2017). Some particles called NI for "Not Identified" did not match our database and could not be determine.

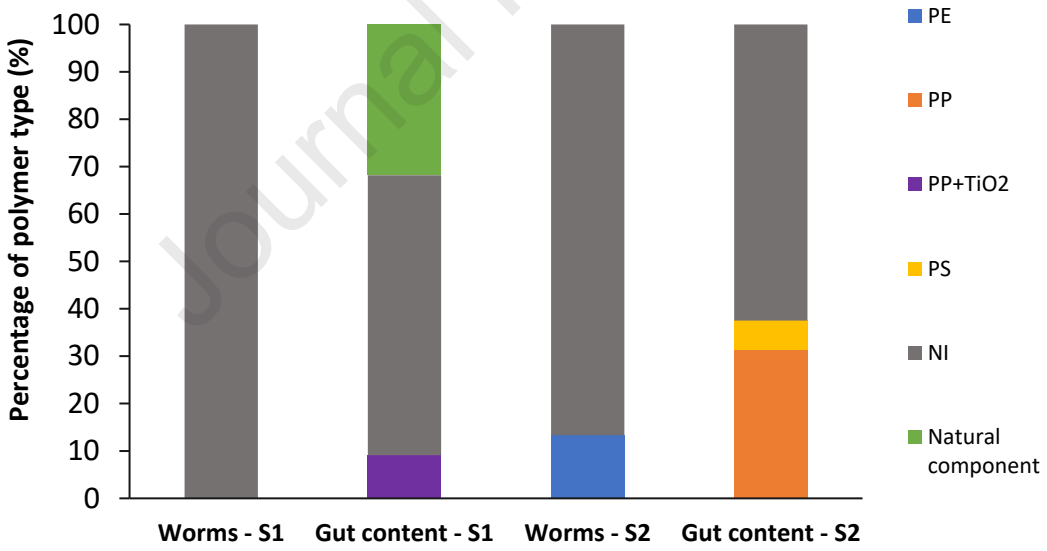


Fig 6. Average percentage composition of the different polymers observed and classified as: unknown, natural components, polypropylene (PP), polyethylene (PE), polystyrene (PS) and polypropylene (PP) associated with titanium dioxide (TiO_2) after Raman analysis of particles from worm tissues and gut content sampled in S1 (A) and S2 (B). NI stands for "Not Identified".

3.2 Laboratory experiment

3.2.1. Evaluation of ingestion and egestion through microscopic analysis

The originality of the present study was to isolate the two parts (anterior and posterior) of the intestinal tract from the worms without modifying the organ morphological structure. In order to assess the amount of MP beads accumulated in worms, direct estimation of fluorescent beads in the gastrointestinal tract through microscopic observation, and pictures analysis for each worm were conducted at each depuration time (T0 to T120). To facilitate result interpretation, a simple visual scale was established (Fig.7 A) based on the number of beads observed in the intestinal tract. Worms were classified as having a “low” bead level when less than 5 beads were observed in the whole digestive tracts; a “medium” bead level when the digestive tract contained between 5 and 15 beads; and finally, a “high” bead concentration when more than 15 beads were identified.

The Fig.7 B shows that immediately after MP water exposure (T0), worms were loaded with microbeads, 83.33% of the worms showing "Medium" level and 16.67% "High" level. During the depuration period, a progressive decrease of bead number in the digestive tract was observed until T60; no worms were classified with “High” bead level whereas 66.67 % and 33.33% of the worms contained "Low" and "Medium" levels of beads level. After 120 min of depuration, it appears that worms were accumulating again the particles as stated with the increased number of beads: 33.33 % contained "High", 33.33 % "Medium" and 33.33 % "Low" bead levels.

After exposure through sediment, Fig.7.C also depicted that after 96h exposure (T0), worms were filled with high quantity of beads (33.33% classified as "High" and 66.67% as "Low" beads levels) that progressively decreased until 30 min (T30) of depuration period (100% of worms with "Low" beads level at T30). Worms exposed through sediment also seemed to demonstrate ingestion of MPs at T60 of depuration time (33.33% of “High”,

33.33% of "Medium" and 33.33% of "Low" beads levels at T60 and 33.33% of "High" and 66.67% of "Low" beads levels at T120).

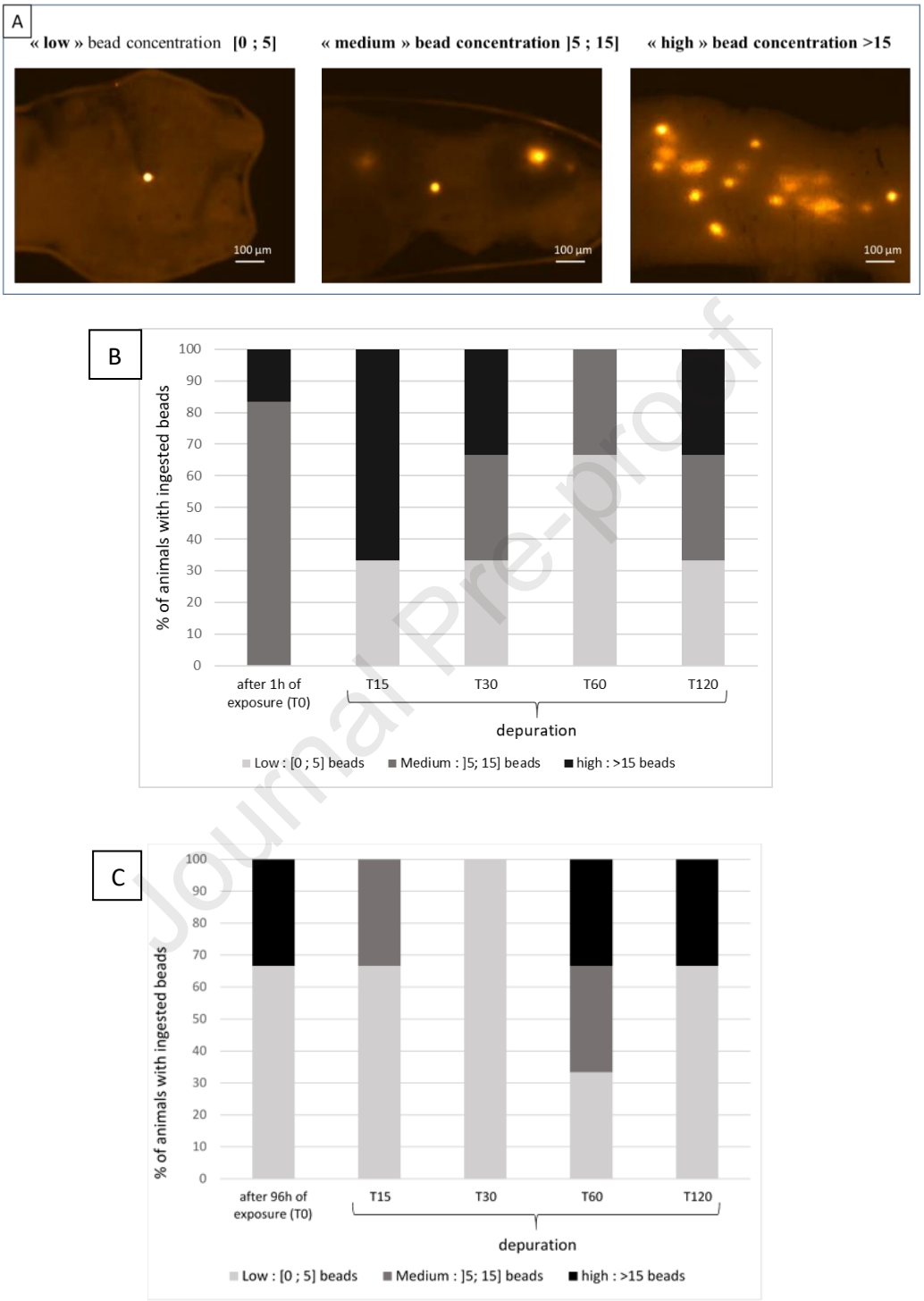


Fig.7 Visualization and ingestion of microbeads by worms *H. diversicolor*. (A) Microscopic observation of fluorescent microbeads at low, medium and high concentrations; Percentage of animals with ingested beads after 1 hour exposure (T0) in water (B) or 96h in sediment (C) followed by different times of depuration (15 min to 120 min)

3.2.2 Localization of microbeads in specific sections of the digestive tract

In order to easily show the hypothesis of particles re-ingestion by *H. diversicolor*, results were presented according to their localization in the digestive tract. The anatomy of the digestive tract of the marine worm *H. diversicolor* is shown in Fig. 8, where the two parts of the tract that were taken from each worm (part A: anterior and part B: posterior) are shown (Lucas and Bertru 1997). After 1h exposure to MPs through water (Fig. 8), the microbeads were found in both anterior part (50%) and posterior part (50%) of the digestive tract.

At T15 and T30 of the depuration time, there is a bead egestion phenomenon, and all the MPs particles that were in the posterior part B at T0 were removed, leaving only beads in the anterior part A from T15 (100% in part A) to T30 (100% in part A). At T60 and T120, the MPs that were in the anterior part A were found in the posterior part B and new particles replaced them in the anterior part A, which could reflect a phenomenon of re-ingestion of the plastic microbeads.

Regarding sediment-borne exposure (Fig. 8), after 96 h of exposure and up to T15 of depuration time, the worms had also an equal repartition of beads in the anterior and in the posterior parts (50% in part A and 50% in part B).

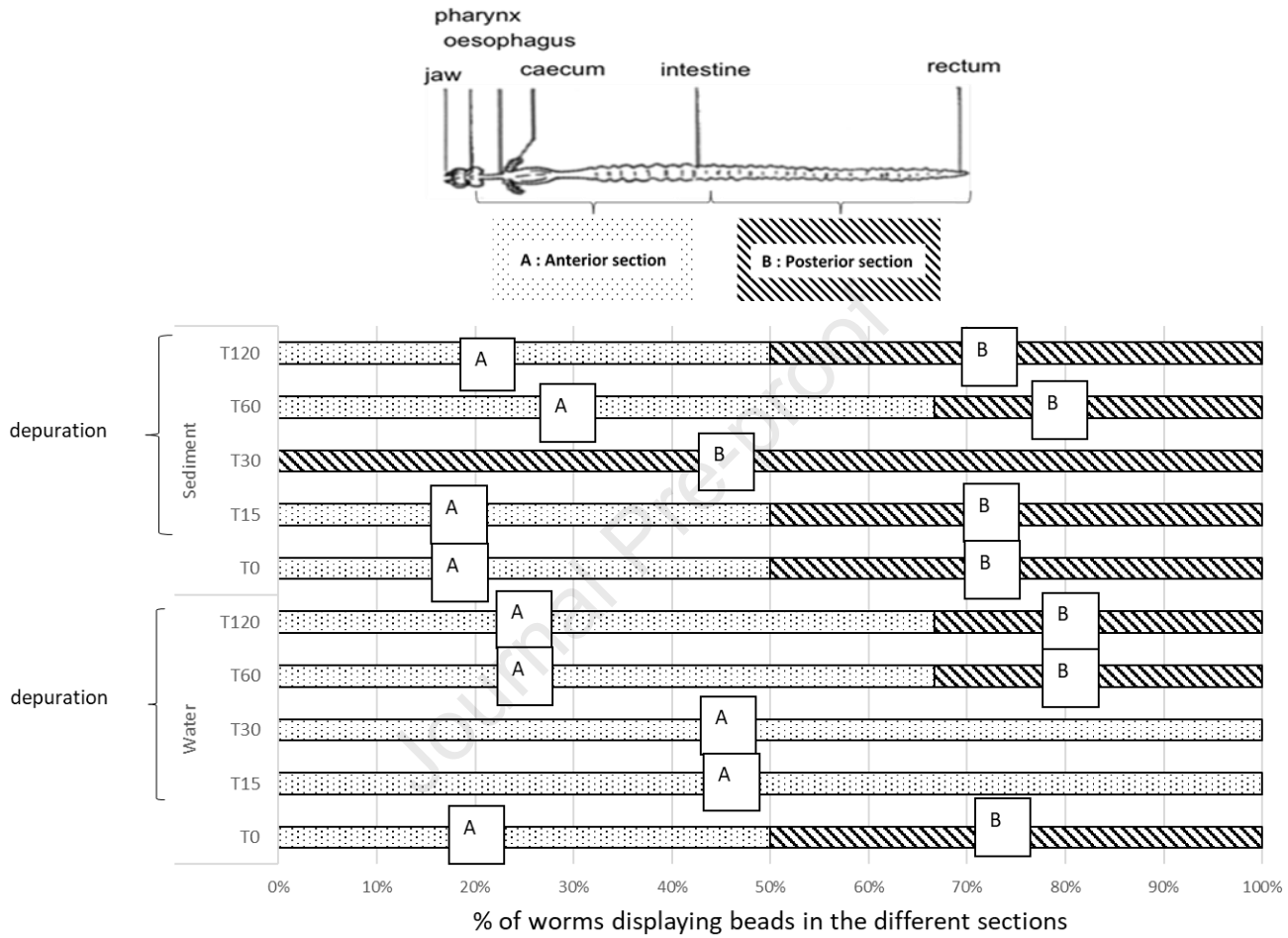


Fig.8 Distribution of polyethylene microbeads in the digestive tract of *H. diversicolor* according to different depuration times after exposure to contaminated water (1 h) or sediment (96 h). This representation allows to evaluate the % of worms displaying beads in the anterior (A) and in the posterior (B) sections.

After 30 min of depuration, all beads were present in the posterior part of the digestive tract (100% B). From T60 to T120, it appeared that a re-ingestion of beads occurred (66.67% in part A and 33.33% in part B at T60, and 50 % in part A and 50% in part B at T120).

4. Discussion

The aim of this study was to get a better understanding of the accumulation, and the ingestion and egestion of MPs in the estuarine worm *H. diversicolor* using a field study to evaluate the potential accumulation of particles, but also with a laboratory exposure and model MPs of PE. The ragworm *H. diversicolor* is a key species of estuaries (McLusky 1989; Masero et al. 1999) and understanding mechanisms of ingestion/egestion in these animals could help predict the potential transfer of MP through the food chain and the consequences on this ecosystem.

To evaluate the ingestion and accumulation of MPs in biological system, previous studies have mainly used tissue digestion (Desforges et al. 2015; Courtene-Jones et al. 2017; Revel et al. 2018; Revel et al. 2019), but it is less suited for visualization of the uptake and identification of ingestion mechanisms. Another possibility is to estimated MPs content in feces as performed by Gonçalves et al. (2018) and Revel et al. (2018). Certain organisms with a small size and a transparent body allow direct identification and/or quantification measurements of MPs through fluorescent microscopy (Windsor et al. 2019; Steer et al. 2017; Hall et al. 2015; Cole et al. 2013).

4.1 Field analysis

The objective of the first part of the study was to evaluate the presence of suspected microplastics and MPs in the tissues and gut content of the estuarine worm *Hediste*

diversicolor sampled in two sites from the Seine estuary, subjected to various pollutions and high hydrodynamism for one of them (S2). Previous laboratory studies showed evidence of microplastic ingestion by benthic organisms (Van Cauwenberghe et al., 2015; Davidson and Dudas, 2016), but very few evaluated the presence of plastic particles in *H. diversicolor* from their natural habitat (Missawi 2020). The mean number of items identified per g of worm in our study is in the range of what was reported by Missawi 2020 with a number of particles ranging from 0.5 ± 0.2 to 3.7 ± 0.2 items g^{-1} depending on the site, but also in the study of Van Cauwenberghe et al. (2015) in *Arenicola marina* collected in the French-Belgiane Dutch coast (with an average of 1.2 ± 2.8 items g^{-1}).

We observed a higher number of suspected microplastics in the gut content of worms and a variety of shapes including foam, films, granules, pellets, fragments but more often fibers compared to other types of particles. No feeding preferences of MPs in terms of colour or shape have been identified in previous studies on worms, but one recent study showed colour preferences for MPs ingestion in clown anemonefish (Okamoto et al. 2022). Previous work has showed that fibers were the most identified MPs in sediments (Wright et al., 2013; Cannas et al., 2017; Ben-Haddad et al., 2022). A primary source of fibers is textile with according to the study of Brown et al 2011 the possible release of more than 1900 microplastic fibers per wash. These fibers can end up in estuaries and marine environments through wastewater discharge (Frère et al., 2017; Gatidou et al., 2019). One study showed that marine habitats located closely from sewage discharge sites presented similar proportions of polyester and acrylic MPs fibers as those used for synthetic clothing. Here, studied sites are located in the Seine estuary which is under various anthropogenic pressures with the proximity of several largely populated cities including Paris. In addition, the blue, green and white fibers could also correspond to fishing line or nets. Once MPs are transferred into the aquatic environment, they can undergo density changes through biofouling, which will

increase their density (Wright et al., 2013). MPs can then be accessible to pelagic but also benthic organisms as they can accumulate in sediment (Ben-Haddad et al., 2022). Fibers represent a threat to benthic organisms as they can induce blockages in the digestive system, be translocated to various tissues of the organism, and potentially accumulate (Wright et al., 2013).

The diversity of shape but also in colors, particularily observed for fragments indicates another source for the particles and fibers analysed such as other anthropogenic activities. Agriculture, industrial and recreational activities involve several types of polymers related to their use, leading to the release of larger plastic debris and fragmentation into the environment (Cole et al., 2011). Le Havre, the city closer to the sampling site is highly industrialised with France's first container harbour. This area is an important cluster for chemistry activity. It is the first petrochemical basin of France involving 2 refining-petrochemical complexes of European scope, and it is responsible of 50% of French plastics and elastomers' production and 80% of the additives and base oils (HAROPA, 2022).

In worm tissues, PE was identified but in gut content, polyethylene and polystyrene were observed. These polymers are the most common plastics used worldwide for packaging and several household materials. Considering the mean number of items found in worms and their gut content, our study showed a tendency to more items in S2 compared to S1. This could be related to the difference in sediments composition between the two sites. In S1 the sediment is compact and sandy whereas in S2, the sediment is soft and sandy-muddy. Although S2 present a higher hydrodynamism than S1, plastic debris are more often seen since they can more easily accumulate and be stuck in tall grass and small ponds of water which could constitute a depositional area. Previous authors have shown the existence of a strong link between the abundance of MPs and organic or fine fraction (< 63 μm) content in

sediments which supports the hypothesis that MPs will accumulate in depositional areas (Mendes et al, 2021).

4.2 Laboratory experiment

In the second part of the study, worms were exposed to a high concentration of MPs ($1.2x10^6$ particles of MPs/mL), not representative of the one found in natural environment but allowing the visualization of the particles and evaluate MPs uptake and depuration which have never been studied on this model.

PE microbeads were detected in the digestive tract, immediately after both exposures through water and sediment, and results showed that particles were more easily ingested when worms were exposed through water, in accordance with Revel et al. (2018). Possibly because of the small size of particles, worms were seen to rapidly ingest them. The feeding mode of animals influence their ability to ingest plastic particles together with their food. *H. diversicolor*, as a "gallery-diffusor" behaviour species, performed two modes of particle transport: biodiffusion in the upper sediment layer and a non-local transport in deeper sediment (Francois et al., 2002), hence creating complex networks of burrows down to 15 cm (Davey 1994). They actively ventilate their burrows that allows constant fluxes of oxygen and nutrients over the sediment–water interface (Kristensen and Hansen 1999) and potentially training and mixing also MPs that could directly be taken up from the sediment or the water column. However, worms exposed through sediment contained more microbeads as compared to water exposed worms, which is in accordance with Porter et al. (2023) who showed that filter feeding *H. diversicolor* contained 15,000 % more fibers than deposit feeding worms. This could be explained by the fact that when deposit feeding occurs, the ragworm emerges partially from its burrow and absorbs food within the sediment or withing the upper layers of the surface.

Particles were observed both in the anterior and the posterior parts of the digestive tube allowing to differentiate ingested particles (present in the anterior part) from egested particles (posterior tract). This observation demonstrates that particles were rapidly ingested and egested but also re-ingested at 120 min of depuration time (for water exposure condition) and 60 min (for sediment exposure condition). This fast turnover of the MPs seems not to allow tissue bioaccumulation, at least for these types of particles. It is stated that *H. diversicolor* ingest sediment particles sizing around 20 µm. The large size of the particles used in this study ranging from 45 to 53 µm could also explain the fast turnover of particles that are hence not accumulated in the guts and digested. Hurley et al., (2017) did not find any microbeads in the guts of the worm *Tubifex tubifex* collected in different sites in the UK, but only fibers and fragments, due to a higher size of MPs microbeads found in the sampling site (124 and 1050 µm). A preferential ingestion of particles associated with organic material has been described in *T. tubifex* (Rodriguez et al., 2001). It is presumably possible that MPs coated with biofilm could be preferentially ingested. Denser polymers (compared to seawater) are more available to benthic species because they tend to sink in the water column (Courtene-Jones et al., 2017) and accumulate on the seafloor and in the sediment. However, MP that are egested out of the animals are presumably covered with organic matter that increase their density and reinforce their interaction with the benthic organisms and hence stimulate their rapid reingestion, as observed in the present study. Future research should be pursued with different particles shapes, polymers and coatings to study the ingestion and retention of MPs.

Polychaetes have already been used to study MPs in relation to their feeding habits and their habitats. Hamzah et al. (2021) demonstrated MPs ingestion by the estuarine polychaetes *Namalycastis* sp., mistaking them for their natural food. Besseling et al. (2013) observed a significant effect of PS MPs on *Arenicola marina* (L.)'s fitness and bioaccumulation related to MP concentration in sediment. Beyond simple ingestion, some worms have also been

shown to be able to fragment the plastics they live on into MPs, thereby becoming MP producers themselves (Jang et al. 2018).

H. diversicolor is a well-known bioindicator in ecotoxicology. This species is widespread in sediments and is a key species of estuarine ecosystems from European to North American coast (Durou et al., 2007). These worms have a limited mobility and could be an interesting species to follow MPs contamination in coastal areas, especially in the sediment since in our study more particles were observed in the gut content. This could be a complementary or alternative strategy to the analysis of large amount of sediment which can be challenging, depending on the sediment composition and high level of organic matter to eliminate, for the evaluation of MPs and also the lack of standardized protocol. In addition, *H. diversicolor* is an important source of food for local fauna (fish, crustaceans and birds) making them an ideal biological model for measuring trophic transfer of MPs and potentiel risks for higher trophic levels (Durou et al., 2007). A previous study conducted on Tubifex worms showed they retained MP for a longer time than for other non-plastic particles of the ingested sediment (add Hurley 2017). This should be investigated further in *H. diversicolor* as it could induce a significant risk for trophic transfer and biomagnification of MPs up the aquatic food chain.

5. Conclusion

In conclusion, this preliminary study was the first in the worm *H. diversicolor* to investigate the mechanisms of ingestion and depuration of model microplastics with an original representation of the results. In addition, this is also the first study evaluating the contamination of worms *H. diversicolor* by plastic particles in the Seine estuary. Our results show that even if the number of suspected microplastics is relatively low in worm tissues, a large majority of individuals were contaminated. Characterization showed polymers of PP and

PE and particles presented various colors with a majority of blue, black and red. Larger particles were observed in the content of worms than in their tissues. Moreover, the gut content of worms contained more suspected microplastics than worm tissues, which could lead a potential trophic transfer in higher organisms from the food chain. Since this species represents a key organism in the trophic web from estuaries, this work provides insight of potential risks towards the food chain. Further studies will have to address this mechanism with MPs that are more representative of natural environments (fibers and fragments) and in more relevant conditions (low concentrations, with microalgae, etc.). Future work should include experiments with several organisms from the trophic chain throughout long term exposures reproducing as much as possible environmentally relevant conditions.

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Figure caption

Fig.1 Ragworms sampling sites in (A) le Havre, France, in (B) the intertidal mudflats (S1 and S2) of the Seine estuary (map data with GPS coordinates: google, 2020).

Fig.2 Presence of plastic-like particles in worms *Hediste diversicolor* sampled at the Seine estuary (le Havre). Mean number of plastic-like particles in worms tissue and gut content/individual from S1 (A) or S2 (B). Percentage of fibers and mean number of plastic-like particles in worm tissues or gut content (depurated sediment)/mg, and mean number of plastic-like particles in blanks.

Fig.3 Proportion of plastic-like items according to colors (A) and depending on the type of sample: worms or their gut content from site 1 (S1)

Fig.4 Proportion of plastic-like items according to colors (A) and depending on the type of sample: worms or their gut content from site 2 (S2)

Fig.5 Proportion of plastic-like items according to size classes found in worms and their gut content sampled at the Seine estuary site 1 (S1) and site 2 (S2)

Fig.6 Average percentage composition of the different polymers observed and classified as: unknown, natural components, polypropylene (PP), polyethylene (PE), polystyrene (PS) and polypropylene (PP) associated with titanium dioxide (TiO_2) after Raman analysis of particles from worm tissues and gut content sampled in S1 (A) and S2 (B). NI stands for “Not Identified”.

Fig.7 Visualization and ingestion of microbeads by worms *H. diversicolor* with (A) photography of the digestive tract of worms to detect the ingestion of microbeads of polyethylene under fluorescent microscopy, and proportion (%) of worms that ingested plastic microbeads after 1h exposure to through water (B) and 96h exposure through contaminated sediment (C) depending on the depuration time (from T0 - 0 min to T120 - 120min). The scale was defined according to the number of MP observed in the digestive tract of worms: "low" is attributed when less than 5 beads were found in animals; "medium" when between 5 and 15 beads were found and "high": when more than 15 beads were observed.

Fig.8 Distribution of polyethylene microbeads in the digestive tract of H. diversicolor according to different depuration times after exposure to contaminated water (1 h) or sediment (96 h). This representation allows to evaluate the % of worms displaying beads in the anterior (A) and in the posterior (B) sections.

- Very high variability in the number of plastic-like particles detected in ragworm *Hediste diversicolor*
- More particles are found in the depurated sediment from the gut
- Model polyethylene microbeads ingested by *Hediste diversicolor* from water or sediment area rapidely egested

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Declaration of interests

☒ 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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