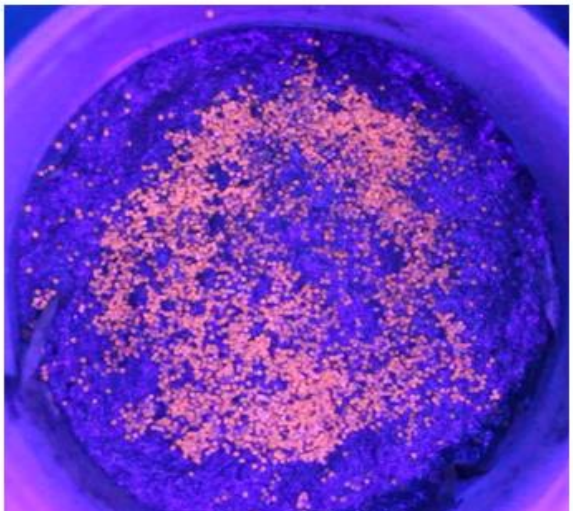
Work Package 4

The potential use of biological agents to filter microplastics from the water column



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1. Introduction

1.1 Estuarine microplastics

Coastal systems are vulnerable to microplastic pollution from land sources via river discharge, wastewater, runoff from roads and direct input from littering and leisure use of the coast[1–3]. Estuarine sediments are a major sink for microplastic particles and various benthic organisms will interact with them. This can have negative effects at the individual, community and ecosystem level, with potential consequences for ecosystem processes and function. Benthic microalgae and invertebrates play significant roles as primary producers and primary consumers that underpin estuarine ecosystem functions. Due to their presence on the sediment bed, they will interact with deposited microplastics that accumulate on the sediment surface, and these interactions may influence the behaviour, transport and fate of microplastic particles[4–6].

To date, we know very little about the transport, fate, and impacts of microplastics in estuarine systems, but predicting microplastic transport and behaviour is highly desirable. Microplastic particle exchange across the sediment-water interface and how their interaction with key benthic biota may influence these dynamics and have the potential to remove significant volumes of microplastics from the water column[7]. Filter feeders that capture particles from suspension will transfer them to the sediment bed, but biofouling and weathering of the particles can also lead to their deposition and accumulation on the bed[8,9]. Deposited microplastics may become resuspended back into the water column and transported onwards through the system, however, they may also be ingested or buried by fauna, or adhere to benthic biofilms which may lead to their long-term retention and burial in the bed[4,10].

The aim of this work package was to examine the controls of microplastic capture and retention by key benthic microbial biofilms and benthic invertebrates and explore the potential for these interactions to be exploited as a mechanism to remove microplastics from the water column.

1.2 Benthic Biofilms

Biofouling effects on microplastic behaviour in the water column are increasingly documented and this will influence the transport of microplastics through estuaries, as well as their settlement to the bed. While the importance of microplastic interactions with sticky biofilms has been recognized[5], far less consideration has been given to benthic microbial biofilms that will interact with deposited microplastics. There remains a distinct lack of empirical evidence of these interactions and how adhesion on the bed may alter the residency times and fate of microplastics on the bed. Benthic biofilms, rich in microphytobenthos, bacteria and extracellular polymeric substances, are well known for their stabilisation of coastal and estuarine sediments, as well as their ability to trap fine sediment particles and contaminants on the bed[11–14].

If biofilms mediate the exchange of microplastics across the sediment-water interface, the spatial and temporal distribution of biofilms across estuarine sediments may be used to predict the trapping, deposition and resuspension of microplastics as they are transported through estuaries. Grazers will consume large quantities of benthic biofilm from the sediment surface, so cohesion between biofilm and microplastics may also be a significant vector for the entry of microplastics into the food web.

1.2 Benthic Invertebrates

Estuarine zooplankton and sessile suspension and deposit feeding fauna may also act as natural microplastics filters by altering the behaviour, fate and bioavailability of estuarine microplastics. Faunal-microplastics interactions therefore have the potential to transfer microplastics particles to the bed, their burial into deeper sediments and their resuspension and onward transport. Several benthic invertebrates ingest microplastics either from the water column or sediment surface, and these organisms may themselves act as a transient sink of microplastics. Filter feeding bivalves that filter large amounts of water can remove and accumulate other contaminants from the water[15], while deposit feeding invertebrates graze on surface biofilms and indiscriminately feed on surface sediments[16,17]. The number of ingested microplastics detected in the natural environment is highly variable, ranging from 0

to over 100 particles ind^{-1}, with differences in ingestion rates influenced by the species feeding mode and life style[4,18–20]. These differences are likely in part due to different ingestion rates and different handling of particles. Microplastic retention can potentially lead to significant accumulation in the organism, which may cause adverse effects, as well as influencing microplastics entry into the food web. However, not all particles will accumulate in the gut, many benthic organisms such as filter feeders can selectively reject particles based on particle size, shape or quality[21–23] without passage through the digestive tract. Some fauna may be more efficient at removing unwanted microplastics from their bodies than others[24,25].

The ingestion, repackaging and excretion of microplastics in (pseudo-) faeces at depth, together with burrowing activity can transport microplastics downwards to deeper sediment layers sequestering them over the long-term[4,10,26], but the fate of these microplastics in the bed will depend on the ingesting organism's position in the bed and where they eject faeces, as well as the sites sedimentation rates and physical and/or biological mixing in the surface layers of sediment. Our understanding the role of different faunal traits, including their feeding mode, lifestyle and processing of microplastics is limited but as these processes may influence microplastics fate and residency time on the bed this warrants investigation.

1.4 Aims

This work package aimed to explore the influence of i) benthic flora and ii) benthic fauna on microplastic behaviour, transport and their potential fate in estuarine sediments. The first experiment focused on the influence of biofilm development on microplastics capture and microplastic resuspension under increasing flow velocities. In addition, as microplastics can be a vector for various other contaminants, the effects of copper (Cu) and Lead (Pb) adsorbed to microplastics on the trapping efficiency of the biofilm was explored.

We hypothesised that i) increasing biofilm growth on the sediment surface would increase MP capture and retention under flow (higher MP erosion thresholds and lower erosion rates) and ii) that heavy metal contamination would influence MP capture and resuspension due to potential negative toxic effects on the biofilm community.

The second experiment explored the influence of key benthic infauna on microplastics capture from the water column, as well as microplastics ingestion and excretion (in faeces and pseudofaeces) across the different species. More specifically, the study assessed i) the removal and uptake of microplastics from the water column by invertebrates from different sediment types (sandy and muddy sediments), exhibiting different feeding modes (filter feeding vs deposit feeding) and ii) the capacity of these organisms to retain or egest microplastic particles.

We hypothesised that i) Benthic invertebrate feeding modes would affect the volume and distribution of microplastics transferred to the bed, and ii) The amount of microplastic ingested and retained in the gut would differ with the organisms feeding and living mode.

2. Methods

2.1 Biofilm experiment

2.1.1 Experimental design & set up

The role of benthic biofilms in trapping and retaining polyamide microplastics was examined using i) a tidal mesocosm incubation to assess the relative trapping of microplastic particles on the surface of sediments exhibiting different levels of biofilm growth, and ii) the erosion microplastics from incubated sediments under flow. To understand the influence of heavy metal contaminated microplastics on the biofilms ability to capture and retain the particles, microplastics from each biofilm treatment were also exposed to Lead II Nitrite or Copper Chloride prior to their use. Copper ($CuSO_4$, Copper Sulfate) and Lead ($Pb(NO_3)_2$, Lead II Nitrate) were selected as associated contaminants.

A total of 36 independent tidal mesocosms were housed in individual water chambers within an outdoor greenhouse under natural light to determine the influence of biofilm growth on microplastics capture and retention on the bed under natural light conditions (Fig 1a). Mesocosms were housed in individual water chambers containing 1.4L of natural, filtered seawater (33 PSU). The pots were submerged during ‘high tide’ periods, and slowly raised out of the chamber to drain from the bottom during ‘low tide’ periods (Fig 1b). Each mesocosm

(pot) had drainage holes on the base of the core, and contained a filter paper to prevent sediment escaping as the pots were submerged and immerged. A regular semidiurnal tide was simulated according to the natural conditions where sediments and biofilms were collected (Tay estuary, Scotland) with this tidal regime controlled via Raspberry Pi and a custom-built program.

The pots (250 mL) were filled to an approximate depth of 7 cm with natural cohesive sediments collected from the Tay estuary, that was pre-sieved (500 µm) to remove macrofauna. As sediment was added, inner cores (ID 55 mm) fitted with mesh bottoms (80 µm mesh; Fig 1c) were carefully inserted into the pots in (Fig 1d) so that its top rim remained flush with the sediment surface once filled and the rim was touching the pot at the bottom side. This enabled the inner core to be removed (intact) later for erosion measurements with the mesh facilitating drainage during the tidal simulations. Sediments were allowed to consolidate overnight and were levelled to the surface of the inner core if required before biofilm rich sediments were added.

Figure 1: a) Outdoor greenhouse at the Scottish Oceans Institute, St Andrews. b) Individual tidal mesocosms submerged in their outer chambers simulating 'high tide' conditions. c) Mesh bottom inserts. d) mesh bottom insert position in the mesocosms.

2.1.2 Biofilm inoculation

Biofilm rich surface sediment (top 2 cm) was collected separately but at the same time from the site to inoculate the mesocosms (hereafter pots) and manipulate biofilm growth in two ways. The surface sediment was spread thinly in large trays and left in the outdoor greenhouse to encourage the microphytobenthos to gather on the surface 2 mm to photosynthesise. The top 2 mm of the biofilm rich surface sediment was then collected from the trays after 24 hr and transferred to a large conical flask topped up to 1L with a nutrient broth (F2 media) to stimulate growth. Sediment slurries (30mL) were prepared in 50mL centrifuge tubes, by adding different proportions of the biofilm rich sediment mix (after the overlying water was removed), to base sediment (largely biofilm-free, deeper sediments). The percentage of biofilm rich sediment (of the total sediment (15 mL) used) were 0, 33, and 100% for the control, low and high biofilm treatments respectively. Control sediments would therefore contain substantially less microphytobenthos, and to this 15 mL gluteraldehyde (2.5% v/v in seawater) was added to the 15 mL of base sediment to eliminate any remaining

microphytobenthos and bacteria present. For the low and high biomass treatments, 15 mL of seawater was added to 15mL of sediment. All slurries were added to the pots at the start of the immersion period on day 1. Further additions of glutaraldehyde (2 mL, controls), and nutrients (F2 media, High and Low biomass treatments) were added on D7, with nutrients applied at full strength (high biomass pots) and half strength (50:50 v/v of F2:Seawater for low biomass pots). This was to inhibit (controls) or further stimulate (high/low biomass) growth.

All biofilm treatments were randomly allocated to pots in the greenhouse using a random number generator with a total of 12 pots treated with i) Control biomass:C_{Bio}, ii) low biomass: L_{Bio} and iii) high biomass: H_{Bio}, respectively. The biofilm was allowed to develop for 14 days before any stressors were applied. The photosynthetic potential of the biofilm (dark adapted Fv/Fm) was measured on days 2, 7 and 14 using a handheld FluorPen (FP 110, PSI [Photon Systems Instruments]) and confirmed that the biofilm was successfully removed from the controls.

2.1.3 Microplastics preparation and additions

All microplastic particles (500 µm, virgin polyamide [PA] particles) were briefly aged in seawater under natural UV (outdoors) and mechanically agitated each night prior to the introduction of metals (13 days). On day 12 the particles were agitated with i) natural seawater ii) seawater containing 10 mg/L Copper (Cu) and iii) seawater containing 10 mg/L Lead (Pb) for 24 hr for the controls (C_{HM}), Cu and Pb treatments respectively. All particles were rinsed and fluorescently stained for 24 hr using a non-toxic Rhodamine WT (2.5% v/v in ethanol) stain to allow greater visualisation of particles during later measurements. Particles were rinsed of excess fluorescent dye and dried prior to their application to the pots (1g per pot). Heavy metals treatments were also randomly allocated across the biofilm treated pots to ensure four replicates of each biomass and metal combination; i) C_{Bio}:C_{HM}, ii) C_{Bio}:Cu, iii) C_{Bio}:Pb, iv) L_{Bio}:C_{HM}, v) L_{Bio}:Cu, vi) L_{Bio}:Pb, vii) H_{Bio}:C_{HM}, viii) H_{Bio}:Cu, ix) H_{Bio}:Pb. Pots were incubated for a further 7 days, with ambient temperature, humidity and light within the greenhouse monitored daily.

2.1.4 Sediment sampling

Pots from each treatment were haphazardly selected and removed from the tidal system 2 hr after 'low tide' to drainage. The inner cores were carefully identified within the pot and small cut off syringes were used to extract six sediment discs (15mm dia, 3mm depth) from the sediment surrounding the inner cores, ensuring the core surfaces remained undisturbed. Four of the sediment samples were pooled for each pot and immediately frozen in liquid Nitrogen for later biochemical analysis. Microphytobenthic biomass (chlorophyll a) and degradation products (pheophytins) were extracted and quantified from freeze-dried sediments using 90% acetone following Jeffery et al[27].

2.1.5 Erosion of microplastics

The inner core of each pot was carefully removed, photographed (natural and under UV light to fluoresce the microplastic particles), and inserted into a small benchtop recirculating flume (1.5m length, EmsRiver). The recirculating flume was fitted with a Vectrino Acoustic Doppler Velocimeter (ADV) to measure the flow velocity (cm sec^{-1}), which was increased in increments from 5–30 cm sec^{-1} for each core. A UV light and a digital SLR camera were set up at the side of the flume directly in front of the sediment core. Experimental runs were performed in the dark, allowing the UV light to fluoresce the microplastic particles to aid visualisation (Fig 2a). The DSLR recorded the full experimental run for each core, allowing the calculation of percentage coverage of microplastics at i) the initial onset of the run (MPs_i), ii) directly after the first flush of water (MPs_f), representing an incoming tide and iii) at each incremental flow increase (MPs_5…..MPS_{30}).

2.1.6 Image capture and processing

The initial biofilm coverage was determined by imaging each core under a UV light to enumerate fluoresced microplastic particles. Image processing was performed in Matlab (R2023a), with the initial coverage calculated from the first 30 frames and the change in microplastics coverage determined for incremental increase in flow. Microplastic erosion thresholds (as bed shear stress, BSS) were defined as the flow velocity at which there was a 5% decrease in surface coverage of microplastics particles (Fig 2b).

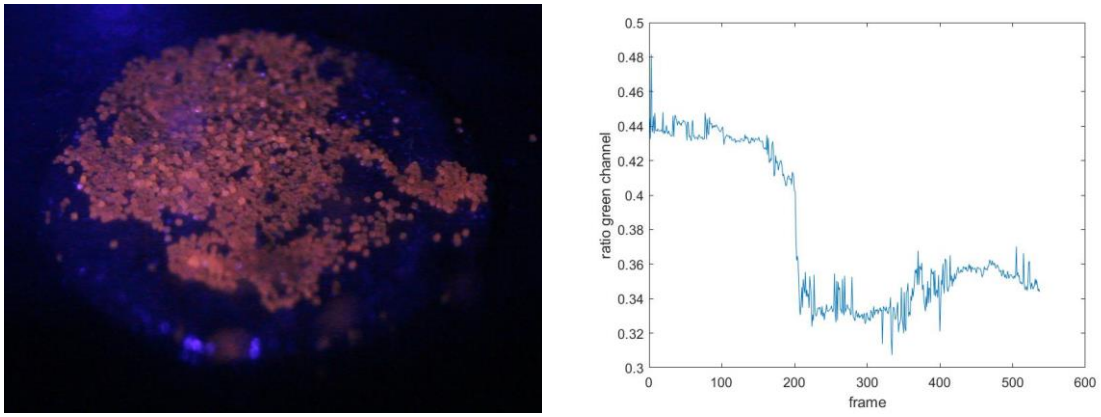

Figure 2: a) the initial (wet) coverage of fluoresced microplastic particles on the surface of the core. b) MatLab output that has the ratio of coverage on the y axis and time on the x axis. The MatLab code was able to identify when the coverage decreased.

2.1.7 Statistical analysis

Statistical analyses were performed using "R" statistical software, (V3.1.1; R Development Core Team 2023) through the R studio graphical interface (v. 2023.03.0) the following packages used; 'dunn.test', 'pastecs', 'viridis', 'effects', 'car' and 'ggplot2'). Heavy metal effects were not found to be significant and was removed from further analyses. As data were not normally distributed a non-parametric equivalent of Analysis of Variance (ANOVA), was conducted. Kruskal-Wallis rank sum test, and a post-hoc dunn's test with a Benjamini-Hochberg adjustment for multiple comparisons., was used to identify differences in the final chlorophyll a content and differences in the erosion threshold of microplastics across the different levels of biomass added.

2.2 Invertebrate experiment

2.2.1 Experimental design & set up

Experimental mesocosms (glass aquaria, 30x20x20cm), were filled with approximately 5-6cm of sediment and 5L of artificial seawater. Two types of sediment were used, sand or mud according to the natural habitat of each species (see below), collected from two sites on

the Humber estuary: Cleethorpes beach (sand) and Skeffling mudflat (mud). Sediment was sieved (2mm) to remove debris and large invertebrates, before it was added to the mesocosms. External air pumps with airstones were used to aerate each mesocosm, with animals housed in single species and multi-species communities. The animals used are commonly found in intertidal estuarine sediments throughout Europe, namely *Arenicola marina* (Lugworm), *Cerastoderma edule* (common cockle), *Perinereis cultrifera (*common ragworm*)* and *Scrobicularia plana (*Pepper shell*)* with the combinations of animals used for the multi-species experiments being i) *Arenicola marina* & *Cerastoderma edule* as these are both commonly found in sandy intertidal habitats across Europe, and ii) *Perinereis cultifera* & *Scrobicularia plana,* often found in muddier intertidal sediments. These benthic invertebrates all live in the upper sediment Layers (up to 20cm depths) and bioturbate, or rework, the sediment around them, however, their feeding mode differ from one another. The bivalve *C.edule* is a common filter feeder that resides up to 5 cm depths in the bed. The bivalve, *S.plana* can filter feed and deposit feed, and can reside up to 20cm into the bed. The worm *A.marina* is a head down, subsurface deposit feeder, that typically resides ~20cm in the bed, and produces characteristic fecal mounds at the sediment surface. *P.cultifera* is an omnivorous common ragworm that feeds and scavenges on crustaceans, worms and molluscs, as well as filter feeding and deposit-feed from sediment surface, and it resides at a depth up to 30 cm in the sediment.

Animals, with the exception of *P.cultifera* were collected at low spring tides from three locations around the Humber estuary: Fraisthorpe beach, Cleethorpes north promenade and Skeffling mudflats. *P.cultifera* were bought from a local tackle shop, individuals were collected locally that same day by bait fishermen. After collection, all animals were transported to the laboratory within an hour in a cool box, where they were inspected for injured or dead individuals. Healthy individuals were transferred to acclimation tanks filled with artificial seawater, sediment and aerated with an external pump. Organisms were acclimated to laboratory conditions for 5 days, during which temperature (17^{o}C), salinity (25 PSU), ammonia, nitrites and nitrates were kept constant, with conditions monitored daily. Water changes (40%) were performed every other day during acclimation. All animals were fed every

3 days with a mixture of spirulina powder and fish food, finely ground and suspended in artificial seawater. Animal behaviour was monitored daily, with all species exhibiting characteristics behaviours such as the production of casts (*A.marina*) and formation of burrows (*A.marina* and *P.cultifera*), burrowing and locomotion (all species), extension of siphons and apparent water filtration (*C.edule* and *S.plana*) as well as the production of visible faeces.

2.2.2 Plastic preparation

A mix of three types of polyamide/nylon (PA, density 1.14 g cm^{-3}) microplastics particles were prepared for the experiment: i) 10 µm diameter nylon fishing line, cut into 100-1000 µm lengths ii) Uniformly sized, 500 µm red plastic nurdles and iii) mixed fragments (30-1000 µm, mean: 361µm), fabricated by blending zip-tie ends cooled with liquid nitrogen (Fig 3). All microplastics were fluorescently stained using non-toxic, water-soluble Rhodamine (WT, 2.5% v/v in ethanol) for 12 hr and rinsed with DI water before being added to the mesocosms.

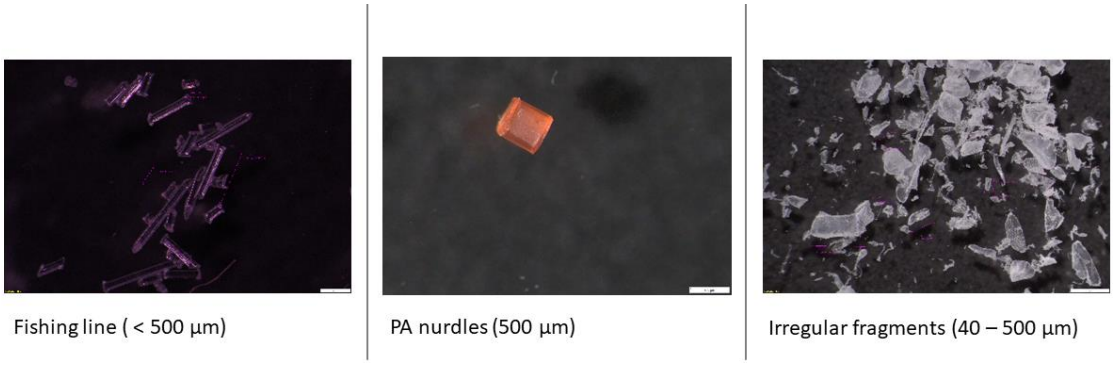

Figure 3: The three types of microplastics used in the experiments were added to each mesocosm at a concentration of 200 g L^{-1}.

2.2.3 Mesocosm experiment

Animals were transferred from acclimation tanks to individual weight boats, weighed (wet weight, grams) and added to experimental mesocosms in varying densities, depending on size of the species (Table 1).

Table 1: Summary of the different experimental mesocosms, sediment and animals used.

Sediment type	Species added	Replicate mesocosms	Number of animals (per mesocosm)	Equivalent density of animals (ind m^{-2})	Number of animals depurated
Single species experiments					
Mud	Scrobicularia *plana*	4	6		4
Mud	*Perinereis cultiforma*	4	5		4
Sand	*Cerastoderma edule*	4	9		4
Sand	*Arenicola marina*	4	3		4

Mixtures of fluorescent PA particles (all three particle types, 1g wet weight) were suspended in 35mL seawater and sonicated prior to the transfer to each mesocosm to ensure particles were not aggregated. Additions to the tanks equated to a concentration of 200mg L^{-1}. This elevated concentration of plastic compared to commonly found plastic concentrations in the wild was chosen to ensure availability to the animals. Microplastics were added 30 mins after the addition of the animals, to allow them to burrow into the bed. The particles were observed sinking to the bottom of the tank after approximately 15-20 min. The animals were then fed (after a further 30 min) with the same food solution used during the acclimation to promote aggregation with the microplastics and aid in their deposition to the bed. For the remaining experimental period, the animals were not fed and no water changes were performed minimise disturbance to the bed. However, water quality (temperature, salinity, ammonia, nitrites/nitrates) was monitored daily for the duration of the experiment. Control tanks were filled with sediment and seawater as above, but no experimental organisms were added.

After five days the experiment was terminated, and subsamples of the water were taken (6x 15ml). Tanks were drained and sediment (3 sediment cores 2.3cm Dia., sliced in 1cm sections) were extracted. Animals were gently retrieved from each mesocosm, with 4 replicates of each species (from different mesocosms) transferred into depuration tanks for 48 hr. Only healthy and uninjured animals were chosen for this additional step. The remaining animals from each mesocosm were immediately frozen intact. All sediment, water and faunal samples were stored at -20°C for later analysis. Control tanks were processed in the same way.

During the depuration stage, each individual was kept in a 500 mL beaker filled with seawater to allow for passage of faeces. After 48 hr the animals were removed, rinsed and frozen as above for later analysis. The faeces produced were collected with a syringe and stored in the fridge at 5°C for later analysis.

2.2.4 Sample preparation

Frozen animals were rinsed with DI water, bivalves were removes from their shell and the outside and inside of the shell was thoroughly rinsed. The soft body tissue was chemically digested using 60 mL Potassium hydroxide (2M) for 24 hr. The digestate was filtered over ash-free cellulose filters (20 µm) and stored in petri dishes for later quantification of microplastics via microscopy. Water samples were directly filtered over cellulose filters and stored the same way. Faeces samples were agitated to break up particles and then filtered over cellulose filters. A number of faecal pellets failed to break up with agitation prior to filtering therefore these were carefully crushed under the microscope to inspect for MP particles inside of them.

2.2.5 Microscopic analysis

An inverted fluorescent microscope in conjunction with a standard bright field microscope was used for all types of samples to inspect for presence of microplastics on the surface of the cellulose filters. A wavelength of ~550 nm excitation, ~570 nm emission (orange channel) was used to excite the Rhodamine stain and provoke fluorescence of the stained microplastic particles. A randomized half of the filter paper was scanned for fluorescent particles and pictures of each item were taken both under fluorescent light and in brightfield

mode. Size, shape and colour (observed in brightfield) were recorded. Particles that visually differed from the Nylon particles used in the experiment were excluded i.e., black or dark coloured fibres, even if they appeared to fluoresce. This autofluorescence (false-positive) is contamination, likely due to the existence of either cellulose (fibres) or residual animal protein, both of which naturally show fluorescence in the orange channel wavelength. Particles smaller than 5µm were excluded because they were too small to be identified. The identity of a subset of suspected Nylon particles was confirmed using FTIR spectroscopy.

2.2.6 Statistical analysis

Statistical analysis and visualization were done using R (v4.0.2 (2020-06-22). As data violated the assumptions of normality for Analysis of Variance (ANOVA), differences in mean number of microplastic particles in the different compartments i) animals (no depuration), ii) animals (depurated) and iii) faeces (depuration faeces) were examined using Kruskal-Wallis rank sum test in combination with post-hoc pairwise Wilcoxon tests.

3. Results

3.1 Biofilm experiment

3.1.1 Biofilm growth

Biofilm growth was successfully manipulated, with the chlorophyll a content of the surface sediments in control (median (range): 21.7 (14.6 – 26.0) µg g^{-1} DW sediment), low (41.5 (35.6 – 54.8) µg g^{-1}) and high (73.1 (37.6 – 110.2) µg g^{-1}) treated pots, all found to be significantly different from one another (Fig 4; $H_2 = 28.8$, $P < 0.001$). There was no significant effect of either heavy metal on the growth of the biofilms.

a **b** **c**

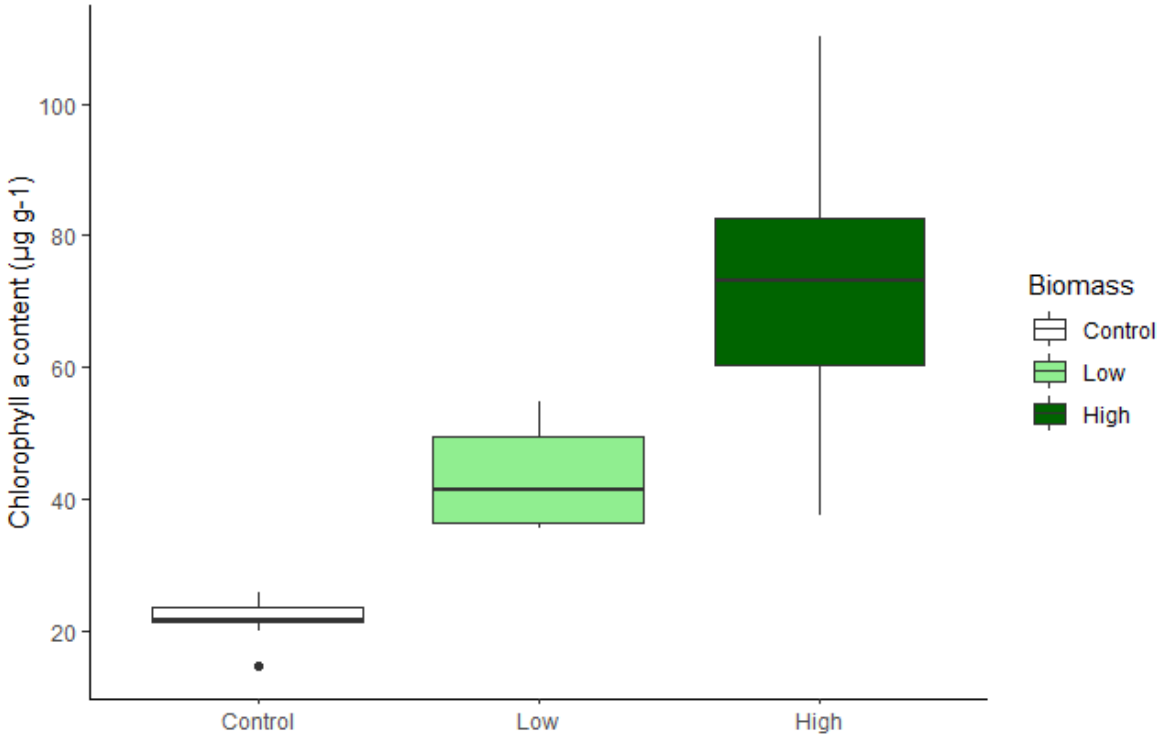

Figure 4: Chlorophyll a content of the surface (3mm) sediment for the different biomass levels; White = Control biomass, Light green = Low biomass, Dark green = High biomass. N=4. Median line, Interquartile range (IQR) are presented. Boxes that do not share the same letter are significantly different from one another.

3.1.2 Erosion of microplastics

Microplastics were captured by the sediments in all pots during the tidal incubations, but there were varying levels of coverage. There was no effect of the heavy metals on the erodibility of the microplastics from the sediment detected. However, significantly higher erosion thresholds were required to remove the microplastic particles from the surface of the sediments containing biofilms (H_2 = 20.1, P < 0.001). Post-hoc dunn's tests revealed that sediments with no biofilm (controls; 0.053 (0.001- 0.119) Nm^{-2}) required less force (Bed shear stress, BSS) to remove the microplastic particles from the surface, compared to sediments containing a low level of biofilm (Low; 0.561 (0.119-0.647) Nm^{-2}) or a high level of biofilm (0.330 (0.053-0.647) Nm^{-2}). No significant difference was detected between the latter two groups (Low & High biomass), likely due to the high variability in the measurements (Fig 5).

a **b** **b**

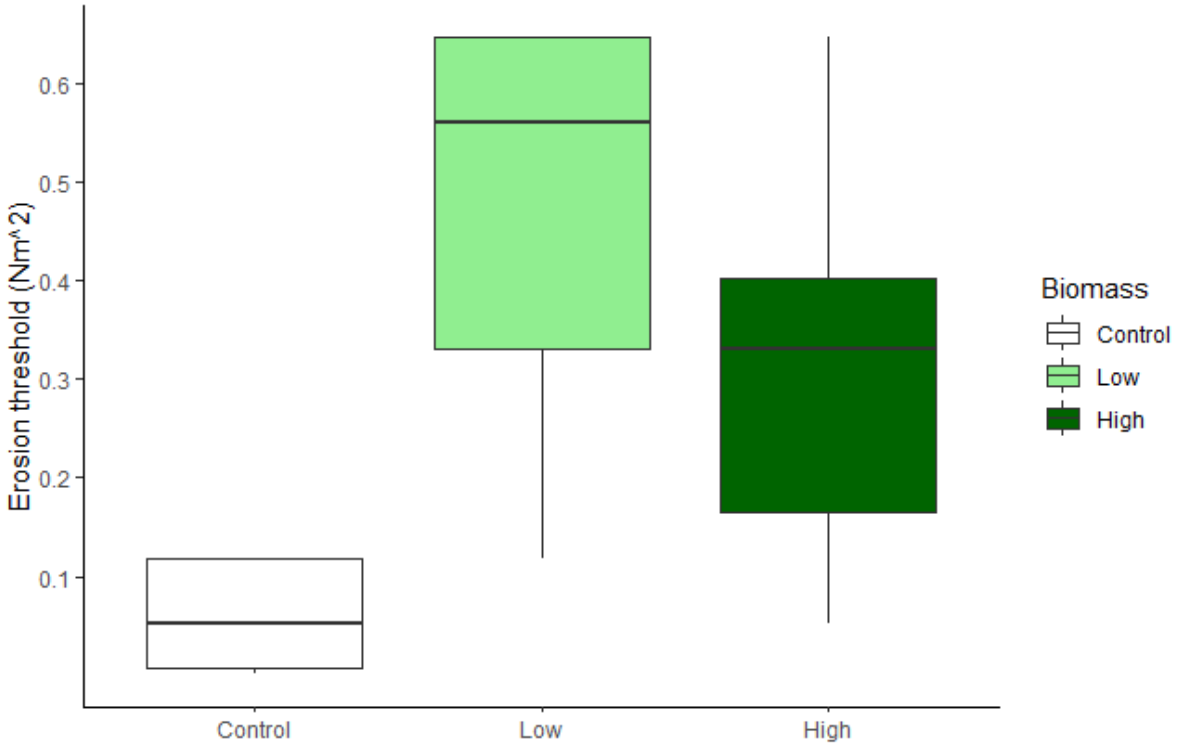

Figure 5: The erosion threshold of the microplastics across sediments containing different biomass levels; White = Control biomass, Light green = Low biomass, Dark green = High biomass. N=4. Median line, Interquartile range (IQR). Boxes that do not share the same letter are significantly different from one another.

3.2 Invertebrate experiment

3.2.1 Ingestion of microplastics

All species used in the experiment ingested fluorescent microplastic particles in varying quantities. The filter feeding bivalve, *C.edule* ingested significantly more microplastics (median of 10 items ind^{-1}) compared to a median of 4 and 2 items ind^{-1} for *A.marina* and *P.cultifera* respectively (Fig 6) with no significant difference between the worms. Despite *S.plana* exhibiting the highest median ingestion rate (12 items ind^{-1}), this was not significantly different from the other species due to the high variability across individuals.

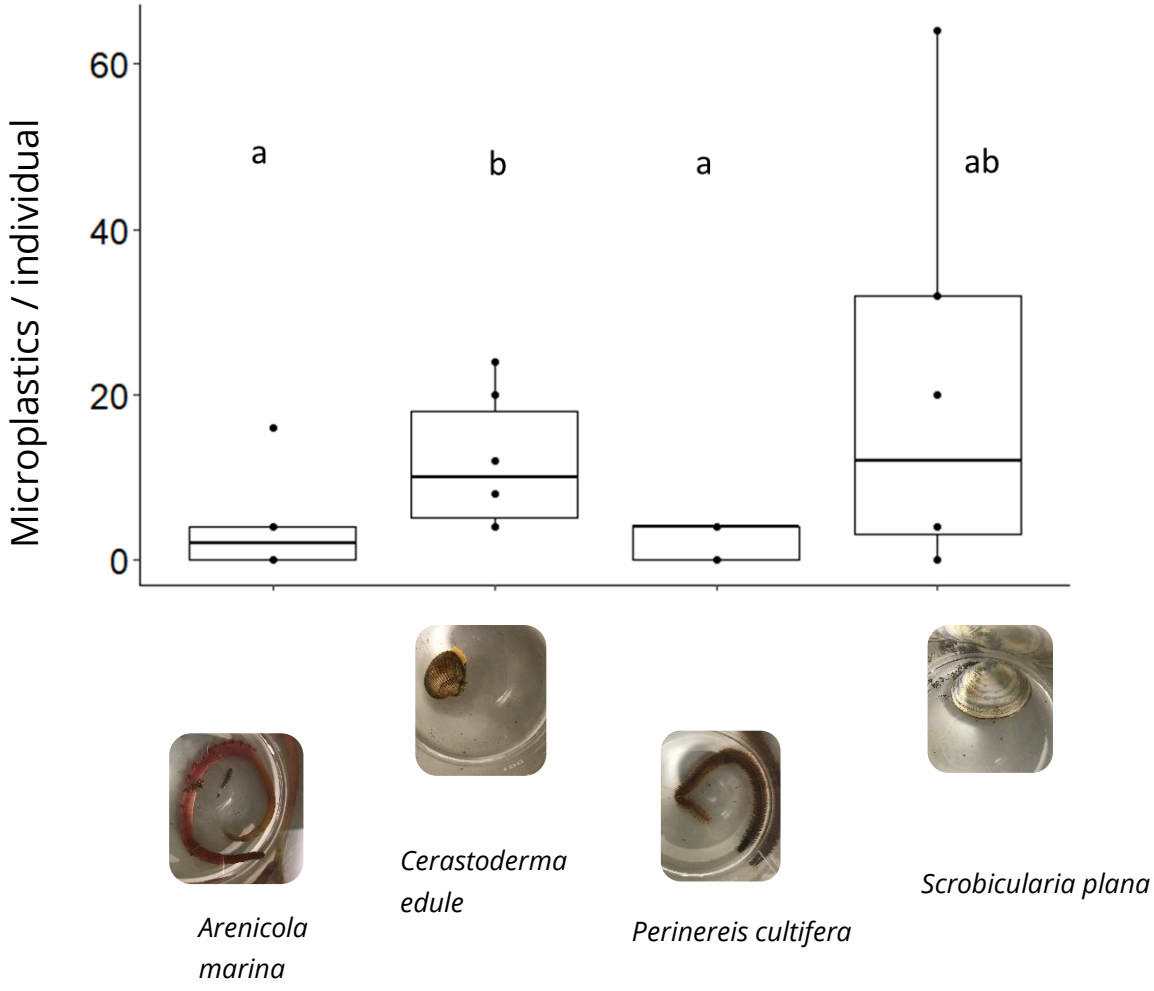

Figure 6: Number of microplastics found inside 4 species of macroinvertebrates from two habitats. Plots that do not share the same letter are significantly different from each other (Kruskal Wallis p< 0.05, Wilcoxon Pairwise comparisons p<0.05, n=6),

The majority of microplastics ingested across all species were irregular fragments: Only a singular item of the "fishing line" fibre was found (in a faeces sample of A.marina, size 707.45µm). Nurdles were only found in *S.plana* (1 item) and in the faeces of *S.plana* (7 items). All others (n=297) were irregular fragments of different sizes (see **Fehler! Verweisquelle konnte nicht gefunden werden.**).

All species exhibited size selectivity, preferentially ingesting microplastics of smaller size (mean ingested size class; 36.49 µm–120.39 µm depending on species), with the largest particle (678 µm) recovered from the faeces of *A.marina*. which is significantly smaller than the mean of particles added to the mesocosms (362.21µm). While there appeared to be larger particles and higher variation between samples in faecal samples compared to animals, this was not significant.

3.2.2 Egestion and bio-packaging (Depuration)

The first observation of faeces and pseudo-faeces produced were after 1-2 hr in the two bivalve species *S.plana* and *C.edule.* After 24 hr there were faeces visible in all species mesocosms and the amount did not visibly increase after a further 24 hr. The bivalves produced well defined pellets whereas the excreted material in the other two species was more diffuse. Examples of faeces pellets produces by *S. plana* and typical microplastics found are provided (Fig 7). No significant difference was observed between animal samples (frozen immediately) and animals that underwent depuration. However, there significantly higher amounts of microplastics were recovered from the faecal samples of the depurated animals (Fig 8).

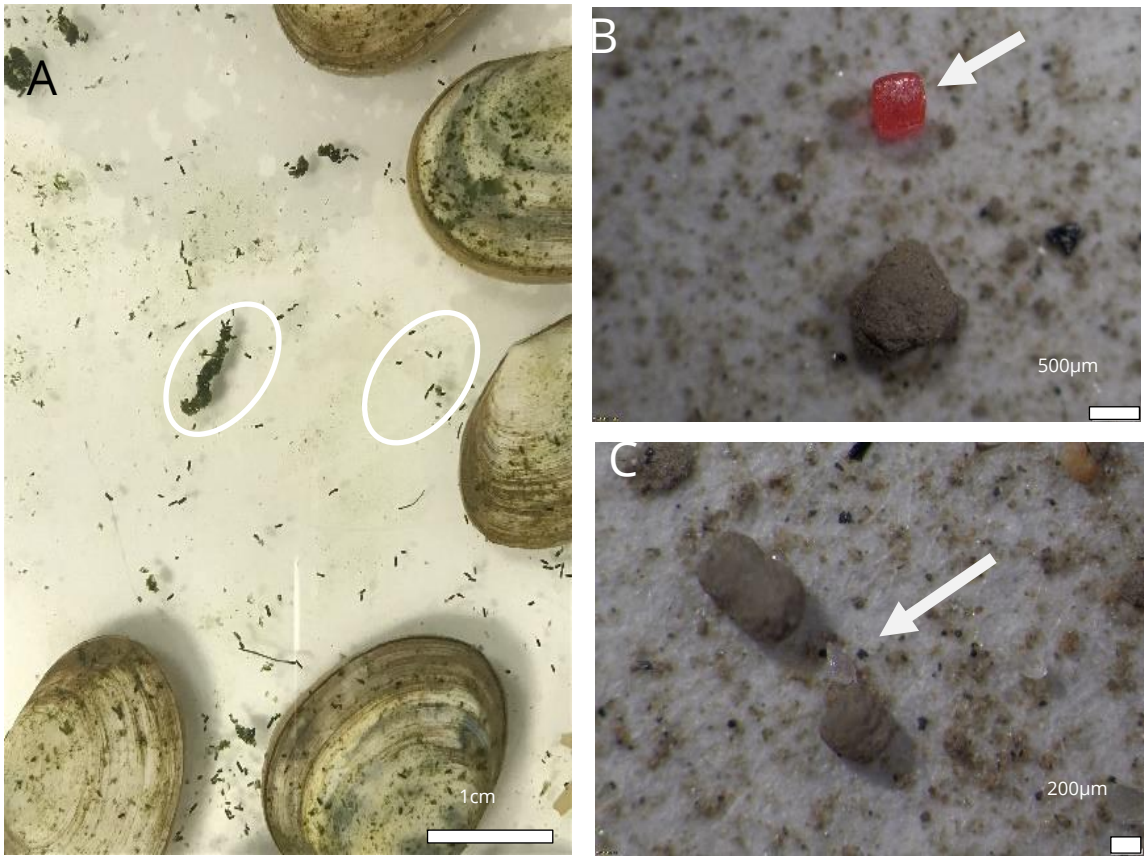

Figure 7: Stereomicroscopic observations during depuration of *S.plana*. A: aggregates of faeces next to *S.plana*. B: Size comparison between a broken faecal pellet and a plastic nurdle. C: Plastic fragment found inside a faecal pellet

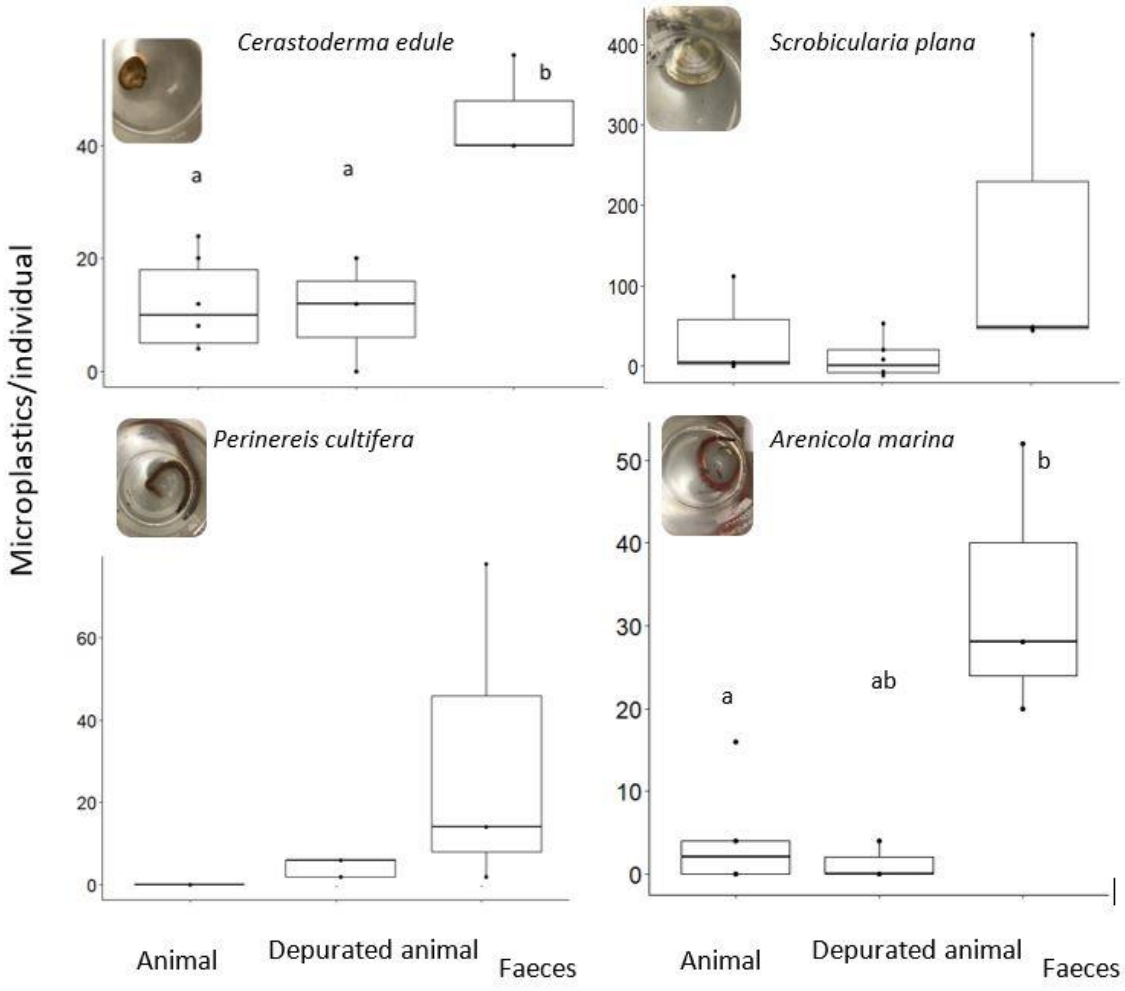

Figure 8: Comparison of the total microplastics detected in non-depurated (Animal), depurated animals and depuration faeces (faeces). Plots that do not share the same letter are significantly different from each other (Kruskal Wallis $p < 0.05$, Wilcoxon Pairwise comparisons $p<0.05$). Plots without letters are not significantly different from one another.

4. Discussion

4.1 Biofilm experiment

The biofilm experiment has demonstrated, for the first time, the potential for biofilm growth on estuarine sediments to mediate the flux of microplastics across the sediment-water interface under flow. While the median erosion threshold of microplastics particles was the highest for low biomass treated cores, there was no significant difference between the low and high biomass cores. This may be due to the growth and development of the high biomass biofilms. For example, higher biomass typically leads to a thicker, and often fluffier biofilm that contains 'loosely' attached material that is easier to erode and slough-off under greater physical disturbance[28]. This may suggest that additional growth of the biofilm does not necessarily mean greater accumulation and retention of microplastics. The hydrodynamic conditions in which biofilms develop, play a key role as higher flow velocities can create thinner, denser and more compact biofilms that are well suited to withstand the greater physical disturbance[29], and this may also influence the retention of microplastics on the bed. Biofilm growth and development, in temperate estuaries, is typically seasonal[30,31], with high microphytobenthic biomass in the spring and summer, often coinciding with calmer conditions such as lower flow velocities[32]. Our findings would therefore suggest that microplastics may be captured and retained on the bed in areas that have sufficient biofilm growth and low flow conditions. Microplastics captured and trapped on the surface of biofilms due to low flow conditions and sticky biofilms may then be more bioavailable to grazers during these periods of higher growth. However, the role of benthic biofilms as a vector of ingestion by benthic invertebrates remains largely unconstrained.

The natural senescence of biofilms and more energetic hydrodynamics later in the year (autumn) would likely release captured microplastics back into the water column as the biofilm dies back and erosion thresholds are exceeded. The fate of these particles is largely unknown, but they may be circulated within estuaries for decades before they are exported or buried[33]. The mode of microplastics erosion may be on a particle-by-particle basis from the

bed (as we observed), or large volumes of microplastics may be resuspended collectively as large sections of biofilm are removed[34,35].

The immobilisation and remobilisation of microplastics associated with benthic biofilms has the potential to influence the spatial and temporal dynamics of microplastics within estuaries, and this knowledge is fundamental for targeting microplastics pollution in estuaries. The onward fate of resuspended microplastics may be influenced by the prevailing flow conditions, the symmetry/asymmetry of the tides and the characteristics of the newly resuspended particles, which was beyond the scope of the current study. Both intertidal sediments and biofilm development, are influenced by the physical disturbance caused by incoming and outgoing tides, freshwater inputs and wave action, therefore a greater understanding of the effects of different hydrodynamic processes on biofilm-microplastics interactions is vital.

While the use of benthic biofilms to remove microplastics from estuarine ecosystems is unlikely, the knowledge of biofilm distributions, seasonal dynamics, their interactions with microplastics and flora-fauna feedbacks may help to identify where and when microplastics may accumulate and when to actively avoid these areas (no dredging activities for example), or manage the area in a way that creates a long term store of microplastics that prevents their export to the marine environment.

4.2 Invertebrate experiment

4.2.1 Ingestion of microplastics

All tested species ingested microplastic particles supporting other studies that have found microplastics in *A.marina*, *S.plana*, *C.edule* in field samples[21,36,37] and in polychaete worms such as *Hediste diversicolor* and other *Perineires ssp*[38,39], which possess a similar lifestyle to *P.cultifera*. However, the findings confirmed previous observations that microplastic ingestion is highly variable across species[40,41]. All species exhibited size selectivity, preferentially ingesting microplastics of smaller size which could not only be problematic for organisms ingesting microplastics (as smaller sizes can be associated with

lower depuration success and higher toxicity[42]), but may alter microplastics size distributions across estuarine habitats as selective burial or release of microplastics occurs.

4.2.2 Egestion and bio-packaging

Higher numbers of microplastics were observed in (pseudo-) faeces, after 48 hr of depuration suggesting the majority of ingested microplastics are not retained or accumulating within the gut of these estuarine invertebrates. Egestion and the ability to selectively reject particles that are not suitable or desirable is common in many species, as it is necessary in a marine environment where animals are exposed to a multitude of "undesired" particles such as sand grains (Ward et al).

Egestion efficiency may depend on particle shape and size. For example, fibres and fibre bundles are more commonly retained than smooth microspheres[43,44]. Although we did not expose the organisms to fibres, there was not significant retention of the fishing line. Egestion can also repackage microplastics into fecal pellets that are deposited on or in the sediment bed, with the former increasing microplastic bioavailability to deposit feeding organisms[45]. This capture and egestion of particles may facilitate their re-entry to the food web, in addition to increasing microplastic deposition and burial in sediments. Further investigation of these processes is warranted.

Interestingly, there was no significant difference in the number of microplastics extracted from that were depurated and animals that were not. Firstly, this could be partially explained by a generally short retention time in the organisms, whereby the non-depurated animals had already passed ingested microplastics at the time of sampling. Secondly, stress during sampling at the end of the experiment could have led to sudden emptying of the gut; which is common and was observed in some individuals of *A.marina*. The overall short egestion time and relatively low number of microplastics retained in the animals highlight the potential inadequacy of "snapshot" sampling for microplastic ingestion studies and will contribute to the variability in microplastic abundance reported in the literature. A repeated sampling approach, long-term monitoring programs, depuration of collected organisms and

the collection of faeces produced during and after animal collection would therefore improve our assessment of microplastics contamination in the environment.

Although filter feeding bivalves such as mussels and oysters, have the potential to ingest large amounts of microplastics, their suitability as indicator species for microplastics pollution has been questioned due to their selective ingestion and egestion[21]. Size and shape selectivity may bias the quantification of microplastics in the environment as different sizes and types of microplastics may not be ingested. Our results of the invertebrate experiment suggest that this size selectivity may be present in benthic estuarine invertebrates, in that the four organisms all egested large volumes of microplastics, and size preferences were apparent. Monitoring the sediments surrounding filter feeder reefs and dense bivalve beds may therefore provide a more comprehensive view of microplastics pollution and the removal potential of benthic organisms.

Although the benthic invertebrates tested largely expelled all microplastics after ingestion, these animals are still effectively removing microplastics from the water column. The transfer to the sediment bed, and fecal deposits within the sediment (likely in many of these animals in their natural habitat) would help sequester microplastics from the environment. However, further studies of the onward fate of the particles are fundamental. The rejection or ingestion-egestion of microplastics may then transfer these to different positions within the bed with faeces and pseudofaeces[4,46] , with burrowing activity resulting in a net burial of particles[4,10]. Furthermore, repackaged microplastics (in fecal pellets) may increase the density of microplastics particles and lead to them sinking to the sediment[46], but pseudofaeces are often low-density and unstable[47] so those microplastics may be easily resuspended with tides and currents.

Even if benthic biofilms and fauna are not retaining microplastics on the surface or internally over the long-term, both appear to be a transient sink to some degree, and they are influencing the redistribution of microplastics. The ingestion of microplastics-rich biofilm, and the depth at which different fauna reside and eject faeces will likely play roles in the fate of this pollutant. Remobilisation may be reduced when particles are transferred below the

depth of sediment mobility, and thus biological redistribution will affect sedimentary microplastic accumulation rates over time.

This study did not identify a particular faunal species or feeding mode that would be good at removing and retaining microplastics from the water column and none appear to be good potential indicator species. However, further studies are required to better understand the cycle of microplastics in and around benthic flora and fauna. It is likely that a selection of different organisms may be required for the long-term monitoring of microplastics pollution in estuaries and to understand the role they may play in immobilising microplastics. The biotic removal microplastics from the entire system seems unlikely but understanding species distributions and the role of different functional traits, how different fauna process microplastics and the fate of ingested (and egested) particles will identify where and when microplastics may accumulate and be released into the water column, allowing future management plans to take account of this pollutant.

5. Conclusion

Benthic biofilms and benthic invertebrates have the potential to influence the transport and fate of microplastics in estuaries but their use as a biological agent to remove microplastics may not yet be feasible. Nonetheless, these benthic organisms are transient sinks; capturing, trapping and redistributing microplastics as they are transported through estuaries. Further studies on the fate of microplastic particles that have interact with sediments, biofilms and sediment dwelling organisms are crucial and a vital step towards managing microplastics pollution in estuarine environments, as these studies have demonstrated that these interactions particle behaviour, movement and fate. These interactions may be particularly important to comprehend in estuaries where sediment management is required; dredging activities or managed realignment, as microplastics accumulation and its effects are poorly resolved.

To our knowledge there no standardized sampling and monitoring program for microplastic for sediments and fauna that is widely adopted and adhered to. Future monitoring of microplastics pollution in estuaries will benefit from methods being

streamlined across studies, as will our understanding of the effects and fate of microplastics in the environment.

6. References

1. Horton, A. A. & Dixon, S. J. Microplastics: An introduction to environmental transport processes. *Wiley Interdiscip. Rev. Water* **5**, e1268–e1268 (2018).
2. Sommer, F. *et al.* Tire Abrasion as a Major Source of Microplastics in the Environment. *Aerosol Air Qual. Res.* **18**, 2014–2028 (2018).
3. Browne, M. A., Galloway, T. S. & Thompson, R. C. Spatial patterns of plastic debris along estuarine shorelines. *Environ. Sci. Technol.* **44**, 3404–3409 (2010).
4. Coppock, R. L. *et al.* Benthic fauna contribute to microplastic sequestration in coastal sediments. *J. Hazard. Mater.* **415**, 125583 (2021).
5. Kalčíková, G. & Bundschuh, M. Aquatic Biofilms—Sink or Source of Microplastics? A Critical Reflection on Current Knowledge. *Environ. Toxicol. Chem.* **41**, 838–843 (2022).
6. Guasch, H. *et al.* Interactions between microplastics and benthic biofilms in fluvial ecosystems: Knowledge gaps and future trends. *Freshw. Sci.* **41**, 442–458 (2022).
7. Cole, M. *et al.* Mussel power: scoping a nature-based solution to microplastic debris. *J. Hazard. Mater.* 131392 (2023) doi:10.1016/j.jhazmat.2023.131392.
8. Mendrik, F., Fernández, R., Hackney, C. R., Waller, C. & Parsons, D. R. Non-buoyant microplastic settling velocity varies with biofilm growth and ambient water salinity. *Commun. Earth Environ.* **4**, 1–9 (2023).

9. Int-Veen, I., Nogueira, P., Isigkeit, J., Hanel, R. & Kammann, U. Positively buoyant but sinking: Polymer identification and composition of marine litter at the seafloor of the North Sea and Baltic Sea. *Mar. Pollut. Bull.* **172**, 112876 (2021).
10. Näkki, P., Setälä, O. & Lehtiniemi, M. Bioturbation transports secondary microplastics to deeper layers in soft marine sediments of the northern Baltic Sea. *Mar. Pollut. Bull.* **119**, 255–261 (2017).
11. Hope, J. A., Paterson, D. M. & Thrush, S. F. The role of microphytobenthos in soft-sediment ecological networks and their contribution to the delivery of multiple ecosystem services. *J. Ecol.* **108**, 815–830 (2020).
12. Hope, J. A. *et al.* Interactions between sediment microbial ecology and physical dynamics drive heterogeneity in contextually similar depositional systems. *Limnol. Oceanogr.* **65**, (2020).
13. Bonnineau, C. *et al.* Role of Biofilms in Contaminant Bioaccumulation and Trophic Transfer in Aquatic Ecosystems: Current State of Knowledge and Future Challenges. in *Reviews of Environmental Contamination and Toxicology Volume 253* (ed. de Voogt, P.) 115–153 (Springer International Publishing, 2021). doi:10.1007/398_2019_39.
14. Mandal, A., Dutta, A., Das, R. & Mukherjee, J. Role of intertidal microbial communities in carbon dioxide sequestration and pollutant removal: A review. *Mar. Pollut. Bull.* **170**, 112626 (2021).
15. Hickey, C. W., Roper, D. S., Holland, P. T. & Trower, T. M. Accumulation of organic contaminants in two sediment-dwelling shellfish with contrasting feeding modes:

Deposit- (Macomona liliana) and filter-feeding (Austrovenus stutchburyi). *Arch. Environ. Contam. Toxicol.* **29**, 221–231 (1995).

16. Fabra, M. *et al.* The plastic Trojan horse: Biofilms increase microplastic uptake in marine filter feeders impacting microbial transfer and organism health. *Sci. Total Environ.* **797**, 149217 (2021).
17. Krause, S. *et al.* Gathering at the top? Environmental controls of microplastic uptake and biomagnification in freshwater food webs. *Environ. Pollut.* **268**, 115750 (2021).
18. Fueser, H., Mueller, M.-T., Weiss, L., Höss, S. & Traunspurger, W. Ingestion of microplastics by nematodes depends on feeding strategy and buccal cavity size. *Environ. Pollut.* **255**, 113227 (2019).
19. Scherer, C., Brennholt, N., Reifferscheid, G. & Wagner, M. Feeding type and development drive the ingestion of microplastics by freshwater invertebrates. *Sci. Rep.* 1–9 (2017) doi:10.1038/s41598-017-17191-7.
20. Bour, A., Avio, C. G., Gorbi, S., Regoli, F. & Hylland, K. Presence of microplastics in benthic and epibenthic organisms: Influence of habitat, feeding mode and trophic level. *Environ. Pollut.* **243**, 1217–1225 (2018).
21. Ward, J. E. *et al.* Selective Ingestion and Egestion of Plastic Particles by the Blue Mussel (Mytilus edulis) and Eastern Oyster (Crassostrea virginica): Implications for Using Bivalves as Bioindicators of Microplastic Pollution. *Environ. Sci. Technol.* **53**, 8776–8784 (2019).

22. Costa, L. L., Arueira, V. F., da Costa, M. F., Di Beneditto, A. P. M. & Zalmon, I. R. Can the Atlantic ghost crab be a potential biomonitor of microplastic pollution of sandy beaches sediment? *Mar. Pollut. Bull.* **145**, 5–13 (2019).
23. Beninger, P. G., Valdizan, A., Decottignies, P. & Cognie, B. Impact of seston characteristics on qualitative particle selection sites and efficiencies in the pseudolamellibranch bivalve Crassostrea gigas. *J. Exp. Mar. Biol. Ecol.* **360**, 9–14 (2008).
24. Wang, Y. *et al.* The uptake and elimination of polystyrene microplastics by the brine shrimp, Artemia parthenogenetica, and its impact on its feeding behavior and intestinal histology. *Chemosphere* **234**, 123–131 (2019).
25. Welden, N. A. C. & Cowie, P. R. Environment and gut morphology influence microplastic retention in langoustine, Nephrops norvegicus. *Environ. Pollut.* **214**, 859–865 (2016).
26. Näkki, P., Setälä, O. & Lehtiniemi, M. Seafloor sediments as microplastic sinks in the northern Baltic Sea – Negligible upward transport of buried microplastics by bioturbation. *Environ. Pollut.* **249**, 74–81 (2019).
27. Jeffrey, S. W. & Humphrey, G. F. New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher plants, algae and natural phytoplankton. *Biochem. Physiol. Pflanz.* **167**, 191–194 (1975).
28. Stoodley, P., Dodds, I., Boyle, J. d. & Lappin-Scott, H. m. Influence of hydrodynamics and nutrients on biofilm structure. *J. Appl. Microbiol.* **85**, 19S-28S (1998).
29. Liu, Y. & Tay, J. H. Detachment forces and their influence on the structure and metabolic behaviour of biofilms. *World J. Microbiol. Biotechnol.* **17**, 111–117 (2001).

30. Moerdijk-poortvliet, T. C. W. *et al.* Seasonal changes in the biochemical fate of carbon fixed by benthic diatoms in intertidal sediments. 550–569 (2018) doi:10.1002/lno.10648.
31. Daggers, T. D., Herman, P. M. J. & van der Wal, D. Seasonal and Spatial Variability in Patchiness of Microphytobenthos on Intertidal Flats From Sentinel-2 Satellite Imagery. *Front. Mar. Sci.* **7**, 1–14 (2020).
32. Savelli, R. *et al.* On biotic and abiotic drivers of the microphytobenthos seasonal cycle in a temperate intertidal mudflat: A modelling study. *Biogeosciences* **15**, 7243–7271 (2018).
33. Tramoy, R., Gasperi, J., Colasse, L. & Tassin, B. Transfer dynamic of macroplastics in estuaries — New insights from the Seine estuary: Part 1. Long term dynamic based on date-prints on stranded debris. *Mar. Pollut. Bull.* **152**, 110894 (2020).
34. Stoodley, P., Cargo, R., Rupp, C. J., Wilson, S. & Klapper, I. Biofilm material properties as related to shear-induced deformation and detachment phenomena. *J. Ind. Microbiol. Biotechnol.* **29**, 361–367 (2002).
35. Telgmann, U., Horn, H. & Morgenroth, E. Influence of growth history on sloughing and erosion from biofilms. *Water Res.* **38**, 3671–3684 (2004).
36. Hermabessiere, L. *et al.* Microplastic contamination and pollutant levels in mussels and cockles collected along the channel coasts. *Environ. Pollut.* **250**, 807–819 (2019).
37. Ribeiro, F. *et al.* Microplastics effects in Scrobicularia plana. *Mar. Pollut. Bull.* (2017) doi:10.1016/j.marpolbul.2017.06.078.
38. James, K. *et al.* Microplastic ingestion by the polychaete community in the coastal waters of Kochi, Southwest coast of India. *Reg. Stud. Mar. Sci.* **62**, 102948 (2023).

39. Porter, A. *et al.* Uptake of microplastics by marine worms depends on feeding mode and particle shape but not exposure time. *Sci. Total Environ.* **857**, (2023).
40. Gouin, T. Towards improved understanding of the ingestion and trophic transfer of microplastic particles - Critical review and implications for future research. *Environ. Toxicol. Chem.* (2020) doi:10.1002/etc.4718 M4 - Citavi.
41. Bour, A., Avio, C. G., Gorbi, S., Regoli, F. & Hylland, K. Presence of microplastics in benthic and epibenthic organisms: Influence of habitat, feeding mode and trophic level. *Environ. Pollut.* **243**, 1217–1225 (2018).
42. Bakir, A., O'Connor, I. A., Rowland, S. J., Hendriks, A. J. & Thompson, R. C. Relative importance of microplastics as a pathway for the transfer of hydrophobic organic chemicals to marine life. *Environ. Pollut. Barking Essex 1987* **219**, 56–65 (2016).
43. Fueser, H., Mueller, M. T. & Traunspurger, W. Rapid ingestion and egestion of spherical microplastics by bacteria-feeding nematodes. *Chemosphere* **261**, (2020).
44. Welden, N. A. C. & Cowie, P. R. Environment and gut morphology influence microplastic retention in langoustine, Nephrops norvegicus. *Environ. Pollut.* **214**, 859–865 (2016).
45. Piarulli, S. & Airoldi, L. Mussels facilitate the sinking of microplastics to bottom sediments and their subsequent uptake by detritus-feeders. *Environ. Pollut.* **266**, 115151 (2020).
46. Khan, M. B. & Prezant, R. S. Microplastic abundances in a mussel bed and ingestion by the ribbed marsh mussel Geukensia demissa. *Mar. Pollut. Bull.* **130**, 67–75 (2018).

47. Bulleri, F., Ravaglioli, C., Anselmi, S. & Renzi, M. The sea cucumber Holothuria tubulosa does not reduce the size of microplastics but enhances their resuspension in the water column. *Sci. Total Environ.* **781**, 146650 (2021).