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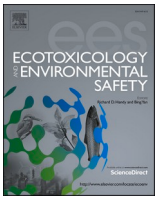

Shrimp and microplastics: A case study with the Atlantic ditch shrimp *Palaemon varians*

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ABSTRACT

Many invertebrate species inhabit coastal areas where loads of plastic debris and microplastics are high. In the current case study, we exemplarily illustrate the principal processes taking place in the Atlantic ditch shrimp, *Palaemon varians*, upon ingestion of microplastics. In the laboratory, shrimp readily ingested fluorescent polystyrene microbeads of 0.1–9.9 μm, which could be tracked within the widely translucent body. Ingested food items as well as micro-particles cumulate in the stomach where they are macerated and mixed with digestive enzymes. Inside the stomach, ingested particles are segregated by size by a complex fine-meshed filter system. Liquids and some of the smallest particles (0.1 μm) pass the filter and enter the midgut gland where resorption of nutrients as well as synthesis and release of digestive enzymes take place. Large particles and most of the small particles are egested with the feces through the hindgut. Small particles, which enter the midgut gland, may interact with the epithelial cells and induce oxidative stress, as indicated by elevated activities of superoxide dismutase and cellular markers of reactive oxygen species. The shrimp indiscriminately ingest microparticles but possess efficient mechanisms to protect their organs from overloading with microplastics and other indigestible particles. These include an efficient sorting mechanism within the stomach and the protection of the midgut gland by the pyloric filter. Formation of detrimental radical oxygen species is counteracted by the induction of enzymatic antioxidants.

1. Introduction

The number of studies demonstrating the uptake of microplastic particles by various marine organisms increased continuously during recent years (e.g. Auta et al., 2017; Bour et al., 2018; de Sá et al., 2018; López-Martínez et al., 2020). Some of them report accumulation of microplastics on the gills of fishes, mollusks, and crustaceans. However, no clear transfer of particles through gill epithelia has been reported yet (Watts et al., 2014, 2016). The principal way of microplastic uptake by marine organisms remains via ingestion. Microplastics have been found in the gastrointestinal tract of numerous aquatic species including fishes (Lusher et al., 2013; Nadal et al., 2016), fish larvae (Steer et al., 2017), zooplankton (Desforges et al., 2015), tropical corals (Hall et al., 2015), and even deep-sea organisms (Taylor et al., 2016). The tiny synthetic particles may easily be mistaken as natural food, such as small plankton organisms, and taken up intentionally or by accident (Bern, 1990). Additionally, microplastics can be transferred across trophic levels via consumption of contaminated prey (Farrell and Nelson, 2013).

Crustaceans inhabiting coastal areas or estuaries are particularly subjected to anthropogenic pollution, including microplastics (Setälä et al., 2016; Sindermann, 2005; Vikas and Dwarakish, 2015). Their feeding mode as scavengers, deposit feeders, suspension feeders, or predators make them vulnerable to microplastic ingestion. Whether ingested microplastics have adverse effects on the health of consumers with different feeding modes depends on the fate of the particles within the organism. Microplastics may simply pass the digestive tract and leave the gut along with the fecal material (Hämer et al., 2014; Cole et al., 2016; Rodríguez-Torres et al., 2020) whereas larger particles (> 100 μm) may be regurgitated through the esophagus (Saborowski et al., 2019). However, very small microplastics in the nanometer size range

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may be resorbed by the epithelia of digestive organs and translocated to adjacent tissues (Browne et al., 2008).

The cellular effects of microplastics are diverse (Sun et al., 2021 and literature cited therein). Ingestion of high-density polyethylene (HDPE) particles by mussels (*Mytilus edulis*) induced inflammatory responses and lysosomal membrane destabilization in the cells of the intestine (von Moos et al., 2012). Similarly, polystyrene microparticles of 5 μm accumulated in the liver of zebrafish (*Danio rerio*) and caused inflammation and cellular oxidative stress through the formation of reactive oxygen species (ROS) (Lu et al., 2016). Oxidative stress, in turn, can have adverse effects on essential cellular compounds such as proteins, lipids, and DNA (Turrens, 2003). In vital cells with functional cellular redox systems, balanced equilibria are established between ROS and ROS-scavenging molecules to protect the cells from oxidative damage (Ray et al., 2012).

The first line of defense against ROS in e.g. epithelial cells is the superoxide scavenging enzyme superoxide dismutase (SOD), which converts the superoxide ion into hydrogen peroxide. In response to increasing superoxide levels, SOD activities are upregulated (Landis and Tower, 2005). Accordingly, elevated enzyme activities can serve as an indicator for oxidative stress. The increase of ROS and antioxidant enzyme activity in response to micro- and nanoplastic uptake has been demonstrated in copepods, rotifers, and bivalves (Jeong et al., 2016, 2017; Paul-Pont et al., 2016; Ribeiro et al., 2017). Particles are taken up into the cells presumably via endocytosis (von Moos et al., 2012; Vogt, 2019). However, the cellular mechanisms causing oxidative stress by microplastics need deeper investigation.

In the current work, we address the effects and reactions in the Atlantic ditch shrimp *Palaemon varians* upon microplastic exposure. This article is conceived as a case study with a crustacean species, providing an overview of the principal processes from the uptake of particles to the internal processing, and the cellular effects they may cause. Our approach comprises feeding of shrimp with differently sized fluorescent microbeads. Their dispersion within the digestive tract and biochemical effects are discussed in view of the anatomy, functional morphology, and cytology of the digestive organs.

2. Material and methods

2.1. Shrimp

Atlantic ditch shrimp (*Palaemon varians*, see also supporting information) were purchased from an aquaristic supplier (Mrutzek Meeres-Aquaristik, Ritterhude, Germany). The shrimp were transferred to the laboratories of the Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, where they were maintained in 10-L aquaria with diluted seawater (salinity 15 PSU). The water was exchanged every other day. Maintenance and feeding were carried out in a temperature-controlled room at 15 °C and a 12/12 h light/dark cycle. The shrimp were fed every other day with plant-based fish food (NovoVert, JBL, Germany) and once a week with freshly hatched *Artemia* nauplii (Great Salt Lake Artemia Cysts, Sanders, USA). The water quality was controlled regularly. Levels of nitrite, nitrate and ammonium were monitored and held below critical concentrations by regular exchanges of the seawater. Only shrimp in the intermolt stage were used for further investigations.

2.2. Microbeads

The microbeads used were Thermo Scientific™ Fluoro-Max fluorescent polystyrene (PS) microspheres. These particles are uniform, easy to observe within organisms, and easy to follow microscopically when translocated between organs. The types of microbeads used varied in terms of size and fluorescent properties and were applied at different concentrations (Table S1) and according to the experimental question as described in the following paragraphs. Stock suspensions were prepared in ultrapure water to eliminate potentially adverse additives and preservatives from the commercial microbead suspension (see supporting information).

2.3. Microscopic investigations

To illustrate the uptake of microbeads and their distribution in the digestive tract of *P. varians*, the shrimp were exposed to high concentrations of fluorescent particles of different sizes (0.1, 2.1, or 9.9 μm, Exp. 1 in Table S1). For each particle type, a 50-mL reaction tube was filled with 35 mL of particle suspension in brackish water (15 PSU). Two shrimp (30–35 mm length) were placed in the tube for 24 h on a rotator mixer at low rotation speed (10 rpm) to keep the particles in suspension. After the incubation, the shrimp were photographed under a Nikon SMZ 25 epifluorescence stereo microscope at the respective filter setting to visualize the distribution of the particles within the digestive tract of the widely translucent animal (Saborowski et al., 2019).

The distribution of the smallest particles (0.1 μm) within the digestive organs, the cellular structure of the midgut gland, and the presence of reactive oxygen species within the cells was visualized by confocal laser scanning microscopy (Leica TCS SP5II, Leica Microsystems CMS GmbH, Wetzlar, Germany). The microscope was equipped with an argon laser (excitation: 488 nm).

Shrimp were incubated in a suspension of 0.1-μm particles as described above. After 24 h, the shrimp were sedated on ice, the midgut gland was dissected and stained with Syto-13 (Molecular Probes, D-23107, 5 mmol·L^{-1} solution in DMSO). The dissected organs were incubated at room temperature for 30 min in darkness in 15 mmol·L^{-1} Na-HEPES and 0.5 mmol·L^{-1} glucose in seawater (pH 8.3) with 10 μmol·L^{-1} Syto-13 (Korez et al., 2020).

Midgut glands of *P. varians*, which were incubated in a suspension of 0.1-μm particles, were also stained with carboxy-2′,7′-difluorofluorescein diacetate (CH_2DFFDA, Molecular Probes, C-13293, 2 mmol·L^{-1} solution in ethanol) to detect reactive oxygen species (ROS) (Rivera-Ingraham et al., 2013). The staining solution was composed of 15 mmol·L^{-1} Na-HEPES and 0.5 mmol·L^{-1} glucose in 250 mmol·L^{-1} NaCl, containing 20 μmol·L^{-1} of CH_2DFFDA. The samples were incubated for 30 min in the dark at room temperature. Prior to imaging, the samples were washed three times in incubation buffer.

The small particles (0.1 μm) emitted in the red spectrum (645–660 nm). The cell nuclei and ROS emitted in the green spectrum (500–520 nm and 517–527 nm, respectively). Photographs were taken with a 40x optical objective and a resolution of 512 × 512 pixels.

Scanning electron microscopy (SEM) was applied to illustrate the ultrastructure of the stomach and the midgut gland of *P. varians*, and to verify the size and shape of the particles used. Stomachs and midgut glands of the shrimp were carefully dissected on ice and the filter press in the proventriculus was exposed. The organs were then dehydrated in an ethanol series: 2 × 15 min in 50% ethanol, 2 × 15 min in 70% ethanol, 2 × 15 min in 90% ethanol, 2 × 15 min in 96% ethanol, 30 min in an ethanol-hexamethyldisilazane (HDMS) solution (ratio 1:1) and, finally, 60 min in pure HDMS-solution (Korez et al., 2020). After air-drying for 24 h, the samples were mounted on SEM stubs with double-sided carbon tape. The microplastic particles were suspended in demineralized water. Droplets of the differently sized particles were first dried on a plastic weighing pan. Then the microplastic particles were sprinkled over the stubs with double-sided carbon tape. The stubs were sputter coated with gold-palladium. The samples were inspected and photographed under the SEM (FEI, Quanta FEG 200).

2.4. Biochemical investigations

2.4.1. Exposure

Twenty-four hours prior to incubation with microplastics, the shrimp were fed with plant-based fish food (NovoVert, JBL, Germany). During the incubation, the animals received no food but only microplastic

particles at the given concentrations (Exp. 2 in Table S1). 500-mL glass flasks were filled with 200 mL of filtered seawater, diluted to a salinity of 15 PSU. A specific amount of the particle stock suspension was added to the flasks to obtain the desired particle concentration. Five randomly selected shrimp were transferred from the aquaria into each of four replicate flasks. The flasks were covered with parafilm to avoid evaporation and contamination. Each flask was equipped with an aeration tube, which was pierced through the parafilm. The aeration suspended the microbeads in the water. The incubation times were 2, 4, 8, 24, and 48 h. Two additional incubations (6 replicates each) served as negative controls using the same setup as described above. In the first negative control, the shrimp received food but no particles. Shrimp were first fed at the start of the incubation and then 24 h later, with 15 mg plant-based fish food per 500-mL glass flask. In the second negative control the shrimp received no particles and no food.

2.4.2. Sample preparation

The shrimp were sedated on ice. The body length of the shrimp was measured, and the midgut gland was dissected in a Petri dish placed on a cooling pad. The midgut gland was transferred into pre-weighed 1.5-mL reaction tubes, weighed, and immediately frozen in liquid nitrogen. Thereafter, the samples were stored at - 80 °C. Untreated animals (0 h of incubation) were randomly taken from the aquaria at the beginning of the feeding trials and processed as described above. The frozen tissues were extracted immediately before the enzyme activities were measured. The individual midgut glands were slowly thawed on ice and homogenized with a conical micro-pestle in 50 μL of ultrapure water. After centrifugation (10 min, 14,000*g*, 4 °C), the supernatants were transferred to new 1.5-mL reaction tubes. Five μL of the extract were diluted 1:20 in SOD homogenization buffer. The SOD homogenization buffer (pH 7.6) was prepared with 100 mL Tris-HCl (20 mmol·L^{-1}) and 50 mL EDTA (1 mmol·L^{-1}) in distilled water (modified after Livingstone et al., 1992). The buffer was filtered (0.2 μm) and stored at 4 °C. Finally, the diluted extracts were vortexed and referred to as 'samples' in the following. The remains of the extracts were stored at - 80 °C. Every step was performed on ice.

2.4.3. Enzyme assays

Superoxide dismutase (SOD) was assayed after Livingstone et al. (1992), with slight modifications and adaptations for shrimp midgut gland tissue samples (for details see the Supporting Information).

2.5. Statistical analyses

The temporal variation of SOD activity in the midgut gland of *P. varians* was analyzed for each food type separately using one-factorial analyses of variance (ANOVA) and Tukey's posthoc tests. Prior to the ANOVA, the data were tested for equal variances using Levene's test. Solely in the case of the 9.9-μm microbeads, the variances were heterogeneous and could not be homogenized by data transformation. Additionally, we compared the overall SOD activity among the treatments by an ANOVA. To account for the heteroscedasticity of the data, as revealed by Levene's test, the significance level was adjusted to $p = 0.01$.

3. Results

The shrimp readily ingested the offered food and the fluorescent microparticles. There was no mortality.

3.1. Dispersion of microplastics in the digestive organs

The largest particles of 9.9 μm (green) were present in the cardiac stomach (Fig. 1a). The 2.1-μm particles (blue) accumulated in the anterior part of the cardiac stomach and advanced further into the dorsal chamber of the pyloric stomach. The smallest particles of 0.1 μm (red) apparently dispersed into the midgut gland, which extends posteriorly from the stomach (Fig. 1b). Excreted feces contained the fluorescent microbeads (Fig. 1c). Smaller fluorescent particles within the fecal pellets were not visible from outside due to shielding by the fecal pellet content.

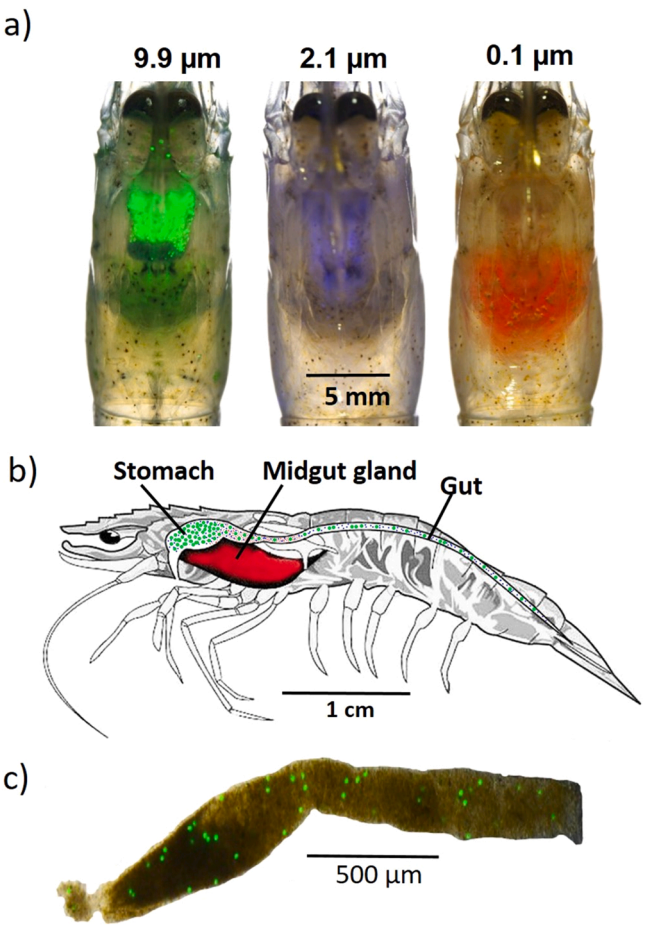

Fig. 1. *Palaemon varians* with ingested fluorogenic microbeads of 9.9 μm (green), 2.1 μm (blue), and 0.1 μm (red) in diameter. a) Appearance of microbeads in the stomach and in the midgut gland. Merged images of transmission and electronically intensified fluorescence spectra. b) Lateral view of the shrimp and schematic illustration of the stomach and the midgut gland and gut with green (9.9 μm), blue (2.1 μm), and red (0.1 μm) microbeads indicated. c) Excreted fecal string with 9.9 μm microbeads. Merged transmission and fluorescence images.

3.2. The stomach and the pyloric filter

The stomach is a complex flexible chitinous capsule. It consists of an anterior cardiac part and a smaller posterior pyloric part. The pyloric stomach contains a fine-meshed filter system (Fig. 2a, b), which fulfils pivotal functions in the separation of digestible compounds and indigestible solid particles. The pyloric filter consists of two inner and two outer valves. The inner valves join dorsally at the so-called filter crest, forming a 'W'-shape in cross section. Each side of the filter is covered by a field of forklike setae, projecting dorsally to the ventrolateral partition (Fig. 2c, d). This separates the dorsal passageway for solids and the ventral passageway for fluids (King and Alexander, 1994). The ventrolaterally projecting filter setae are connected by longitudinal ridges. Between the longitudinal ridges four to six pyloric filter setae are bundled, thus forming transversal channels. The pyloric filter setae are studded with densely packed setules. The gaps between the setules have a width of approximately 170 nm. The chyme produced in the stomach

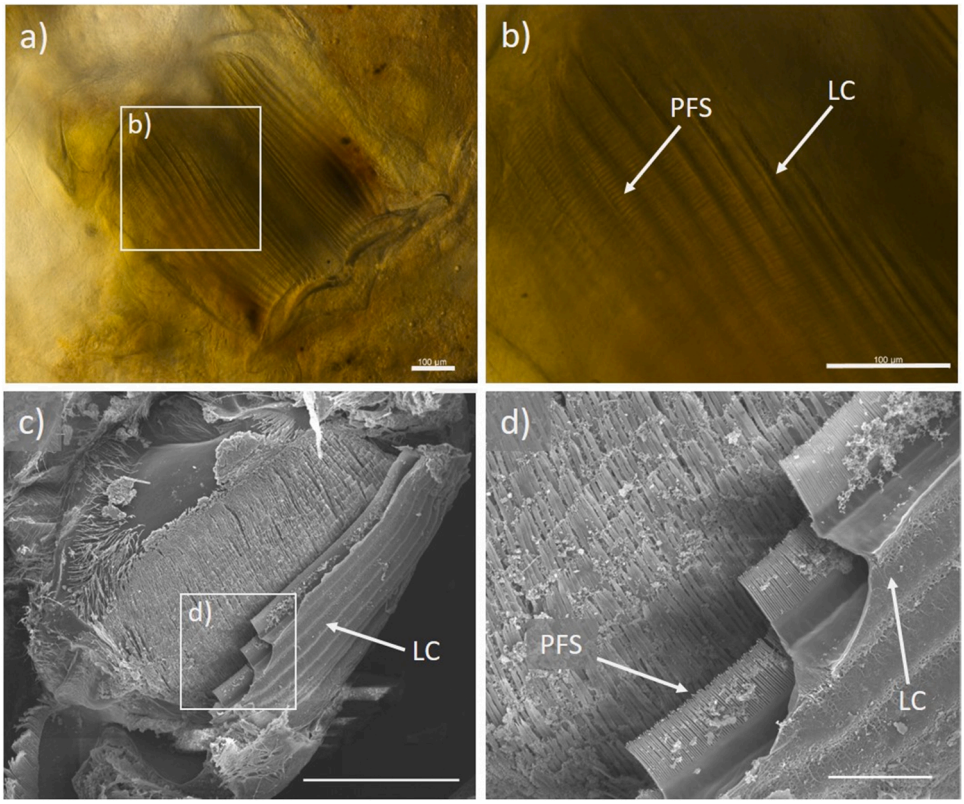

Fig. 2. The pyloric filter of *Palaemon varians*. a,b) Transmitted light microscopic view on the pyloric filter. c,d) Scanning electron microscopic details of the pyloric filter with longitudinal costae (LC) bearing vertical pyloric filter setae (PFS). Scale bars in a and b: 100 µm, c: 300 µm, d: 50 µm.

is pressed through the pyloric filter. The fluid fraction of the chyme enters the midgut gland where nutrients are resorbed while solids are retained and directed into the gut for egestion.

3.3. The midgut gland

The midgut gland (syn. hepatopancreas) consists of numerous blind ending tubules with a monolayer of cells forming the tubule wall. The nuclei of the cells, stained with Syto-13, indicate the arrangement of the tubule with their distal tips and the cellular monolayer surrounding the tubule lumen (Fig. 3). The diameter of the tubules is up to 200 µm. The tubules become wider towards the proximal regions of the midgut gland. The scanning electron micrograph shows longitudinally broken tubules and membranous fragments of the cell monolayer. The tubule lumen extents to about 50 µm. Specific cells cannot be identified, but large vacuoles of the B-cells are suggested.

Individual microbeads of 0.1 µm could not be resolved within the midgut gland. However, several hours after feeding on these particles, agglomerations of fluorescent particles appeared adjacent to the midgut gland tissue (Fig. 4). Their shape was cylindrical with a length of up to 500 µm and a width of up to 160 µm.

Incubation of midgut gland tissue with CH_2DFFDA revealed distinct green fluorescent spots in the cells of the midgut gland tubules (Fig. 5). The lumen of the tubule showed a weak and diffuse fluorescence.

3.4. SOD-activity

The SOD activity increased significantly after exposure to 0.1-µm microbeads (ANOVA: $F_{4,17} = 4.17$; $p = 0.02$ – Fig. 6a). At the beginning of the incubation period, the initial average (± SEM) SOD activity was 518 ± 140 U·g^{-1}. After 4 h the activity increased to 953 ± 155 U·g^{-1} and reached the maximum of 1120 ± 79 U·g^{-1} after 8 h. After 24 h (1045 ± 120 U·g^{-1}) and 48 h (1003 ± 95 U·g^{-1}) the activities slightly decreased.

Shrimp feeding on 2.1-µm microbeads showed a significant increase in SOD activity (ANOVA: $F_{4,17} = 4.98$; $p < 0.01$ – Fig. 6b). The SOD activity increased steadily from 725 ± 60 U·g^{-1} at the beginning of the incubation period to 1337 ± 115 U·g^{-1} after 24 h of incubation. After 48 h of incubation the SOD activity was slightly lower at 1015 ± 93 U·g^{-1}.

Exposure to 9.9-µm microbeads also resulted in an increase in SOD activity (ANOVA: $F_{4,17} = 6.21$; $p < 0.01$ – Fig. 6c) with the activity being highest at 1076 ± 44 U·g^{-1} after 8 h of incubation. The heterogeneous variances (Levene's test: $F_{4,17} = 3.54$; $p = 0.03$) increased the probability of erroneously accepting the differences in SOD activity as statistically significant. However, the variances within the groups were small and the initial increase in SOD activity was steep, suggesting clearly elevated activities after 8 h of incubation. After 24 h of incubation, the SOD activity declined to 765 ± 107 U·g^{-1} and to 711 ± 29 U·g^{-1} after 48 h of incubation.

Feeding on commercial fish food had no effect on the SOD activity of the shrimp (ANOVA: $F_{4,17} = 0.34$; $p = 0.85$ – Fig. 6d). The SOD activities varied only little between the incubation times from 726 ± 90 U·g^{-1} at the beginning of the incubation period and 855 ± 60 U·g^{-1} after 24 h of incubation. Similarly, the control without food and without particles showed no significant variation in SOD activity (ANOVA: $F_{4,17} = 1.65$; $p = 0.21$ – Fig. 6e). The activities varied from 768 ± 74 U·g^{-1} at the beginning of the incubation, increased towards 1030 ± 83 U·g^{-1} after 24 h, and decreased again to 944 ± 15 U·g^{-1} at the end of the incubation.

No differences in the overall SOD activity were detected among the treatments ($F_{4,105} = 2.75$; $p = 0.03$) when the significance level was adjusted to $p = 0.01$ to account for the heteroscedasticity of the data.

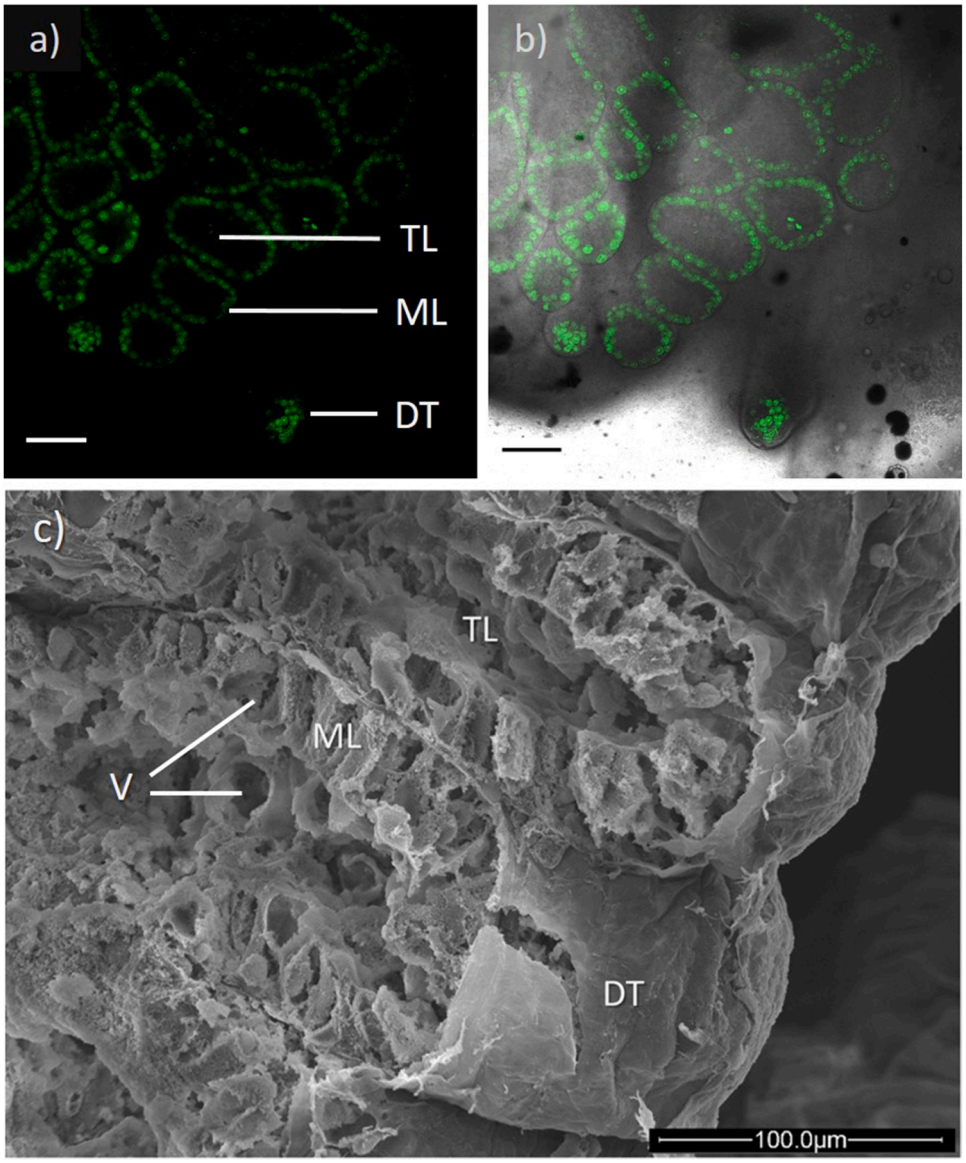

Fig. 3. Midgut gland tubules of *Palaemon varians*: fluorescent image of a) Syto-13 dyed cell nuclei (green) and b) fluorescent image merged with brightfield image of midgut gland. c) Scanning electron micrograph (SEM) of longitudinally broken midgut gland tubule showing the tubule lumen (TL), the cell monolayer (ML), the distal tip of the tubule (DT), and vacuoles (V) of B cells. No microplastics were administered. All scale bars represent 100 µm.

4. Discussion

Microplastics have no nutritional value, except a more or less evolved biofilm on the surface (Michels et al., 2018). Nevertheless, numerous reports showed that a huge range of marine invertebrates, including crustaceans, mollusks, chaetognaths, tunicates, and cnidarians do ingest microplastic particles in the µm size range accidentally or intentionally (Cole et al., 2013), among them mysid shrimp and caridean shrimp (Setälä et al., 2014; Devriese et al., 2015; Korez et al., 2020). However, the effects of microplastic ingestion are only marginally understood and seem to differ between consumers, potentially because of morphological and physiological variations among species.

4.1. Exposure and uptake of particles

The Atlantic ditch shrimp, *Palaemon varians*, lives in brackish lagoons, estuaries, and salt marshes from Scandinavia to the Mediterranean, where it faces anthropogenic pollution. Microplastic concentrations in its area of distribution range from 0 to 70 items per m^3 in the water column and 22–702 items per kg dry sediment (Table S2). Small items in the size range < 100 µm dominated the microplastic samples in the coastal areas of the North Sea (Lorenz et al., 2019). The size range of the collected particles depends on the mesh size of the sieves, which was 20 µm in the study by Lorenz et al. (2019). Smaller particles mostly passed the filter, while particles < 100 µm as well as fragments thereof are in the size range of potential food items and resemble the administered 9.9-µm particles in our experiment.

Investigations on the microplastic burden of wild individuals of *P. varians* are lacking. However, studies on mussels and brown shrimp from the same area showed up to 6.9 plastic items per gram (Table S3). Moreover, previous laboratory studies showed that *P. varians* readily ingest microplastic beads (9.9 µm) and fibers of up to 200 µm (Saborowski et al., 2019). The present observations confirm the uptake of microplastics of different size by *P. varians*. Moreover, they indicate that microplastics are taken up by the shrimp in misperception of food, rather than accidently along with the food as shown for the uptake of sand grains by the brown shrimp *Crangon crangon* (Schmidt et al., 2021). In the current study, no food was offered simultaneously with the

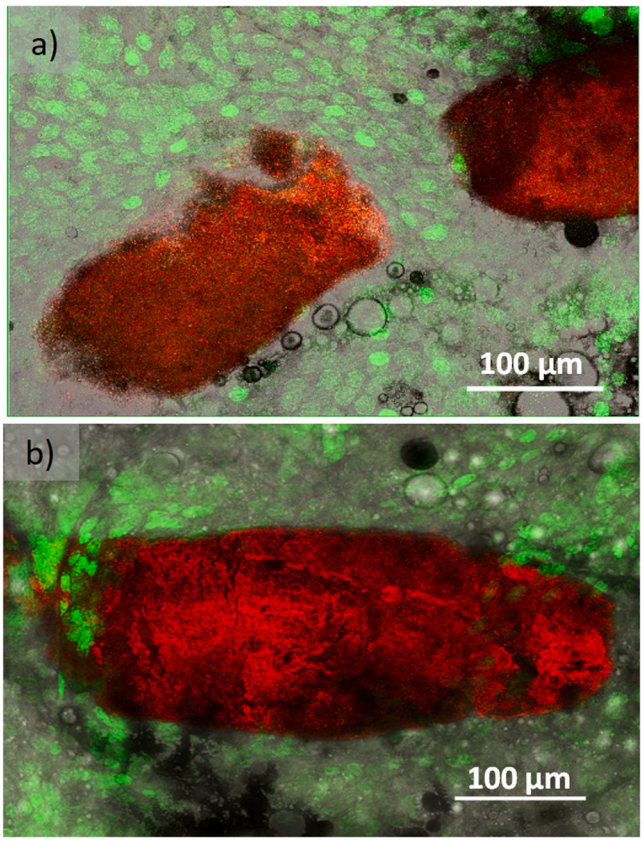

Fig. 4. Confocal laser scanning micrographs showing two aspects (a, b) of Syto-13 stained cell nuclei (green) and agglomerations of microparticles (0.1 µm, red) adjacent to the midgut gland tissue. Merged images of transmission, green, and red spectra.

microplastics.

Administration of differently sized fluorescent microbeads indicates that the largest particles (9.9 µm) were retained in the stomach and subsequently egested via the gut, whereas smaller particles (2.1 and 0.1 µm) apparently translocated into the surrounding tissue. Leaching of the fluorescent dye from the microbeads, as observed by Schür et al. (2019) did not occur in our study. This is probably due to the different nature of the particles. The particles used in our study are internally dyed while the particles used by Schür et al. (2019) were surface-dyed through carboxylate surface modifications.

This distribution of the particles inside the shrimp can be explained by the anatomy and function of the digestive tract. The digestive tract of crustaceans is subdivided into foregut, midgut, and hindgut. The foregut is composed of the esophagus and the stomach. The midgut consists of the midgut gland (hepatopancreas) and the anterior part of the gut. The hindgut terminates in the anus. The stomach and the midgut gland are connected via a short-paired duct. The foregut, including the stomach, and the hindgut are of ectodermal origin and are protected against mechanical and chemical damage by a chitinous layer. Simultaneously, this chitinous layer prevents passive or active translocation of particles into the surrounding tissue. Nutrients are made available for metabolism only when the macerated and predigested chyme passes from the stomach into the midgut gland, which is the principal organ of nutrient resorption and digestive enzyme synthesis.

The midgut, including the midgut gland, is of endodermal origin, thus lacking a protective chitinous layer (Ceccaldi, 1989; Sousa and Petriella, 2006). Once entering the midgut gland tubules, ingested microplastics may interact with the epithelial cells of the midgut gland, which are basically resorption cells (R-cells), fibrillary cells (F-cells), and blister-like cells (B-cells). The R-cells facilitate nutrient resorption and nutrient storage (Ceccaldi, 1989). Similarly, B-cells may be involved in the translocation of microplastics. They contain a distinctive vacuole, which grows while the cell matures. The vacuoles are believed to collect substances and molecules, which ought to be degraded and resorbed or egested, i.e. denatured enzymes or cellular waste (Vogt, 2019; Štrus et al., 2019). The epithelium of the midgut gland is the most vulnerable site of the digestive system where microplastics can translocate into the tissue to induce cytotoxic responses.

In other taxa, such as the bivalve *Mytilus edulis*, microplastics are ingested through the esophagus as well. The particles were taken up into the stomach and transported into the digestive gland. There, they accumulated in the lysosomal system and caused a strong inflammatory response demonstrated by the formation of granulocytomas and lysosomal membrane destabilization (von Moos et al., 2012). Microplastic ingestion was reported for a variety of species with different focus on the uptake and the cellular effects of particles. Recent reviews summarize the current knowledge (e.g. Wang et al., 2019; Coyle et al., 2020; Prinz and Korez, 2020).

4.2. *Effects and cellular responses*

Many studies have shown adverse effects of ingested micro- and nanoplastics on invertebrates including reduced feeding rates, energy reserves, fitness, and fecundity, as well as immune suppression (e.g.

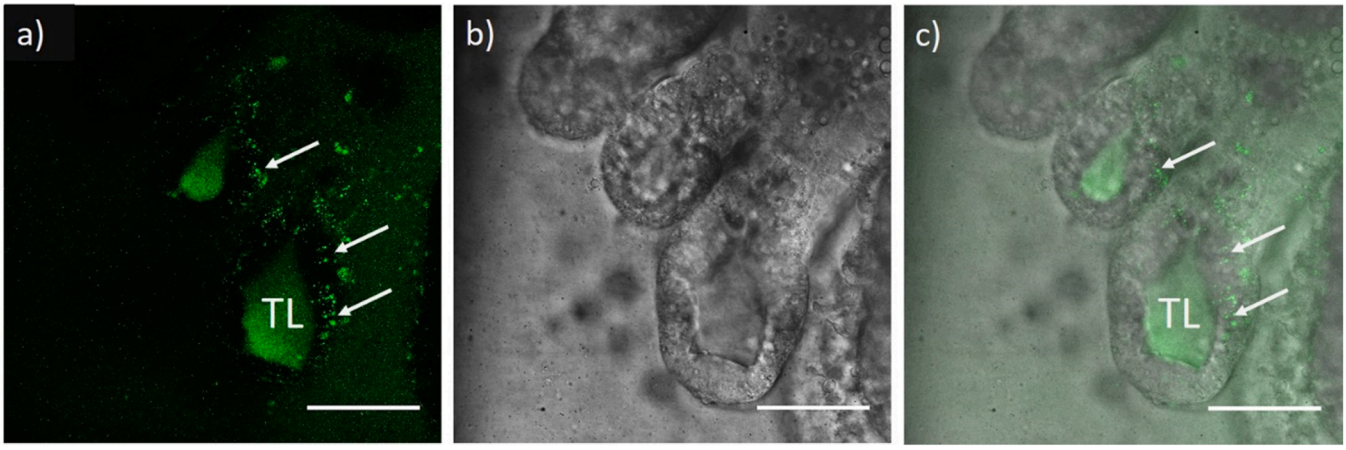

Fig. 5. Detection of reactive oxygen species with the fluorescent probe CH_2DFFDA in midgut gland tissue of *Palaemon varians*. a) Upon oxidation, CH_2DFFDA is converted into the fluorescent derivate difluorofluorescein (DFF). b) Transmission photograph and c) merged fluorescence and transmission images. Arrows indicate spots of DFF derived fluorescence. Scale bars represent 100 µm.

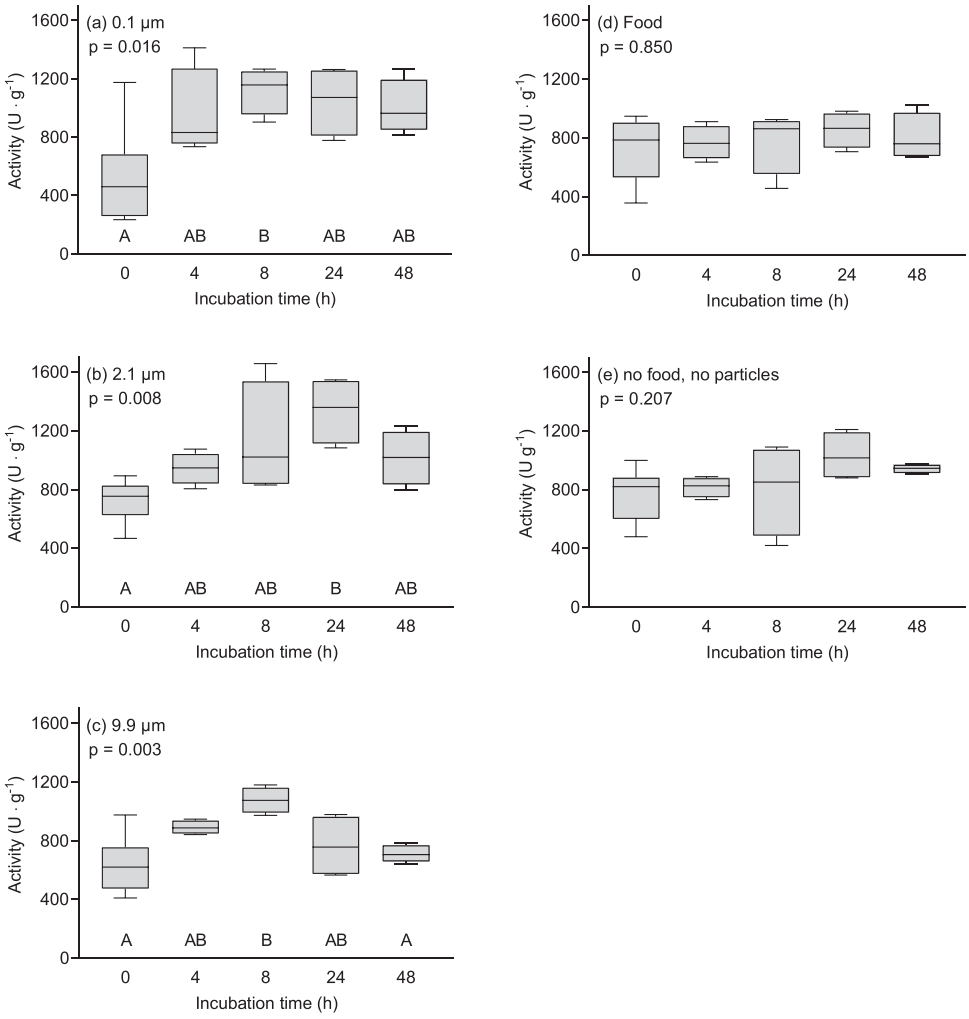

Fig. 6. Activities of superoxide dismutase in the midgut glands of *Palaemon varians* exposed to microbeads (a, b, c) and two controls; d) plant based commercial food, and e) treatment where both food and particles were absent. Different letters indicate statistically significant differences between groups ($p < 0.05$, $n = 4$).

Besseling et al., 2013; Cole et al., 2013; Wright et al., 2013; Li et al., 2016; Auta et al., 2017). However, in experiments the load of microplastics often exceeds environmentally detected concentrations (Cunningham and Sigwart, 2019). Thus, adverse effects may be attributed to a high exposure, potentially inducing false satiation and malnutrition. Moreover, in some polymers the cellular response is induced by the plastic additives or leachates rather than by the microparticles themselves (Zimmermann et al., 2020). To minimize the risk of such misinterpretation, we diluted and washed the particles with pure water and removed additives as well as possible.

Other studies report little or no effects of microplastics on invertebrates, such as e.g. sea urchin larvae (Kaposi et al., 2014), marine isopods (Hämer et al., 2014), amphipods (Bruck and Ford, 2018), and decapods (Carreras-Colom et al., 2018). It appears reasonable that effects of microplastics differ between species. For example, species, which commonly take up considerable quantities of indigestible particles with their food, such as diatom frustules, plant fibers, or sand grains, may be better prepared to cope with ingested microplastics (Saborowski et al., 2019; Korez et al., 2020; Schmidt et al., 2021).

Studies addressing the cytotoxic potential of particles in invertebrates are sparse. Incorporation and translocation of microplastics into cells of the digestive tract of the blue mussel (*Mytilus edulis*), were accompanied by encapsulation of particles and the formation of eosinophil granulocytomas, an inflammatory response, known to occur after parasite infestation (Lowe and Moore, 1979; von Moos et al., 2012). Polystyrene particles induce oxidative stress in rotifers and activate by phosphorylation two factors of the mitogen-activated protein kinases (MAPKs) pathway, JNK and p38 (Jeong et al., 2016).

In the present investigation, SOD-activities increased rapidly already after few hours of exposition of the shrimp in microplastic suspension. These findings indicate a significant production of reactive oxygen species, as confirmed by the ROS marker CH_2DFFDA. SOD-activity as immediate cellular reaction responds rapidly to an imbalance of the oxidative state (Timofeyev et al., 2006). These results are in agreement with recent studies by Jeong et al. (2016, 2017) who showed in rotifers and copepods, that small polystyrene particles (0.05 µm) entailed higher ROS production and higher antioxidant activities than larger particles. This indicates that antioxidant enzymes are suitable cellular markers of oxidative stress occurring after ingestion of microplastics and that smaller particles, especially nanoparticles, cause stronger cytotoxic effects than larger ones (Jiang et al., 2008; Lee et al., 2013; Jeong et al., 2016). Upon ingestion and translocation of these particles into the digestive organs, the cells may identify them as pathogens and react by generating destructive superoxide ions (O_2^-) through e.g. NADPH-oxidase activation (Inada et al., 2012; Brandes et al., 2014). In this way, microplastic particles might have an indirect hazardous effect on cells by activating their endogenous defense mechanisms. Upon chronic exposure, this may entail damage of cellular compounds such as membranes and DNA. Additionally, chronic activation of endogenous defense mechanism may deplete resources needed for other

physiological processes, such as growth and reproduction, potentially leading to fitness reduction.

The largest particles (9.9 μm) also caused increases in SOD activity although they are assumed not to enter the midgut gland. Possibly, these particles were at least partly fragmented by the action of the gastric mill. The grinding of the food may generate very small particles or even nanoparticles, which are not visible by light microscopy and able to enter the midgut gland. This process has been shown in Antarctic krill, langoustine, and freshwater amphipods (Dawson et al., 2018; Cau et al., 2020; Mateos-Cárdenas et al., 2020).

The overall SOD activity did not vary among the treatments although the temporal development of the SOD activities differed fundamentally between the particle treatments and the control treatments. The initial increase and the subsequent decline in SOD activity resulted in a considerable variation of the overall SOD activity in the particle treatments (but not in the control treatments), which prevented the detection of statistically significant differences in SOD activity among the treatments.

4.3. Protection against microplastics

Ideally, the first line of protection should be the selective refusal of particles as discussed for bivalves in view of microplastic uptake (Ward et al., 2020). Since selective refusal is not the case in *P. varians*, the shrimp have to rely on internal selective mechanisms. Beads of 9.9 μm diameter passed the digestive tract and were egested with fecal material, although some of them might have been fragmented. Fibrous microplastics first remained in the stomach but are regurgitated after a few hours through the esophagus (Saborowski et al., 2019). Accordingly, the stomach and its various morphological features are crucial for the processing of the ingested food and the allocation of nutrients and indigestible items.

The fine-meshed chitinous filter system at the base of the pyloric stomach functions as a selective barrier to prevent the passage of larger particles into the midgut gland. A paired duct connects the pyloric stomach with the midgut gland and another duct leading into the gut functions as the pyloric passage for solids. Indigestible particles, which are not regurgitated but are too big to pass the pyloric filter, are directly passed into the hindgut for excretion. The pyloric filters of crustaceans with outer and inner valve assembled with setae and setules seem to have taxon-specific mesh sizes of 100–200 nm (Korez et al., 2020). In the shrimp *Penaeus merguiensis* it is assumed that particles smaller than about 170 nm may pass the filter barrier (King and Alexander, 1994).

In *P. varians*, particles of 9.9 μm in diameter were too large to pass the pyloric filter. Similarly, 2.1 μm sized microbeads were retained by the filter setae, whereas 0.1 μm particles seem to pass the filter and enter the midgut gland. Nevertheless, a vast number of these particles seems to be also retained by the pyloric filter. The aggregations that we observed adjacent to the midgut gland tissue have a similar diameter as the fecal strings of the shrimp. Therefore, it is likely, that these aggregations were separated from the gut nearby the junction to the pyloric stomach during the dissection of the midgut gland. The smallest particles of 0.1 μm diameter were too small to be resolved by light microscopy. However, previous studies showed that indigestible particulate matter accumulated in the vacuoles of the midgut gland of crayfish and green tiger shrimp (Loizzi, 1971; Al-Mohanna and Nott, 1986). Shortly after feeding on food spiked with colloidal gold or thorium dioxide, the indigestible particles accumulated in the vacuoles of the B-cells. Within 24 h, the enlarged vacuolar content was released into the lumen of the midgut gland tubules and excreted with the feces. In this way, excretion of the particles through the feces appears likely (Štrus et al., 2019; Vogt, 2019), preventing or reducing the translocation of particles to tissues and organs. This mechanism would efficiently protect the cells from detrimental accumulation of indigestible microparticles.

5. Conclusions

Aquatic invertebrates are in many ways affected by microplastics. The Atlantic ditch shrimp, *P. varians*, readily and indiscriminately ingest microplastics. The conceptual model (Fig. 7) illustrates the fate of microplastics and other indigestible material upon ingestion in shrimp. Large items, such as microplastic fibers or bivalve shell fragments or polychaete bristles derived from food, remain in the stomach and may be discarded through the esophagus by regurgitation. Smaller particles, such as the experimentally administered 9.9-μm beads, are passed from the stomach into the gut for defecation. The fine-meshed pyloric filter prevents the passage of these particles into the midgut gland, which is the principal organ of nutrient resorption. The smallest particles (0.1 μm), however, can pass the pyloric filter and advance into the midgut gland, where they cause oxidative stress as indicated by the occurrence of radical oxygen species in epithelial tissue and the rapid increase of the anti-oxidative enzyme superoxide dismutase. Accordingly, *P. varians* shows efficient mechanical and biochemical protection mechanisms to reduce the uptake of indigestible material and to counteract ROS formation.

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CRediT authorship contribution statement

R. Saborowski: Conceptualization, Supervision, Data curation, Writing – original draft, review & editing. **Špela Korez:** Investigation, Methodology, Data collection and curation, Writing – review & editing. **Sarah Riesbeck:** Investigation, Methodology, Data collection. **Mara Weidung:** Investigation, Methodology, Data collection. **Ulf Bickmeyer:** Methodology, Writing – review & editing. **Lars Gutow:** Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

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

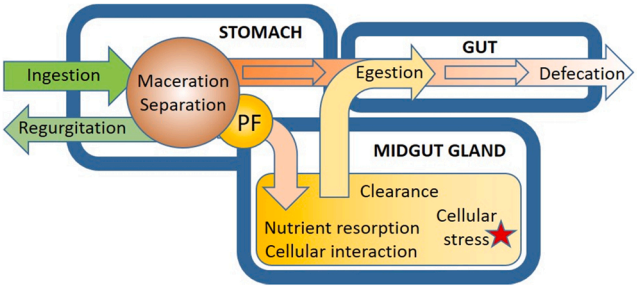

Fig. 7. Conceptual model of the fate of microparticles in the digestive tract of the Atlantic ditch shrimp, *Palaemon varians*. Ingested material (food in the wider sense) is macerated in the stomach. Large indigestible items like shell fragments, spines, or fibers are regurgitated through the esophagus. Smaller indigestible items are retained by the pyloric filter (PF) and directed into the gut for defecation. The liquid share of the chyme and smallest particles pass the pyloric filter and enter the midgut gland tubules. Here, nutrient resorption and interaction with the epithelial cells takes place, potentially causing cellular stress by reactive oxygen species. Indigestible components of the chyme, including smallest particles, are pushed back from the midgut gland tubules into the gut and defecated as well.

Data availability

All data will be made available upon request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.113394.

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