

METHODOLOGY

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Development of a toolbox for the analysis of microplastic-tissue interactions in two benthic freshwater organisms

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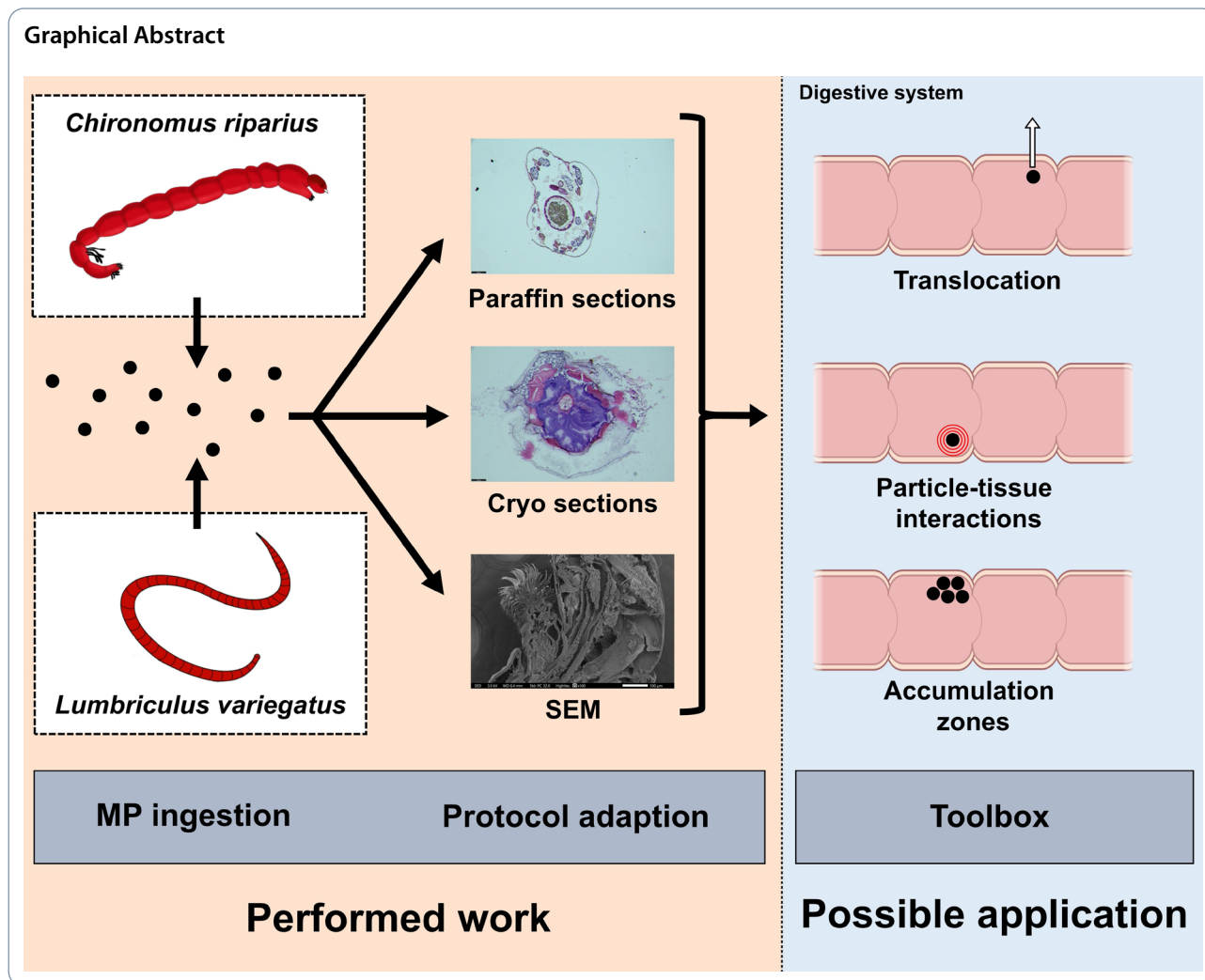
Abstract

Freshwater sediments represent a sink for microplastics (MP) and, hence, especially benthic invertebrates are exposed to these particulate contaminants. Therefore, ingestion of MP by this group of organisms is likely leading to a variety of toxic effects. However, it is still unclear whether these effects of MP are related to the simple passage of the gut for instance at contact or accumulation zones or after translocation of the particles into tissues. To address this issue, histological analyses need to be performed. However, available protocols for these investigations commonly rely on chemicals/solvents that dissolve plastic/polymers and are thus not compatible with MP exposure experiments. Hence, our goal was the adaptation of existing protocols that allow the analysis of MP-tissue interactions in two model systems for benthic freshwater invertebrates *Lumbriculus variegatus* (Blackworm) and *Chironomus riparius* (Harlequin fly). We established and optimized protocols for paraffin sectioning and cryosectioning, as well as for SEM (scanning electron microscopy) analysis. In these adapted protocols we did not use plastic damaging reagents. Resulting sections (paraffin and cryo) showed intact tissue and allowed detection of the fluorescence labelled polystyrene (PS) MP inside the digestive system. Furthermore, they represent a basis for classical histological staining, histochemistry and immunohistochemistry. Cryosections additionally allow for a variety of spectroscopic methods such as MALDI-MSI, Raman or FTIR to be applied. The SEM approach provides high magnification and three-dimensional images that enable ultrastructural analysis of the location of MP in the digestive system of both organisms. In conclusion, our adapted protocols can be used as a toolbox for detailed MP-tissue investigations.

Keywords *Chironomus riparius*, *Lumbriculus variegatus*, SEM, Paraffin sectioning, Cryosectioning, Histology

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Introduction

Due to their wide range of application, plastics are indispensable in our modern society [1, 2]. However, waste management is still a major issue and therefore large amounts of plastic litter end up in the environment every year [3, 4]. Once in the environment plastics undergo degradation and fragmentation [5] which can lead to the formation of microplastics (MP), typically defined as plastic particles < 5 mm [6]. MP are present in all environmental compartments [7] and therefore can be found in oceans [8, 9], freshwater habitats [10, 11], soils [12, 13], and even in the atmosphere [14, 15].

In particular, freshwater sediments are discussed to act as a sink for MP [16, 17]. Hence, benthic invertebrates, like *C. riparius* and *L. variegatus* might be predominantly exposed to these particulate contaminants. The ingestion of MP by both organisms has already been shown [18, 19]. Further, due to their important role in the ecosystem [20–22], both species are relevant for general ecotoxicology [23–25] and have therefore already been used in

MP-related ecotoxicological studies [18, 19]. Here, toxic effects such as intestinal damage, depletion of energy reserves, oxidative stress or inflammation have been reported, for instance [18, 19]. However, the exact drivers of the toxicity remain ambiguous as the fate of MP once ingested remains unclear. In particular, it is unknown whether the effects of MP are related to direct contact with the tissue during simple passage of the gut or if the effects only occur after translocation of the particles into the tissue.

For investigation of particle-tissue interactions and for the localization of the particles inside the digestive system, histological methods need to be applied. For example, paraffin sections could be used for a variety of techniques such as classic histological stainings, histochemistry or immunohistochemistry (for investigation of stress markers) to study histopathological effects of MP. Frozen (cryo)sections could also be used for the same techniques as paraffin sections at the expense of tissue integrity. Nonetheless, they can additionally be used

for a variety of spectroscopic measurements like mass spectrometry imaging (MSI), Raman-, or Fourier transform infrared (FTIR)- imaging [26–28] for MP detection as well as analysis of the tissue. For investigation at the ultrastructural level, electron microscopic analysis is necessary and here scanning electron microscopy represents a powerful tool, allowing high magnification, three dimensional images.

However, most of the existing protocols for histological investigations rely on plastic damaging solvents. For example, protocols for paraffin sectioning include xylene as intermedium during embedding procedures [29]. This solvent also dissolves certain plastics such as PS, even by short times of exposure [30] and is therefore not usable in studies working with specific MP. Further, classical staining procedures for paraffin- and cryosections like haematoxylin and eosin (H&E) staining use xylene as an intermedium [31]. Protocols for SEM preparation of the sample frequently include acetone series for sample dehydration [32, 33] that also damages specific MP and hence should not be used for sample preparation in MP related studies. While protocols avoiding acetone (by performing the dehydration steps with ethanol) already exist [34] workflows for the investigation of MP in the digestive system using SEM remain missing. Although protocols with alternative, plastic-compatible solvents [30, 35] have been developed for marine mussels, protocols for histological examinations of benthic freshwater invertebrates like *C. riparius* and *L. variegatus* are not reported so far. Further, sediment dwelling organisms such as *L. variegatus* and *C. riparius* come with the additional challenge of ingested sediment particles that can lead to tissue processing issues like damage of microtome blades and thereby to disruption of tissues during sectioning. In addition, an analysis of surface alterations of MP particles after exposure to the optimized reagents using SEM is missing so far, to verify the compatibility of the adapted protocols. Hence, our goal was to develop protocols compatible with paraffin sectioning, cryosectioning, and SEM preparation, that enable a detailed analysis of MP-tissue interactions without harming the MP particles.

Addressing this issue of missing methodical approaches for the detailed MP-tissue analysis, we developed a toolbox for *C. riparius* and *L. variegatus* after MP ingestion. Therefore, we adapted three methodologies that are commonly used for particle-tissue analysis for the two benthic freshwater species. In detail, we adapted histological protocols for paraffin- and cryosectioning in combination with the classic H&E (hematoxylin and eosin) staining for both organisms. Additionally, we established protocols for SEM analysis for studying particle-tissue interactions on the ultrastructural level.

Methods/ experimental

The experiments were conducted with *L. variegatus* and larvae of *C. riparius* (fourth instar), both deriving from an established culture at the Chair of Animal Ecology I of the University of Bayreuth and were performed inside a climate chamber under the following conditions: 20 ± 0.1 °C, 14 h:9 h light: dark cycle with 30 min dusk/dawn. Initial egg clutches of *C. riparius* were provided by Dr. Philipp Egeler (ECT Oekotoxikologie, Flörsheim am Main, Germany) and *L. variegatus* originally derived from the University of Koblenz (Federal Institute for Hydrology, Unit G3, biochemistry, ecotoxicology).

Animal husbandry

Rearing of *C. riparius* was conducted in a self-build breeder that was located inside a Rubarth P 850 climate cabinet (Rubarth Apparate GmbH, Laatzen, Germany) with the following settings: Temperature 20 ± 0.5 °C, air circulation 50%, light intensity 25%, day-night cycle 12 h. Basins filled 1/3 with quartz sand (Quarzwirke GmbH, Frechen, Germany) and 2/3 with artificial Elendt M4 Medium [36, 37] were used for rearing of the larvae. The M4 medium was exchanged every other week and constantly enriched with oxygen by a pump. During rearing, larvae were fed with Novo Bel fish flakes (JBL GmbH & Co. KG, Neuhofen, Germany) *ad libitum*.

L. variegatus were reared in glass beakers containing quartz sand and an artificial medium following the guidelines of the OECD [23] inside a climate chamber under the following conditions: 20 ± 0.1 °C, 14 h:9 h light: dark cycle with 30 min dusk/dawn. The glass beakers were covered with glass disks that were used for preventing extensive evaporation. Further, the medium was renewed every other week and constantly enriched with oxygen by a pump. Worms were also fed *ad libitum* with Novo Bel fish flakes (JBL GmbH & Co. KG, Neuhofen, Germany).

Method development

Before performing the main microplastic exposure experiments we performed prior experiments for method development to find the most suitable protocols. For these experiments we also used fourth instar larvae of *C. riparius* and specimens of *L. variegatus* (without size-synchronisation). Despite the establishment of the protocols for the paraffin sections, cryosections as well as for the SEM sample preparation, we further tested wood chips as an alternative sediment to quartz sand during rearing of the organisms. The latter has been applied, as we frequently observed disrupted tissue sections and damaged blades in previous experiments when using quartz sand as a sediment. Therefore, we reared specimens of *C. riparius* (starting from the egg packages) in 1 l Weck® glass beaker (J. Weck GmbH u. Co. KG, Wehr-Ölfingen, Germany). The beakers were filled with M4

and either quartz sand or in wood chips (Chipsi classic, J. Rettenmaier & Söhne GmbH+Co. KG, Rosenberg, Germany) as a sediment. Just as in the main culture we also exchanged the M4 every other week and constantly enriched it with oxygen by a pump. Feeding also occurred *ad libitum* with Novo Bel fish flakes (JBL GmbH & Co. KG, Neuhofen, Germany). When reaching the fourth instar the specimens were prepared for paraffin sections (as described in 2.6 Paraffin embedding of *C. riparius*).

Microplastic exposure

The PS-MP particles were manufactured by the Kunststoff-Zentrum Selb (FSKZ e. V., Würzburg, Germany) by spray drying using a 2% solution of red fluorescent PS granules (Magic Pyramid Brücher & Partner KG, Frechen, Germany) labelled with rhodamine B (for size distribution see Fig. S1) [38, 39]. The polystyrene (PS) particles were spherical with a $d_{90} = 10.4 \mu\text{m}$ (characterised by measurements using the particle analyser Microtrac Sync, Microtrac Retsch GmbH, Haan, Germany)). We chose fluorescence labelled particles rather than coloured microplastics as we wanted to be able to reliably detect the MP after the ingestion as a differentiation between coloured MP and ingested heterogeneous coloured food particles would be challenging. Here it is important to mention, that fluorescent particles can be used in studies for investigation of uptake and potential accumulation or distribution of MP. However, as many fluorescent dyes are toxic, fluorescent labelled particles should not be used in ecotoxicological studies, due to potential leaching of the dye [40–42]. In our study we only used these particles for method development purposes, to be able to easily locate them after ingestion. Nonetheless, instead of fluorescence labelling, the MP could also be identified using spectroscopic methods like FTIR or Raman.

Exposure of *C. riparius*

For the exposure experiments of *C. riparius*, ten freshly laid egg packages derived from the established culture were removed and placed in a 1 l Weck® glass beaker (J. Weck GmbH u. Co. KG, Wehr-Ölfingen, Germany). This glass beaker was filled with M4 medium and wood chips (Chipsi classic, J. Rettenmaier & Söhne GmbH+Co. KG, Rosenberg, Germany) as sediment. Larvae were fed *ad libitum* with Novo Bel fish flakes (JBL GmbH & Co. KG, Neuhofen, Germany) until reaching the fourth instar. Then they were used for the MP exposure experiments that were conducted in the same climate chamber under the same conditions as described above. The exposure experiments were performed as follows. Briefly, the exposure experiments were performed in 6-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) where each single well was filled with 10 ml

of the treatment medium. Then, for each treatment six wells were filled with five larvae each, resulting in a total of 30 larvae per treatment. Into each well two drops of a Novo Bel-M4 medium mixture were added to provide food supply. We used two different treatments that were a control treatment (just M4 medium) and a MP treatment (M4 medium with 1 g/l PS and 10 μl 20% Tween20 (Merck Schuchardt OHG, Hohenbrunn, Germany) per 100 ml solution as an emulsifier). For the MP exposure we prepared 60 ml of a PS-stock solution with the beforementioned concentration of 1 g/l which was then used for distributing over the replicates. Here we want to emphasize that this concentration of MP is much higher than in the environment and also that the use of tween as a detergent can lead to artifacts in effect studies, due to potential toxicity. However, as our main goal was the development of new methods and no MP-effect evaluation, we decided to use this approach, to guarantee the ingestion of MP. The experiment was terminated after 48 h.

Exposure of *L. variegatus*

For the exposure experiments of *L. variegatus*, 60 specimens were taken from the established culture and bisected approximately 1.5 cm from the tail for size synchronisation [23]. The detached tails were then placed in new 290 ml Weck® glass beakers (J. Weck GmbH u. Co. KG, Wehr-Ölfingen, Germany) for two weeks that were filled with wood chips (Chipsi classic, J. Rettenmaier & Söhne GmbH+Co. KG, Rosenberg, Germany) and the *L. variegatus* medium to allow regeneration and defecation of quartz sand. Afterwards, they were used for the exposure experiments which lasted 48 h in new 290 ml Weck® glass beakers (J. Weck GmbH u. Co. KG, Wehr-Ölfingen, Germany). These experiments were also conducted inside a climate chamber under the following conditions: $20 \pm 0.1^\circ\text{C}$, 14 h:9 h light: dark cycle with 30 min dusk/dawn. Here we also used two different treatments that were a control treatment (just *L. variegatus* medium) and a MP treatment (*L. variegatus* medium with 1 g/l PS and 10 μl 20% Tween20 per 100 ml solution as an emulsifier). Therefore, the control beakers were filled with 200 ml of *L. variegatus* medium, and the MP beakers were filled with 200 ml of the MP-treatment solution (200 mg of PS in 200 ml of *L. variegatus* medium with additional 20 μl of 20% Tween20 as an emulsifier). Afterwards, for each treatment two 290 ml Weck® glass beakers (J. Weck GmbH u. Co. KG, Wehr-Ölfingen, Germany) where each filled with 15 specimens of *L. variegatus* making up a total of 30 specimens/treatment. Here the MP-solutions for the two glass beakers were weighed in separately. Just as in the exposure experiment of *C. riparius* we also added food during the exposure experiment (two drops of a Novo Bel – *L. variegatus* medium mixture).

Table 1 Dehydration series and first steps in paraffin for fixation and embedding of *C. riparius*

70% EtOH	2 × 30 min
96% EtOH	2 × 30 min
98% EtOH	2 × 30 min
Isopropanol 100%	2 × 30 min
Paraffin	30 min
Paraffin	2 h
Paraffin	Over night

Table 2 Dehydration series and first steps in paraffin for fixation and embedding of *L. variegatus*

70% EtOH	2 × 15 min
80% EtOH	2 × 15 min
90% EtOH	2 × 15 min
98% EtOH	2 × 15 min
Isopropanol 100%	3 × 20 min
Paraffin	Over night
Paraffin	2 h

Paraffin embedding of *C. riparius*

Right after the MP exposure, ten larvae of each treatment randomly chosen from the wells were transferred to new trays containing M4 medium that were cooled on ice. After 10 min, when movement of larvae was slowed down, 96% EtOH was pipetted into the medium, until larvae were motionless. Afterwards, they were transferred to 4% buffered paraformaldehyde (16% PFA (MP Biomedicals Germany GmbH, Eschwege, Germany) mixed with phosphate buffered saline (PBS, pH=7.5)) and were fixed over night at 4 °C. This was followed by three rinsing steps in PBS buffer for 15 min each, a dehydration series with ethanol and isopropanol at room temperature as well as three steps in molten paraffin (Paraplast Plus®, Sigma Aldrich, Merck KGaA, Darmstadt, Germany) at 60 °C (detailed steps in Table 1). Most paraffin embedding protocols use xylene as an intermediate [29]. However, this reagent would lead to dissolving of the PS plastic particles. Therefore, we adapted the protocol by using isopropanol as an intermediate as it is miscible with ethanol as well as with paraffin.

Then the larvae were embedded in Tissue-Tek® stainless steel embedding molds (Sakura Fintek, Alphen aan den Rijn, Netherlands) containing fresh molten paraffin. Therefore, always five larvae exposed to the same treatment were placed in one reservoir and oriented the same way. Afterwards the embedding molds were placed on ice for cooling and subsequently hardening of the paraffin cubes. The blocks stayed for another night inside a refrigerator at 4 °C for final hardening.

Paraffin embedding of *L. variegatus*

First preparation steps were the same as for *C. riparius*. After the MP exposure, ten specimens of each treatment

were randomly chosen from the Weck® glass beakers and instantly transferred to a new tray containing the *L. variegatus* medium on ice. When movement of the worms was slowed down, we pipetted 96% EtOH into the medium, until larvae were motionless. Then they were transferred to 4% buffered PFA and stayed in it over night at 4 °C inside a refrigerator. After three washes in PBS buffer for 15 min each, the dehydration series with alcohol at room temperature as well as two steps in paraffin at 60 °C were performed (detailed steps can be seen in Table 2).

After the last step in paraffin, the worms were embedded in Tissue-Tek® stainless steel embedding molds containing new paraffin. Therefore, just as for *C. riparius*, always five specimens exposed to the same treatment were placed in one mold and oriented the same way for better sectioning results. Afterwards the embedding molds were placed on ice for cooling and subsequently hardening of the paraffin cubes. The blocks stayed for another night inside a refrigerator at 4 °C for final hardening.

Sectioning of paraffin samples

Sectioning of the paraffin blocks was performed with a sledge microtome (Reichert-Jung, Mod. Hn 40, Cambridge Instruments GmbH, Nussloch, Germany) using disposable blades type 818 (Leica Biosystems Nussloch GmbH) at a thickness of 5 µm. Here, longitudinal as well as cross sections were performed for each organism and each treatment. Afterwards the sections were transferred to a water bath containing autoclaved distilled ultrapure water at room temperature, where they were then loaded onto Epredia superfrost microscope slides (Gerhard Menzel GmbH, Braunschweig, Germany). Following this step, the sections were again transferred to a water bath containing warm (40 ± 1 °C) autoclaved distilled ultrapure water to allow the sections to straighten. Then the loaded microscope slides rested on a heating plate (C. Gerhardt GmbH & Co. KG, Königswinter, Germany) at 40 ± 1 °C and subsequently passed one hour in a heating cabinet (Mettmert GmbH+Co. KG, Schwabach, Germany) at 50 °C for drying.

Haematoxylin and eosin staining

The dried sections of *C. riparius* and *L. variegatus* of both treatments were deparaffinized and stained with the H&E staining for general overview, determination of quality and identification of the tissue. It was performed the same for both study organisms and started with deparaffinization of the tissue. Therefore, the slides were first incubated in warm isopropanol 100% (60 °C) and subsequently in isopropanol 100% at room temperature, 1 × 15 min each. This deparaffinization step is usually performed with xylene which would again lead to serious damage of the PS-MP particles [30]. Therefore, we

substituted this step with isopropanol. The deparaffinization step was followed by rehydration of the samples which occurred via 10 min in 98% EtOH twice, followed by an EtOH series of decreasing concentrations (90%, 80%, and 70%). During this EtOH series, the slides were immersed in the following concentrations of EtOH until no more flow marks were visible. Afterwards, the samples were immersed several times in tap water, just right before they passed 10 min in Mayer's hemalum solution acid (Carl-Roth GmbH+Co. KG, Karlsruhe, Germany). The slides were again briefly washed in tap water and subsequently passed 10 min in another basin filled with tap water for bluing. Then the slides were counterstained with eosin (0.1% esin Y (C.I. 45380; Carl-Roth GmbH+Co. KG, Karlsruhe, Germany) in aqueous solution) for two minutes, again briefly washed in tap water, followed by dehydration via an increasing EtOH series (70%, 80%, 90%, 98% EtOH until no more visible flow marks). Lastly, they stayed in Neo-Clear (Sigma Aldrich, Merck KGaA, Darmstadt, Germany) as an intermediate for 10 min twice. During method development we also tried isopropanol as an intermediate (for all other mounting media than Neo-Clear), but better results were achieved with Neo-Clear and hence the latter one was used for the protocol during the main experiment. Here, both intermediates were used as a substitute for the commonly used xylene. Finally, the slides were mounted with microscope coverslips (Carl-Roth GmbH+Co. KG, Karlsruhe, Germany). For mounting we tried a variety of mounting media during the early method development including Neo-Mount (Sigma Aldrich), Mowiol (Carl-Roth GmbH+Co. KG), Kaisers glycerol gelatin (Merck KGaA), euparal (Carl-Roth GmbH+Co. KG), cedarwood oil (Carl-Roth GmbH+Co. KG), Eukitt neo® (ORSAtec GmbH), 5% carboxymethylcellulose (CMC) (Sigma Aldrich), 6% gelatin (VWR International, Leuven, Belgium) as well as HYDRO-matrix (MICRO TECH LAB, Graz, Austria). This was necessary, as the frequently used Eukitt® is xylene based and therefore not usable for investigations that are related to specific plastic polymers. The best results during early testing were achieved when using Neo-Mount and hence this mounting medium was also used for mounting of the specimens derived from the main exposure experiment.

Comparison with xylene-based protocols

To be able to compare the quality of the tissue integrity and the staining of our newly adapted protocols, we also performed a paraffin embedding using the same protocol as mentioned in 2.6 and 2.7 but with xylene instead of the isopropanol steps. Further, also the H&E staining was slightly different than described in Chap. 2.9. Firstly, the deparaffinization was performed with xylene (2×10 min), followed by 10 min in isopropanol once as

an intermediate. Then, after the ethanol dehydration series the slides stayed in isopropanol for 10 min twice before they passed another two steps of 10 min in xylene. Lastly, they were mounted using Eukitt® (ORSAtec GmbH, Bobingen, Germany).

Cryosectioning

The protocol for cryosectioning was the same for both study organisms. Ten specimens of each treatment were immediately frozen in liquid nitrogen just after terminating the exposure. Then they were bisected and afterwards always two organisms were embedded in embedding molds (Tissue-Tek Cryomolds, 15 mm x 15 mm x 5 mm, Sakura Fintek, Alphen aan den Rijn, Netherlands) containing an embedding medium. During method development we tested a variety of embedding media including different concentrations of gelatin (4–12% gelatin (VWR International, Leuven, Belgium) in 10 ml milli-q-water), CMC (2–8% CMC (Sigma Aldrich) in 10 ml milli-q-water), 1:1 mixtures of 6% gelatin and different CMC concentrations (3, 4, and 5% CMC), 1% tragacanth (Kremer Pigmente GmbH & Co. KG., Aichstetten, Germany), and 1% gum arabic (Kremer Pigmente GmbH & Co. KG.). Here 6% gelatin showed the best results and was hence used for embedding of the specimens deriving from the main exposure experiment. The cryomolds with sample and embedding medium were then placed on a brass plate on dry ice for gentle freezing of the embedding medium which also enabled rearranging of the samples if necessary. After hardening, embedded samples were stored at -80 °C until sectioning. Sectioning was conducted with a Leica CM3050 S cryostat (Leica Biosystems Nussloch GmbH, Nussloch, Germany) at -20 °C chamber temperature, -24 °C object temperature, and 20 µm thickness using carbon steel microtome blades (Feather C35 microtome blades, pfm medical AG, Cologne, Germany). The optimal sectioning settings were previously evaluated during method development by testing different chamber temperatures (ranging from -18 to -30 °C) and different object temperatures (ranging from -18 to -30 °C), as well as different thicknesses ranging from 10 µm to 25 µm. The sections were loaded on Eupredia superfrost microscope slides and again stored at -80 °C until staining with H&E. H&E staining for cryosections was performed as described previously for the paraffin sections, but without the deparaffinization steps.

Microscopic analysis of paraffin sections and cryosections were performed using a Leica M205 FCA fluorescence stereomicroscope (Leica Biosystems Nussloch GmbH, Nussloch, Germany) equipped with a Leica DBC6200 camera (Leica Biosystems Nussloch GmbH, Nussloch, Germany) and the Leica Application Suite X software (Leica Biosystems Nussloch GmbH, Nussloch, Germany). This device was also used for

taking fluorescence images. Fluorescence images were also taken of control sections to verify potential contamination or background fluorescence.

SEM of *C. riparius* and *L. variegatus*

For the SEM analysis of both study organisms, we also used 10 individuals per treatment and performed critical point drying for sample preparation. This drying method was based on a protocol of Leica [43] and started by placing specimens in 30% EtOH on ice for anaesthesia. Afterwards the tissue was fixed by placing them in 2.5% glutaraldehyde (25% glutaraldehyde (GA) (Carl Roth GmbH+Co. KG, Karlsruhe, Germany) mixed in 0.1 M phosphate buffer, pH=7.2, supplemented with 1.8% sucrose) over night. After washing them in 0.1 M phosphate buffer (same as used for diluting glutaraldehyde) for 10 min thrice, an EtOH drying series was conducted. Therefore, they stayed in 60% EtOH for 10 min twice, 10 min in 70% EtOH twice, 10 min in 80% EtOH twice, 10 min in 90% EtOH twice, 10 min in 96% EtOH twice, and lastly 10 min in 98% EtOH twice. Then the samples were transferred to the sample holder of the Leica EM CPD300 (Leica Biosystems Nussloch GmbH, Nussloch, Germany). The sample holder was inserted into the critical point dryer instrument and covered with 98% EtOH, ensuring that the individuals were completely submerged. Afterwards the program with the following conditions was started for *C. riparius*: Stirrer on, 50% seed; auto on; CO₂ In (speed=slow, delay=120 s); Exchange (speed=1, cycles=18); Gas out (heat=slow, speed=slow 20%) [43]. The program lasted 4 h 52 min and subsequently the larvae were stored in a desiccator.

For drying of *L. variegatus* in contrast we used settings that were initially developed for the sludge worm *Tubifex tubifex* [44]. Here the settings were: Stirrer on, 50% seed; auto on; CO₂ In (speed=slow, delay=120 seconds);

Exchange (speed=5, cycles=14); Gas out (heat=med., speed=med.). This program lasted 1 h 28 min and dried specimens were also stored in a desiccator until mounting on stubs for sputtering. The dried samples were then prepared using classical dissection tools. Here longitudinal, cross, as well as diagonal sections were prepared. Afterwards they were mounted on 0.5" SEM Pin Stubs (6 mm length, Agar Scientific Ltd, Stansted, Essex, United Kingdom) with double-sided adhesive conductive carbon tape (Plano GmbH, Wetzlar, Germany) for sputtering. Sputtering was performed with a Leica EM ACE600 (Leica Biosystems Nussloch GmbH, Nussloch, Germany). Dried specimens were first sputtered with a 2 nm layer of carbon and subsequently with a 2 nm layer of platinum. SEM analyses were conducted with a JSM-IT500 SEM (JEOL (Germany) GmbH, Freising, Germany) equipped with a LaB6 cathode electron gun.

Results

We were able to adapt and optimize all methods by replacing the plastic damaging reagents in existing protocols. Therefore, they can now be used for the analysis of MP-tissue interactions.

Paraffin sections

The adapted embedding protocol resulted in equal section quality compared to the classic protocol with xylene (Fig. 1). In addition, the adaption of the H&E staining did not reduce the staining quality when substituting the xylene steps by isopropanol as well as the mounting media (Fig. 1).

In general, the paraffin embedding and sectioning of *C. riparius* led to sectioning results with intact tissue (Fig. 2). Here we were able to obtain sections with tissue integrity for cross as well as for longitudinal sections. The main structures visible were the salivary glands, fat

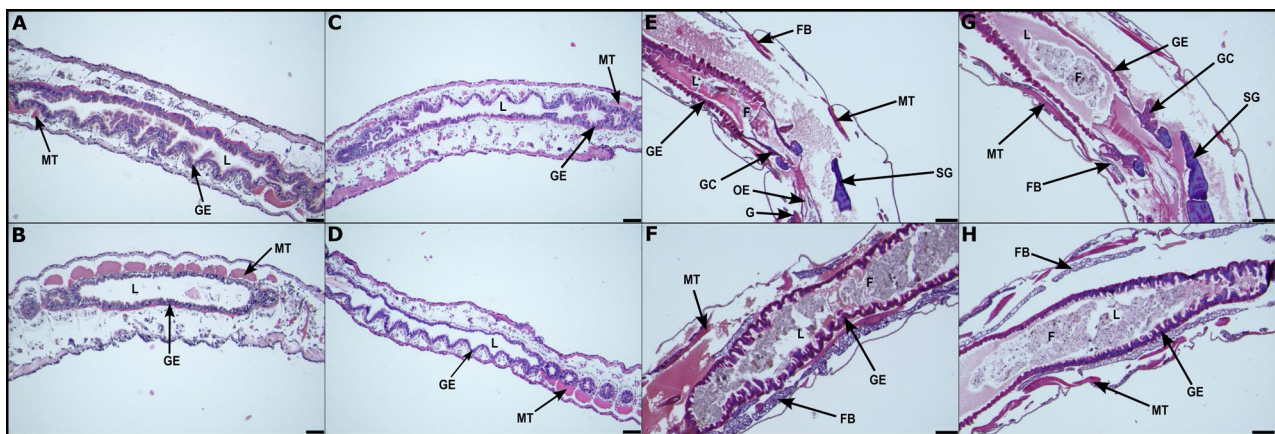


Fig. 1 Longitudinal sections of *L. variegatus* (A to D) and *C. riparius* (E to H) embedded in paraffin and H&E stained with a protocol containing either isopropanol (A, B, E, F) or xylene (C, D, G, H). GE = gut epithelium, FB = fat body, GC = gastric caeca, SG = salivary gland, OE = oesophagus, MT = muscle tissue, G = ganglion, F = food bolus, L = lumen. Scale bar = 100 µm

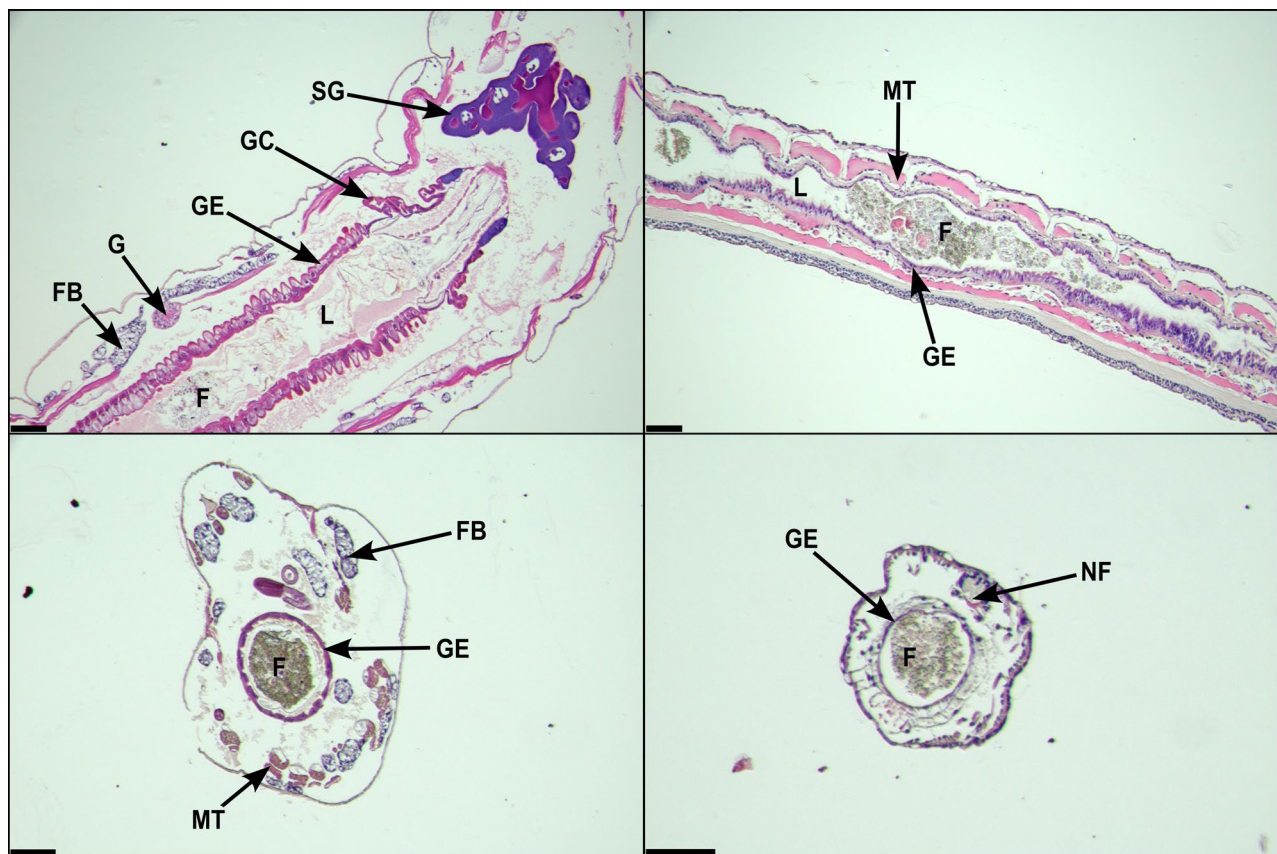


Fig. 2 Paraffin sections of *C. riparius* (left) and *L. variegatus* (right) exposed to 1 g/L PS. For proof of MP presence see Fig. 5 and Fig. S2. **Top:** longitudinal sections, **Bottom:** cross-sections. The sections were stained with H&E. SG = salivary gland, GE = gut epithelium, FB = fat body, MT = muscle tissue, GC = gastric caeca, G = ganglion, NF = nerve fiber, F = food bolus, L = lumen, Scale bar = 100 µm

Table 3 Compatibility list of tested solvents and mounting media for the H&E staining

Medium	Compatible with H&E	PS fluorescence
Ethanol*	Yes	Yes
Isopropanol*	Yes	Yes
Neo-Clear*	Yes	Yes
Xylene	Yes	No
Neo-Mount*	Yes	Yes
Mowiol	No	Yes
Kaisers Glycerin gelatin	No	Yes
Euparal	Yes	No
Cedarwood oil	Yes	No
Eukitt	Yes	No
Eukitt Neo	Yes	No
Gelatin 6%	No	Yes
CMC 5%	No	Yes
Hydro Matrix	No	Yes

Solvents (above dotted line) and mounting media (below dotted line) that were tested during the adaption of the H&E staining protocol. Shown are the compatibilities with the staining process itself and the compatibility with the fluorescence labelled PS particles (labelled with rhodamine B) when tested individually. The asteriks indicate solvents or mounting media that were suitable for our staining protocol

body, gut epithelial cells, as well as the nervous system. The paraffin embedding of *L. variegatus* also led to sectioning results with intact tissues (Fig. 2). Here intact sections were achieved for cross as well as for longitudinal sections. Further, adaption of the H&E staining protocol resulted in adequate stained sections giving a good overview of the tissue (Fig. 2).

Here the main visible tissues were the gut epithelial cells, muscle cells, the nervous system as well as blood vessels. There were no visible differences in the section quality between the two treatments for both study organisms.

Neo-mount was the only mounting media that met the requirements. These requirements were compatibility with our H&E staining protocol, as well as compatibility with the fluorescence labelled PS particles (Table 3). All other mounting media than Neo-mount either led to interference with the staining (for example wash out of the eosin or milky haze of the mounting medium after drying) or led to a loss of the fluorescence of the PS particles (Table 3).

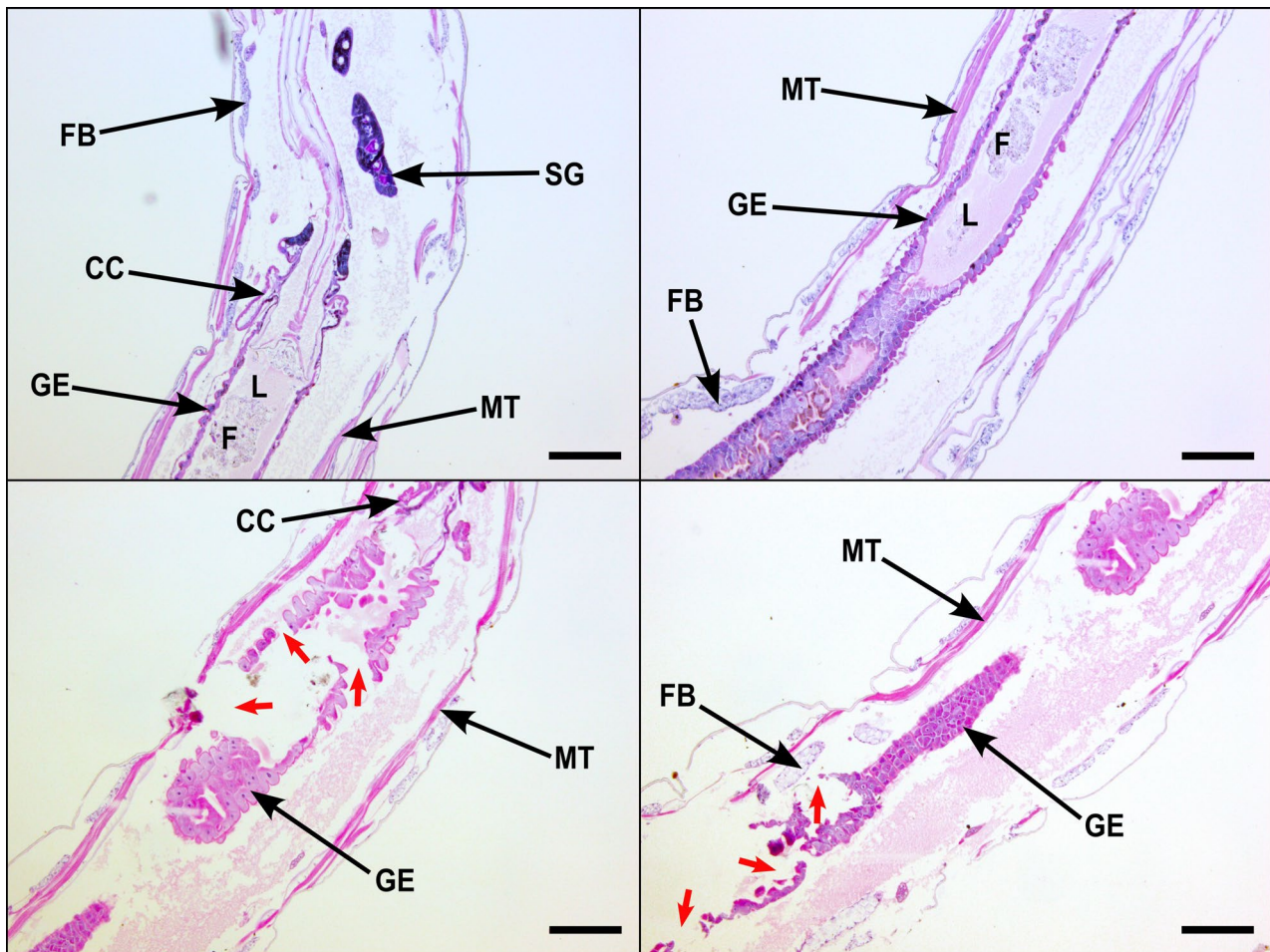


Fig. 3 **Top:** Paraffin sections (longitudinal) of *C. riparius* larvae reared without quartz sand as a sediment. **Bottom:** Paraffin sections (longitudinal) of *C. riparius* larvae reared in quartz sand as a sediment. **Left:** Midgut region with transition to oesophagus including the caeca. **Right:** posterior part of the midgut. Red arrows indicate disruptions in the gut epithelium. The staining varies in the intensity between the top and the bottom images due to exchange of the staining solutions and does not result from rearing in different sediments. SG = salivary gland, GE = gut epithelium, FB = fat body, MT = muscle tissue, GC = caeca, L = lumen, F = food bolus. Scale bar = 200 μ m

Impact of sediment on section quality

Sections of specimens that were reared in quartz sand as a sediment frequently showed disrupted tissue, whereas larvae reared without the quartz sand only rarely showed disruptions (Fig. 3). Therefore, *C. riparius* and *L. variegatus* that were used for the PS exposure experiments were not reared in quartz sand but in wood chips.

Artifacts during drying of microscopic slides

A few sections of *C. riparius* showed tissue alterations in the midgut region (Fig. 4). Here ballooning of the midgut could be observed. Thereby, the bloated midgut also showed damaged tissue like disruption of the gut epithelium (Fig. 4). These kinds of artefacts were visible, when the sections stayed too long on the heating plate after mounting on the microscopic slides or when the temperature was too high but were not observed for sections of *L. variegatus*.

Detection of fluorescent particles

The fluorescent PS particles could be observed in the digestive tract of paraffinized sections (e.g., sections before the staining process) of both study organisms when exposed to the PS treatment (Fig. 5, A to C and G to I). No fluorescence was visible in organisms exposed to the control treatment.

While adaption of the H&E staining led to good staining results of tissues for both study organisms and both treatments, the fluorescent PS particles were no longer visible in the sections after staining. However, when testing the single reagents of the H&E staining individually on the particles, the fluorescence was still given (Table 3, see ethanol, isopropanol, Neo-Clear and Neo-Mount). Furthermore, fluorescence images of sections right after the deparaffinization step (via isopropanol) still showed fluorescent particles but with a reduced intensity (Fig. 5, D to F and J to L).

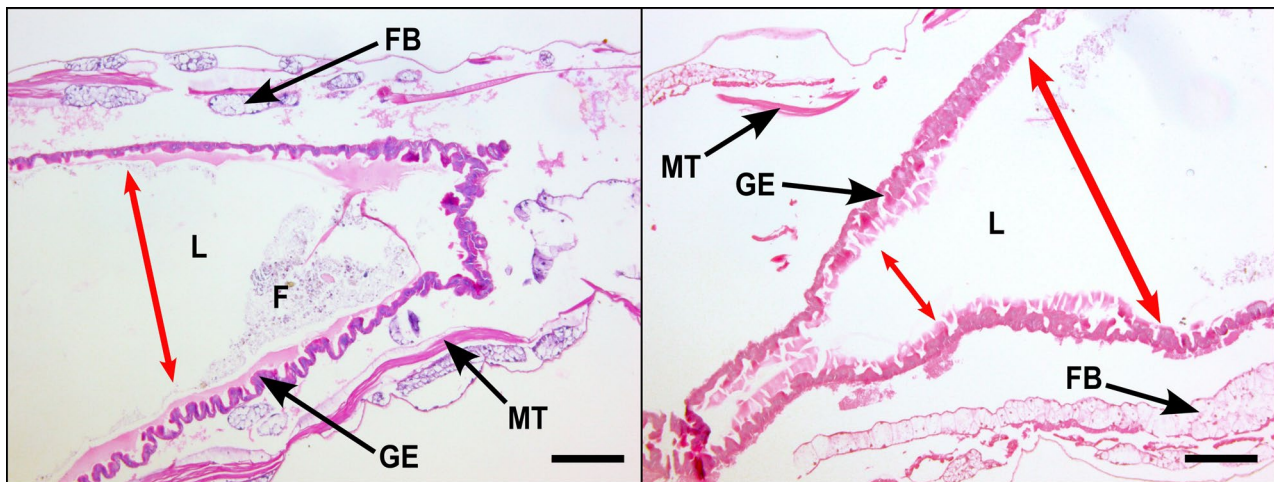


Fig. 4 Tissue sections of *C. riparius* showing ballooning in the midgut region (red arrows) during the drying process after mounting. GE = gut epithelium, FB = fat body, MT = muscle tissue, L = lumen, F = food bolus. Scale bar = 200 μ m

Still, brightfield images of paraffin section of organisms exposed to the MP treatment proved the presence of spherical PS-particles in the digestive tract for both species (Fig. S2), whereas these spherical particles were absent in the digestive tract of control organisms.

Cryosections

For cryosectioning, best sectioning results for both study organisms were achieved when embedded in 6% gelatin and sectioned at -24°C (chamber temperature) and -20°C (object temperature) with a thickness of 20 μ m (Fig. 6). Here, the tissue was intact and no difference in section quality was visible between the reference group and the PS exposed specimens. Further, the H&E staining led to a staining result that allowed distinguishing the different structures of the tissue sections. Here, also no differences could be seen in the quality of the sections between the two treatments.

All of the other tested embedding media frequently led to disrupted sections and were therefore not considered to be used for our protocol (Fig. S3). Here damage occurred in all tissues and the overall tissue integrity was lower compared to the sections of specimens embedded in 6% gelatin. A high amount of sections embedded in a different embedding medium than 6% gelatin even showed a complete loss of their initial shape (Fig. S3, C & I). Therefore, larvae for the main experiments were embedded in 6% gelatin.

The rhodamine B labelled PS particles could also be observed in cryosections of both study organisms when using the fluorescence binocular (Fig. 7). In contrast to the paraffin sections however, the fluorescence of the particles was still visible after staining with H&E. Still, the fluorescence intensity was much higher before than after staining with H&E. This was the same for both study

organisms *C. riparius* and *L. variegatus*. Further, the fluorescence signals aligned with the particle locations and no fluorescence signals were seen in cryosections of the control treatment.

SEM analyses

The sample preparation of *C. riparius* using the CPD method led to drying results without major drying artefacts and could therefore be used for further analysis. Dried samples were successfully sectioned longitudinally (Fig. 8). SEM images of CPD dried *C. riparius* enabled insights into the specimens and especially into the opened digestive tract (Fig. 8). Here we were able to detect MP particles in a high amount in the MP exposed specimens and further we were also able to get high magnification images of the MP-peritrophic membrane interaction zone (Fig. 8B). Further, even very delicate structures were visible and showed a good conservation (Fig. 8D). No cross-contamination (e.g., MP particles in control organisms) was observed.

Sample preparation of *L. variegatus* using the CPD approach also led to dried specimens without significant drying artefacts. Therefore, we were able to also prepare these specimens for the SEM analysis by sectioning them longitudinal. Preparation of *L. variegatus* specimens turned out to be more complicated compared to *C. riparius* due to their smaller diameter. Still, resulting SEM images led to overview of the digestive system without major disruption or sectioning artefacts (Fig. 9) just as for *C. riparius*. Therefore, also here analyses of the gut epithelium and the lumen were possible. Here we were also able to detect MP particles in the digestive tract of MP exposed specimens and no cross-contamination (e.g., MP particles in control organisms) was observed. Further

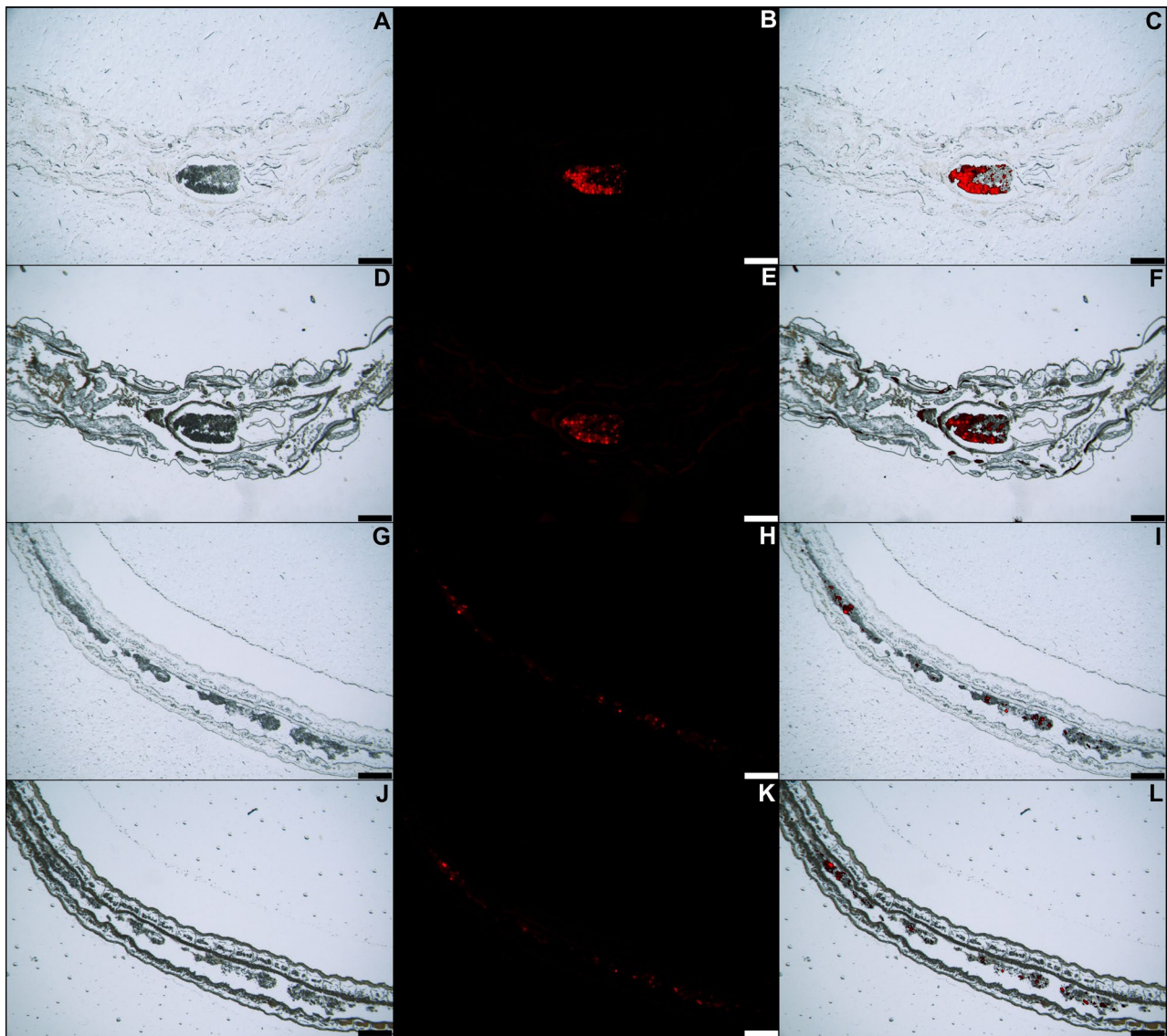


Fig. 5 Paraffin sections of *C. riparius* (A to F) and *L. variegatus* (G to L) that were exposed to 1 g/l PS. Sections are either still paraffinized (i.e., before removal of paraffin, A to C and G to I) or right after the paraffin removal steps in isopropanol (D to F and J to L) and are unstained. **Left:** images with transmission light. **Middle:** fluorescent images showing the ingested particles inside the digestive tract. **Right:** overlay images of the brightfield and the fluorescence images. Fluorescent images were taken with the same light-exposure time. Scale bar = 250 μ m

we managed to take high magnification images of the MP-gut epithelium interaction zone (Fig. 9D).

Discussion

We present established protocols for the two study organisms *C. riparius* and *L. variegatus* with the purpose of histological analysis of MP in the digestive system. This included the establishment of protocols for paraffin sections, cryosections as well as SEM analysis.

Paraffin sections

The paraffin embedding protocol led to intact tissue sections for both organisms. One main factor influencing the quality of the sections was the usage of wood chips

as a sediment in comparison to the quartz sand which is commonly used as a sediment [23–25]. The occurrence of quartz sand inside the digestive tract of *C. riparius* and *L. variegatus* that is related to their sediment dwelling lifestyle, frequently led to damage of the microtome blades and therefore to disrupted tissue sections. However, when rearing them in wood chips this damage did not occur. Additionally, the high amount of PS particles in the digestive system also did not negatively influence the section quality for neither of the study organisms. Here it is important to mention, that our adapted protocols could also be used in experiments with lower, more environmental relevant MP-exposure concentrations. However, then the likelihood of finding a region with MP

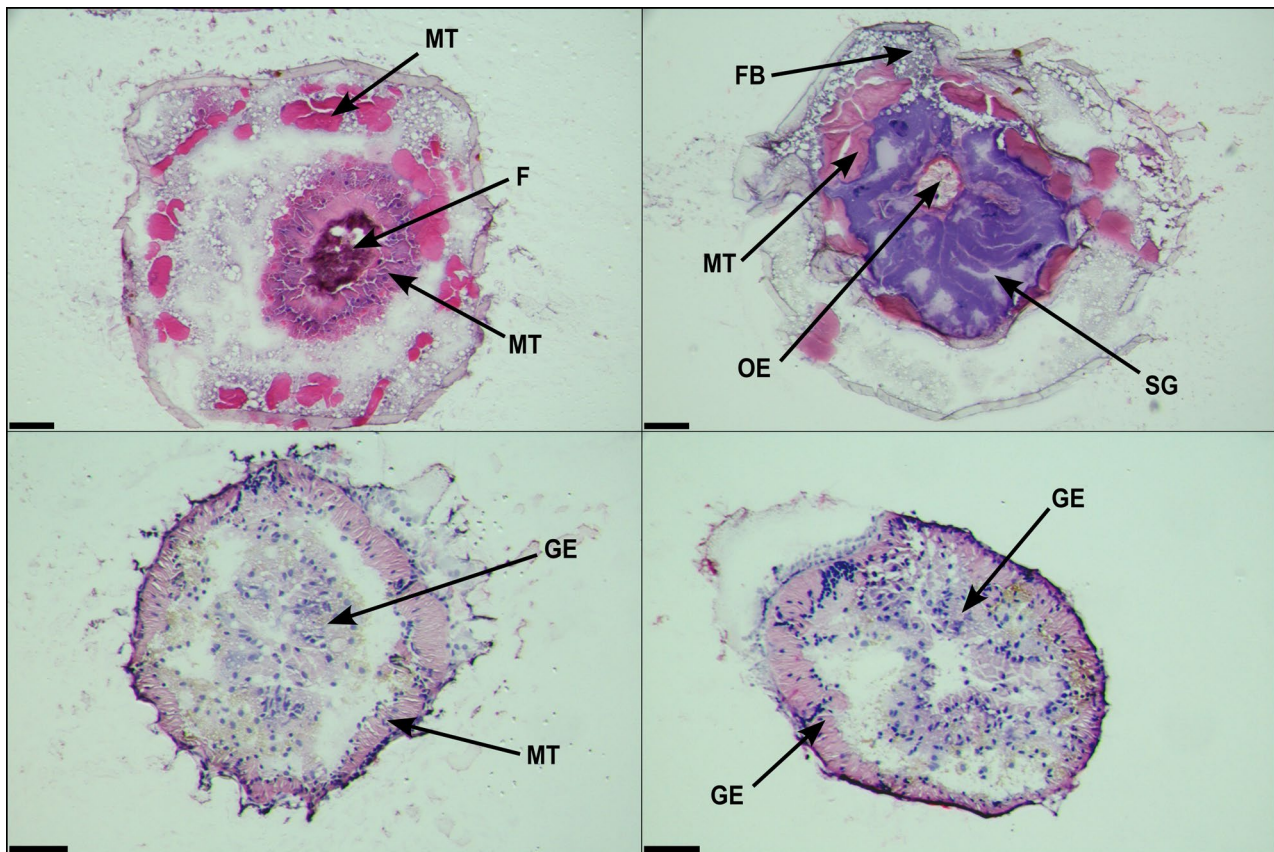


Fig. 6 Cryosections of *C. riparius* (top) and *L. variegatus* (bottom) embedded in 6% gelatin. Specimens were sectioned with a crosswise orientation and stained with H&E. SG = salivary gland, MT = muscle tissue, GE = gut epithelium, OE = oesophagus, F = food bolus. Scale bar = 100 μ m

particles would be lower. Therefore, for the development of the methods, we used a high MP-exposure concentration. Exchanging xylene by isopropanol in the embedding protocol still led to intact sections without any loss in quality. This exchange was necessary for being able to work with PS-MP, as xylene can lead to damage of certain MP such as PS [30] whereas isopropyl alcohol does not. Hence, using xylene as an intermedium in our protocol could have resulted in damage of the PS-MP, extensive loss of the fluorescence already during the embedding or even the complete dissolving of the MP. The exchange of xylene by isopropanol has already been performed by a study of Gonçalves et al. [30] where they also reported preservation of MPs and intact tissues for mussels of the genus *Mytilus*. However, in their protocol they did not use ethanol for dehydration but different concentrations of isopropanol, to avoid potential polymer dissolution [30]. Our SEM analyses of the PS-MP compatibility, did not show a dissolution of PS particles when exposed to ethanol (Fig. S5). Another possible solvent that can be used in exchange for xylene would be butanol, as successfully shown by a study of De Marco et al. [35]. In this study, they excised gills of the mediterranean mussel *Mytilus galloprovincialis* and prepared tissue sections

showing satisfying results. As shown, alternative protocols for paraffin sectioning have been adapted for mussels, but not for other organism groups. Still, protocols for paraffin embedding and sectioning must be adapted for each organism individually to achieve satisfying sectioning results.

While there was no loss in sectioning quality in our adapted workflow when using isopropanol as an intermedium, disrupted sections occasionally occurred. However, it is likely that these disruptions are caused by variances in the humidity during the actual sectioning process, which is a factor that can influence the sectioning process since with higher humidity, sections are more likely to stick to the stage of the microtome. Thereby they can also be damaged when transferring them to the water bath. In addition, we also observed that when the temperature of the heating plate was increased, or when the sections stayed too long on it, this also led to damage of the sections.

The exchange of xylene by isopropyl alcohol for deparaffinization in the H&E staining also showed staining results that were comparable with the staining performed with the protocol using xylene. The waiver of xylene has the additional advantage that xylene is highly toxic,

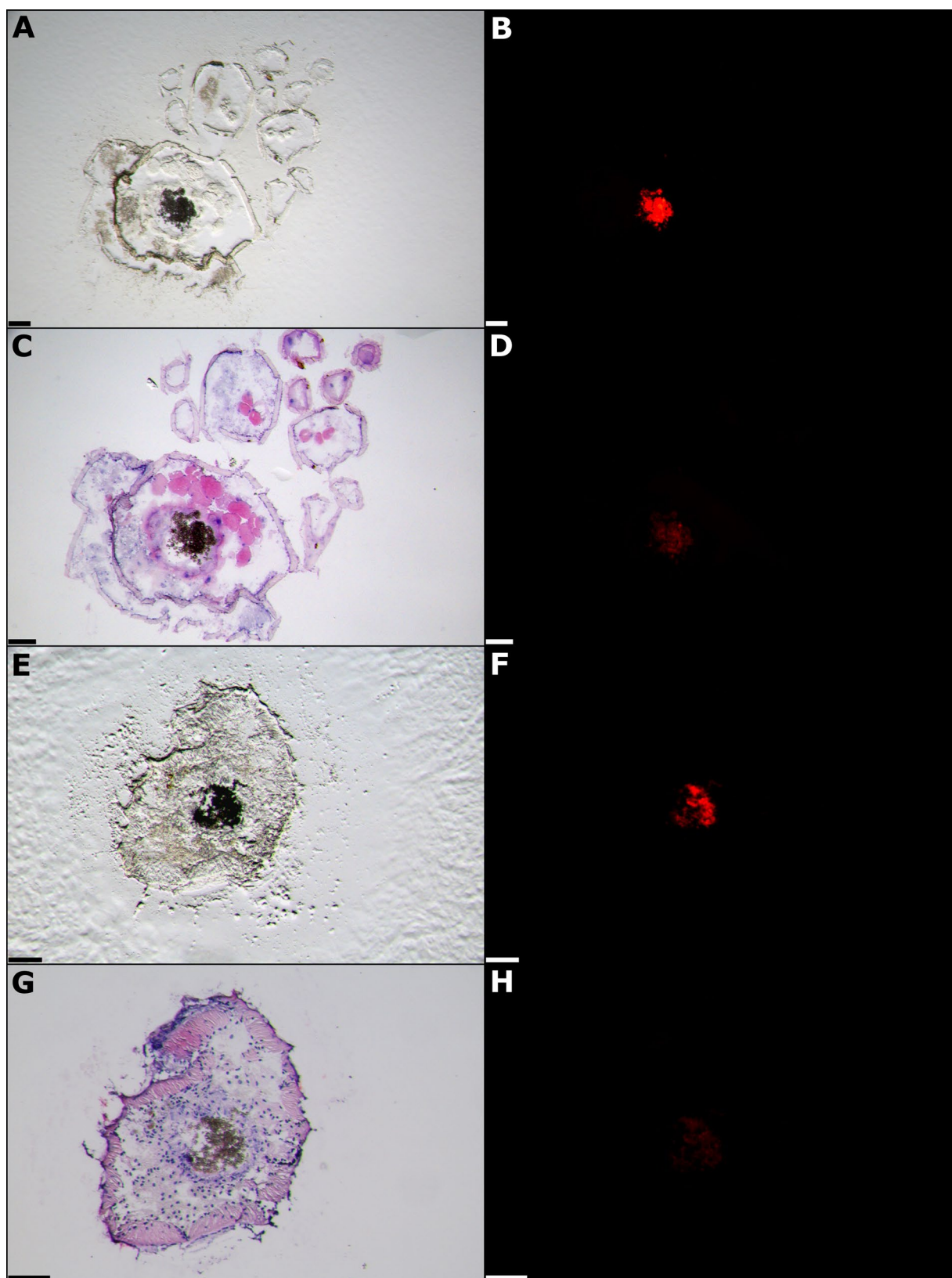


Fig. 7 Cryosections of *C. riparius* (A to D) and *L. variegatus* (E to H) embedded in 6% gelatin before (A, B, E, and F) and after (C, D, G, and H) staining with H&E. **Left:** bright-field images. **Right:** Fluorescence images. The rhodamine B marked PS particles show a loss in the fluorescence signal after staining with H&E. Scale bar = 100 µm

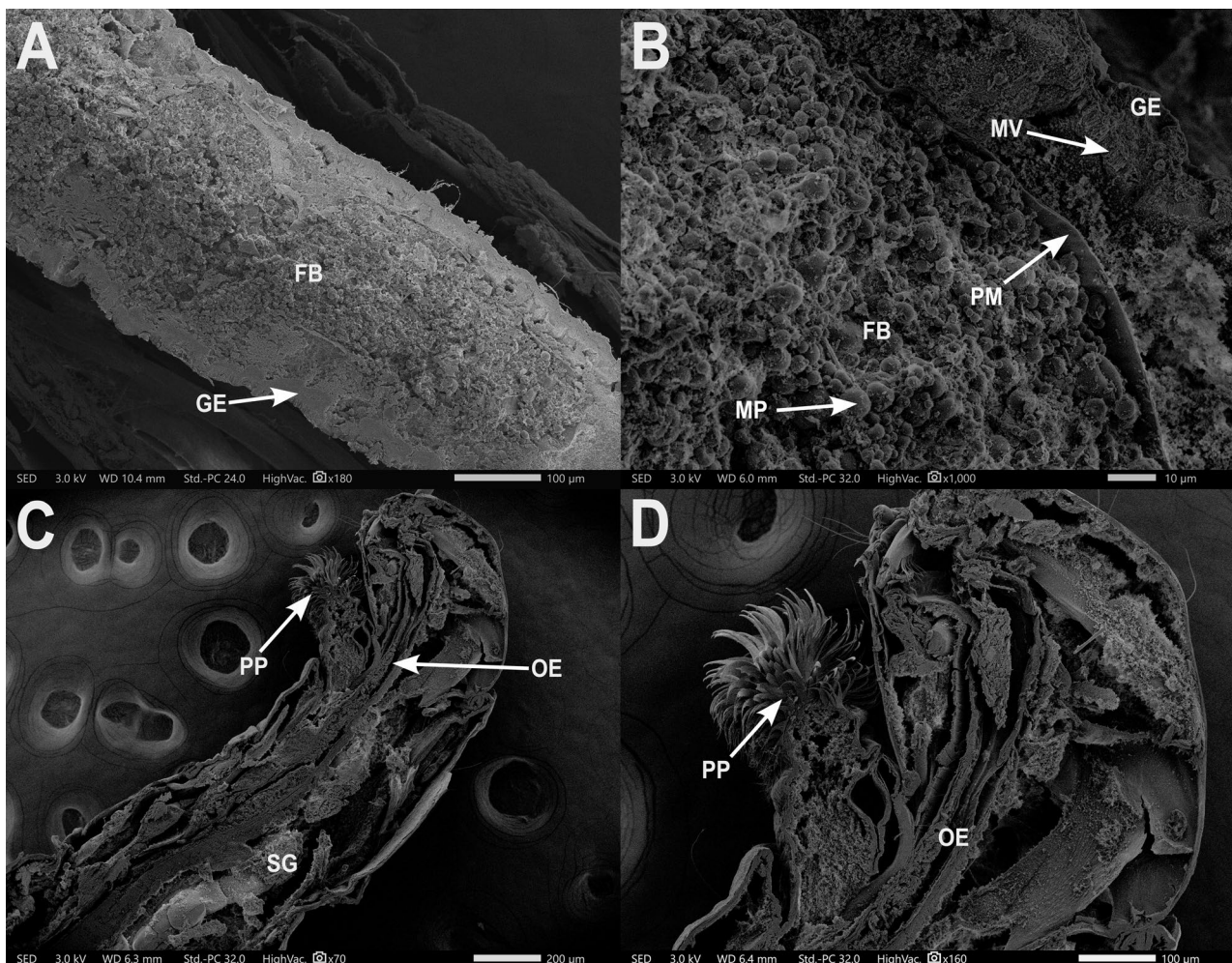


Fig. 8 SEM images of the general intestine morphology of *C. riparius*. Specimens were dried with CPD and bisected longitudinally. **(A)** Open midgut of PS exposed larvae. The food bolus is spiked with PS and encased by the peritrophic membrane. **(B)** Close up image of the midgut region of a PS exposed larvae with the peritrophic membrane representing the border between the food bolus and the epithelial cells. The ingested PS-MP are visible and no differences in the shape or size of the particles compared to the untreated PS-MP can be seen. **(C)** Longitudinal section of the head area. Here insights into the oesophagus are possible. **(D)** Magnified image of the same longitudinal section of the head area as in image **(C)**. PM=peritrophic membrane, GE=gut epithelium, PP=parapods, OE=oesophagus, SG=salivary gland, FB=food bolus, MP=microplastics, MV=microvilli

whereas isopropanol is not. Therefore, we support the recommendation of using isopropanol instead of xylene when working with MP, as already proposed by other studies that have not worked with MP [45, 46]. Another substitute for xylene can be coconut oil as a recent study by Bright et al. [47] has shown, but its compatibility with our organisms and MP remains to be evaluated. In addition, we also had to use Neo-Mount as a mounting medium instead of the frequently used Eukitt®, a general-purpose fast drying mounting medium [48] due to the presence of xylene in the latter one, which once again would have affected the detection of the PS-MP. The substitution of the mounting medium resulted in equally stained sections. Therefore, we were able to achieve a good overview of the tissues, and we could use the protocol for our analyses. However, for long term storage

we observed drying out of the slides. To avoid this, the slides should be sealed with nail polish or any other sealing fluid. During establishment of the protocol, we tried a variety of mounting media including Eukitt neo®, CMC, cedarwood oil, Kaisers glycerine gelatin, euparal, gelatin, mowiol, and hydro matrix. However, none of these led to promising results, as they were either not compatible with the MP particles fluorescence or with the H&E staining. For example, when using our protocol, parts of the staining were washed out (when mounting with Kaisers glycerine gelatin or Hydro Matrix) or a milky haze occurred after mounting (when mounting with gelatin or CMC) which might be caused by remaining water on the slides [49]. Further, mounting with mowiol led to insufficient staining with H&E. When it comes to the PS fluorescence, issues occurred during screening for

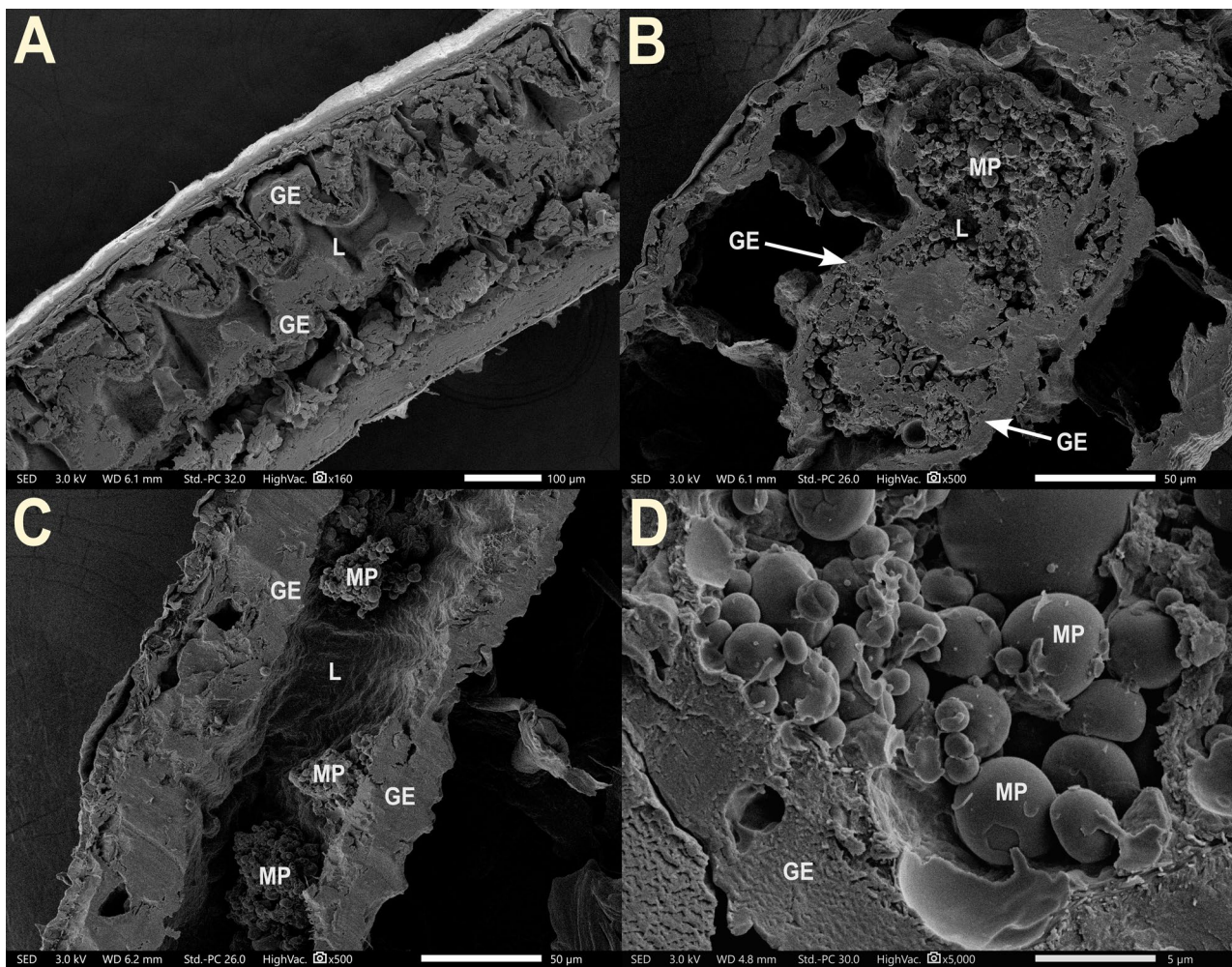


Fig. 9 SEM images of the general intestine morphology of *L. variegatus*. Specimens dried with CPD and bisected longitudinal. **(A)** Digestive tract without ingested food particles. **(B)** Area of digestive tract that is filled with food bolus. The food bolus is spiked with PS. **(C)** Part of the digestive tract filled with clusters of food and MP. **(D)** High magnification image of the gut epithelium-MP interaction zone showing the ingested PS particles where no differences in the shape or size of the particles compared to the untreated PS-MP can be seen. GE=gut epithelium, L=lumen, MP=microplastics, F=food bolus

fluorescent particles when using the mounting media euparal, cedarwood oil, as well as Eukitt Neo. This was either due to loss of the fluorescence of the particles, or issues with overlapping of fluorescence emissions of the mounting media itself in the same range as the particles. That shows the importance of adapting the H&E staining protocol for one's own requirements.

While we were able to obtain well-stained paraffin sections with our adapted protocol, the fluorescence of the rhodamine B marked PS particles was only visible in unstained sections and no longer visible after the staining process. Nonetheless, brightfield images of paraffin sections of both species exposed to the PS treatment showed spherical PS-particles, that were visible in the digestive tract and that were absent in the control group. This suggests that the fluorescence might be washed out during the process of staining. This hypothesis is also supported by our results of the cryosections. Here we also

detected fluorescent PS particles in the unstained sections, whereas the stained sections showed a reduction in the fluorescence. The fact that these particles still showed a little fluorescence might be explained by the missing deparaffinization step in the H&E staining protocol of the cryosections. Further, fluorescence images of the sections right after the deparaffinization process indicated, that there is no dislocation of the MP as the fluorescence signals align with the location of the particles that can be seen in the brightfield images (Fig. 5). Furthermore, besides the fluorescence aligning with the particles there was no potential leached dye detectable in the surrounding tissue. Hence, only fluorescence dye accompanied with MP was visible. In further studies we recommend MP-particles with a covalent bound fluorescence label to avoid leaching of the dye.

Cryosections

Embedding with 6% gelatin led to the best results for the cryosectioning of both organisms. Still, compared to paraffin sections, cryosections generally show inferior tissue integrity compared to paraffin sections [50]. This tendency was also observable for our results. Still cryosections are fastly produced and further are advantageous when it comes to immunohistochemistry (IHC). In IHC the conservation of antibodies is of high importance, which can be achieved the best when using unfixed cryosections. In contrast when using paraffin or fixed sections, the antigenicity can be hindered [51]. In the last decade, tissue sections have further been used for a variety of spectroscopic measurements [52, 53]. Here it is important that the samples are not previously fixed as the fixation process links peptides and proteins together that cannot be reversed completely [54] and thereby it limits the amount of the chemical information that is attainable by spectroscopic measurement such as MALDI-MSI [55]. So, the fixation process would negatively affect the outcome of the results. Nonetheless, fixation of the cryo samples using glutardialdehyde for example would lead to even better results in sectioning (Fig. S4). Still, our goal was to establish a protocol allowing fast production of tissue sections that could be further used for a variety of spectroscopic analyses like MALDI-MSI, Raman or FTIR (e.g. Ritschar et al. [28]) and hence we did not include a fixation step for our cryo samples. This is also the reason, why we did not consider the common standard reagent Tissue-Tek® O.C.T. (Sakura Fintek, Alphen aan den Rijn, Netherlands) as an embedding medium, as we observed reduced signal intensities and inhomogeneities across tissue sections for spectroscopic and mass spectrometric measurements. The adaption of cryosections to spectroscopic measurements has already been shown by a variety of studies [268]. For example, in one of our previous studies cryosections of *Eisenia fetida* have been used for measurements with FTIR and MALDI-MSI for chemical analysis of the same tissue section [28] and in another study MALDI-MSI measurements were applied on the zebrafish *Danio rerio* and the water flea *Daphnia magna* for mapping of lipids in the body [26].

SEM analyses

Many classical sample preparation protocols for SEM analysis contain acetone during the dehydration steps [32, 33]. However, acetone can lead to surface changes or possibly complete dissolving of certain MP [56, 57] and consequently it cannot be used for analysing specific tissue-MP interactions. Therefore, we based our dehydration protocol on EtOH. The preparation of the dried samples using the classical dissection tools allowed us to open the body and to thereby get insights into the digestive system. Using our SEM sample preparation protocol,

we were able to maintain tissue integrity, and we were even able to maintain integrity of very fine and filigree structures such as microvilli, that were clearly visible at a high magnification during the electron microscopic investigations of the digestive tract. Further, we were able to observe ingested PS-MP in the digestive tract of both study organisms, allowing the analysis of the MP-tissue contact zone to study possible interactions.

When comparing SEM images of untreated PS particles to PS particles that were exposed to the single reagents separately, no differences could be seen visually under SEM between the particles (Figs. S5, S6), at least within the time frame that they were also exposed to the respective reagents during the adapted protocols. Further, the PS particles that can be seen in the opened digestive tract of both organisms during SEM observations (Figs. 8 and B and 9 and D) did not show any visual changes in size and surface compared to untreated particles (Fig. S5). Therefore, we suggest our protocol do not affect them significantly. While we achieved good results with our dissection approach, other studies reported issues, especially when working with hard shell organisms like crustaceans [58–60]. Therefore, they used paraffin-carving, another sample preparation technique for studying the internal anatomy [58–60]. Still, this method has not only been used for crustaceans exclusively, but was also applied to the SEM analysis of spiders [61]. For this technique, the whole organisms (or the structure of interest) is first fixed and embedded in paraffin, the excessive paraffin on the sides of the specimen is removed, the samples are deparaffinized and then they are treated as usual for drying [58]. This procedure might be a good alternative when the dissection protocol described in our work might not be applicable to the organism of interest or smaller specimens. Another preparation method for smaller specimens was described for copepod nauplii [62]. They introduced a novel fracturing device that requires frozen samples. These samples are placed on the device that consists of a brass base and two brass plates that can freely slide in the space of the base. Then the samples are fractured by pressing a sharp knife in between the two movable brass plates, the so called “fracturing line” [62]. This method led to very high accuracy fractions, and could also be applied to other organisms, especially for smaller ones. Still, our approach has the advantage that it can be performed faster than the paraffin carving approach, and it does not require specialised equipment such as the fracturing device.

Conclusion

While most protocols for the detailed analysis of tissues rely on plastic damaging reagents, we were able to adapt protocols for three different methodological approaches that allow analysis of PS-MP exposed specimens of *C.*

riparius and *L. variegatus*. These benthic freshwater invertebrates come with the additional challenge of sediment in their digestive tract, that can lead to damage during sample preparation. Our adapted methods preserve tissue integrity and allow detailed histological investigations of the two organisms. Further, the established methods can be used as a basis for studies of particle-tissue interactions and open possibilities for further investigations. For example, paraffin and cryosections can be used for a wide range of histological stainings, histochemical or immunohistochemical approaches to investigate histopathological effects of MP. Cryosections can be further used for a variety of imaging techniques such as Raman, FTIR, or MALDI-MSI, to analyse the lipid or protein composition of the tissue. In addition, these spectroscopic measurements even allow the detection of unlabelled MP and hence can be used in more environmentally realistic scenarios. Lastly, SEM analyses enable ultrastructural investigation and could be expanded by further EDX (energy dispersive X-ray spectroscopy) analysis for analysis of the tissue's elemental composition.

Abbreviations

MP	Microplastics
PS	Polystyrene
SEM	Scanning electron microscopy
H&E	Hematoxylin and eosin
MALDI	Matrix-assisted laser desorption/ionization
MSI	Mass spectrometry imaging
FTIR	Fourier transform infrared
EtOH	Ethanol
CMC	Carboxymethylcellulose
PFA	Paraformaldehyde
PBS	Phosphate-buffered saline
GA	Glutaraldehyde
CPD	Critical point drying
EDX	Energy dispersive X-ray spectroscopy

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s43591-025-00171-4>.

Supplementary Material 1

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Author contributions

CL conceptualized the study. JS and MS performed the experiments, analysed the data and prepared the figures. SR and JS wrote the manuscript draft. SR supervised method development. CL and AR provided instruments and expert advice. All authors reviewed and approved the final manuscript.

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Data availability

The data used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests

The authors declare no competing interests.

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