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TECHNICAL REPORT

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Low-cost microplastic visualization in feeding experiments using an ultraviolet light-emitting flashlight

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Abstract

Microplastics are pollutants threatening the health of marine, freshwater and terrestrial organisms. To analyze whether an organism is able to ingest microplastics, the organism is usually fed with expensive fluorescent microbeads and placed under a fluorescence microscope for microplastic detection. However, such equipment cannot be afforded by many laboratories. Therefore, we developed a low-cost method to study the ingestion and egestion of low-cost, fluorescent microplastic fragments and fibers by aquatic invertebrates. During our feeding experiments, we exposed the freshwater snail *Radix balthica* to artificial biofilms containing 15% microplastic fragments and fibers, respectively. We then used an ultraviolet (UV) flashlight to irradiate the microplastics. Hence, we could directly observe the microplastics in the food, during ingestion and egestion as well as in the snail feces. Our method, thereby, allowed us to analyze the snails' behavior (during ingestion and egestion), the microplastics' distribution in the snail feces and the microplastics' shape after the snails' digestion. Furthermore, we observed that the snails ceased to egest microplastics after 76 hr following microplastic ingestion. However, chemical digestion of the snails 6 days after microplastic exposure revealed that microplastics were still present in *R. balthica*, emphasizing the microplastics' persistence in the animals' bodies. This is the first study combining the usage of low-cost fluorescent microplastics with an inexpensive UV flashlight for microplastic detection during microplastic ingestion and egestion by living organisms. Our new method is not only cost-effective, but also fast and can be performed by a wide range of researchers without special previous training.

KEYWORDS

affordability, black light, gastrointestinal passage, gastropods, synthetic polymers

1 | INTRODUCTION

Microplastics (plastic particles <5 mm) are polluting marine (Erni-Cassola, Zadjelovic, Gibson, & Christie-Oleza, 2019), freshwater (Luo et al., 2019) and terrestrial (Scheurer & Bigalke, 2018; Zhang & Liu, 2018) ecosystems worldwide. So far, microplastics have been found in various aquatic (Germanov, Marshall, Bejder, Fossi, & Loneragan, 2018)

and terrestrial (Smiroldo, Balestrieri, Pini, & Tremolada, 2019; Zhao, Zhu, & Li, 2016) vertebrates and invertebrates (Akindele, Ehlers, & Koop, 2019; Machado, Kloas, Zarfl, Hempel, & Rillig, 2018; Panebianco, Nalbone, Giarratana, & Ziino, 2019; Wright, Thompson, & Galloway, 2013). As microplastics are considered emerging anthropogenic pollutants, their potential effects are usually assessed in ecotoxicological studies. In such studies, for instance, study organisms are exposed to high microplastic concentrations to examine whether microplastics affect organism development (Lo & Chan, 2018; Nobre et al., 2015) or neurotransmission (Oliveira, Ribeiro, Hylland, & Guilhermino, 2013). Usually, expensive fluorescent microbeads are fed to the study organisms as such microplastics can be commercially purchased (Gonçalves, Martins, Sobral, Costa, & Costa, 2019; Phuong et al., 2016). However, to analyze whether microplastics may influence an animal's behavior and to estimate possible ecosystem-wide consequences of potential behavioral changes, it is necessary to conduct ecological studies (Green, Boots, O'Connor, & Thompson, 2017; Gutow, Bartl, Saborowski, & Beermann, 2019; Tosetto, Williamson, & Brown, 2017). In contrast to ecotoxicological laboratories, ecological research facilities usually have limited access to expensive equipment (such as expensive fluorescent microbeads and a fluorescence microscope) for microplastic detection in animal samples. Hence, it is important to develop protocols that are inexpensive while still allowing for fast and reliable microplastic detection during microplastic feeding experiments. The usefulness of low-cost materials in ecological research has previously been highlighted (Suzuki & Sasaki, 2010). In this technical report, we therefore present a novel low-cost method which enables the experimenter to make low-cost fluorescent microplastics (see also Gutow et al., 2019; Gutow, Eckerlebe, Giménez, & Saborowski, 2016) visible by irradiating them from different angles using a portable ultraviolet (UV) flashlight while such microplastics are offered to aquatic study organisms (i.e., freshwater snails) in glass beakers or aquaria. Thereby, our method allows the experimenter to simultaneously observe the microplastics and the study organisms together in an aquarium. In our study, we observed microplastic ingestion and egestion by the freshwater snail *Radix balthica* during a feeding experiment. We then analyzed the duration of the gastrointestinal passage of microplastics (polystyrene fragments and polyacryl fibers) in *R. balthica*, how the microplastics were distributed in the snail feces and whether the microplastics' shape changed after digestion. A large amount of microplastics in the environment are fragments deriving from macroplastic abrasion (Wagner et al., 2014) and fibers which are released from clothes during washing and which can be ingested by aquatic organisms (Jemec, Horvat, Kunej, Bele, & Kržan, 2016). Furthermore, we investigated whether microplastic distribution on experimentally offered food slides was homogeneous and whether microplastics were present in the water column inside the glass beakers used during the feeding experiment. So far, a similar UV lamp has only been used in a previous study to observe the distribution of microplastics in chemical solutions (Carr, Liu, & Tesoro, 2016). Hence, we hypothesized that using a UV flashlight during microplastic feeding experiments may help in visualizing microplastic ingestion and egestion by *R. balthica*.

2 | METHODS

2.1 | Microplastic preparation

We obtained blue fluorescent microplastic (polystyrene, PS) fragments by manually shredding fluorescent plastic granules in a glass blender. Before shredding them, we put the granules into Falcon tubes and cooled them down in a freezer (−80°C). During shredding, we put ice as well as distilled water into the blender to prevent it from heating up. Then, we dried the plastic mixture at 35°C for 48 hr. Subsequently, we sieved the microplastics through stacked sieves with the bottom sieve having a mesh size of 200 μm. Thus, we used only the microplastic fragments for our feeding experiments which had a maximum size of 200 μm. This size was confirmed by digital microscopy (VHX-2000 series, Keyence, Osaka, Japan). We chose this maximum size, as a pilot study indicated that *Radix balthica* could ingest sediment grains of the same size. The low-cost fluorescent plastic granules that we used to obtain microplastic fragments were purchased from a decoration shop (Magic Pyramid Brücher & Partner KG, Frechen, Germany), and are an alternative to expensive fluorescent microbeads (Gutow et al., 2016) often used in microplastic effect studies (Cole et al., 2016; Yu et al., 2018). Furthermore, we obtained green fluorescent microplastic fibers by cutting fluorescent polyacryl wool (Magic Pyramid Brücher & Partner KG) into small pieces (with lengths between 30 and 2,000 μm) using scissors. Those fiber lengths are similar to the lengths of polyacryl fibers that were previously fed to the marine snail *Littorina littorea* (Gutow et al., 2016). We confirmed the microplastics' size by digital microscopy. Although the raw plastic material (blue PS granules and green polyacryl wool) that we shredded and cut to obtain the microplastics showed strong colors, the

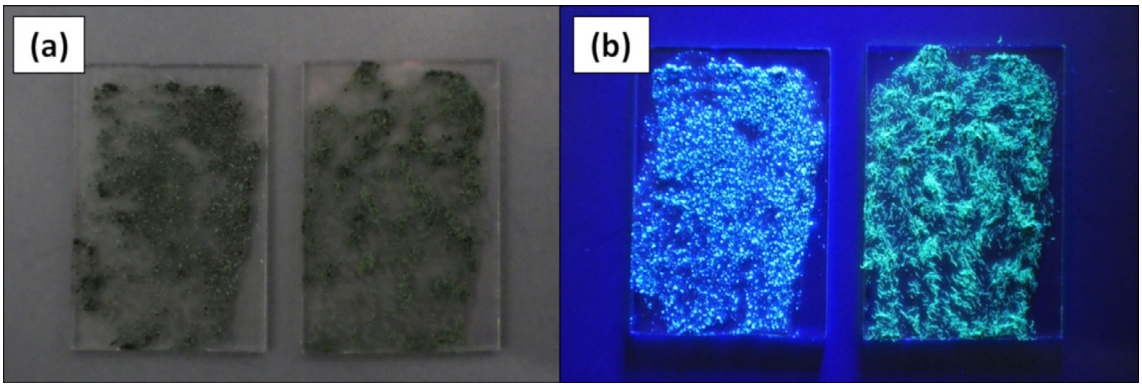

FIGURE 1 Artificial biofilms on 'food slides' which were spiked with fluorescent blue polystyrene microplastic fragments (left slide) and green polyacryl fibers (right slide). Pictures were taken without (a) and with (b) the ultraviolet (UV) flashlight

microplastics were not as colorful and could not easily be detected by the naked eye. However, the microplastics became visible when we irradiated them with a portable UV flashlight at 395 nm which was equipped with 51 LEDs (Vansky; Vansky Network Technology Co., Ltd [Vanskytek]; Shenzhen, China; Figure 1).

2.2 | Study organisms

We used individuals of the freshwater gastropod *Radix balthica* (Linnaeus, 1758) for our microplastic feeding experiments. The snails were sampled at the Lahn River (coordinates: 50°19′52.9″N, 7°40′08.5″E; Rhineland-Palatinate, Germany). We fed 15 snails with individual artificial biofilms containing microplastic (polystyrene) fragments and three snails with biofilms containing (polyacryl) microplastic fibers. All individuals used for the microplastic fragment feeding experiment had equal sizes with a mean shell length of 1.3 ± 0.05 cm (mean ± SE, $n = 15$ snails). The three snails that we fed microplastic fibers had a mean shell length of 1.5 ± 0.06 cm (mean ± SE). We measured the shell length with calipers as the distance between the shell apex and the shell lip. *R. balthica* is a grazer that feeds on biofilms and inhabits lotic freshwaters. It is prey for leeches and fish (Ahlgren & Brönmark, 2012). No permission or ethical approval for using the snail *Radix balthica* as a study organism was necessary for our study.

2.3 | Feeding experiment

After a maintenance period of 7 days (in an aquarium which was equipped with stones covered in natural biofilm and with air stones), finishing off with a 24-hr starvation period, we exposed 15 individual *Radix balthica* to artificial biofilms containing a 15% microplastic fragment concentration and three individual *Radix balthica* to biofilms containing a 15% microplastic fiber concentration, respectively. To do so, we placed artificial biofilms which we prepared by covering glass slides (area: 3.8 cm × 2.6 cm = 9.88 cm^2) with a mixture of 12 mg gelatine, 12 mg fragmented Spirulina Tabs (Sera, Heinsberg, Germany), and 4.24 mg microplastic (PS) fragments or 4.24 mg microplastic (polyacryl) fibers (see Figure 1) in individual 400 mL glass beakers filled with filtered Lahn River water. Hence, 15% of the offered food consisted of microplastic fragments or fibers. We had previously filtered the water through cellulose acetate filter paper with a pore size of 4–7 μm (Whatman 595, GE Healthcare GmbH, Buckinghamshire, UK) to prevent any contamination and to remove any suspended matter that might serve as snail food. The water that we put into the glass beakers was taken from the site at Lahn River where the snails were sampled. The feeding started at 9 a.m. on May 14, 2019 and lasted for 4 hr during which we placed individual snails in the glass beakers. We cleaned each snail with filtered water and placed it in a new glass beaker without food at 1, 3 and 5 p.m. on the same day. As the snails were exposed to a natural day/night rhythm, we left them overnight without again checking their fecal egestion and without placing them into new beakers. One day later (9 a.m., May 15, 2019) we again individually cleaned the snails and placed them in a new glass beaker every 2 hr (not between 5 p.m. and 9 a.m. in the morning) until 5 p.m. on May 17, 2019. Whenever snails were transferred to new beakers, their feces were collected for subsequent analyses (see Section 2.4). To observe the snails' microplastic ingestion and egestion, we used a portable UV flashlight. Due to the microplastics' strong fluorescence, we could easily observe the oral ingestion of microplastics and their egestion by *R. balthica* (Video S1; Figure 2).

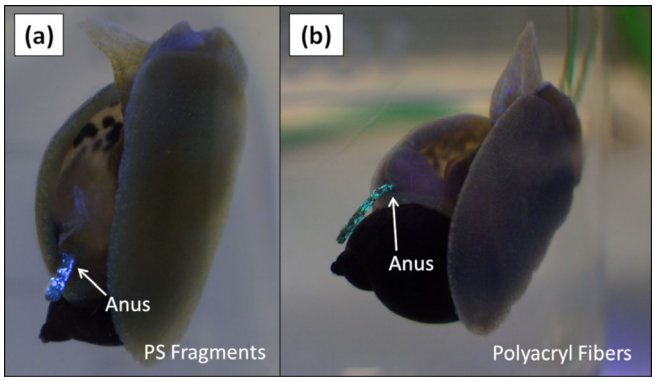

FIGURE 2 Blue polystyrene (PS) fragments (a) and green polyacryl fibers (b) are egested by *Radix balthica* and are irradiated with the ultraviolet (UV) flashlight

2.4 | Detection of microplastics in gastropod feces

After microplastic exposure, we observed microplastic fragments and fibers in the gastropod feces as the microplastics showed a strong fluorescence in the shine of the portable UV flashlight. We put the feces into a glass Petri dish, placed them under a digital microscope and used the portable UV flashlight to make the microplastics visible. The angle in which we held the UV flashlight could easily illuminate all areas of interest inside the Petri dish. Then, we assessed fluorescence of the feces that resulted from microplastic fragment ingestion and egestion on May 14, 2019 at 1, 3 and 5 p.m.; as well as on May 15, 2019 at 9 a.m, 11 a.m., 1 p.m., 3 p.m. and 5 p.m. In the same manner, we examined the feces until 5 p.m. on May 17, 2019. On May 20, 2019 at 2 p.m. we analyzed the feces for fluorescence again. We categorized fluorescence intensity resulting from microplastic fragment ingestion in the snail feces using values ranging from 3 (very strong fluorescence) to 0 (no fluorescence, Figure 3). Similar classifications based on fluorescence intensity are regularly performed in ecotoxicological studies (Stengel, Zindler, & Braunbeck, 2017). A strong fluorescence represented high microplastic fragment egestion (a high microplastic amount in the feces), while low fluorescence represented low microplastic egestion by the snails. As we fed microplastic fibers to only three snails, we did not categorize their fecal fluorescence intensity. We conducted all statistical tests with the software STATISTICA 10 (Statsoft, Tulsa, Oklahoma).

2.5 | Chemical digestion of the snails' soft tissue

On May 20, 2019, we euthanized all snails using 70% ethanol, we removed their shells and cleaned their bodies with ultrapure water. Then, we stored them in individual glass vials. Subsequently, we cleaned the soft tissue of all snails with ultrapure water again and individually dissolved them in 10 mL of a hydrogen peroxide solution (H_2O_2, 34.5–36.5%) and in 10 mL of 10 M KOH for 24 hr at room temperature. Then, we neutralized the KOH with formic acid and filtered each sample onto a cellulose acetate filter with a pore size of 0.45 µm (Whatman, Dassel, Germany) using a vacuum filtration unit. We ran blanks in parallel to the digestion and to the filtration to exclude any contamination from our analysis. We placed the filters into small aluminum bowls, covered them with aluminum foil and dried them in an oven at 50°C for 24 hr. Then, we analyzed each filter for fluorescence using the UV flashlight and the digital microscope to check whether microplastic fragments and fibers had remained in the snails' bodies.

2.6 | Experimental setup

We used our method to observe how the microplastics were distributed in the glass beakers, and to investigate whether the biofilms showed a homogeneous fluorescence (indicating a homogeneous microplastic distribution in the snails' food). Using the UV flashlight revealed that no microplastics were floating in the water column

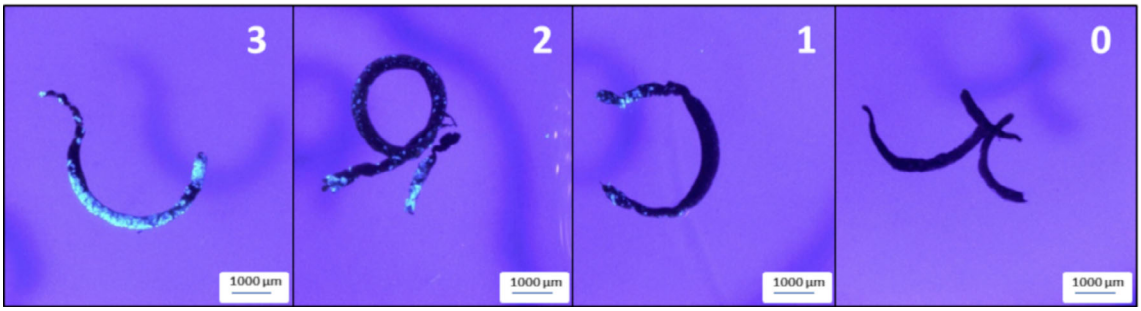

FIGURE 3 The four categories of fecal fluorescence intensity ranging from 3 (high fluorescence intensity), to 2 (medium fluorescence intensity), 1 (low fluorescence intensity), and 0 (no fluorescence)

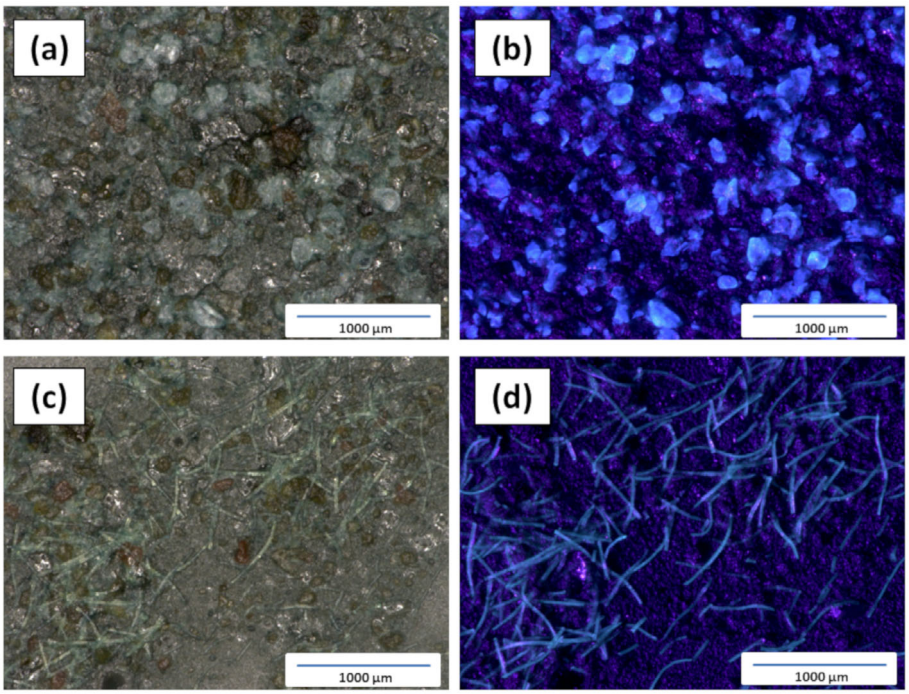

FIGURE 4 Close view of artificial biofilms which were homogeneously spiked with fluorescent polystyrene microplastic fragments (a, b) and polyacryl fibers (c, d). Pictures were taken under the digital microscope in bright field (a, c) and with ultraviolet (UV) flashlight illumination (b, d)

inside the beakers and that the distribution of microplastic fragments and fibers on the offered food slides was homogeneous (Figure 4).

3 | RESULTS

3.1 | Feeding experiment

We observed that the snails readily ingested the microplastic fragments and fibers. Moreover, we could directly observe the microplastics in the gastropod feces while the feces were egested by the snails (see Figure 2). This not only confirmed that the microplastics passed through the snails' digestive system, but also showed how fast the microplastics were egested. The snails were fed microplastic contaminated biofilms on May 14, 2019 from 9 a.m. until 1 p.m. and had already egested feces containing microplastic fragments when the beakers were inspected for fecal fluorescence at 1 p.m. on May 14, 2019 (Figure 5). The number of snails that had egested feces was not always consistent throughout the microplastic fragment feeding experiment, ranging from three to 15. During the experiment, one of the 15 snails died. Hence, only 14 snails were analyzed for fecal fluorescence intensity in the subsequent analysis. The feces containing the microplastics fell off the gastropods' bodies and immediately sank to the bottom of the glass beaker, which we observed multiple times during the experiment.

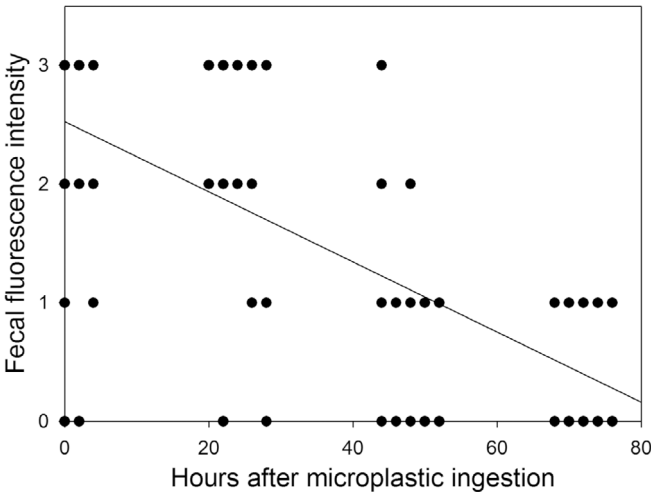

FIGURE 5 Fecal fluorescence intensity (see Section 2.4) resulting from microplastic fragment egestion from May 14 at 1 p.m. (0 hr after microplastic ingestion) until May 17 at 5 p.m. (76 hr after microplastic ingestion)

3.2 | Detection of microplastics in gastropod feces

The amount of microplastic fragments that the snails egested with their feces gradually decreased from May 14 until May 17, as shown by decreasing fecal fluorescence. The snails needed 76 hr (from 1 p.m. on May 14, 2019 until 5 p.m. on May 17, 2019) for microplastic fragment egestion (Figure 5). On May 20, 2019 the snails' feces showed no fluorescence, meaning that the snails did not egest any microplastics after 5 p.m. on May 17.

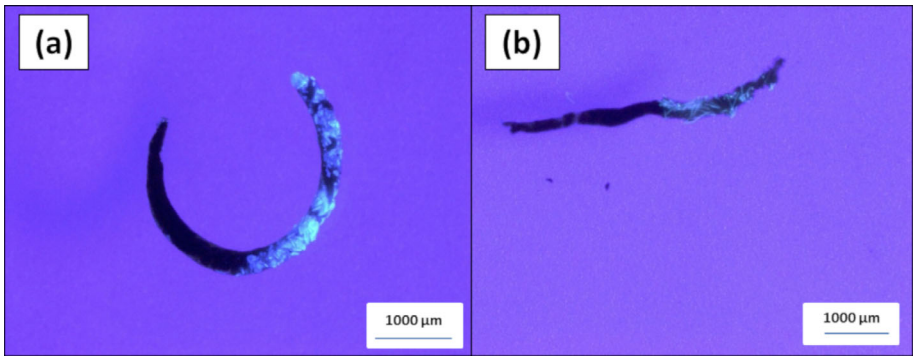

FIGURE 6 Gastropod feces in which microplastic fragments (a) and fibers (b) are just present in a certain fraction of the feces

We tested the number of hours after microplastic ingestion and fecal fluorescence intensity for normality using Shapiro–Wilk tests. As the data were not normally distributed, we then calculated the Spearman correlation coefficient between the number of hours after microplastic ingestion and fecal fluorescence intensity, and obtained a significant negative correlation ($r = -0.67$, $p < .05$; $n = 144$ records of fecal fluorescence intensity).

In the feces of the three snails which we exposed to microplastic fibers, we also observed fecal fluorescence which gradually decreased until 5 p.m. on May 17, 2019. Again, we observed no fecal fluorescence on May 20, 2019. However, one snail that consumed microplastic fibers died. We observed that sometimes microplastics were only present in a certain fraction of the fecal matter (Figure 6). When analyzing the feces of *R. balthica* individuals that we collected in the field and that we did not feed with microplastics during preliminary tests, we also observed a clear mark in the feces separating sediment grains from softer food items (such as algae). In the snail feces, the microplastic fibers did not form clumps and we did not observe any fringes of the fibers following snail digestion (Figure 7).

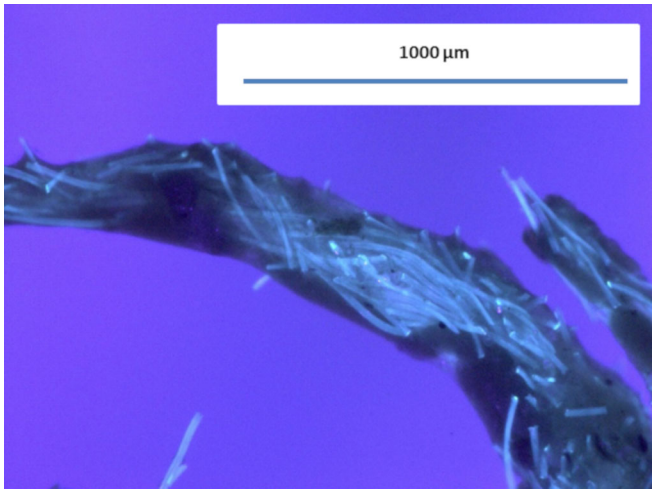

FIGURE 7 Microplastic fibers in the snail feces which did not clump together and which did not show any fringes after the snails' digestion

3.3 | Chemical digestion of the snails' soft tissue

Although all snails had stopped to egest any microplastic fragments after 76-hr-post microplastic ingestion, we detected fluorescent microplastic fragments on 13 out of 15 filters after the snails' chemical digestion. On five filters, microplastic fragment numbers ranged from 1 to 5 fragments per filter. However, five filters showed a relatively high number of microplastic fragments and three filters (among which was one filter resulting from the snail that had died during the microplastic fragment feeding experiment) were heavily covered by microplastics (Figure 8). Regarding the three snails that consumed microplastic fibers, the filter resulting from the dead snail showed one fiber, another filter showed 3 fibers, and one filter showed 27 fibers.

4 | DISCUSSION

With our new method, we propose the usage of low-cost materials to obtain ecologically relevant data on microplastic ingestion and egestion by living organisms. While our method may not be used by labs that have access to expensive equipment (such as a fluorescence microscope) and to expensive materials (e.g., fluorescent microbeads), our experimental approach is interesting for labs doing research on various biological disciplines (marine, freshwater, terrestrial, fundamental, applied and behavioral biology) and that do not have access to such cost-intensive tools for microplastic detection. Another advantage of our low-cost method for microplastic detection is that it can be used for microplastic feeding experiments in aquaria and terraria, and that no special previous knowledge and training is required. Using our novel method, we found that *R. balthica* egested most

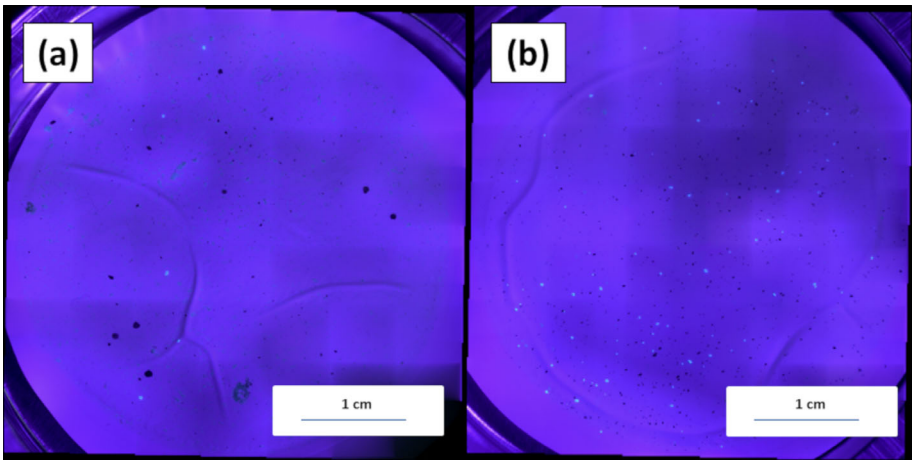

FIGURE 8 A cellulose acetate filter resulting from chemical digestion of the snails with a relatively high number of fluorescent microplastic fragments (a) and with a very high number of microplastic fragments (b)

microplastics within the first 44 hr after microplastic ingestion as feces showed the strongest fluorescence at that time and that microplastic egestion lasted for 76 hr, after which microplastics could still be found in the snails' bodies. Due to the microplastics' and the animals' small size in some studies, ingestion and egestion of fluorescent microbeads in organisms such as copepods is usually observed under a fluorescence microscope (Cole et al., 2016). The disadvantage is that organisms have to be placed in a Petri dish to examine them under a fluorescent microscope and that they are potentially stressed, not showing their natural behavior. For instance, it cannot be excluded that stress caused by the organism's placement in the Petri dish may lead to behavioral changes in for example, microplastic ingestion or egestion speed. A less stressful alternative would be feeding the copepod with microplastics in a glass beaker. According to a previous microplastic feeding study (Cole et al., 2016), copepod fecal pellets have a length of ca. 450 μm. The microplastic fragments that we used for our feeding experiment had a maximum length of 200 μm and were easily observed in the glass beaker without using any microscope for magnification (see Figure 2). If the copepod fecal pellets were loaded with microplastics as shown by Cole et al. (2016), the pellet would strongly fluoresce in the shine of the UV flashlight in a glass beaker. Hence, the fecal egestion of microplastics by animals as small as copepods could be observed in a spacious beaker using our method. In a larger glass beaker and in aquaria, the animals' stress is reduced as there is more space, more water and therefore presumably higher oxygen levels. To investigate whether larger animals such as gastropods consume experimentally offered fluorescent microplastics, the animals are often not observed during microplastic ingestion and egestion, but euthanized and prepared as squash preparations (Gutow et al., 2016) and tissue samples (Farrell & Nelson, 2013) before placing them under a fluorescence microscope. Regarding microplastic detection in an animal's feces, many labs do not have access to a fluorescence microscope but to a regular microscope and our method provides a low-cost alternative to observe microplastics in feces, thereby confirming an organism's microplastic ingestion.

The rapid occurrence of microplastics in the snail feces and the rapid feces sedimentation suggest that microplastics in gastropod feces are quickly available to benthic organisms. A similar vertical microplastic transfer has already been shown in marine copepods (Cole et al., 2016). In the field, gastropod feces together with egested microplastics may be eaten by other aquatic organisms. Furthermore, the occurrence of the offered microplastic fragments and fibers in the snails' feces suggests that the microplastics do not easily block the digestive tract of *R. balthica*. The same has been suggested for a marine gastropod species (Gutow et al., 2016). However, the majority of *R. balthica* individuals used in our study was not able to egest all consumed microplastic fragments and fibers during fecal egestion. Therefore, it cannot be fully excluded that at some point during their digestion, the snails' digestive tract may have been blocked by accumulating microplastics. That was shown by the strong fluorescence (of microplastics) on the filters after chemical digestion of the snails' soft tissues 6 days after microplastic exposure. Microplastics emit toxic leachates (Hermabessiere et al., 2017) and long microplastic retention in the snails may expose the snails to higher levels of harmful chemicals. Apparently, the microplastic fibers in *R. balthica* did not form clumps as indicated by the lack of clumping fibers on the filters after chemical digestion of the snails' tissues. In other aquatic invertebrates, it has been shown that microplastic fibers may clump together in an animal's intestines which may impede the animals' microplastic egestion (Murray & Cowie, 2011). That was not the case in *R. balthica*. Moreover, the microplastics were partly separated from softer

food items in the snail feces, suggesting that they were occasionally separated from the softer biofilm components during the snails' digestion. The microplastics did not change their shape after digestion, as indicated by the lack of fringes of egested polyacryl fibers.

As polystyrene is a polymer of low density (1.04–1.11 g/cm^3; Avio, Gorbi, & Regoli, 2017) and as polyacryl fibers can easily float in the water column (Gutow et al., 2016), we used the UV flashlight to investigate whether the water column inside the beakers showed fluorescence. Fluorescence of the water column was not observed, thereby confirming the suitability of our experimental setup. The information that could be obtained using the UV flashlight regarding the suitability of our experimental setup was important for our study design as floating microplastics would not have been available to the grazing snails anymore, thereby changing the microplastic concentration available for ingestion during the experiment. However, the presence of microplastics on a snail's shell suggests that microplastics from the artificial biofilm covering the glass slides may have attached to the edge of the snail's shell opening (Video S1). We conclude that our novel method holds many advantages as it facilitates microplastic detection during microplastic feeding experiments in ecological studies using aquatic as well as terrestrial organisms.

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CONFLICT OF INTEREST

We declare that there are no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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