

Environmental Chemistry

Are Fragrance Encapsulates Taken Up by Aquatic and Terrestrial Invertebrate Species?

Sebastian Kuehr,^{a,b} H. Windisch,^a C. Schlechtriem,^{a,b,c,*} G. Leon,^d G. Gasparini,^d and S. Gimeno^e^aFraunhofer Institute for Molecular Biology and Applied Ecology IME, Schmallenberg, Germany^bDepartment Chemistry and Biology, "Ecotoxicology" Work Group, University of Siegen, Siegen, Germany^cRWTH Aachen University, Aachen, Germany^dCorporate R&D Division, Firmenich, Satigny, Switzerland^eLegal and Compliance, Global Registration Services, Firmenich Belgium, Louvain La Neuve, Belgium

Abstract: The uptake potential of fragrance encapsulates by aquatic or terrestrial organisms was investigated. Because of their size of <5 mm and their polymeric nature, fragrance encapsulates fall under the Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection definition of microplastics. After use, fragrance encapsulates enter the sewer system and reach the sewage treatment plant (STP), where >90% of them are likely to be removed by sorption to the sludge. When the STP-generated sludge is used as fertilizer for agricultural soils, this may lead to potential exposure of terrestrial invertebrates to fragrance encapsulates, especially those feeding on particles of a similar size as the fragrance encapsulates. Two aquatic (*Corbicula fluminea* [water exposure] and *Hyalella azteca* [water and dietary exposure]) and one terrestrial invertebrate (*Eisenia andrei* [soil exposure]) species were exposed to 50 mg/L (or mg/kg) double fluorescence-labeled fragrance encapsulates (diameter 5–50 µm). The results showed that fragrance encapsulates are available to aquatic and terrestrial invertebrates but that species-specific differences regarding the ability to ingest fragrance encapsulates may exist. The benthic grazer *H. azteca* showed no ingestion of fragrance encapsulates, whereas the capsules were readily ingested and egested by the unselective freshwater filter feeder *C. fluminea* as well as the terrestrial decomposer *E. andrei*. No signs of bioaccumulation of fragrance encapsulates were indicated by microscopic assessment. *Environ Toxicol Chem* 2022;41:931–943. © 2021 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

INTRODUCTION

Fragrance encapsulates in consumer products are used in detergents and fabric conditioners as a technology to provide a long-lasting fragrance experience. They are present in 10 to 20% of laundry detergents and in approximately 60% of the fabric softeners sold in Europe (Postle et al., 2018). Concentrations of fragrance encapsulate shell material in final products range typically from 0.01% in liquid laundry detergents to 0.1% in fabric softeners (International Association for Soaps, Detergents and Maintenance Products, 2018). Fragrance encapsulates consist of a droplet of fragrance oil surrounded by a thin polymeric shell (<1 µm thickness) and have a diameter of 5 to

50 µm (Postle et al., 2018). These are designed to break when dry and on friction, allowing slow diffusion of the fragrance oil during the washing process. A direct benefit is the reduced use and emission of fragrance oils. This is due to a greater retention efficiency of the fragrance encapsulates containing the fragrance oils versus the oils added directly into the products, where there is loss in the wastewater during washing, rinsing, and drying. As a consequence, this has led to the reduction in use and emissions of fragrance oils by 33% for the same or improved consumer benefit (International Association for Soaps, Detergents and Maintenance Products, 2018). To achieve this, the technology requires the use of polymeric shell material that constitutes 2 to 7% of the weight of the fragrance encapsulates. Because of their size (tens of micrometers in diameter) and polymeric nature, fragrance encapsulates fall under the definition from the Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection for microplastics as synthetic polymer fragments with a size <5 mm (Kershaw, 2015). However, the thermoset polymer constituting the shell differs drastically from the thermoplastic polymer of

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* Address correspondence to christian.slechtriem@ime.fraunhofer.de

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other microplastics. Because currently the majority of fragrance encapsulates are made of melamine formaldehyde, they are not biodegradable. The reduction of the thickness of the shells of the most recent fragrance encapsulate generations was a step toward the reduction of the use of polymeric material. The next challenge is how to replace the polymeric shell of fragrance encapsulates by biodegradable materials as required by the regulations to further minimize the impact on the environment (European Chemicals Agency, 2020).

After use, fragrance encapsulates are generally released via the sewer to reach the sewage-treatment plant (STP) or released directly via the gray water into the environment and represent <0.03% of the released microplastics, as calculated by Cai et al. (2021). Unlike the large majority of the thermoplastic particles (low- and high-density polyethylene, diphenyl ethylenes, and polypropylene) which are rigid particles of various sizes and shapes with a density <1 g/cm³ that tend to float and may enter the aquatic environment suspended in effluents (Carr et al., 2016), fragrance encapsulates with a density >1 g/cm³ have the tendency to sink in the water column. When passing through the STP, only a minor percentage may reach the aquatic environment (Corradini et al., 2019); and it has been estimated that >90% of microplastics and nanoparticles are likely to be removed and captured in the sludge (Li et al., 2018; Mitrano et al., 2019). When the sewage sludge is not incinerated or disposed of in landfills, it may be treated and applied to agricultural soils. On average in Europe and North America, 30 and 50%, respectively, of the STP-generated sludge is used as fertilizer for agricultural purposes and in Ireland even >70% (Coors et al., 2016; Corradini et al., 2019). Therefore, this practice may represent a source of fragrance encapsulates which are potentially ingested by terrestrial organisms (Schmidt et al., 2006), albeit a very minimal source of microplastics (0.03% as estimated by Cai et al. [2021]). In a fate modeling exercise, these authors predicted the European average concentration of fragrance encapsulate polymeric shells in sludge-treated soil to be 210 µg/kg. The highest European estimated concentration was 2700 µg/kg, in Croatia (Cai et al., 2021).

Research has been conducted to understand whether microplastics present in the environment are available to aquatic and terrestrial invertebrates. Ingestion of thermoplastic microplastics has been observed in laboratory experiments, such as exposure of earthworms to polyethylene particles with a size of <50 to 150 µm at a concentration of 500 mg/kg soil (Huerta Lwanga et al., 2016; Rillig et al., 2017). Other experiments have shown that aquatic species like mussels (exposed to 4–6 µm polyethylene particles at concentrations of 2 and 4 mg/L [Fernández & Albentosa, 2019]), amphipods (exposed to polyethylene with 10–100,000 particles/L [Au et al., 2015]), and fish (dietary exposure of polyethylene microbeads and microfibers [Grigorakis et al., 2017]) are able to ingest microplastics (Au et al., 2015; Grigorakis et al., 2017; Von Moos et al., 2012). Due to their large specific surface, microplastics adsorb organic pollutants like polycyclic aromatic hydrocarbons, polybrominated diphenyl ethers, polychlorinated biphenyls, and heavy metals and may thus represent a vector that may allow

the transfer and even the bioaccumulation of these pollutants (Chua et al., 2014; Holmes et al., 2012). A further potential risk is posed by observed mechanical and inflammatory damage and negative effects on tissues. Such effects were observed, for example, for intestinal tissues of fish after 10-day exposure of adult zebrafish (*Danio rerio*) to polyamide, polyethylene, polypropylene, or polyvinyl chloride particles (~70 µm) at 1 mg/L (Lei et al., 2018). Similar effects were observed after chronic exposure of adult *D. rerio* to 0.5 and 50 µm polystyrene microparticles for 14 days at concentrations of 100 and 1000 µg/L (Jin et al., 2018). Also, after exposure of 3-month-old marine medakas (*Oryzias melastigma*) to polystyrene particles (10 µm) at concentrations of 2 to 200 µg/L, similar observations were noted (J. Wang et al., 2019).

However, investigations on the bioavailability and bioaccumulation potential of micro-/nanoparticulate material are challenging. In contrast to chemicals dissolved in the water phase, particles like microplastics or nanomaterials often tend to precipitate out of suspension and do not necessarily lead to a constant exposure of aquatic organisms (Gouin, 2020). Furthermore, the tissue uptake and elimination processes are not driven by mechanisms like passive diffusion through the tissues or transporters as for solutes (Felix et al., 2017; Schultz, 1976). Thus, as for poorly soluble substances for which guidelines have been developed (Organisation for Economic Co-operation and Development, 2019), the assessment of particles requires the development of new approaches as well as testing and assessment strategies as already initiated by the European Centre for Ecotoxicology and Toxicology of Chemicals (2019, 2020). Some studies have investigated the uptake of microplastics and their accumulation in the intestine of various organisms. Usually, a rapid elimination of the microplastics during the following depuration phase was observed (Au et al., 2015; Dawson et al., 2018; Fernández & Albentosa, 2019; Grigorakis et al., 2017; Von Moos et al., 2012), and only in a few studies were indications for real incorporation and translocation of microplastics between different tissue or cells found. For instance, translocation of microplastics to tissue has been observed in blue mussel (*Mytilus edulis*) exposed to polyethylene particles (<80 µm) at concentrations of 2.5 g/L (Von Moos et al., 2012). Also, translocation of microplastics to the circulatory system has been observed in this mussel species after 3 h of exposure to 3- and 9.6-µm polystyrene particles at 0.51 g/L (Browne et al., 2008). While the fate of fragrance encapsulates in the environment has been modeled (Cai et al., 2021), so far a differentiated investigation on the eventual uptake of fragrance encapsulates in organisms is not available.

In the present study, we investigated the potential of fragrance encapsulates to be taken up by terrestrial and aquatic organisms. Bioavailability studies were conducted with three invertebrate species with different feeding behaviors using fluorescence-labeled fragrance encapsulates. The freshwater bivalve *Corbicula fluminea* was used as a representative filter feeder, whereas the benthic freshwater amphipod *Hyalella azteca* allows investigation of the aqueous and dietary exposure pathways. Both species have already been used to study the accumulation of nanomaterials (Kuehr, Kaegi, et al., 2020;

Kuehr, Klehm, et al., 2020; Kuehr, Meisterjahn, et al., 2020) and microplastics (Au et al., 2015) and have been proposed as suitable test organisms for the bioaccumulation assessment of nanomaterials (Kuehr, Kosfeld, & Schlechtriem, 2021). In addition, they may be a suitable test species for particles in the micrometer range (Kuehr, Kosfeld, & Schlechtriem, 2021). Terrestrial systems have rarely been examined with respect to the fate and effects caused by microplastics, despite the fact that the contamination level may be several times higher compared to aquatic systems (de Souza Machado et al., 2018). Thus, the earthworm *Eisenia andrei* was used in the present study as a representative of terrestrial invertebrates.

MATERIALS AND METHODS

All tests for the investigation on the uptake and fate of fragrance encapsulates in organisms were conducted with an uptake phase followed by a depuration phase, as recommended by Burns and Boxall (2018).

Test species

Aquatic amphipods of the species *H. azteca* used in the present study were taken from the stock culture of Fraunhofer Institute for Molecular Biology and Applied Ecology (IME) in Schmallenberg, Germany. The culturing procedure was described in detail by Kuehr et al. (2018). Only male individuals with an age of at least 8 weeks and an approximate length of 0.3 cm were used. Males were distinguished as described by Schlechtriem et al. (2019). Amphipods were transferred to clean water without food for 4 days before test start to ensure that they accepted the diets applied during the bioavailability test.

Freshwater bivalves *C. fluminea* used in the present study were taken from the husbandry of Fraunhofer IME. Individuals were originally collected at a floodplain shoreline from the river Niers near Wachtendonk (47669, Germany) and kept in 1.5-m³ glass microcosms. The culture procedure is further described in Kuehr, Meisterjahn, et al. (2020). A 2-week acclimation phase was required before the organisms were used in the exposure

tests. Only individuals with a shell length (anterior–posterior) of 2.5 (± 0.5) cm, typically representing an age of 2 to 3 years were used in the present study. All organisms were kept in a 20-L glass aquarium before test start for 4 days without feeding, to allow defecation.

The earthworms *E. andrei* were collected from the stock culture of Fraunhofer IME. Worms originated from Regenwurmfarm Tacke and were cultured according to guideline 317 (Organisation for Economic Co-operation and Development, 2010). The culture was synchronized (developmental stage) with individuals in an age range between 2 months and 1 year. Only individuals already possessing a clitellum were used for the study.

Test item

The test item was a pink thick slurry consisting of fragrance encapsulates suspended in water (7% shells containing 29% benzyl benzoate suspended in 64% water). Fluorescence-labeled fragrance encapsulates were used in the bioavailability studies. The capsules consist of a polymeric shell surrounding the oil content. Both components of the fragrance encapsulates had been labeled with fluorescent markers (Figure 1). Schür et al. (2019) underlined that care should be taken during the fluorescence-based examination of microplastic localization, especially with regard to the potential release of fluorescence dye from microplastics and artifacts caused by their accumulation in lipid vesicles. Therefore, double-labeled fluorescent fragrance encapsulates were used in the present study. The outer shells were permanently labeled with red fluorescent dye carboxytetramethylrhodamine (TAMRA)–cadaverin, which was linked covalently to the polymeric material. The green fluorescent dye boron-dipyrromethane (BODIPY) is dissolved in the oil within the shells of the capsules. By investigating both markers using fluorescence microscopy, the structural integrity and stability of the test material were confirmed, and we were able to detect any potential leak of the oil after breakage of the shells. The fragrance encapsulates were prepared according to procedures in the literature (Gasparini et al., 2020; León et al., 2017; Paret et al., 2019).

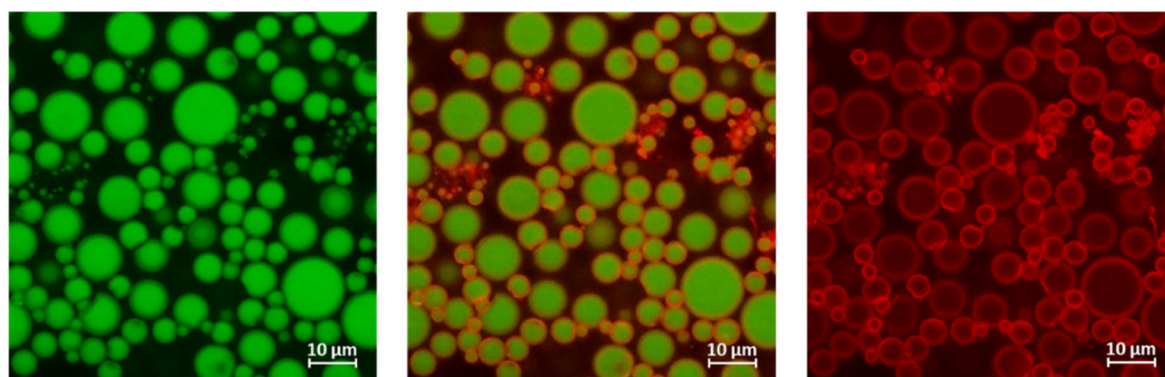


FIGURE 1: Fluorescence microscopy of double-labeled fluorescent fragrance encapsulates (pure slurry sample), showing individual spherical fragrance encapsulates. The colors result from the fluorescence of the respective dyes: boron-dipyrromethane in the core oil phase (left, green channel) and carboxytetramethylrhodamine-cadaverine in the polymeric shell (right, red channel). A superimposition of both channels is shown in the center.

Melamine-formaldehyde resin (1.12 g; Urecoll SMV; BASF), 0.12 g of methylated melamine-formaldehyde resin (CYMEL®; 9370 Resin), colloidal stabilizer (4.90 g, poly[acrylamide 20%, acrylic acid 80%] 20% w/w in water), and water (42.0 g) were introduced into a 200-ml reactor at room temperature. The resulting solution was mixed with an anchor-shaped paddle, and 0.20 g of acetic acid were added, bringing the pH value to 5.0. The reaction mixture was stirred at room temperature for 1 h. Benzyl benzoate (29.2 g, containing ~1 mg of BODIPY, corresponding to a concentration of ~40 µg/ml of the dye in the oil phase; Sigma-Aldrich Chemie) and polyisocyanate (0.58 g; Takenate® D-110N; Mitsui Chemicals, containing ~1 mg of TAMRA-cadaverine [mixed isomers]; Biotium) were added, and the resulting mixture was emulsified with an Ultra-Turrax® at 19 500 rpm for 2 min. The emulsion was stirred for 2 h at 80 °C, 15.3 g of Salcare SC60 (solution 3% w/w) were added, and the reaction mixture was stirred for an additional 1 h at 80 °C. Then, 6.00 g of a 50% w/w of ethylene urea solution were added, and the heating system was stopped. Once the dispersion reached room temperature, 0.12 g of xanthan gum were added; and the stirring was maintained for 1 h. The final pH of the resulting suspension was corrected to 6.5 using NaOH 30% w/w in water. A slurry containing double-labeled fragrance encapsulates was obtained as a pink dispersion.

The capsule size was in the range of 10 to 25 µm, with <5% of capsules being <5 µm, as determined by flow particle image analysis (Sysmex FPIA3000; see Supporting Information, Figure SI 7). The pH of the suspension was 6.83.

The fragrance encapsulates were examined in ultrapure water (purified using an ELGA Pure Lab Ultra water purification system, <18 MΩcm) and tap water by dynamic light scattering (DLS) for their hydrodynamic diameter. The DLS measurements were carried out as described by Zeumer et al. (2020), and further details are presented in the Supporting Information. Zeta potential was measured using a zetasizer (Zetasizer Nano Series; Malvern Instruments) and a disposable folded capillary cell (DTS1070; Malvern Panalytical). Measurements were carried out in triplicate, each consisting of 10 to 100 runs of measurement at 25 °C with an equilibration time of 120 s. Zeta potential was determined in ultrapure water and tap water.

The concentration in soil of 50 mg/kg (slurry containing 7% of shells) used in tests with earthworms corresponds to the highest worldwide concentration of shells (3600 µg/shells) in sludge-treated soil as estimated by Cai et al. (2021). The same slurry preparation with double-labeling of shells and content was used for the water exposures. However, the highest water concentration estimated by Cai et al. (2021) was only 0.13 µg/L, in Belgium. This is approximately a factor of 25,000 times lower than the tested concentrations for the amphipod and the bivalve, but this high concentration was necessary to allow detection of any potential uptake from the water column.

Test media

All tests with aquatic organisms were conducted in aerated and purified tap water sourced from the Schmollenberg district. The supply (water production plant) is sourced by small springs

and percolation. The on-site purification and dechlorination processes consisted of filtration with activated charcoal, passage through a limestone column, and aeration to the point of oxygen saturation. To avoid copper contamination, plastic water pipes were used in the test facilities. Further characteristics of the water used can be found in Kuehr, Diehle, et al. (2021), where the same water was used at the same time as the aquatic studies of the present study.

The earthworm tests were carried out by using a defined test substrate (Table 1), as recommended in technical guideline 317 (Organisation for Economic Co-operation and Development, 2010), which was prepared to carry out the experiment. Pulverized calcium carbonate was added (3 g/kg substrate) to the mixed and wetted substrate to adjust the pH to 6.0 (± 0.5) before use.

Production of fragrance encapsulate-enriched experimental diets

Two sets of experimental diets were prepared for the bioavailability test with *H. azteca* (dietary exposure) as described by Kuehr, Kaegi, et al. (2020), containing nominal concentrations of 1 and 50 mg test item/kg. In brief, 500 mg of agar-agar (Roth) were dissolved in hot ultrapure water, and the respective amount of test item slurry was added to obtain the nominal concentrations. The suspension of the fragrance encapsulates in the agar-agar solution was gently heated and stirred. After 1 min of stirring, 1.5 g milled TetraMin® flakes were added to the suspension and stirred for 1 additional min. The suspension was transferred to silicon ice trays to form cubes of 1 ml volume before the curing process at 4 °C.

Bioavailability tests with *H. azteca*. The fluorescence-labeled fragrance encapsulates were applied in two tests with *H. azteca* via aqueous or dietary exposure. For aqueous exposure the slurry was mixed directly into the water column. The test was performed with two treatments at nominal concentrations of 1 and 50 mg test item/L. During the exposure phase of the bioavailability test, 120 individuals were exposed for 2 days under static conditions in a 3-L glass beaker filled with 2 L medium and fed ad libitum with a control diet (DECOTABs containing TetraMin®) as described by Kuehr, Kaegi, et al. (2020). Organisms were sampled at the end of the exposure phase (48 h), and the remaining organisms were rinsed with clean tap water and transferred to a clean system for the depuration phase lasting 2 days. Further individuals were sampled at the end of the depuration phase. Each sample consisted of five single organisms. The sampled organisms were rinsed with tap water before being blotted dry with paper tissue. Control organisms were sampled as

TABLE 1: Composition of artificial substrate

Substrate component	Percentage
Sphagnum peat, air-dried, finely ground	10%
Kaolinite, air-dried	20%
Industrial quartz sand, air-dried	70%

reference from the stock culture following the same procedure and time intervals. The samples were stored in a freezer ($\leq -18^{\circ}\text{C}$) until analysis.

The second test (dietary exposure) was conducted with the same experimental setup as for aqueous exposure. However, the control diet (DECOTABS containing TetraMin®) was enriched with the test item to be used as the experimental diet allowing a clean dietary-only exposure, avoiding leaching of the test item into the water. The diet cubes were stored at $\leq 4^{\circ}\text{C}$ until use. The integrity of the capsules embedded in the diet was confirmed via fluorescence microscopy (Supporting Information, Figure SI 1). One diet cube (equivalent to a volume of 1 ml) was added (sliced into smaller bits) per day to the 3-L beaker containing 120 organisms. Uneaten food was removed daily and replaced by a fresh diet cube. During the depuration phase the amphipods were fed control diet cubes. Samplings occurred as described.

Bioavailability test with *C. fluminea*. The bioavailability test with the freshwater bivalve *C. fluminea* was conducted at a nominal concentration of 50 mg test item/L. A predilution of the slurry (1:50) was applied to the water column of the test vessel (10 L of tap water) at test start without any further supply of food to reach the target concentration. The test item was dispersed manually using a stainless steel rod, and 45 specimens were added to the test vessel. During the present study the water was mixed by gentle stirring using a stainless steel rotor at approximately 35 rpm (RZR 1; Heidolph). Because of the rapid ingestion of the test item, the duration of the uptake phase of the static test was set to 12 h. Organisms ($n = 3$) were sampled after 6 and 12 h of exposure. At the end of the uptake phase, remaining organisms were transferred to a new vessel containing clean water for the depuration phase. The basic standard food (400 mg/L ground stinging-nettle leaves) for the bivalve culture was added. After 12 and 24 h of depuration, further organisms ($n = 3$) were sampled. After sampling, the individuals were rinsed in clean tap water and dissected as described by Kuehr, Meisterjahn, et al. (2020). The four tissues—mantle, foot, viscera, and muscle—were stored separately in a freezer ($\leq -18^{\circ}\text{C}$) until examination. The different tissues were examined by fluorescence microscopy. For part of the sampled material (three replicates, each consisting of two organisms), fluorescence measurements of tissue extracts were carried out immediately after sampling (for preparation, see section, *Fluorescence measurement in digested mussel soft tissue*) using a multimode microplate reader (Synergy H1; Biotek). In addition, released feces and pseudofeces were collected and stored under the same conditions for examinations by fluorescence microscopy.

Bioavailability test with *E. andrei*. The bioavailability test with *E. andrei* was conducted with a test concentration of 50 mg test item/kg substrate. For the test, the humidity of the substrate was adjusted to be equivalent to 45% of the water holding capacity of the test substrate. This is in the range of 40 to 60%, as specified in guideline 317 (Organisation for Economic Co-operation and Development, 2010).

The fluorescence-labeled fragrance encapsulates were suspended in the water, which was used for wetting the substrate. The suspension was evenly distributed by careful mixing of the substrate with a bricklayer's trowel until the substrate was homogeneously wetted. Spiked substrate samples were analyzed by fluorescence microscopy to confirm that during application the capsules remained intact. Control substrate, containing no capsules, was prepared and used as test substrate during the depuration phase. A total of 46 glass vessels (150 ml with a diameter of 5 cm and a height of 9 cm) were used as test containers. Each vessel contained 50 g (dry wt) of substrate and a single worm, representing one replicate. The containers were covered with perforated aluminum foil to allow gas exchange and access of light. The exposure phase lasted 48 h. The worms were sampled at the end of exposure and gently rinsed with water. One group of three organisms was immediately dissected into the carcass and viscera (gut), which were investigated by fluorescence microscopy to assess the distribution of the previously ingested capsule material. The other group of three organisms was allowed to purge their gut overnight on wetted filter paper. The organisms and excreta were then collected for fluorescence microscopy. Remaining organisms exposed during the uptake phase were transferred to fresh vessels with uncontaminated substrate for depuration. After 48 h of depuration the remaining organisms were sampled and analyzed as described.

During our study, representative substrate samples of 50 g were collected at test start, end of uptake, and end of depuration. Collected samples were stored in a freezer ($\leq -18^{\circ}\text{C}$) until analysis by fluorescence microscopy. However, microscopic investigations were hampered by the nontranslucent character of the test medium.

Fluorescence microscopy of tissue samples

The localization of the fragrance encapsulates in tissue samples collected during the different experiments was determined by fluorescence microscopy using a DMI6000B Microscope (Leica). The dye-specific wavelengths used for the excitation and emission are summarized in Table 2.

The tissue distribution and the integrity of the ingested capsule material in the organisms were analyzed. *Hyalella azteca* were assessed as whole organisms. For *C. fluminea* individual tissues (foot, mantle, muscle, and viscera) and released feces and pseudofeces were analyzed. In *E. andrei*, carcass and viscera (gut) were assessed separately from purged or unpurged worms, as well as the feces from purged worms.

Fluorescence measurement in digested mussel soft tissue

A subset of dissected tissue samples obtained from the exposure study with freshwater bivalves was further digested using Proteinase K to extract the test item with fluorophores. The fluorescence measurements were carried out using a microplate reader (Synergy H1; Biotek) as described by Kuehr et al. (2021). The digestion method was carried out as described by Kuehr, Meisterjahn, et al. (2020). After incubation for

TABLE 2: Specificities of dyes in tested fragrance encapsulates used for fluorescence detection

Dye	Localization	λ max _{ex}	λ max _{em}	Recorded range λ	λ excitation	Filter type
TAMRA	Shell material	557	583	605 \pm 30	546	CY3
BODIPY	Shell content oil	505	510	480 \pm 20	470	L5

max_{ex} = maximum excitation; max_{em} = maximum emission; TAMRA = carboxytetramethylrhodamine; BODIPY = boron-dipyrromethane.

4 h, aliquots of 200 μ l of the digestion solution of each sample were transferred to a well plate (Nunc™ F96 MicroWell™ Black polystyrene Plate; ThermoFisher) and measured using the specific extinction and emission wavelengths of each dye (Table 2). In addition, aliquots of all samples were measured after dilution four times with ultrapure water to minimize potential matrix effects.

RESULTS AND DISCUSSION

Characterization of the fragrance encapsulates

The fragrance encapsulates contained in the slurry were labeled with two fluorescent markers: a red dye (TAMRA) linked to the shell of the fragrance encapsulate and a green dye (BODIPY), which was dissolved in the oil contained inside the fragrance encapsulates. Intact fragrance encapsulates displayed fluorescence from both fluorescent dyes in a typical spherical shape, as depicted in Figure 1. It is possible to see the fluorescence from red dye localized on the fragrance encapsulate shells, whereas the green dye is localized in the inner part.

The hydrodynamic diameter of the fragrance encapsulate was determined by DLS to be 34.32 and 25.55 μ m in ultrapure and tap water, respectively. This is in accordance with the data provided by the supplier using flow particle image analysis and indicates a low level of aggregation under the test conditions. The measured zeta potential was -52.6 mV in ultrapure water and -24.6 mV in tap water. The integrity of the fragrance encapsulates collected during exposure after freezing–thawing and in the experimental diet was confirmed by fluorescence microscopy. A diluted sample (concentration 100 mg/L) of test medium was analyzed to ensure that storage of the exposed matrix did not change the fragrance encapsulate condition. In comparison with fresh material, thawed material showed clustering effects after freezing for 48 h at ≤ -18 °C (Supporting Information, Figure SI 1, middle pictures, observation based on one replicate), but the integrity of the fragrance encapsulate was not affected. Also, the high temperature (83 ± 5 °C) required for the preparation of the experimental diet used for the feeding study with *H. azteca* did not degrade or rupture the fragrance encapsulate (Supporting Information, Figure SI 1).

Bioavailability tests with *H. azteca*

The amphipod *H. azteca* is an omnivorous grazer and an excellent model organism for bioaccumulation tests (Kosfeld et al., 2020; Othman & Pascoe, 2001; Raths et al., 2020; Schlechtriem et al., 2019). Because the fragrance

encapsulates applied in the present study tend to sediment under aquatic conditions, the benthic organism is especially suitable for testing the uptake of the test item under realistic exposure conditions.

During the bioavailability test, test material was applied at two concentrations (1 and 50 mg slurry/L). These are not environmentally relevant concentrations but were selected to ensure exposure and possible ingestion by the test organisms, as well as potential body burdens that allow adequate detection. Aqueous exposure did not affect the fragrance encapsulate integrity as shown by the fluorescence measurement of red and green channel signals of capsule shells and oil content, respectively. It can be assumed that the oil contained in the fragrance encapsulates did not leak during our study. During exposure, the organisms showed no behavioral changes. Based on microscopic analysis of the amphipods sampled after 48 h of exposure, no ingestion of fragrance encapsulates was observed for both exposure concentrations (Figure 2). However, fragrance encapsulates were observed to adhere to the carapace (clustering along the rims and cleaves of limbs) of the amphipod, which came into contact with the sedimented material at the bottom of the test vessel. For organisms sampled at the end of the 48-h depuration phase, only single fragrance encapsulates were detected on the body surface, indicating that adhering capsules were only loosely attached to the organisms' body surface (Supporting Information, Figure SI 2). Following sedimentation, fragrance encapsulates might have also been ingested by the organisms grazing at the bottom of the test vessel. However, no ingestion of suspended fragrance encapsulate from aqueous test media was observed for *H. azteca*. This might be explained by the size of the fragrance encapsulates, which are potentially too large (~ 10 – 40 μ m) to be taken up from the water by the gills. This is in accordance with the results of Kuehr, Kaegi, et al. (2020), where no uptake of TiO₂ particles with a hydrodynamic diameter of approximately 1.9 μ m by *H. azteca* was observed during a bioconcentration test in tap water.

During the dietary exposure study, fragrance encapsulates embedded in the experimental diet were applied. The use of diet cubes containing fragrance encapsulates ensured exposure of the amphipods exclusively via the dietary pathway. Following feeding of the experimental diet, no fragrance encapsulates could be identified in the digestive tract, which might be explained by selective grazing of the amphipods, as previously observed (Hudson et al., 2019; F. Wang et al., 2004). In the present study, only in a few samples were fragrance encapsulates detected, and they were attached to the carapace of the organisms collected at the end of the uptake phase (Figure 3).

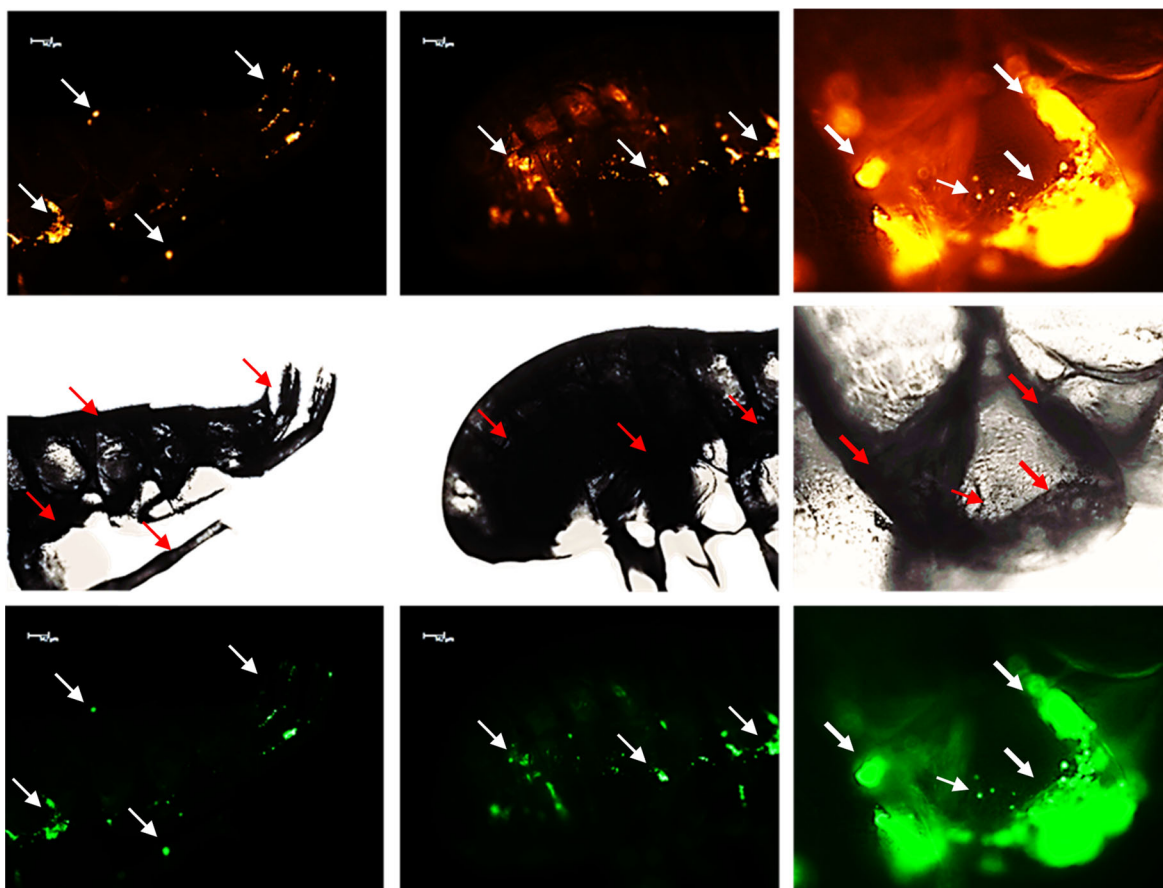


FIGURE 2: Fluorescence microscopy of *Hyalella azteca* exposed with suspended fragrance encapsulates of the higher-concentration treatment (50 mg slurry/L) for 48 h; left and middle panels, $\times 5$ magnification; right photos, detail of middle panel with $\times 20$ magnification; upper row, red channel; middle row, bright field; lower row, green channel. Arrows indicate (aggregated) fragrance encapsulates.

In conclusion, *H. azteca* is obviously not capable of ingesting fragrance encapsulates of the type tested in the present study, independently of whether they are dispersed in water or are part of a mixed diet. Interestingly, food particles of similar size (e.g., algal plugs) are usually consumed by *H. azteca*, and it can only be speculated why ingestion of the tested fragrance encapsulates did not occur. As described, limited numbers of single fragrance encapsulates or small clusters were observed loosely adhering to the carapace surface. Residues would be completely removed by the end of the next molting cycle at the latest. The potential of fragrance encapsulates being transferred along the food chain thus seems to be low considering the grazing activity of benthic invertebrates.

Bioavailability test with *C. fluminea*

The freshwater bivalve *C. fluminea* is a benthic filter feeder, and because of its high filtration rates, it is a suitable organism to assess the bioaccumulation of dispersed or suspended material (McMahon, 2000). As a neozoic invasive species, *C. fluminea* is easily accessible in many European freshwater systems and has been successfully used for laboratory studies.

Because of the rapid ingestion of fragrance encapsulates observed in a preliminary study, the exposure of the test organisms was limited to a time span of 12 h. The presence of the test item in the water column with a nominal concentration of

50 mg test item/L did not induce any avoidance behavior due to acute toxic or triggering effects. However, the filtration behavior was increased, which was due to the relatively high presence of particulate matter leading to increased production of feces and pseudofeces. These findings are in accordance with previous observations of Wegner et al. (2012) with blue mussels exposed to nanostructural polystyrene and Kuehr, Meisterjahn, et al. (2020) with *C. fluminea* exposed to TiO_2 nanoparticles and to gold nanoparticles (AuNPs). Also, in these studies increased filtration activity and release of feces or pseudofeces were observed (Kuehr, Meisterjahn, et al., 2020; Wegner et al., 2012). During the chronic exposure of *C. fluminea* to 5 mg AuNPs/L by Kuehr, Diehle, et al. (2021), increased mortality was observed, which was explained by the high energy demand required for the increased filtration activity and the loss of nutrients and energy by the continuous excretion of pseudofeces.

The examination of the different mussel tissues dissected in the present study showed that the digestive tract was the only compartment containing considerable amounts of fragrance encapsulates which was detected by fluorescence microscopy. Again, this is in accordance with the results of Kuehr, Meisterjahn, et al. (2020), who examined different tissues of *C. fluminea* which was previously exposed to different nanomaterials. In the latter study, the main part of the measured

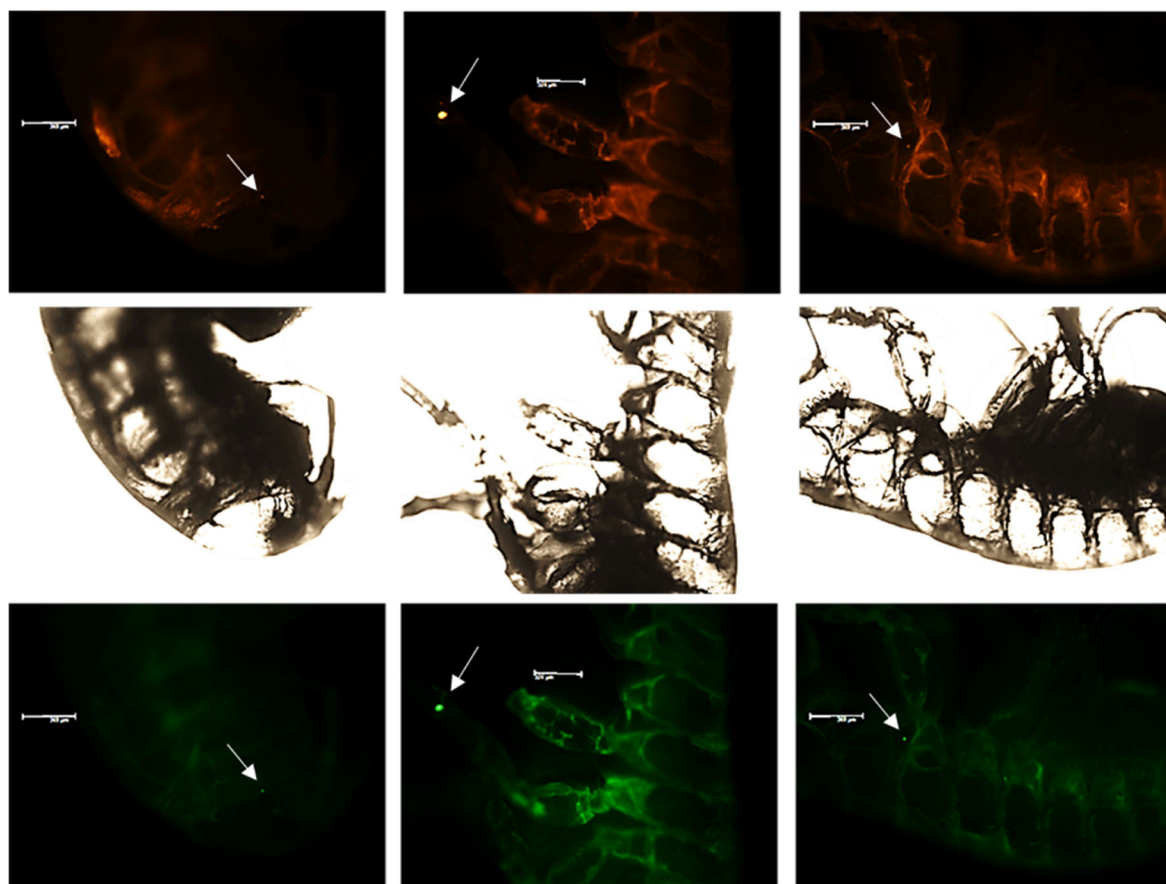


FIGURE 3: Fluorescence microscopy of *Hyalella azteca* exposed via the diet for 48 h with the higher-concentration treatment (50 mg slurry/L); all panels $\times 5$ magnification (different replicates); upper row, red channel; middle row, bright field; lower row, green channel. Autofluorescence of organism in both red and green channels can be observed. White arrows show observed presence of (aggregated) fragrance encapsulates.

body burden resulted from ingested material that was concentrated in the digestive tract. Nevertheless, the ingested material was quickly and effectively eliminated during a following depuration phase (Kuehr, Meisterjahn, et al., 2020).

In the present study, fluorescence measured in the viscera was stronger in organisms collected after 6 h of exposure than in organisms sampled after 12 h, indicating that the test material was efficiently ingested and thus removed from the surrounding medium (Figure 4). This led to a decrease in concentration of the suspended material in the test system, potentially limiting the uptake of the test item toward the end of the exposure phase. A fast elimination of the previously ingested microcapsules was shown during the depuration phase. In fact, already after 12 h no fragrance encapsulates could be detected in the digestive tract (Figure 4).

Only limited contaminations of other compartments or tissues were observed. On the surface of the foot tissue, for example, a plug of agglomerated fragrance encapsulate was visible after 12 h of exposure (Supporting Information, Figure SI 3). This material was probably part of excreted (pseudo)feces (Figure 5), which showed a strong fluorescence for both markers, indicating that the integrity of the fragrance encapsulates was not affected when being “digested” by the mussels. The faintest signals were observed for mantle and

muscle tissue, indicating that no incorporation of ingested fragrance encapsulates occurred during the test (Supporting Information, Figure SI 4). This was confirmed by fluorescence measurements of the digested mussel soft tissue resulting in weak fluorescence signals that were overlaid by the natural autofluorescence of the digested tissue. Although fragrance encapsulates are in the micrometer range, the results of the present study are still in agreement with those of Kuehr, Meisterjahn, et al. (2020), who could not find any indications of incorporation of nanomaterials that were previously ingested by *C. fluminea*.

The bioavailability study with *C. fluminea* has shown that the tested fragrance encapsulates are bioavailable for a filter-feeding organism, like freshwater bivalves, when applied as suspended particles. The fragrance encapsulates are readily ingested as dispersed particulate matter from the water column but are immediately egested thereafter in pseudofeces without any interference of the content. Despite the fast and effective elimination, there might still be a risk of secondary poisoning for predators feeding on contaminated individuals, as described by Farrell & Nelson (2013) for *M. edulis* and *Carcinus maenas*. However, compared to the high content of fragrance encapsulates in the gut, only low contamination levels were observed in the entire organisms.

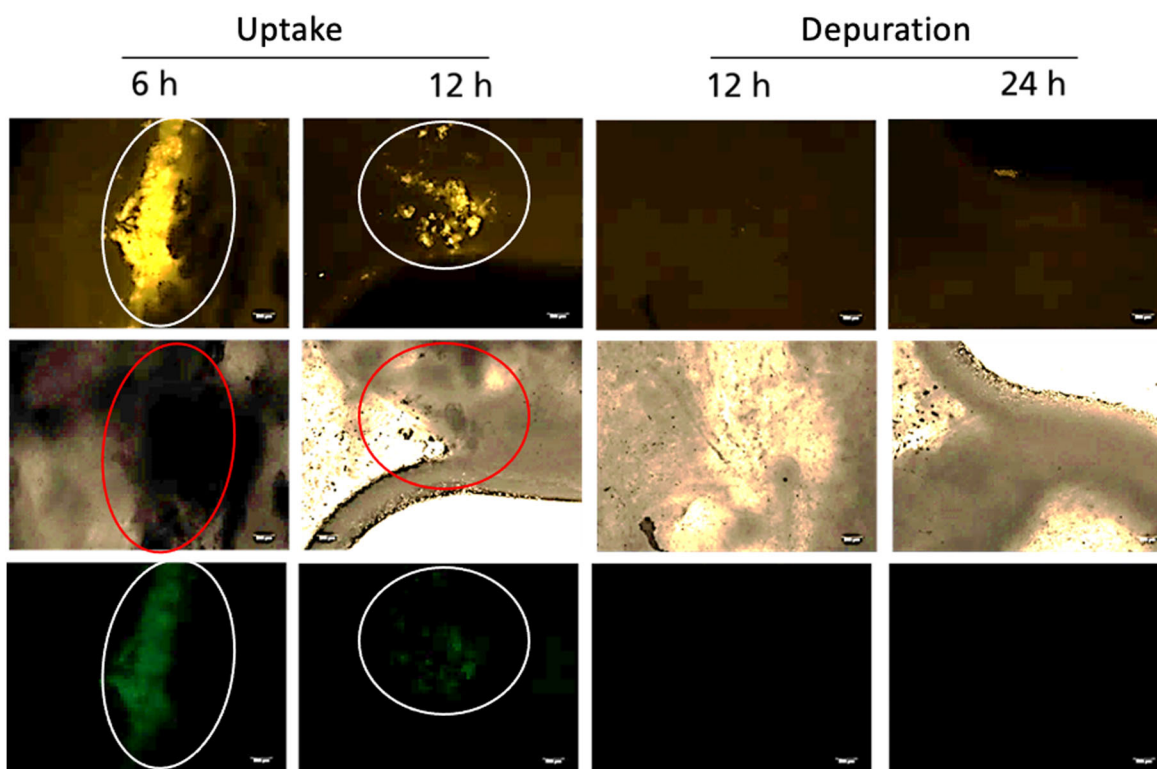


FIGURE 4: Fluorescence microscopy of squeeze preparations of viscera from *Corbicula fluminea* exposed to 50 mg slurry/L; all pictures with $\times 5$ magnification; upper row, red channel; middle row, bright field; lower row, green channel. The circles indicate fluorescence from fragrance encapsulates taken up by the bivalves and being located in the bivalves' viscera.

The filtration activity and the release of (pseudo)feces were increased in response to the high load of particulate matter in the test system. Further investigation of how chronic exposure with fragrance encapsulates (microplastics) may affect the condition of freshwater bivalves, considering the loss of nutrients and energy caused by the continuous excretion of (pseudo)feces, is needed. The high accumulation of fragrance encapsulates in the feces may lead to a transfer of the particles to higher trophic levels by organisms feeding on it (Basen et al., 2012; González & Burkart, 2004). This needs to be further developed with respect to ecosystem health.

The results clearly indicate that freshwater bivalves such as *C. fluminea* may play a role regarding the movement of fragrance encapsulates in aquatic ecosystems by transfer of the fragrance encapsulates into their (pseudo)feces, which

represents a potential exposure pathway for other species (Kuehr, Diehle, et al., 2021). As test organisms in laboratory studies they can provide important information on the bioavailability and accumulation of fragrance encapsulates in the benthic community.

Bioavailability test with *E. andrei*

The compost worm *E. andrei* is typically used for terrestrial bioaccumulation studies, and potential effects of the fragrance encapsulates when applied to the terrestrial environment have been assessed (Gerdt, 2015; Lwanga et al., 2017; Rillig et al., 2017). Because both tests with aquatic organisms were performed with a final concentration of 50 mg slurry/L, the same concentration-equivalent was chosen for this test with 50 mg slurry/kg soil.



FIGURE 5: Fluorescence microscopy of mussel feces collected at the end of the uptake phase; all pictures with $\times 20$ magnification; left panel, red channel; middle panel, bright field; right panel, green channel. The high concentration of fragrance encapsulates in the fecal samples cause the strong fluorescence emission. No individual fragrance encapsulates are visible.

Application of the fragrance encapsulates was challenging because monitoring of the capsule dispersal in the test substrate was hampered by the nontranslucent character of the test medium. However, the analysis of representative samples by fluorescence microscopy confirmed that the fragrance encapsulates were successfully applied and available to be ingested by the worms. The integrity of the capsules in the test medium was confirmed over the entire exposure phase because intact capsules were observed on the moistened substrate following exposure (Supporting Information, Figure SI 5).

At the end of the uptake phase of 48 h, spots of fluorescence were detected by fluorescence microscopy in the carcass of the worms (Figure 6). However, the analysis of control worms showed the same pattern, which turned out to be induced by endoparasitic nematodes that are commonly associated with organisms inhabiting moistened terrestrial substrate and that display high autofluorescence (Campos-Herrera et al., 2006; Poinar, 1978). The same observations were shown for dissected purged worms, which also displayed the same autofluorescent structures.

No capsules could be detected in the unpurged gut system. This might be explained by the limited uptake of fragrance encapsulates that was not high enough to allow detection, for example, because of the dense structure of the soil matrix also taken up, which may have impaired the analysis of the sample (low light transmission). To confirm this hypothesis, feces of purged organisms were analyzed on a squeezed preparation to

facilitate the detection of capsules in the dense, light-absorbing matrix (Figure 7; Supporting Information, Figure SI 6). The presence of fragrance encapsulates was detected in a fraction of the fecal samples, which confirms that fragrance encapsulates were ingested by *E. andrei*. However, the fragrance encapsulates were efficiently eliminated by defecation, which indicates that there was low interaction between fragrance encapsulates and the organisms' tissue or high affinity of the fragrance encapsulates to the organic matter in the soil matrix. This would be in accordance with results described by Carbone et al. (2016), who observed that CeO₂ and SnO₂ nanoparticles showed only low bioavailability and no bioaccumulation in *E. fetida* after exposure via soil and that the amount of organic matter in the soil strongly impacts the fate of the NPs. In addition, Lwanga et al. (2016) observed that the earthworm *Lumbricus terrestris* showed a size-selective egestion for polyethylene microplastics with different sizes. Thus, further investigations should be carried out to elucidate which characteristics alter the bioavailability of fragrance encapsulates for earthworms. Within the egested fecal samples collected during our study, fragrance encapsulates appeared as isolated clusters, showing that the integrity of the capsules was not degraded during our study. In contrast to the study with *C. fluminea*, no strong accumulation of fragrance encapsulates was observed in any of the fecal samples ($n=3$) from the purged worms; thus, no further risk is to be expected from the worms' feces for coprophagous terrestrial organisms.

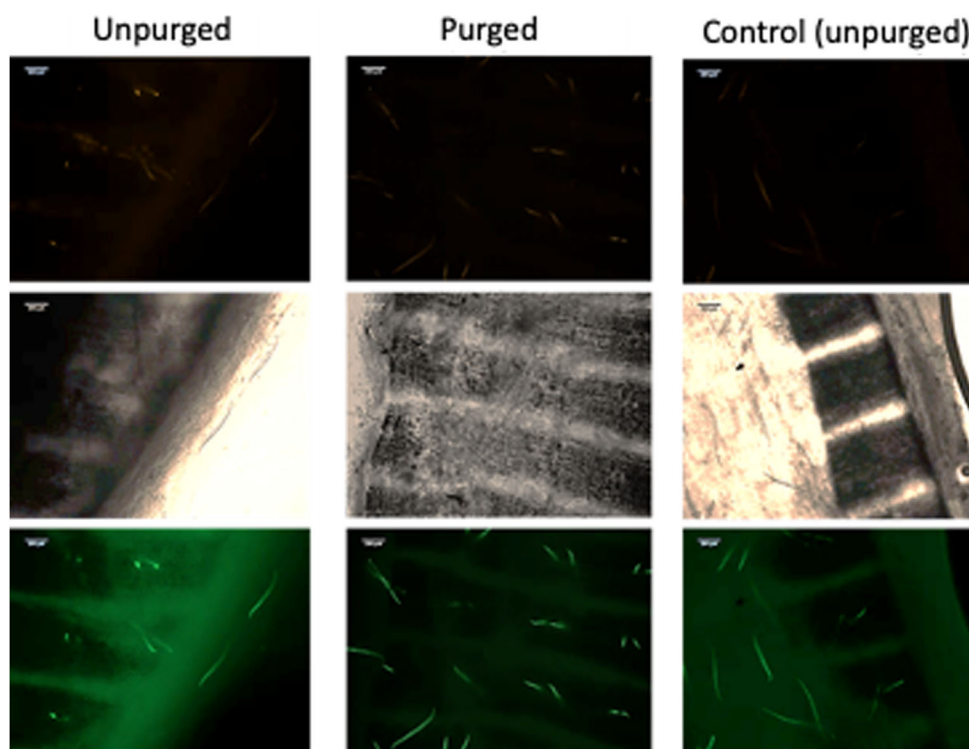


FIGURE 6: Fluorescence microscopy of squeeze preparations of carcass from unpurged (left) and purged (middle) *Eisenia andrei* at the end of the uptake phase and exemplary control carcass (unpurged); all pictures with x5 magnification; upper row, red channel; middle row, bright field; lower row, green channel. The visible fluorescence (in both the red [top panels] and green [bottom panels]) channels is the result of the autofluorescence of endoparasitic nematodes, and no fragrance encapsulates can be located.

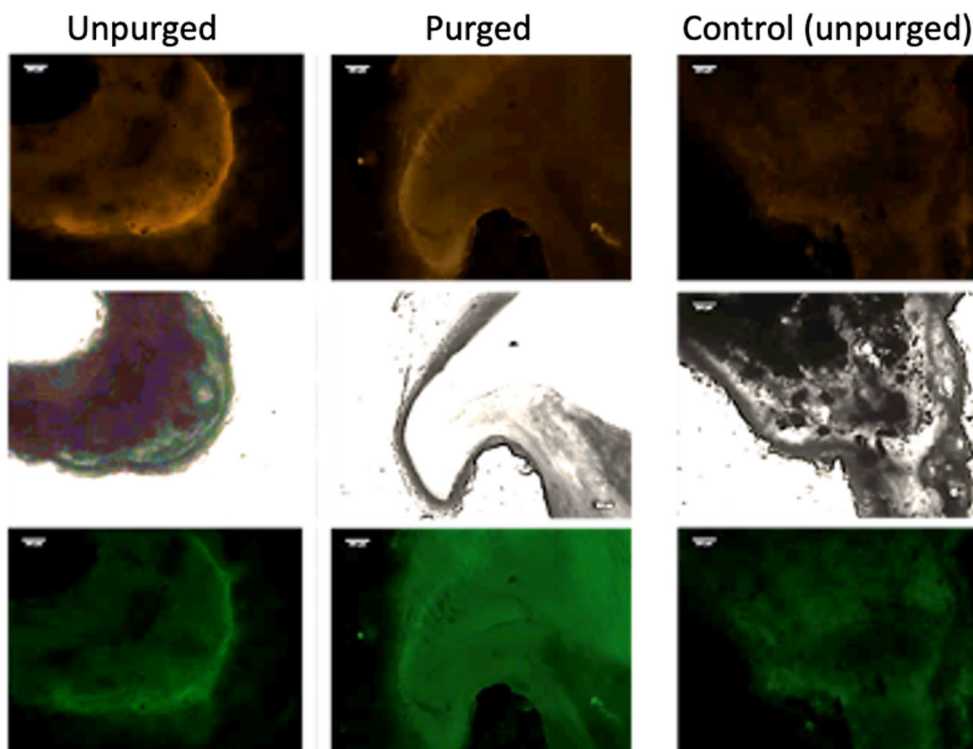


FIGURE 7: Fluorescence microscopy of squeeze preparations of viscera and from unpurged (left) and purged (middle) *Eisenia andrei* at the end of the uptake phase and exemplary control viscera (unpurged); all pictures with $\times 5$ magnification; upper row, red channel; middle row, bright field; lower row, green channel. Only autofluorescence is visible, and no fragrance encapsulates could be identified.

Evaluation of fluorescence measurements

The double labeling of the fragrance encapsulates allowed tracking of the fate of the capsules to prove the structure and integrity of the fragrance encapsulates during the present studies. In all experiments the integrity of the fragrance encapsulates was maintained over the duration of the experiment; there was no leakage of the content. In addition, neither the polymeric nor the content oil was bioaccumulated in any of the organisms.

The double labeling allowed visualization and localization of the fragrance encapsulates. It was concluded that no real bioaccumulation following incorporation (in contrast to simple ingestion/uptake) into tissue and cells was observed. Further methods and techniques such as microcomputed tomography, as described by Kuehr, Klehm, et al. (2020), could be applied to investigations on *H. azteca*. This method allows the examination of organisms or media that are opaque to light and thus limit the analytical options using fluorescence-labeled test items. The use of radiolabeled materials may solve this problem by allowing a more precise quantitative analysis after combustion analysis of spiked soil and samples. This may further allow localization of the ingested material within the test organisms by the method of autoradiography, as described by Raths et al. (2020) for ^{14}C -laurate in *H. azteca*.

CONCLUSIONS

In the present study, we investigated the potential of fragrance encapsulates labeled with double fluorescent

markers to be taken up by aquatic or terrestrial organisms in a test setup with high concentrations of fragrance encapsulates. With a size of approximately 5 to 50 μm , fragrance encapsulates are in the same size range as the food particles of many invertebrates such as bivalves, amphipods, and earthworms. The results of the present study show that fragrance encapsulates are available to aquatic and terrestrial invertebrates but that species-specific differences regarding the ability to ingest fragrance encapsulates may exist. The benthic grazer *H. azteca* showed no ingestion of fragrance encapsulates when present in the water column or when offered in the diet. In contrast, the test item was readily ingested and egested by the unselective freshwater filter feeder *C. fluminea*. As for the terrestrial decomposer *E. andrei*, no signs of bioaccumulation of whole microcapsules or fragments were indicated by microscopic assessment. However, to allow firm conclusions on the accumulation of fragrance encapsulates in invertebrates to be drawn, analytical methods could be further improved, especially regarding the experiments in soil. This would enable a more accurate and quantitative analysis of the test media and tissue concentrations. Additional experiments of longer exposure durations at lower fragrance encapsulate concentrations and experiments with treatments using aged fragrance encapsulates (e.g., after passing through a model STP) could be conducted. Also, testing with other species should be of benefit, to allow a more precise conclusion on the impact of fragrance encapsulates on invertebrates.

Supporting Information—The Supporting Information are available on the Wiley Online Library at <https://doi.org/10.1002/etc.5202>.

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Disclaimer—Firmenich is a producer of fragrance encapsulates.

Data Availability Statement—Data, associated metadata, and calculation tools are available from the corresponding author (christian.schlechtriem@ime.fraunhofer.de).

REFERENCES

- Au, S. Y., Bruce, T. F., Bridges, W. C., & Klaine, S. J. (2015). Responses of *Hyalella azteca* to acute and chronic microplastic exposures. *Environmental Toxicology and Chemistry*, 34(11), 2564–2572. <https://doi.org/10.1002/etc.3093>
- Basen, T., Gergs, R., Rothhaupt, K.-O., & Martin-Creuzburg, D. (2012). Phytoplankton food quality effects on gammarids: Benthic–pelagic coupling mediated by an invasive freshwater clam. *Canadian Journal of Fisheries and Aquatic Sciences*, 70(2), 198–207.
- Browne, M. A., Dissanayake, A., Galloway, T. S., Lowe, D. M., & Thompson, R. C. (2008). Ingested microscopic plastic translocates to the circulatory system of the mussel, *Mytilus edulis* (L.). *Environmental Science and Technology*, 42(13), 5026–5031. <https://doi.org/10.1021/es800249a>
- Burns, E. E., & Boxall, A. B. A. (2018). Microplastics in the aquatic environment: Evidence for or against adverse impacts and major knowledge gaps. *Environmental Toxicology and Chemistry*, 37(11), 2776–2796. <https://doi.org/10.1002/etc.4268>
- Cai, Y., Lin, J., Gimeno, S., Begnaud, F., & Nowack, B. (2021). Country-specific environmental risks of fragrance encapsulates used in laundry products. *Environmental Toxicology and Chemistry*. <https://doi.org/10.1002/etc.5168>
- Campos-Herrera, R., Trigo, D., & Gutiérrez, C. (2006). Phoresy of the entomopathogenic nematode *Steinernema feltiae* by the earthworm *Eisenia fetida*. *Journal of Invertebrate Pathology*, 92(1), 50–54.
- Carbone, S., Hertel-Aas, T., Jøner, E. J., & Oughton, D. H. (2016). Bio-availability of CeO₂ and SnO₂ nanoparticles evaluated by dietary uptake in the earthworm *Eisenia fetida* and sequential extraction of soil and feed. *Chemosphere*, 162, 16–22.
- Carr, S. A., Liu, J., & Tesoro, A. G. (2016). Transport and fate of microplastic particles in wastewater treatment plants. *Water Research*, 91, 174–182. <https://doi.org/10.1016/j.watres.2016.01.002>
- Chua, E. M., Shimeta, J., Nugegoda, D., Morrison, P. D., & Clarke, B. O. (2014). Assimilation of polybrominated diphenyl ethers from microplastics by the marine amphipod, *Allochrestes compressa*. *Environmental Science & Technology*, 48(14), 8127–8134.
- Coors, A., Edwards, M., Lorenz, P., Römbke, J., Schmelz, R. M., Topp, E., Waszak, K., Wilkes, G., & Lapen, D. R. (2016). Biosolids applied to agricultural land: Influence on structural and functional endpoints of soil fauna on a short- and long-term scale. *Science of the Total Environment*, 562, 312–326. <https://doi.org/10.1016/j.scitotenv.2016.03.226>
- Corradini, F., Meza, P., Eguiluz, R., Casado, F., Huerta-Lwanga, E., & Geissen, V. (2019). Evidence of microplastic accumulation in agricultural soils from sewage sludge disposal. *Science of the Total Environment*, 671, 411–420. <https://doi.org/10.1016/j.scitotenv.2019.03.368>
- Dawson, A., Huston, W., Kawaguchi, S., King, C., Cropp, R., Wild, S., Eisenmann, P., Towns, K., & Bengtson Nash, S. (2018). Uptake and depuration kinetics influence microplastic bioaccumulation and toxicity in Antarctic krill (*Euphausia superba*). *Environmental Science & Technology*, 52(5), 3195–3201.
- de Souza Machado, A. A., Kloas, W., Zarfl, C., Hempel, S., & Rillig, M. C. (2018). Microplastics as an emerging threat to terrestrial ecosystems. *Global Change Biology*, 24(4), 1405–1416. <https://doi.org/10.1111/gcb.14020>
- European Centre for Ecotoxicology and Toxicology of Chemicals. (2019). *The ECETOC conceptual framework for polymer risk assessment (CF4polymers)*. Technical report 133-1.
- European Centre for Ecotoxicology and Toxicology of Chemicals. (2020). *TR 133-2 The applicability of analytical tools, test methods and models for polymer risk assessment*. Technical report 133-2.
- European Chemicals Agency. (2020). *Response to comments document (RCOM) on the Annex XV dossier proposing restriction on intentionally-added microplastics*. Non-confidential. ECHA/RAC/RES-O-0000006790-71-01/F.
- Farrell, P., & Nelson, K. (2013). Trophic level transfer of microplastic: *Mytilus edulis* (L.) to *Carcinus maenas* (L.). *Environmental Pollution*, 177, 1–3. <https://doi.org/10.1016/j.envpol.2013.01.046>
- Felix, L. C., Ortega, V. A., & Goss, G. G. (2017). Cellular uptake and intracellular localization of poly (acrylic acid) nanoparticles in a rainbow trout (*Oncorhynchus mykiss*) gill epithelial cell line, RTgill-W1. *Aquatic Toxicology*, 192, 58–68. <https://doi.org/10.1016/j.aquatox.2017.09.008>
- Fernández, B., & Albentosa, M. (2019). Insights into the uptake, elimination and accumulation of microplastics in mussel. *Environmental Pollution*, 249, 321–329. <https://doi.org/10.1016/j.envpol.2019.03.037>
- Gasparini, G., Semaoui, S., Augugliaro, J., Boschung, A., Berthier, D., Seyfried, M., & Begnaud, F. (2020). Quantification of residual perfume by Py-GC-MS in fragrance encapsulate polymeric materials intended for biodegradation tests. *Molecules*, 25(3), Article 718.
- Gerdts, G. (2015). *Mikroplastik in ausgewählten Kläranlagen des Oldenburgisch-Ostfriesischen Wasserverbandes (OOWV) in Niedersachsen*.
- González, M. J., & Burkart, G. A. (2004). Effects of food type, habitat, and fish predation on the relative abundance of two amphipod species, *Gammarus fasciatus* and *Echinogammarus ischnus*. *Journal of Great Lakes Research*, 30(1), 100–113. [https://doi.org/10.1016/S0380-1330\(04\)70333-0](https://doi.org/10.1016/S0380-1330(04)70333-0)
- Gouin, T. (2020). Toward an improved understanding of the ingestion and trophic transfer of microplastic particles: Critical review and implications for future research. *Environmental Toxicology and Chemistry*, 39(6), 1119–1137. <https://doi.org/10.1002/etc.4718>
- Grigorakis, S., Mason, S. A., & Drouillard, K. G. (2017). Determination of the gut retention of plastic microbeads and microfibers in goldfish (*Carassius auratus*). *Chemosphere*, 169, 233–238.
- Holmes, L. A., Turner, A., & Thompson, R. C. (2012). Adsorption of trace metals to plastic resin pellets in the marine environment. *Environmental Pollution*, 160, 42–48.
- Hudson, M. L., Costello, D. M., Daley, J. M., & Burton, G. A. (2019). Species-specific (*Hyalella azteca* and *Lymnea stagnalis*) dietary accumulation of gold nano-particles associated with periphyton. *Bulletin of Environmental Contamination and Toxicology*, 103(2), 255–260. <https://doi.org/10.1007/s00128-019-02620-2>
- Huerta Lwanga, E., Gertsen, H., Gooren, H., Peters, P., Salánki, T., Van Der Ploeg, M., Besseling, E., Koelmans, A. A., & Geissen, V. (2016). Microplastics in the terrestrial ecosystem: Implications for *Lumbricus terrestris* (Oligochaeta, Lumbricidae). *Environmental Science and Technology*, 50(5), 2685–2691. <https://doi.org/10.1021/acs.est.5b05478>
- International Association for Soaps, Detergents and Maintenance Products. (2018). *Fragrance encapsulation technology in detergents in the framework of a potential REACH restriction on microplastics*.
- Jin, Y., Xia, J., Pan, Z., Yang, J., Wang, W., & Fu, Z. (2018). Polystyrene microplastics induce microbiota dysbiosis and inflammation in the gut of adult zebrafish. *Environmental Pollution*, 235, 322–329.
- Kershaw, P. (2015). *Sources, fate and effects of microplastics in the marine environment: A global assessment*. International Maritime Organization.
- Kosfeld, V., Fu, Q., Ebersbach, I., Esser, D., Schauerte, A., Bischof, I., Hollender, J., & Schlechtriem, C. (2020). Comparison of alternative methods for bioaccumulation assessment: Scope and limitations of in vitro depletion assays with rainbow trout and bioconcentration tests in the freshwater amphipod *Hyalella azteca*. *Environmental Toxicology and Chemistry*, 39(9), 1813–1825.

- Kuehr, S., Diehle, N., Kaegi, R., & Schlechtriem, C. (2021). Ingestion of bivalve droppings by benthic invertebrates may lead to the transfer of nanomaterials in the aquatic food chain. *Environmental Sciences Europe*, 33(1), 1–16.
- Kuehr, S., Kaegi, R., Maletzki, D., & Schlechtriem, C. (2020). Testing the bioaccumulation potential of manufactured nanomaterials in the freshwater amphipod *Hyalella azteca*. *Chemosphere*, 263, Article 127961.
- Kuehr, S., Klehm, J., Stehr, C., Menzel, M., & Schlechtriem, C. (2020). Unravelling the uptake pathway and accumulation of silver from manufactured silver nanoparticles in the freshwater amphipod *Hyalella azteca* using correlative microscopy. *NanoImpact*, 19, Article 100239. <https://doi.org/10.1016/j.impact.2020.100239>
- Kuehr, S., Kosfeld, V., & Schlechtriem, C. (2021). Bioaccumulation assessment of nanomaterials using freshwater invertebrate species. *Environmental Sciences Europe*, 33(1), Article 9. <https://doi.org/10.1186/s12302-020-00442-2>
- Kuehr, S., Meisterjahn, B., Schröder, N., Knopf, B., Völker, D., Schwirn, K., & Schlechtriem, C. (2020). Testing the bioaccumulation of manufactured nanomaterials in the freshwater bivalve *Corbicula fluminea* using a new test method. *Environmental Science: Nano*, 7(2), 535–553. <https://doi.org/10.1039/c9en01112a>
- Kuehr, S., Schneider, S., Meisterjahn, B., Schlich, K., Hund-Rinke, K., & Schlechtriem, C. (2018). Silver nanoparticles in sewage treatment plant effluents: Chronic effects and accumulation of silver in the freshwater amphipod *Hyalella azteca*. *Environmental Sciences Europe*, 30(1), Article 7. <https://doi.org/10.1186/s12302-018-0137-1>
- Lei, L., Wu, S., Lu, S., Liu, M., Song, Y., Fu, Z., Shi, H., Raley-Susman, K. M., & He, D. (2018). Microplastic particles cause intestinal damage and other adverse effects in zebrafish *Danio rerio* and nematode *Caenorhabditis elegans*. *Science of the Total Environment*, 619–620, 1–8.
- León, G., Paret, N., Fankhauser, P., Grenno, D., Erni, P., Ouali, L., & Berthier, D. L. (2017). Formaldehyde-free melamine microcapsules as core/shell delivery systems for encapsulation of volatile active ingredients. *RSC Advances*, 7(31), 18962–18975.
- Li, X., Chen, L., Mei, Q., Dong, B., Dai, X., Ding, G., & Zeng, E. Y. (2018). Microplastics in sewage sludge from the wastewater treatment plants in China. *Water Research*, 142, 75–85. <https://doi.org/10.1016/j.watres.2018.05.034>
- Lwanga, E. H., Gertsen, H., Gooren, H., Peters, P., Salánki, T., van der Ploeg, M., Besseling, E., Koelmans, A. A., & Geissen, V. (2017). Incorporation of microplastics from litter into burrows of *Lumbricus terrestris*. *Environmental Pollution*, 220, 523–531.
- McMahon, R. F. (2000). Invasive characteristics of the freshwater bivalve *Corbicula fluminea*. In Claudi, R. & Leach, J., (Eds.), *Nonindigenous freshwater organisms: Vectors, biology and impacts* (pp. 315–343). Lewis.
- Mitrano, D. M., Beltzung, A., Frehland, S., Schmiedgruber, M., Cingolani, A., & Schmidt, F. (2019). Synthesis of metal-doped nanoplastics and their utility to investigate fate and behaviour in complex environmental systems. *Nature Nanotechnology*, 14(4), 362–368. <https://doi.org/10.1038/s41565-018-0360-3>
- Organisation for Economic Co-operation and Development. (2010). Test No. 317: Bioaccumulation in terrestrial oligochaetes. *OECD guidelines for the testing of chemicals*.
- Organisation for Economic Co-operation and Development. (2019). *Guidance document on aquatic toxicity testing of difficult substances and mixtures*. Series on testing and assessment, No. 23. ENV/JM/MONO (2000)6/REV1.
- Othman, M. S., & Pascoe, D. (2001). Growth, development and reproduction of *Hyalella azteca* (Saussure, 1858) in laboratory culture. *Crustaceana*, 74(2), 171–181.
- Paret, N., Trachsel, A., Berthier, D. L., & Herrmann, A. (2019). Developing multi stimuli-responsive core/shell microcapsules to control the release of volatile compounds. *Macromolecular Materials and Engineering*, 304(3), Article 1800599.
- Poinar, G. O. (1978). Associations between nematodes (Nematoda) and oligochaetes (Annelida). *Proceedings of the Helminthological Society of Washington*, 45(2), 202–210.
- Postle, M., Daly, E., La Vedine, M., & Upson, S. (2018). *IFRA Europe 2018. Survey of fragrance encapsulation systems*. Risk & Policy Analysts (RPA) J10007 IFRA Survey. International Fragrance Association.
- Raths, J., Kuehr, S., & Schlechtriem, C. (2020). Bioconcentration, metabolism, and spatial distribution of ¹⁴C-labeled laurate in the freshwater amphipod *Hyalella azteca*. *Environmental Toxicology and Chemistry*, 39(2), 310–322. <https://doi.org/10.1002/etc.4623>
- Rillig, M. C., Ziersch, L., & Hempel, S. (2017). Microplastic transport in soil by earthworms. *Scientific Reports*, 7(1), Article, 1362. <https://doi.org/10.1038/s41598-017-01594-7>
- Slechtriem, C., Kampe, S., Bruckert, H.-J., Bischof, I., Ebersbach, I., Kosfeld, V., Kotthoff, M., Schäfers, C., & L'Haridon, J. (2019). Bioconcentration studies with the freshwater amphipod *Hyalella azteca*: Are the results predictive of bioconcentration in fish? *Environmental Science and Pollution Research*, 26(2), 1628–1641. <https://doi.org/10.1007/s11356-018-3677-4>
- Schmidt, J. E., Christensen, N., Batstone, D. J., Trably, E., Lyberatos, G., Stamatiadou, K., Komaros, M., Metzger, L., Amellal, N., Watson, J., García, K., Ayuso, S., & Patureau, D. (2006). Safe recycling of sewage sludge on agricultural land-biowaste. *Process Safety and Environmental Protection*, 84(4), 253–257. <https://doi.org/10.1205/psep.05168>
- Schultz, S. G. (1976). Transport across epithelia: Some basic principles. *Kidney International*, 9(2), 65–75.
- Schür, C., Rist, S., Baun, A., Mayer, P., Hartmann, N. B., & Wagner, M. (2019). When fluorescence is not a particle: The tissue translocation of microplastics in *Daphnia magna* seems an artifact. *Environmental Toxicology and Chemistry*, 38(7), 1495–1503.
- Von Moos, N., Burkhardt-Holm, P., & Köhler, A. (2012). Uptake and effects of microplastics on cells and tissue of the blue mussel *Mytilus edulis* L. after an experimental exposure. *Environmental Science and Technology*, 46(20), 11327–11335. <https://doi.org/10.1021/es302332w>
- Wang, F., Goulet, R. R., & Chapman, P. M. (2004). Testing sediment biological effects with the freshwater amphipod *Hyalella azteca*: The gap between laboratory and nature. *Chemosphere*, 57(11), 1713–1724. <https://doi.org/10.1016/J.CHEMOSPHERE.2004.07.050>
- Wang, J., Liu, X., Liu, G., Zhang, Z., Wu, H., Cui, B., Bai, J., & Zhang, W. (2019). Size effect of polystyrene microplastics on sorption of phenanthrene and nitrobenzene. *Ecotoxicology and Environmental Safety*, 173, 331–338. <https://doi.org/10.1016/j.ecoenv.2019.02.037>
- Wegner, A., Besseling, E., Foekema, E. M., Kamermans, P., & Koelmans, A. A. (2012). Effects of nanoplastyrene on the feeding behavior of the blue mussel (*Mytilus edulis* L.). *Environmental Toxicology and Chemistry*, 31(11), 2490–2497. <https://doi.org/10.1002/etc.1984>
- Zeumer, R., Hermsen, L., Kaegi, R., Kühr, S., Knopf, B., & Schlechtriem, C. (2020). Bioavailability of silver from wastewater and planktonic food borne silver nanoparticles in the rainbow trout *Oncorhynchus mykiss*. *Science of the Total Environment*, 706, Article 135695. <https://doi.org/10.1016/j.scitotenv.2019.135695>