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Hazard/Risk Assessment

Moving Toward Standardized Toxicity Testing Procedures with Particulates by Dietary Exposure of Gammarids

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Abstract: Ecotoxicological effect assessment of particulate materials and sparingly soluble substances is an emerging field. Current standard toxicity tests of aquatic organisms are based on soluble substances which are added to the aqueous phase. Although soluble substances distribute homogeneously, particles can form aggregates, resulting in inhomogeneous distribution and unpredictable exposure. Therefore, test scenarios need to be adapted to overcome these uncertainties. We present a dietary particle exposure tool for the toxicity testing of sparingly soluble substances or particles in combination with a standardizable food source for gammarids based on decomposition and consumption tablets (DECOTABs). Four food supplements in the DEOCOTAB formulation were compared to test their influence on the energy reserves of gammarids. Although feeding rate was constant for most supplements, mortality and energy reserves revealed clear differences. Tabs supplemented with algae-based phyll or animal protein–based trout food best met all of the requirements. Fluorescent plastic microparticles (10–65 µm) were homogenously distributed and stable in the DECOTABs. Constant feeding was observed, and the number of ingested microparticles by *Gammarus roeseli* was quantified in relation to the consumed food. The developed method provides a realistic and methodologically reliable uptake from the oral pathway and allows the quantification of inner exposition via feeding rate, providing a promising tool for standardized dietary exposure scenarios with particles. *Environ Toxicol Chem* 2021;40:1463–1476. © 2021 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

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INTRODUCTION

In the context of environmental risk assessment, it is necessary to conduct standardized ecotoxicological bioassays that can be linked to realistic exposure scenarios (Connon et al. 2012). Such investigations are well established for many organisms from aquatic environments and are available as Organisation for Economic Co-operation and Development guidelines, mainly for cladocera, algae, and fish. However, the present guidelines target the exposure with soluble substances, limiting their applicability for toxicity testing of particles or sparingly soluble substances. In contrast to soluble substances, particles or sparingly soluble substances have other chemical properties, resulting in entirely different distribution in the test medium and therefore affecting uptake routes (Rufli et al. 1998; Hartmann et al. 2015; European Centre for Ecotoxicology and Toxicology of Chemicals 2018; Eitzen et al. 2019). In addition, reliable methods for prediction of resulting particle distributions and exposure are lacking (Rist and Hartmann 2018; Eitzen et al. 2019). This lowers the general comparability of the aquatic ecotoxicological studies on particle effects (Hartmann et al. 2015) and stresses the need for ecotoxicological bioassays that account for particle-specific properties. With respect to sparingly soluble substances, it is more likely that exposure via the food is the main uptake route compared to the surrounding water column (Cole et al. 2011; Bundschuh et al. 2019; Toussaint et al. 2019), which is thereby often neglected.

Therefore, a testing procedure which allows a standardized and systematic testing of particulates or chemicals via the oral pathway is needed (Bundschuh et al. 2019). Such a dietary exposure system would need to fulfill specific prerequisites to ensure a standardized and quantifiable uptake of the substances during the feeding process. This includes comparability

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in production, homogeneity of the substrate, stability, and minimal weight variation during the test. Further prerequisites are a homogenous distribution of the sparingly soluble substances in the food without aggregate formation, adjustable dosimetry, and minimal leaching of embedded sparingly soluble substances. Also, constant feeding by the test organisms is necessary to allow quantification of embedded and ingested particulates.

Decomposition and consumption tablets (DECOTABs; Kampfraath et al. 2012) can meet these prerequisites and have been successfully used as a food source in aquatic toxicity tests (Straub et al. 2017; Raths et al. 2020). It was recommended by the authors, and applied by several studies, that DECOTABs can be amended by adding food supplements for specific applications or loading them with chemicals during production (Kampfraath et al. 2012). Some exposure studies have already used loaded tabs (Zhai et al. 2018) or similar matrices (Imhof et al. 2013; Hämer et al. 2014; Imhof and Laforsch 2016; Fenoy et al. 2020; Yardy and Callaghan 2020). Therefore, DECOTABs are a promising tool for a standardized toxicity testing procedure with organisms from different functional feeding groups such as shredders and grazers.

Decomposition and consumption tablets already meet some of the stated prerequisites such as minimal weight variation and quantifiable uptake, which helps to ensure a high comparability between different experimental runs (Kampfraath et al. 2012). Nevertheless, a deeper examination concerning the needs of the targeted organism regarding nutritional value and survival by adding food supplements is still required. Subsequent upcoming prerequisites are stability of the supplemented DECOTABs, the distribution of the particles in the DECOTABs, and the leaching of particles to the surrounding medium.

In the present study, we focus on a river organism because many sensitive organisms of lotic environments are underrepresented in established guidelines (Feiner et al. 2016). Suitable organisms for ecotoxicity testing of riverine ecosystems are *Gammarus* spp., which are already often examined and known to be sensitive to many pollutants (Gerhardt et al. 2011; Brock and van Wijngaarden 2012). The characteristics of *Gammarus* spp., like a wide trophic repertoire, foraging plasticity, and migration ability, make this keystone species in the food web a representative benthic river organism (Gerhardt et al. 2011; Boeker and Geist 2015). In addition, a basic guideline for ecological testing with gammarids is provided by the US Environmental Protection Agency (2016).

With the objective of moving toward a standardized protocol for toxicity testing with gammarids, we first evaluated the suitability of DECOTAB formulations with different food supplements as a food source for *Gammarus roeseli*. This was tested based on food source properties, gammarid response in feeding behavior, and energy content. In a second step, to adapt the protocol for testing with particulates, we evaluated the applicability of the test system as a dietary exposure tool of particles or with regard to aggregates and homogeneous distribution sparingly soluble substances by testing the practicability of loading the adjusted food source with microparticles and the constant and quantifiable supply of particles over the oral exposure pathway.

MATERIAL AND METHODS

Evaluation of DECOTABs with food supplements

To examine a nutritionally valuable and standardizable food source for toxicity testing with gammarids, *G. roeseli* were fed over the course of 21 d with different formulations based on the DECOTAB as originally described by Kampfraath et al. (2012). This formulation, filled with cellulose and 4 DECOTAB formulations modified with specific food supplements, was tested for variability in weight, based on size and initial dry weight, and stability over time. Stability in water was measured during a watering experiment for 3 and 4 d. Next to mortality and the feeding behavior of the gammarids, their energy reserves were monitored by the measurement of lipids, glycogen, and glucose.

The DECOTABs

Formulation and preparation. The DECOTABs (Kampfraath et al. 2012) were produced with 80 mL distilled water and 1.6 g agar (Sigma-Aldrich). This mixture was heated for approximately 1.5 min until it foamed and homogenized with an agitator. Then 4.8 g cellulose (Sigma-Aldrich) was added and homogenized for 1 min. The mixture was poured into 50 cylindrical molds of 1 cm diameter and 0.5 cm height (custom-built stainless steel device; Chair of Process Systems Engineering, Technical University of Munich). The supernatant was scraped using an even scraper. After 15 min in the refrigerator at 6 °C, the DECOTABs were removed from the molds and dried in a drying cabinet (U 40; Memmert) at 45 °C for 24 h. After this, the DECOTABs were placed into a desiccator for 30 min, and initial dry weight (dw_I) was measured with a Sartorius R200D Analytical Balance (Sartorius; 0.01 ± 0.02 mg). Before the transfer to the experiment, the DECOTABs were prewetted in the test medium for 48 h in the climate chamber at 13 ± 0.5 °C.

For the modified DECOTABs, the same procedure was used, but 70% of the cellulose weight was replaced with the particular food supplement. This results in 1.44 g cellulose and 3.36 g of the food supplement added to 80 mL distilled water. The following 4 DECOTAB formulations with specific food supplements were tested in addition to the cellulose-tab, solely containing cellulose and agar: phyll-tabs (ground flakes of the algae-based fish food Phyll; Tetra), gammarus-tabs (powdered dried gammarids; Dehner), trout food-tabs (ground pellets of the commercial trout food Advance, 0.2–0.3 mm; Alltech Coppens), and beech-tabs (dried and powdered beech leaves; Figure 1).

Variability (size, volume, dry wt). To evaluate the effect of drying and prewetting on the DECOTABs and to evaluate dry weight stability, 150 cellulose-tabs were weighed at different steps of handling using a fine scale (Sartorius; 0.01 ± 0.02 mg). Initial wet weight (ww_I) of freshly prepared but not dried

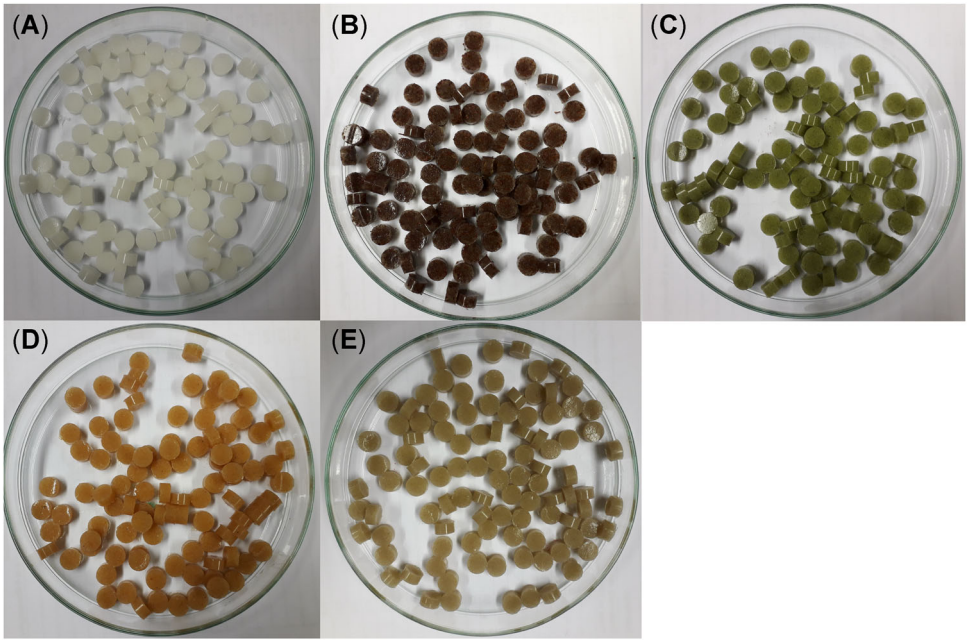

FIGURE 1: Prepared decomposition and consumption tablets before first drying. (**A**) Cellulose-tabs, (**B**) beech-tabs, (**C**) phyll-tabs, (**D**) gammarus-tabs, and (**E**) trout food-tabs.

DECOTABs was measured for 3 production iterations ($n = 50$) to evaluate wet weight variation. In addition, the dw_I and the final dry weight (dw_F) after 48 h prewetting were measured to examine the dry weight variation within and between the dry weight states. Further, the wet weight after prewetting of dried DECOTABs for 24 and 48 h was measured to verify whether their ability to absorb the water again.

Stability in water. Weight loss of the different DECOTAB formulations in water without further impact was monitored to evaluate the stability during bioassays. Therefore, 14 DECOTABs of each food supplement were weighed to examine dw_I, transferred into tap water, and prewetted for 2 d in the climate chamber (13 ± 0.5 °C) prior to the experiment. To mimic the food replacement cycle of the bioassay with gammarids, the experimental procedure was as follows. After prewetting, 7 DECOTABs of each food supplement were placed into glass beakers (1 L) filled with 500 mL tap water, 3 glass stones for hiding, but no gammarids and kept in a climate chamber with a 16:8-h light:dark cycle and 13 ± 0.5 °C. After 4 d, the DECOTABs were replaced for another 3 d by the remaining 7 DECOTABs. After the experiment, the DECOTABs were dried again for 24 h, placed into a desiccator for 30 min, and weighed with a Sartorius R200D Analytical Balance (0.01 ± 0.02 mg) to measure the dw_F. The difference between dw_I and dw_F as a percentage per day gives the stability for each DECOTAB formulation and allows comparisons between the treatments. In addition, the dry weight difference in milligrams per day was used as an adjustment factor for the feeding rate of the gammarids.

The bioassay. *Gammarus roeseli* were caught from the River Moosach at the Aquatic Systems Biology Unit in Freising, Germany, with plant-filled traps and trout food as bait in November 2019. Subsequently, gammarids were size-selected (9.9 ± 1.5 mm, $n = 534$) by sieve passage (Beggel et al. 2016), and their body size was determined as described below (see *Gammarid length and dry wt*).

To assess their natural energy reserve state, 100 *G. roeseli* were directly transferred to liquid nitrogen and stored at −20 °C until further analysis.

For acclimatization to the test conditions, 500 gammarids were evenly allocated to the 5 treatments and transferred to aerated 5-L glass beakers with 5 L of tap water and glass stones. Acclimatization occurred in a climate chamber for 1 wk with a 16:8-h light:dark cycle and 13 ± 0.5 °C. The 100 gammarids per treatment were fed ad libitum with 10 DECOTABs of the formulation corresponding to the treatment. For determination of energy reserves after the acclimatization period (acclimatization state), 20 randomly chosen gammarids from each treatment were transferred to liquid nitrogen and stored at −20 °C until further analysis.

After the acclimatization period, 3 randomly chosen gammarids were placed into each of the 21 glass beakers (1 L) per treatment filled with 500 mL tap water, 3 glass stones for hiding, and one dried and preweighed DECOTAB. The experiment took place under the same conditions as the acclimatization period, except aeration. Instead, water change was conducted once per week and oxygen content, conductivity, temperature, and pH were monitored. All parameters were constant throughout the experiment, and oxygen concentration was always >7.8 mg/L. Gammarids were fed with one DECOTAB of the corresponding formulation per experimental beaker in an alternating exchange interval of 4 and 3 d. Because the remains of approximately 67 ± 7% of the DECOTABs were found in the glasses after 3 or 4 d, ad libitum feeding can

be anticipated. Mortality was controlled daily, and dead gammarids were removed from the beaker and stored at –20 °C until further examination.

After 1, 2, and 3 wk, 7 replicates were removed from the experiment; and the corresponding individuals were transferred to liquid nitrogen and stored at –20 °C until measurement of the energy reserves. In addition, 42 gammarids were starved for 16 d, then transferred to liquid nitrogen, and stored at –20 °C until further examination of the energy reserves from starvation state.

Feeding rate. To determine the feeding rate, DECOTABs were dried for 24 h to measure dw_I, then they were watered again for 48 h prior to the transfer to the experimental beakers. The DECOTABs were replaced by new ones in an alternating cycle of 4 and 3 d. The used DECOTABs were again dried for 24 h to determine dw_F. The feeding rate (FR) per day and gammarid (gd) was calculated with dw_I and dw_F of the specific DECOTAB, adjusted by the number of feeding days and the mean
milligrams of weight loss per day (MWL) calculated from the stability measurement:

$$FR\left[\frac{mg}{gd \times day}\right] = \frac{\frac{dw_I(mg) - dw_F(mg)}{\text{feeding days}} - MWL\left[\frac{mg}{day}\right]}{n\ (\text{living gammarids})} \quad (1)$$

Gammarid length and dry weight. Prior to further processing of the gammarids, samples were defrosted, and pictures were taken with a stereomicroscope (M3Z; Wild Heerbrugg; equipped with an SC180 camera; Olympus). The size of the gammarids was determined by measuring the length from the base of the first antenna to the end of the uropod by a polyline along the gut using an image analysis system (CellSens Entry, Ver 1.18; Olympus) according to Burgherr and Meyer (1997). After the measurements, gammarids were dried at 45 °C for 24 h, placed into a desiccator for 30 min, and weighed with a Sartorius R200D Analytical Balance (0.01 ± 0.02 mg) to examine gammarid dry weight.

Energy reserve determination. The energy storage assay followed the original protocol by van Handel (1985) and the modifications by Charron et al. (2014), with some further adjustments. The assay for lipids and the carbohydrates glycogen and glucose is described in brief; a schematic view is available in the Supplemental Data. Dried gammarids were frozen with liquid nitrogen in a 1.5-mL centrifuge tube and ground to a fine powder with a stainless-steel pistil. Powdered gammarids were suspended in 900 µL methanol. Aliquots were transferred into new 1.5-mL tubes for the lipid assay and the glucose/glycogen assay. Energy reserves were calculated according to de Coen and Janssen (1997) using the energy per milligram of storage substance with 39 500 mJ/mg lipids and 17 500 mJ/mg glucose or glycogen.

Lipid assay. Chloroform was added to the lipid aliquot (2:1 ratio) to extract the lipids from the powder. After mixing thoroughly, the suspension was cooled for 20 min at 4 °C, and 600 µL were transferred to glass test tubes. The suspension was heated for 5 min at 95 °C until the solvent evaporated. After adding 200 µL sulfuric acid (95% v/v), the suspension was heated again at 95 °C for 10 min and cooled in an ice bath. By adding 5 mL vanillin-phosphoric acid a color change from colorless to pink was achieved. Photometric measurement was performed using a UVIKON 930 photometer at 525 nm against the reagent as blank. The amount of absorption shows the amount of lipids on the basis of a calibration curve. Lipids were calculated in micrograms per milligram dry weight.

Glucose and glycogen assay. Sodium sulfate (2%, 200 µL) was added to the glucose/glycogen aliquot to precipitate the glycogen. After mixing thoroughly, the suspension was cooled for 20 min at 4 °C and centrifuged for 4 min at 11 000 *g* (Centrifuge 5430R; Eppendorf). The supernatant with the glucose was transferred into a new 1.5-mL tube. The remaining glycogen pellet was resuspended with 400 µL distilled water. Then, 400 µL from each suspension were separately transferred to glass test tubes. After the addition of 5 mL anthron reagent, the suspensions were heated at 95 °C for 17 min. A color change from yellow to green allowed a photometric measurement. Photometric measurement was performed using a UVIKON 930 at 625 nm against the reagent as blank. The amount of absorption shows the amount of glucose and glycogen on the basis of a calibration curve. Glycogen and glucose were calculated in micrograms per milligram dry weight.

DECOTABs as an oral exposure tool for particles

Decomposition and consumption tablets were examined as a dietary exposure tool for particle testing. Therefore, a suspension of red fluorescent polystyrol microparticles (10–65 µm) was prepared and added to the DECOTABs during preparation. These particles were tracked in sections of the DECOTABs by fluorescence microscopy, and the concentration per milligram and volume was calculated. Afterward, gammarids were exposed to blue fluorescent microparticle-filled phyll-tabs, and the inner particle concentration was monitored by gut dissection and subsequent fluorescence microscopy. Spherical fluorescent polystyrol nanoparticles of 1000 nm were likewise embedded in phyll-tabs and imaged to get first impressions of nanoparticle distribution.

Particle distribution in the DECOTABs. To receive red fluorescent polystyrene microplastic particles with a fraction of 10 to 65 µm, 200 mg of fluorescent microparticles generated by centrifugal milling (0–125 µm, Ultra Centrifugal Mill Type ZM 200; Retsch) were suspended in 54 mL ethanol (96%, undenatured) and centrifuged according to Correia and Loeschner (2018) for 44 s at 8.5 *g* to separate particles at 65 µm. The supernatant was transferred into new centrifuge tubes and centrifuged again for 1 min at 95 *g* (Labofuge 400; Heraeus Instruments). Supernatant with the 10- to 65-µm particles was removed by pipetting, and the pellet was resuspended in 8 mL ethanol to a concentration of approximately 31 particles/µL.

The size distribution (Supplemental Data, Figure 1SI) was verified using a Mastersizer S longbed (Malvern).

Stock suspension (3200, 1600, or 160 µL) was added during the DECOTAB production after the homogenization of the formulation for 1 min (for detailed production, see above, *The DECOTABs*). The suspension was again homogenized for approximately 30 s and poured into the molds. Further DECOTAB handling was as described.

One to 3 horizontal sections with 50 µm thickness from 2 dried DECOTABs for the lowest and middle and 3 from the highest concentration were generated with a microtome cryostat HM 505 E (Microm) and from one DECOTAB per concentration in the vertical direction. Sections were mounted on microscopic slides with the use of VECTASHIELD Antifade Mounting Medium (Vector Labs) and imaged under a Leica DMi8 with a CoolLED Pe4000 light source (Leica Microsystems) with green fluorescent protein excitation for green phyll-tab fluorescence and rhodamine (Rhod) excitation for red particle fluorescence. Images were exported, and extended depth-of-field was calculated from the approximately 20 Z-planes to project all particles into one single layer using FiJi (Schindelin et al. 2012) and the method of Forster et al. (2004). Fluorescence channels were merged and stitched using the method of Preibisch et al. (2009). The number of fluorescent microplastic particles was counted to particles per cubic millimeter and particles per milligram dry weight, and their distribution within the sections was analyzed.

For an exemplary examination of the distribution of polystyrene beads in the DECOTABs, 250 µL of a 5 wt% polystyrol 1000 nm (nominal mean, mean diameter 1294 nm) fluorescent beads solution (BS-Partikel) was added during phyll-tab production. One horizontal section with resulting 10 µm thickness was cut with the microtome (adjusted to 2 µm slice thickness) and imaged with the Leica Thunder imaging system (DM6B-Z microscope, DFC9000GT camera, and LAS X software, Ver 3.0.2.7506) with large-volume computational clearing.

Particle leaching from the DECOTABs. Six particle-loaded phyll-tabs with polystyrene microparticles with a fraction of 10 to 65 µm, were dried, transferred into tap water, and prewetted for 2 d in the climate chamber (13 ± 0.5 °C) prior to the experiment. After prewetting, the loaded phyll-tabs were placed into glass beakers (1 L) filled with 500 mL tap water, 3 glass stones for hiding, but no gammarids and kept in a climate chamber with a 16:8-h light:dark cycle and 13 ± 0.5 °C. After 4 d, the DECOTABs were removed and the water was filtered through a 0.8-µm filter (47 Ø; Merck Millipore). Beakers and filter stations were flushed twice with distilled water to remove particles attached to the glass. The whole surface of each filter was examined with a fluorescence microscope (Laborlux S; Leitz; equipped with a DP74 camera with CellSens Standard, Ver 1.18) to count the leached particles.

Uptake of the particles via DECOTABs. Phyll-tabs were loaded with blue fluorescent polystyrene microparticles of 10 to 60 µm with 1% particle weight of tab weight, resulting in approximately 40 000 particles per tab. Estimation is based on particle concentration and the amount of stock suspension used. Loaded phyll-tabs were used in an exposure experiment with *G. roeseli* to characterize the particle uptake and compare it with the feeding rate. Therefore, gammarids were exposed to 1) particle-free phyll-tabs as a negative control, and 2) particle-loaded phyll-tabs as a positive control. This experiment was conducted under the same conditions as described in above (see *The Bioassay*), only the starvation during the acclimatization was conducted as recommended by the US Environmental Protection Agency (2016).

After 1 wk of acclimatization, 3 randomly chosen individuals were placed in each beaker with 500 mL tap water and 3 glass stones for hiding. Dried and preweighed DECOTABs were changed every day to measure the feeding precisely. Also, gammarids were transferred to new beakers in daily rhythm to minimize the uptake of leached particles from the surrounding medium. Every day for 2 wk, 5 replicates were taken from the negative and the positive controls, and the gammarids were anesthetized with carbon dioxide and fixed in 7.5% formaldehyde with 7.5 g/100 mL glucose. Used particle-loaded phyll-tabs were dried and weighed again. Feeding rate was calculated as described above (see *The Bioassay*) and compared between the negative and positive controls. Guts were removed from fixed gammarids, mounted on microscopic slides with VECTASHIELD Antifade Mounting Medium, and imaged with a stereomicroscope (M3Z; Wild Heerbrugg; equipped with an SC180 camera; Olympus) to measure the gut length with CellSens Entry (Ver 1.18). The ingested particles were counted under fluorescence excitation at 340 to 380 nm (Laborlux S; Leitz; equipped with a DP74 camera with CellSens Standard, Ver 1.18). Further, feeding rate and particles per centimeter of gut were set in relation to validate whether the inner particle concentration can be calculated by the measurement of the mean mass eaten alone.

Statistical analysis

Statistical analyses were conducted with Rstudio (RStudio 2015). Normal distribution was tested with the Shapiro-Wilk test, followed by the robust Fligner test for homogeneity of variance. Gammarid dry weight per millimeter body length was normally distributed and homogeneous in variance; therefore, further analysis was conducted with analysis of variance. All other endpoints including feeding, lipids, glucose content, glycogen content, and energy reserves as well as ingested particles were tested with the Kruskal-Wallis test, followed by a post hoc pairwise Wilcoxon test with the Benjamini-Hochberg correction (Benjamini et al. 1998). Differences in risk for mortality were tested by survival analysis with the Kaplan-Meier model and log-rank test using Jamovi (Jamovi Project 2019) based on the R language. Particle distribution in the DECOTABs was examined based on the XY coordinates with PAST, Ver 4.01 (Hammer et al. 2001) and point pattern analysis with nearest neighbor classification and wrap-around edge correction. Clustered points give ratio $R < 1$, Poisson patterns give R of approximately 1, whereas overdispersed points give $R > 1$. Correlation of particles per centimeter of gut and

feeding rate was conducted with Rstudio with the Kendall method. A correlation coefficient of $r = 0$ implies no correlation, whereas $r = 1$ or -1 shows a strong positive or negative correlation, respectively. The p values for summarized comparisons are given as a minimum p value for nonsignificant comparisons and a maximum p value for significant comparisons.

RESULTS

The DECOTABs

Variability (size, volume, weight). The size of the freshly prepared DECOTABs was 10 mm in diameter and 5 mm in height before drying. When dried, they shrank to 4.0 to 4.5 mm in diameter and 2.0 to 2.5 mm in height but nearly retained their shape. The production iteration of cellulose-tabs revealed variable ww_I (8.5% relative standard deviation) within the iterations ($p = 0.004$), but the dw_I remained stable ($p = 1.000$) and was on average $10.5 \pm 0.5\%$ of the ww_I.

A comparison of the dry weight of the cellulose-tabs with the other formulations revealed that the cellulose-tabs varied more in dry weight than the modified tabs ($p < 0.001$; Supplemental Data, Table 1SI). Trout food-tabs were lightest, and variation of the dry weight was lowest. The dry weight of phyll-, gammarus-, and beech-tabs was nearly the same as for the cellulose-tabs ($p = 0.061$); but the dry weight variation of phyll-tabs was 50% smaller than the variation of the cellulose-tabs. Beech- and gammarus-tabs had medium dry weights and variations 75% lower than the cellulose-tabs.

If watered again, the DECOTABs readsorbed water and reached a maximum wet weight of 60% compared to the ww_I independent of the watering duration ($p = 0.461$). When dried a second time after watering, the dw_F of the DECOTABs was still 10.1% of the ww_I and thus, the dry weight before and after watering was the same ($p = 0.747$).

Stability in water. All DECOTABs were relatively stable in water, with a daily weight loss between 2.0 ± 2.2 and $8.1 \pm 1.8\%$ (Supplemental Data, Table 1SI). Cellulose- and beech-tabs revealed the lowest weight loss per day ($p < 0.001$). The least stable DECOTABs were those with gammarus as a supplement ($p = 0.003$). Phyll- and trout food-tabs lost weight to the same extent of approximately 6.3% ($p = 0.324$) and are thus in the middle between the other DECOTABs. Remarkably, the trout food-tabs had the lowest variation in weight loss per day.

The Bioassay

Mortality. Feeding the gammarids with cellulose-tabs resulted in the lowest risk for mortality over the course of 3 wk, with 11.5% after 21 d (Supplemental Data, Figure 2SI). The risk for mortality was also very low for gammarids fed with phyll-tabs (23.3%, $p = 0.061$). Feeding gammarids with trout food-tabs resulted in nearly the same low risk for mortality as those fed with phyll-tabs (26.4%, $p = 0.684$). Although the survival for gammarids fed with beech-tabs was similar to phyll- and trout food-tab-fed gammarids in the first 2 wk, the risk for mortality increased to 61.6% after the experimental duration of 21 d. Feeding gammarids with gammarus-tabs resulted in high mortality of approximately 40% already within the first 2 wk. At the end of the experiment a similar high risk for mortality compared with beech-tab-fed gammarids of 53.4% was seen ($p = 0.334$).

Feeding rate. All gammarids accepted the offered DECOTAB formulations as a food source, though no tab was fully consumed after feeding periods of 3 or 4 d. Constant feeding rates were observed in the 3 treatments with beech-tabs ($p = 1.000$, 0.31 ± 0.29 mg/d), trout food-tabs ($p = 0.140$, 0.34 ± 0.41 mg/d), and phyll-tabs ($p = 0.059$, 0.40 ± 0.49 mg/d). This resulted in an equal amount of food eaten after 21 d ($p = 0.410$) of 12.60 ± 5.59 mg in sum for trout food-tab-fed, 12.60 ± 2.32 mg in sum for phyll-tab-fed, and 10.00 ± 4.43 mg in sum for beech-tab-fed gammarids.

Although, the highest DECOTAB mass was consumed from the gammarus-tabs with 27.10 ± 11.10 mg ($p < 0.001$), the feeding rate was very variable and decreased or increased over time ($p < 0.001$), with minimum feeding of 0.352 ± 0.240 mg/d in week 1 to a maximum feeding of 1.99 ± 1.11 mg/d in week 2 (Supplemental Data, Figure 3SI). Likewise, the feeding rate on the cellulose-tabs increased from the beginning (0.44 ± 0.29 mg/d) until the end (2.39 ± 0.69 mg/d, $p < 0.001$) of the experiment. After 21 d, gammarids were in sum consuming the second highest amount of the cellulose-tabs ($p = 0.052$, 25.30 ± 7.66 mg).

Additional weight loss occurred for the cellulose-tabs because they lose material if touched under water, for example, by the gammarids while swimming or feeding. Also, gammarids were observed pulling bigger pieces of the grounded beech leaves out of the beech-tabs but not ingesting them afterward. This weight loss was not calculable and is still included in the results; thus, it rather mirrors a shredding rate than a consumption rate.

Gammarid dry weight. The dry weight per millimeter of gammarid length decreased for the beech-tab-fed gammarids within 21 d ($p = 0.045$) and resulted in the lowest dry weight per millimeter at the end of the experiment (Table 1). Reduction in dry weight per millimeter over time was also observed for cellulose-tab-fed gammarids ($p = 0.044$), but at the end of the experiment they still had the same dry weight as the other treatments. The gammarids fed with phyll- ($p = 0.865$), gammarus- ($p = 0.577$), or trout food-tabs ($p = 0.664$) did not change in milligrams of dry weight per millimeter within the 3 wk (Table 1) and had comparable dry weight per millimeter at the end of the experiment ($p = 0.135$). Overall, the dry weight per millimeter of all 5 treatments was between the dry weight per millimeter of the natural and the starved states ($p = 0.126$). Only the natural-state gammarids had a higher weight than the starved ones ($p < 0.001$).

Energy reserve determination

Energy reserves. The energy reserves per milligram of dry weight were the same for all acclimatization treatments and the

TABLE 1: Mean and standard deviation of the dry weight ratio and the energy storage substances including energy reserves for gammarids in natural, starvation, and acclimatization state (0 d), and after 3 wk (21 d)[a]

Time (days)	Treatment	Dry weight ratio (mg/mm)	Glucose (µg/mg dry wt)	Glycogen (µg/mg dry wt)	Lipid (µg/mg dry wt)	Energy reserves (mJ/mg dry wt)
	Natural state	0.50 ± 0.12	13.90 ± 8.18	9.32 ± 6.52	76.10 ± 32.00	3395 ± 1293
	Starvation state	**0.38 ± 0.12**	**7.46 ± 3.97**	9.10 ± 4.28	**59.20 ± 21.50**	**2628 ± 853**
0	Cellulose	0.47 ± 0.09	***31.50 ± 14.30***	***36.30 ± 15.50***	**56.70 ± 14.70**	*3428 ± 872*
	Beech leaves	0.45 ± 0.10	***27.40 ± 16.50***	***21.00 ± 11.90***	68.80 ± 17.80	*3558 ± 863*
	Phyll	0.44 ± 0.08	***21.00 ± 10.60***	***18.20 ± 7.33***	*74.40 ± 25.00*	*3625 ± 1097*
	Gammarus	0.50 ± 0.13	*16.00 ± 10.70*	*13.50 ± 7.16*	69.50 ± 26.20	3260 ± 1173
	Trout food	0.46 ± 0.10	***22.20 ± 10.30***	***18.20 ± 7.75***	*81.70 ± 29.90*	*3935 ± 1234*
21	Cellulose	0.41 ± 0.09	*19.00 ± 10.60*	***30.60 ± 12.70***	**54.00 ± 13.40**	3000 ± 575
	Beech leaves	0.36 ± 0.09	**6.37 ± 2.97**	11.90 ± 5.03	**48.40 ± 9.44**	**2232 ± 412**
	Phyll	0.45 ± 0.11	*19.50 ± 10.90*	***15.90 ± 6.98***	66.00 ± 17.10	*3225 ± 777*
	Gammarus	0.49 ± 0.11	***28.40 ± 20.00***	***18.60 ± 8.69***	72.30 ± 28.70	3679 ± 1368
	Trout food	0.42 ± 0.13	***22.40 ± 11.60***	***16.30 ± 6.42***	*76.40 ± 17.00*	*3695 ± 836*

[a]Bold values indicate significant difference from natural state, italic values indicate significant difference from starvation state, underlined values indicate difference from acclimatization state.

natural-state gammarids ($p = 0.130$). The starved gammarids had approximately 35% less energy reserves than the natural-state gammarids and the gammarids acclimatized with beech-, trout food-, phyll-, or cellulose-tabs ($p = 0.007$). In contrast, those fed with gammarus had with 3260 ± 1173 mJ/mg dry weight the same energy reserves as both states ($p = 0.108$). Beside the differences in the natural and starvation states, the energy reserves between the treatments were the same for the acclimatization state ($p = 0.193$; Supplemental Data, Figure 4SI) at approximately 3500 ± 235 mJ/mg dry weight.

Comparing the treatments after 3 wk, only the phyll- and the trout food-tab-fed gammarids still had 30% more energy reserves per milligram of dry weight than the starved gammarids ($p = 0.035$). The treatments with gammarus- and cellulose-tabs led to energy reserves in the gammarids between the natural ($p = 0.560$) and starvation ($p = 0.067$) states. It has to be noted that beech-tab-fed gammarids lost 40% of their energy reserves compared to the acclimatized gammarids ($p = 0.002$) and had the lowest energy reserves out of the 5 treatments (Table 1). This results in lower energy reserves than the natural state ($p = 0.031$) and is comparable to starved organisms ($p = 0.551$).

Glucose. All treatments, including the natural-state gammarids, had from 90 to 320% more glucose per milligram of dry weight than the starved ones ($p = 0.001$; Table 1). The gammarids acclimatized with cellulose-tabs or those with beech leaves, phyll, or trout food had at least 50% more glucose than those in the natural state ($p = 0.011$); but those acclimatized on gammarus-tabs had the same glucose content as those in the natural state ($p = 0.806$). The gammarids acclimatized on the cellulose-tabs had the most glucose per milligram of dry weight, approximately 40% more compared to the other acclimatization treatments ($p = 0.065$), except for the gammarids acclimatized with beech-tabs ($p = 0.501$), which had only slightly lower glucose content. Gammarus-tab-acclimatized gammarids had 16.0 ± 10.7 µg glucose/mg dry weight and therefore the lowest glucose content (Table 1).

After 3 wk, glucose content in gammarids was stable for 3 out of 4 treatments ($p = 0.113$). The gammarids fed with trout food- or gammarus-tabs had 80% more glucose per milligram of dry weight than those in the natural state ($p = 0.028$) and 235% more than those in the starvation state ($p < 0.001$). In addition, phyll-tab-fed gammarids had 21.0 ± 10.6 µg glucose/mg dry weight and, thus, 180% more glucose than those in the starvation state ($p < 0.001$) and the same as those in the natural state ($p = 0.087$). Overall, gammarids from all 3 treatments had similarly high glucose content ($p = 0.463$).

The glucose content of the beech-tab-fed gammarids was reduced by up to 80% ($p = 0.002$) and, thus, was the same as those in the starvation ($p = 0.616$) and lower than those in the natural state ($p = 0.033$). Also, gammarids fed with cellulose-tabs contained after 3 wk 40% less glucose than after acclimatization ($p = 0.018$) and consequently the same amount as those in the natural state ($p = 0.125$) yet still more glucose than the starved gammarids ($p < 0.001$). Overall, beech-tab-fed gammarids contained 6.4 ± 3.0 µg glucose/mg dry weight and had the lowest glucose content compared to the other treatments at the end of the experiment ($p = 0.007$).

Glycogen. In sum, natural-state and starved gammarids had the same glycogen content ($p = 0.969$) of approximately 9.2 ± 5.4 µg glycogen/mg dry weight, and 4 out of the 5 acclimatization treatments had more than twice the amount of glycogen than both states ($p < 0.001$). The glycogen content (13.5 ± 7.2 µg/mg dry wt) of gammarus-tab-fed gammarids was lowest but still the same as for those in the natural state ($p = 0.066$). Glycogen per milligram of dry weight was highest for gammarids acclimatized with cellulose-tabs ($p < 0.001$).

After 3 wk, the cellulose-fed gammarids contained 30.6 ± 12.7 µg glycogen/mg dry weight and had still the highest glycogen content compared to the other treatments ($p = 0.013$), including the natural and starvation states ($p = 0.003$). As with the glucose content, the gammarids fed with trout food-, gammarus-, or phyll-tabs had all approximately 84 ± 14% more glycogen than those in the natural and

starvation states ($p = 0.003$). In contrast, the beech-tab-fed gammarids were not different in their glycogen content from natural-state and starved gammarids ($p = 0.221$), having the lowest glycogen content compared to the other treatments (Table 1). Overall, glycogen content was stable in all 5 treatments over the course of 3 wk ($p = 0.074$).

Lipids. Gammarids from the natural state and acclimatization fed with trout food- or phyll-tabs had approximately 30% more lipids per milligram of dry weight than those from the starvation state ($p = 0.042$; Table 1). In contrast, the lipid content of gammarids acclimatized with cellulose-, gammarus-, or beech-tabs was the same as for those in the starvation state ($p = 0.080$). Beside these differences, the 5 treatments were mainly similar ($p = 0.061$), except trout food-tab- and cellulose-tab-fed gammarids ($p = 0.024$). This is because trout food-tab-fed gammarids contained the most lipids (81.7 ± 29.9 µg/mg dry wt), and those fed with cellulose-tabs contained the lowest amount (56.7 ± 14.7 µg/mg dry wt).

After 3 wk, the lipid content of the gammarids was stable within the 4 treatments ($p = 0.487$). Only the beech-tab-fed gammarids lost 30% lipids per milligram of dry weight over the course of 3 wk ($p = 0.026$). The lipid content of gammarids fed with beech- or cellulose-tabs was approximately 51.2 ± 11.4 µg/mg dry weight, which was the lowest and similar to the starvation state ($p = 0.444$). The gammarus-, trout food-, or phyll-tab-fed gammarids had same amount of lipids per milligram of dry weight as those in the natural state, but only trout food-tab-fed gammarids had a higher lipid content than those in the starvation state ($p = 0.024$; Table 1). Nevertheless, the gammarids of these 3 treatments had together the highest amount of lipids per milligram of dry weight ($p = 0.217$) of approximately 66 ± 17.1 to 76 ± 17 µg lipids/mg dry weight.

DECOTABs as oral exposure tool for particles

Phyll-tabs were loaded with red fluorescent polystyrene microparticles of a size range from 10 to 65 µm. Randomly selected DECOTABs were sliced into 50-µm layers to evaluate the particle distribution and concentration. Further, adult *G. roeseli* were fed with the particle-loaded DECOTABs, and the feeding rate as well as the particles per centimeter of gut were measured to examine the usability of the DECOTABs as a dietary exposure tool.

Particle distribution in the DECOTABs. Analysis of the particle distribution in the particle-loaded DECOTABs revealed a random distribution or overdispersion. In all vertical slices ($n = 1$–3 per concentration), the particles were found distributed in a random pattern over the entire height of the DECOTAB ($p = 0.129$) for all tested particle concentrations (Figure 2; Supplemental Data, Figure 5SI). Overdispersion occurred in only one (1600 µL) out of the 7 sections ($p = 0.017$, $R = 1.123$).

In 50% of the horizontal sections at each concentration ($n = 1$–3 per concentration), particle distribution was random ($p = 0.062$; Supplemental Data, Figure 5SI) and 42% revealed overdispersion ($p = 0.004$–0.042). Only one horizontal section out of 14 contained clustered but not aggregated particles ($p < 0.001$, $R = 0.827$).

Because the particles were randomly distributed or overdispersed, it was possible to calculate the number of particles per DECOTAB dry weight out of the number of particles per slice of 50 µm thickness. The addition of 3200 µL of the particle stock suspension to the DECOTAB formulation resulted in a final particle concentration of 456 ± 124 particles/mm³ and 381 ± 103 particles/mg dry weight of the DECOTAB. Preparation with 1600 µL reduces the particle concentration to 243 ± 48 particles/mm³ and 203 ± 40 particles/mg dry weight, which is a reduction of 53% compared to the highest concentration and corresponds to the added amount of particles. Also, the addition of 160 µL (5% of the highest amount added) resulted in 17 ± 8 particles/mm³ and 14 ± 6 particles/mg dry weight, which is approximately 3.6% of the highest concentration.

The section from the tab loaded with fluorescent 1000-nm beads revealed also a homogeneous particle distribution, which has to be verified with further statistics. At first sight, neither agglomerations nor clustered particles were observed (Figure 3).

Particle leaching from the DECOTABs. No leached particles were detected after the prewetted DECOTABs were left for 4 d in water.

Uptake of the particles via the DECOTABs. The feeding rate of gammarids exposed with microparticle-loaded phyll-tabs was like the feeding rate of gammarids fed with particle-free tabs ($p = 0.192$). Further, the feeding rate in both treatments did not change over time ($p = 0.127$ for control, $p = 0.057$ for particle treatment), except for the first day when feeding rate was higher ($p = 0.035$; Figure 4A).

As expected, no particles were detected in the gammarids fed with particle-free tabs (negative control, $p = 1.000$), whereas a significant amount of particles (70 ± 35 particles/cm gut) was found in the guts of the gammarids fed with particle-loaded tabs ($p = 0.042$; Figure 4B) within 1 d. The number of particles per centimeter of gut was stable over time ($p = 0.189$) and proportional to the feeding rate ($p < 0.001$, $r = 0.33$).

DISCUSSION

Evaluation of DECOTABs with food supplements

We were able to identify 2 food supplements that allow an increase of the nutritional value of the cellulose-tabs originally published as DECOTABs by Kampfraath et al. (2012) to facilitate their use as suitable food in ecotoxicological short- and long-term bioassays. Best-suited supplements for *G. roeseli* were the algae-based phyll and the animal protein–based aquaculture trout food. This was demonstrated by dietary properties and behavioral responses including mortality and constant feeding of gammarids as well as the characteristics of

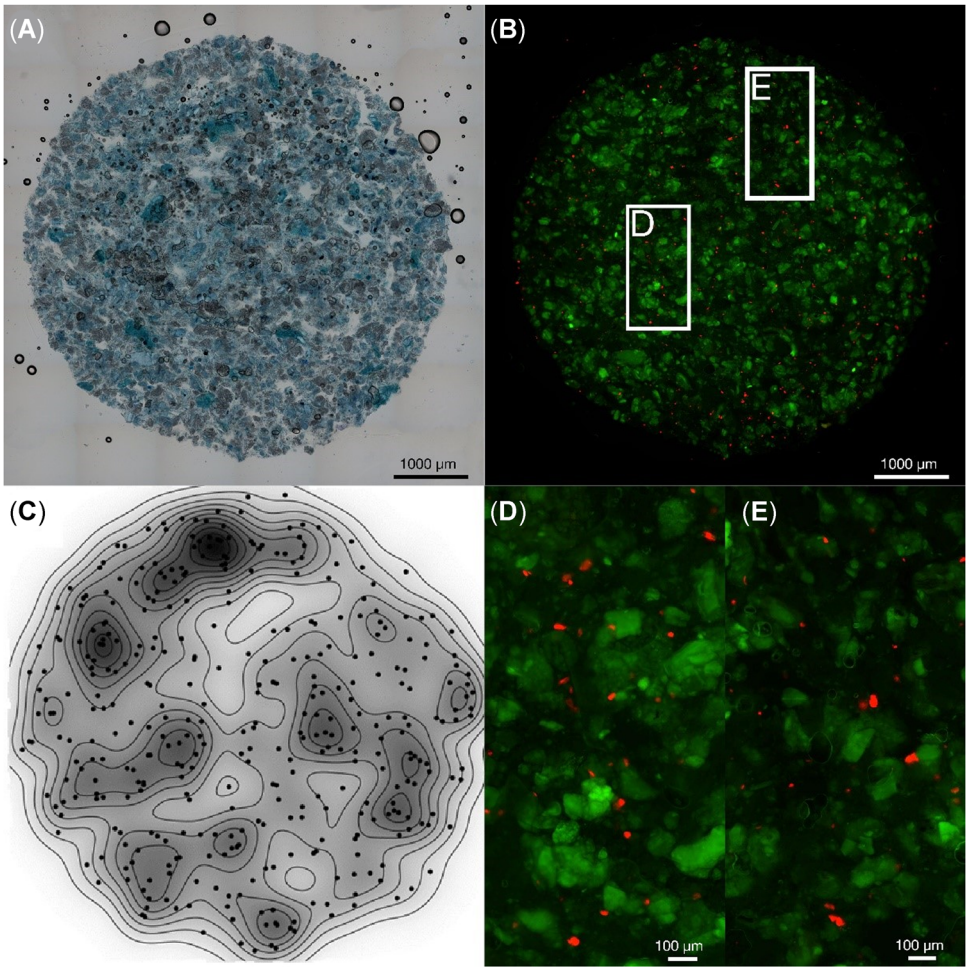

FIGURE 2: Particle distribution in one horizontal section of 50 µm thickness from a phyll-tab filled with 3200 µL of the prepared 10- to 65-µm microplastic suspension. This section contained 376 particles. (**A**) Brightfield image of the phyll-tab with red fluorescent polystyrol particles. (**B**) Fluorescence microscopic image of phyll-tab with red fluorescent particles with extended depth-of-field calculation of 15 Z-sections. (**C**) Nearest neighbors method plot. Dots mark particle position, and gray scaling shows particle density. (**D**, **E**) Magnification of (**B**).

the supplemented DECOTABs themselves. Important prerequisites of using the tabs in standardized dietary exposure experiments such as stability, low dry weight variation, the homogenous distribution of embedded particles, and the possibility to calculate the feeding rate with high accuracy make them a suitable tool for controlled ecotoxicological experiments.

The main need for the supplementation of the cellulose-tabs was to increase the nutritional value and adapt it to the needs of shredding organisms like gammarids to minimize a loss in fitness and mortality. The nutritional value was determined by the change of the organismal wide level of lipids, glucose, and glycogen. While glucose and lipids are primarily metabolized by gammarids during starvation for 14 d, glycogen seems to be utilized only when glucose is nearly depleted (Semsar-Kazerouni et al. 2020). The cellulose-tabs were not able to meet the nutritional demand because gammarids lost weight per millimeter when feeding on them for 3 wk and had to use their glucose reserves, although they were fed ad libitum. The same was true for the gammarids fed with tabs supplemented with beech leaves, although conditioned leaf discs are an established standard food in *Gammarus* spp. assays (Blockwell et al. 1998; Gergs and Rothhaupt 2008; Blarer and Burkhardt-Holm 2016). This observed reduction of the glucose level was similar to reduction after a starvation period of 16 d in our experiments and was likewise observed by Charron et al. (2014) after a starvation period of 23 d.

Phyll-, gammarus-, or trout food-tabs were chosen as alternative food supplements because they are based on algae or animal proteins. Although gammarids fed nearly twice as much on the gammarus- than on the trout food- or phyll-tabs, they did not build up more energy reserves. This indicates that gammarus as a food supplement provides a lower food quality compared to the algae-based phyll or animal protein–based trout food, which might be compensated by higher feeding rates (Bärlocher and Kendrick 1975; Gergs and Rothhaupt 2008; Agatz et al. 2014). Next to the lower food quality, a fast increasing risk for mortality up to >50% within the 21 d when fed with gammarus-tabs highly exceeded the acceptable range.

In contrast to the cellulose-tabs or the supplementation with beech leaves or ground and dried gammarids, trout food or

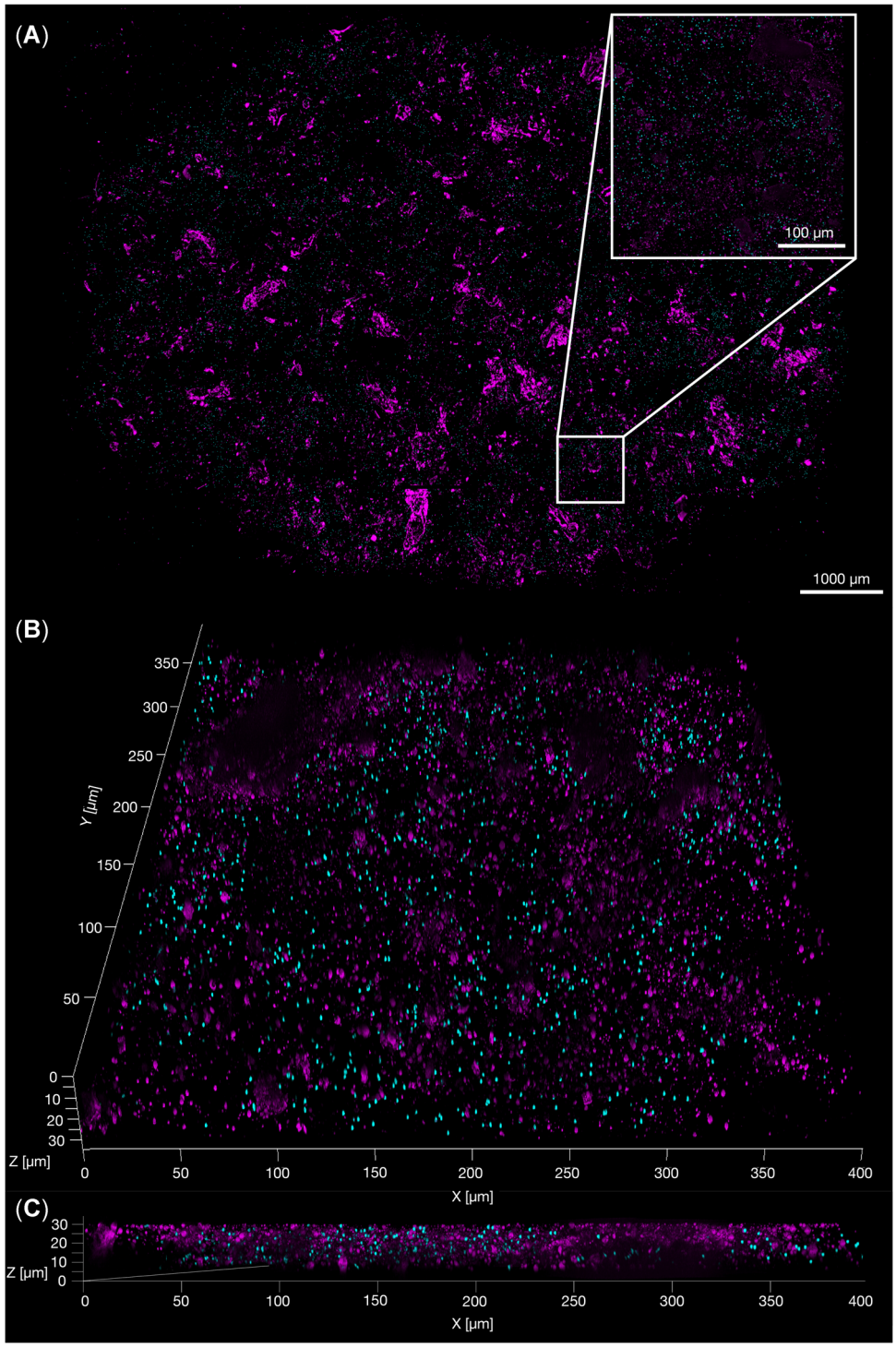

FIGURE 3: Exemplary examination of one horizontal slide (10 µm) of a decomposition and consumption tablet loaded with 1000-nm beads. Fluorescence images were taken under a Leica Thunder imaging system (DM6B-Z microscope, DFC9000GT camera, and LAS X software, Ver 3.0.2.7506) with large-volume computational clearing. Cyan fluorescent protein (CFP) excitation was used for the blue fluorescent particles (cyan) and Texas red (TxRed) excitation for the underlying red fluorescence of the phyll-tab (magenta). (**A**) Overview image with maximum projection imaged with a ×20 HC PLAPO CS2 20×/0.75 IMM UV objective. Cutout represents a magnified extract with 2 × 2 tiles imaged with a ×63 HC PL FLUOTOAR 63×/1.10 IMM and 81 Z-planes. (**B**,**C**) Volume visualization (3D) of a small 2 × 2 area of the slide imaged with a ×63 HC PL FLUOTOAR 63×/1.10 IMM and 81 Z-planes.

phyll proved suitable for long-term feeding of *G. roeseli*. After feeding on DECOTABs with both supplements, the gammarids were able to build up energy reserves higher than starved gammarids, and the feeding rate was constant over 21 d. Another remarkable fact is the slowly increasing and low risk for mortality up to a maximum of 25% after 3 wk, which is particularly crucial in establishing valid control groups in any ecotoxicological testing.

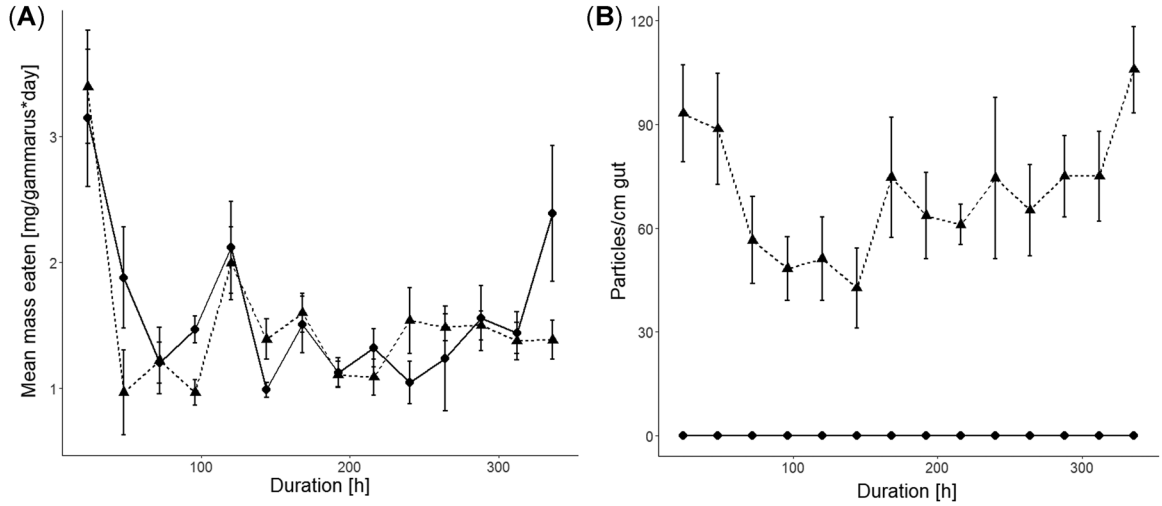

FIGURE 4: (**A**) Feeding rate of the provided decomposition and consumption tablets by one gammarid per day and (**B**) mean particle count in gammarid gut over the course of 2 wk. Feeding rates for the first 3 d were calculated with a batch-specific tab standard weight (initial dry wt = 33.4 ± 0.2 mg, $n = 330$).

The addition of food supplements can lead to a varying weight loss when exposed to water or a variation in dry weight after production, which interferes with the aim of a high standardization. Moreover, stability in dry weight of the food tabs during exposure is essential for the application of embedded substances and the determination of feeding rates. Although beech- and cellulose-tabs were the most stable ones compared to the other formulations when submerged into water, both revealed an incalculable weight loss when gammarids were feeding on them. The dried cellulose-tabs were very brittle and had a tendency to lose material solely through touching by the gammarids. The beech-tabs still contained larger leaf parts, which were entirely removed from the tab by the gammarids but not ingested. In contrast, the tabs supplemented with phyll, gammarids, or trout food revealed a consistent dry weight after production; and no additional weight loss was observed during feeding experiments with organisms. This would make phyll, trout food, and gammarus suitable as food supplements with an acceptable material loss in the aquatic environment of only 6 to 8% if only the stability of the DECOTABs is considered as an important prerequisite. In contrast, beech and cellulose were inappropriate in this respect.

An additional positive aspect of trout food or phyll is the commercial availability of these supplements. On the one hand, this ensures a constant quality and subsequently reproducible feeding rates. On the other hand, it greatly increases comparability between different exposures and laboratories. The latter is a strong advancement in contrast to the common use of conditioned leaf discs as a food source. Herein, a strong variation in food quality can occur because conditioning is typically performed by using stream water (Blarer and Burkhardt-Holm 2016), organically enriched dechlorinated water (Blockwell et al. 1998), or bacterial cultures (Agatz and Brown 2014). Because of the high nutritional value gammarids can gain from the tabs with phyll or trout food as a supplement, no pretreatment of the DECOTABs is necessary, making their use easier and more convenient. Overall, supplementation of the DECOTABs with phyll or trout food promises the best conditions for standardized toxicity testing.

DECOTABs as an oral exposure tool

During aqueous exposure, every particle or other sparingly soluble substance distributes differently (Cole et al. 2011; Oliveira and Almeida 2019), leading to fundamental problems in toxicity testing of an inhomogeneous distribution in the medium and the wrong assumption of equilibrium between the medium and the organism (European Centre for Ecotoxicology and Toxicology of Chemicals 2018; Eitzen et al. 2019). Actually, the uptake of a particle in aqueous exposure–oriented experiments is mainly random (Cole et al. 2011; Bartonitz et al. 2020) and therefore not calculable. This is further complicated by the often incomplete or unfeasible tracking of the particles in the exposure system (Tiede et al. 2009; Correia and Loeschner 2018; Triebskorn et al. 2019). We wanted to explore an alternative way to aqueous exposure focusing on the oral exposure of organisms with their food, which bypasses these toxicity-testing problems targeting particles or sparingly soluble substances. Moreover, oral exposure is a more realistic pathway for the uptake of such substances because they tend to adsorb to surrounding material (Werner et al. 2002; Bundschuh et al. 2019). Different approaches of particle exposure via a food matrix can be found, for example, for the mudsnail *Potamopyrgus antipodarum* (Imhof 2013; Imhof and Laforsch 2016), the amphipod *Gammarus pulex* (Imhof 2013), and the isopod *Idotea emarginata* (Hämer et al. 2014), however without deeper evaluation of the properties of the matrix and their respective influences on the uptake of the tested substances. Therefore, we demonstrated the fulfilled prerequisites for such an oral exposure tool regarding homogenous distribution of

particles as well as their permanent fixation in the matrix. Also, the necessary predictable and constant particle exposure based on the feeding rate was verified.

We were able to prove the applicability of loaded tabs as a dietary exposure tool by adding fluorescent polystyrene microparticles with a size of 10 to 65 μm. Examination of tab slices revealed homogenous distribution of the microparticles without aggregation in phyll-tabs. The same seems true for 1000-nm PS beads, as shown by exemplary visualization in the present study. Because of the standardized production procedure and formulation of the DECOTABs, it was possible to ensure a comparable and quantifiable exposure of *G. roeseli* with particles based on the feeding rate calculation and the particle concentration in the DECOTAB. Particle preparation before addition to the tabs is a remaining challenge that still should be harmonized and standardized (Hartmann et al. 2015; Eitzen et al. 2019), but no complex particle presuspension seems nessecary for particles like those used in the present study where an oral exposure tool is used.

The exposure of *G. roeseli* with microparticle-loaded DECOTABs led to a constant inner particle concentration resulting from a steady and fast ingestion as well as from a continuous egestion of the particles with the food. Complete egestion of particles with a size of 10 to 65 μm from the gut was observed, corresponding with the limited transfer of this size of particles into cells (Triebskorn et al. 2019). For the particles in the nanometer size class, such a transfer and resulting accumulation are more likely and need to be examined. Finally, we were able to verify that the dietary exposure as performed by Zhai et al. (2018) and Yardy and Callaghan (2020) solves the often claimed inhomogeneous distribution and unpredictable exposure of the particles in aquatic toxicity tests.

Further, we were able to link the feeding on the microparticle-loaded tab with the amount of particles in the gut of the gammarids, enabling a more realistic dose–response assessment. The inner particle concentration can be determined without a direct examination of the gut content, which can only be quantified at the end of the experiment (e.g., Straub et al. 2017). Instead, the inner particle concentration, which is more relevant for the observed effects than the outer particle concentration (European Centre for Ecotoxicology and Toxicology of Chemicals 2018), becomes continuously calculable via the feeding rate. This gains an adjustable dosimetry for each experiment, which is of great advantage not only for a standardized particle testing via the oral pathway but also for a more realistic dose–effect assessment for particulate substances.

Nevertheless, leaching of particulates or other embedded chemicals from the DECOTAB into the surrounding medium should be considered because it can affect the exposure pathway. The DECOTABs suggested in the present study did not leach any particles when watered for several days, whereby it must be recognized that gammarids are shredding their food during feeding and, thus, DECOTAB pieces with particles can spread in the test vessel. However, in our experiments, uptake via this route was negligible. Concerning the exposure with soluble substance via DECOTABs or if particles with adsorbed pollutants are embedded, a validation of potentially leached substance via chemical analysis of the medium is highly recommended because the partitioning between food source, medium, and organism might vary (Moermond et al. 2013; US Environmental Protection Agency 2016).

Another essential prerequisite for oral application is that the embedded substance does not influence the uptake by the organism. This could be proven as the embedded microparticles did not change the feeding of the gammarids on the particle-loaded phyll-tabs compared to particle-free ones. We observed a higher feeding rate on day 1, which can be interpreted as a response to starvation during the acclimatization period. This consequently leads to the conclusion that the acclimatization and the test periods should be conducted with a constant ad libitum feeding. Despite the compensatory feeding after starvation, the feeding rate normalized within 1 d and remained constant with and without microparticles during the entire test period.

Recommendations for future studies using DECOTABs as an advanced food source and/or oral exposure tool

With regard to the applicability in further studies, we want to give some recommendations concerning test duration, feeding time, and tab handling as well as the possibility of using the tabs for other organisms.

The test duration is mainly dependent on the feeding behavior of the test organism. The organisms should feed at least several days on the tabs to allow a reliable detection of tab dry weight changes. For 1-d exposures, the weighing error and weight loss are sometimes higher than the feeding of the gammarids, which may lead to undetectable effects on the feeding behavior (Bartonitz et al. 2020). Further, if the transfer of particles is intended, a certain amount of time is necessary to allow subsequent exposure of the particles. In addition, experiments with particulates often require a longer duration because no acute toxicity has been shown so far (Triebskorn et al. 2019).

The maximum feeding time on one DECOTAB can be extended. In our experiment, the DECOTABs were stable for over 4 d. However, to enable ad libitum feeding, the DECOTAB should be replaced on a regular basis depending on their dry weight before they are eaten completely. We replaced them after 3 or 4 d, which corresponds to a weight loss of 11.5 ± 3.6 mg with 3 untreated gammarids (~30% of our phyll-tab dry wt). Considering the overall duration of the experiment, no constraints exist if the DECOTABs are replaced regularly considering the above-mentioned minimum and maximum exposure times of a single DECOTAB. Most important for measuring the feeding rate is the use of the individual tab weight before and after the experiment because this increases the sensitivity of the endpoint instead of using an average standard weight. Calculation of feeding rate should include the mean daily weight loss of the tabs and the initial and final dry weight for every single tab. We also recommend the examination of a suitable food supplement for the investigated organism including stability of the resulting tab.

Although not specifically tested in the present study, it is likely that supplemented DECOTABs can also be used to feed organisms other than *G. roeseli* (Kampfraath et al. 2012). Decomposition and consumption tablets with and without supplements were already successfully used to feed other amphipods, like *Gammarus fossarum* (Straub et al. 2017) or in experiments with *Hyalella azteca* (Raths et al. 2020) and microbes (Hunting et al. 2017); but standardization of production and homogeneous distribution of the particles and tabs as a sufficient nutrient supply were not extensively tested. Also, mixtures containing similar ingredients and supplements were successfully used to provide food for the caddisfly *Allogamus mortoni* (Fenoy et al. 2020). Consequently, we assume the suitability of the DECOTABs supplemented with either phyll or trout for gammarids or even another species-dependent supplement during short- and long-term exposures for multiple functional feeding groups, particularly for shredder and grazer organisms in aquatic and terrestrial environments.

CONCLUSION

In the present study we describe a step toward a more standardized operational framework for toxicity testing procedures with particulate substances during short- and long-term bioassays with aquatic organisms. Focusing on the oral exposure pathway, we were able to provide an alternative to inhomogeneous aqueous particle exposure, which is difficult to standardize because of inhomogeneous distribution and unpredictable uptake. Moreover, a more realistic dose–response assessment of dietary particle exposures over the course of the entire experiment is feasible by combining the advantages of the homogenous distribution of particles and sparingly soluble substances in a stable food matrix with the possibility of a reliable feeding rate determination and consequently controllable exposure.

Supplementation of DECOTABs to meet the requirements of a test organism is already common practice. However, the deeper evaluation of the nutritional value in the present study provides one of the most standardized and verified food sources which is adapted to the specific needs of gammarids. It allows short- and long-term bioassays while maintaining a healthy state of the test organisms. Regular application of such supplemented DECOTABs in ecotoxicity testing procedures will improve the determination of feeding rates as a sensitive endpoint because of the high stability and low dry weight variation of the tabs. It also will increase comparability between studies by providing a constant health status and lowering possible stress of the gammarids, resulting in more realistic endpoint measurement and effect assessment. By varying the supplementation, tabs have a great potential of being used in toxicity tests with several organisms from different functional feeding groups.

Future research should focus on establishing clear relationships between the bioavailable fraction of particles or sparingly soluble substances in aquatic environments and observed effects. Studies concerning the partitioning of particles and other sparingly soluble substances to digestible sources are rare as well as particle concentration in the organism and subsequently real particle exposure for each functional feeding group. We further emphasize the mandatory comparison between anthropogenic plastic particles and natural particles, to avoid the confusion of mechanical and chemical effects. In addition, the uptake pathways (oral, dermal, pulmonal) of particles and sparingly soluble substances clearly must be considered in ecotoxicological research because exposure from the water column is negligible, and guidelines must be updated accordingly (Bundschuh et al. 2019; Oliveira and Almeida 2019). Our approach provides a template for further studies in this direction.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at https://doi.org/10.1002/etc.4990.

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Author Contributions Statement—A. Götz: conceptualization, data curation, formal analysis, investigation, methodology, visualization, writing–original draft, review and editing; J. Geist: conceptualization, supervision, resources, writing–review and editing; H.K. Imhof and S. Beggel: conceptualization, formal analysis, methodology, supervision, writing–review and editing.

Data Availability Statement—Data, associated metadata, and calculation tools are available from the corresponding author (sebastian.beggel@tum.de).

REFERENCES

Agatz A, Ashauer R, Brown CD. 2014. Imidacloprid perturbs feeding of *Gammarus pulex* at environmentally relevant concentrations. *Environ Toxicol Chem* 33:648–653.

Agatz A, Brown CD. 2014. Variability in feeding of *Gammarus pulex*: Moving towards a more standardised feeding assay. *Environ Sci Eur* 26:15.

Bärlocher F, Kendrick B. 1975. Leaf-conditioning by microorganisms. *Oecologia* 20:359–362.

Bartonitz A, Anyanwu IN, Geist J, Imhof HK, Reichel J, Graßmann J, Drewes JE, Beggel S. 2020. Modulation of PAH toxicity on the freshwater organism *G. roeseli* by microparticles. *Environ Pollut* 260:113999.

Beggel S, Brandner J, Cerwenka AF, Geist J. 2016. Synergistic impacts by an invasive amphipod and an invasive fish explain native gammarid extinction. *BMC Ecol* 16:32.

Benjamini Y, Hochberg Y, Stark PB. 1998. Confidence intervals with more power to determine the sign: Two ends constrain the means. *J Am Stat Assoc* 93:309–317.

Blarer P, Burkhardt-Holm P. 2016. Microplastics affect assimilation efficiency in the freshwater amphipod *Gammarus fossarum*. *Environ Sci Pollut Res Int* 23:23522–23532.

Blockwell SJ, Taylor EJ, Jones I, Pascoe D. 1998. The influence of fresh water pollutants and interaction with *Asellus aquaticus* (L.) on the feeding activity of *Gammarus pulex* (L.). *Arch Environ Contam Toxicol* 34:41–47.

Boeker C, Geist J. 2015. Effects of invasive and indigenous amphipods on physico-chemical and microbial properties in freshwater substrates. *Aquat Ecol* 49:467–480.

Brock TCM, van Wijngaarden RPA. 2012. Acute toxicity tests with *Daphnia magna*, *Americamysis bahia*, *Chironomus riparius* and *Gammarus pulex* and implications of new EU requirements for the aquatic effect assessment of insecticides. *Environ Sci Pollut Res Int* 19:3610–3618.

Bundschuh R, Bundschuh M, Otto M, Schulz R. 2019. Food-related exposure to systemic pesticides and pesticides from transgenic plants: Evaluation of aquatic test strategies. *Environ Sci Eur* 31:3290.

Burgherr P, Meyer EI. 1997. Regression analysis of linear body dimensions vs. dry mass in stream macroinvertebrates. *Arch Hydrobiol* 139:101–112.

Charron L, Geffard O, Chaumot A, Coulaud R, Jaffal A, Gaillet V, Dedourge-Geffard O, Geffard A. 2014. Influence of Molting and Starvation on Digestive Enzyme Activities and Energy Storage in *Gammarus fossarum*. *PLoS ONE* 9(4):e96393.

Cole M, Lindeque P, Halsband C, Galloway TS. 2011. Microplastics as contaminants in the marine environment: A review. *Mar Pollut Bull* 62:2588–2597.

Connon R, Geist J, Werner I. 2012. Effect-based tools for monitoring and predicting the ecotoxicological effects of chemicals in the aquatic environment. *Sensors* 12:12741–12771.

Correia M, Loeschner K. 2018. Detection of nanoplastics in food by asymmetric flow field-flow fractionation coupled to multi-angle light scattering: Possibilities, challenges and analytical limitations. *Anal Bioanal Chem* 410:5603–5615.

de Coen WM, Janssen CR. 1997. The use of biomarkers in *Daphnia magna* toxicity testing. IV. Cellular energy allocation: A new methodology to assess the energy budget of toxicant-stressed *Daphnia* populations. *J Aquat Ecosyst Stress Recov* 6:43–55.

Eitzen L, Paul S, Braun U, Altmann K, Jekel M, Ruhl AS. 2019. The challenge in preparing particle suspensions for aquatic microplastic research. *Environ Res* 168:490–495.

European Centre for Ecotoxicology and Toxicology of Chemicals. 2018.

Feiner M, Beggel S, Geist J. 2016. Miniature circulatory systems: A new exposure system for ecotoxicological effect assessments in riverine organisms. *Environ Toxicol Chem* 35:2827–2833.

Fenoy E, Moyano FJ, Casas JJ. 2020. Warming and nutrient-depleted food: Two difficult challenges faced simultaneously by an aquatic shredder. *Freshw Sci* 39:393–404.

Forster B, van de Ville D, Berent J, Sage D, Unser M. 2004. Complex wavelets for extended depth-of-field: A new method for the fusion of multichannel microscopy images. *Microsc Res Tech* 65:33–42.

Gergs R, Rothhaupt KO. 2008. Feeding rates, assimilation efficiencies and growth of two amphipod species on biodeposited material from zebra mussels. *Freshw Biol* 53:2494–2503.

Gerhardt A, Bloor M, Mills CL. 2011. *Gammarus*: Important taxon in freshwater and marine changing environments. *Int J Zool* 2011:1–2.

Hämer J, Gutow L, Köhler A, Saborowski R. 2014. Fate of microplastics in the marine isopod *Idotea emarginata*. *Environ Sci Technol* 48:13451–13458.

Hammer Ø, Harper DAT, Ryan PD. 2001. PAST: Paleontological statistics software package for education and data analysis. *Palaeontol Electronica* 4:9.

Hartmann NB, Jensen KA, Baun A, Rasmussen K, Rauscher H, Tantra R, Cupi D, Gilliland D, Pianella F, Riego Sintes JM. 2015. Techniques and protocols for dispersing nanoparticle powders in aqueous media—Is there a rationale for harmonization? *J Toxicol Environ Health B Crit Rev* 18:299–326.

Hunting ER, Barmentlo SH, Schrama M, van Bodegom PM, Zhai Y, Vijver MG. 2017. Agricultural constraints on microbial resource use and niche breadth in drainage ditches. *PeerJ* 5:e4175.

Imhof HK, Ivleva NP, Schmid J, Niessner R, Laforsch C. 2013. Contamination of beach sediments of a subalpine lake with microplastic particles. *Curr Biol* 23:R867–R868.

Imhof HK, Laforsch C. 2016. Hazardous or not—Are adult and juvenile individuals of *Potamopyrgus antipodarum* affected by non-buoyant microplastic particles? *Environ Pollut* 218:383–391.

Jamovi Project. 2019. Jamovi. Sydney, Australia.

Kampfraath AA, Hunting ER, Mulder C, Breure AM, Gessner MO, Kraak MHS, Admiraal W. 2012. DECOTAB: A multipurpose standard substrate to assess effects of litter quality on microbial decomposition and invertebrate consumption. *Freshw Sci* 31:1156–1162.

Moermond CTA, Korkaric M, Agerstrand M, Kase R. 2013. Reliability and relevance evaluation of ecotoxicity studies for use in risk assessment of chemicals. Working version.

Oliveira M, Almeida M. 2019. The why and how of micro(nano)plastic research. *Trends Analyt Chem* 114:196–201.

Preibisch S, Saalfeld S, Tomancak P. 2009. Globally optimal stitching of tiled 3D microscopic image acquisitions. *Bioinformatics* 25:1463–1465.

RStudio. 2015. *RStudio*. Boston, MA, USA.

Raths J, Kuehr S, Schlechtriem C. 2020. Bioconcentration, metabolism, and spatial distribution of ^{14}C-labeled laurate in the freshwater amphipod *Hyalella azteca*. *Environ Toxicol Chem* 39:310–322.

Rist S, Hartmann NB. 2018. Aquatic ecotoxicity of microplastics and nanoplastics: Lessons learned from engineered nanomaterials. In Wagner M, Lambert S, Besseling E, Biginagwa FJ, eds, *Freshwater Microplastics: Emerging Environmental Contaminants?* Vol 58. Springer Open, Cham, Switzerland, pp 25–49.

Rufli H, Fisk PR, Girling AE, King JM, Länge R, Lejeune X, Stelter N, Stevens C, Suteau P, Tapp J, Thus J, Versteeg DJ, Niessen HJ. 1998. Aquatic toxicity testing of sparingly soluble, volatile, and unstable substances and interpretation and use of data. Task Force of the European Centre for Ecotoxicology and Toxicology of Chemicals. *Ecotoxicol Environ Saf* 39:72–77.

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J-Y, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. 2012. Fiji: An open-source platform for biological-image analysis. *Nat Methods* 9:676–682.

Semsar-Kazerouni M, Boerrigter JGJ, Verberk WCEP. 2020. Changes in heat stress tolerance in a freshwater amphipod following starvation: The role of oxygen availability, metabolic rate, heat shock proteins and energy reserves. *Comp Biochem Physiol A Mol Integr Physiol* 245:110697.

Straub S, Hirsch PE, Burkhardt-Holm P. 2017. Biodegradable and petroleum-based microplastics do not differ in their ingestion and excretion but in their biological effects in a freshwater invertebrate *Gammarus fossarum*. *Int J Environ Res Public Health* 14:774.

Tiede K, Hassellöv M, Breitbarth E, Chaudhry Q, Boxall ABA. 2009. Considerations for environmental fate and ecotoxicity testing to support environmental risk assessments for engineered nanoparticles. *J Chromatogr A* 1216:503–509.

Toussaint B, Raffael B, Angers-Loustau A, Gilliland D, Kestens V, Petrillo M. 2019. Review of micro- and nanoplastic contamination in the food chain. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* 36:639–673.

Triebskorn R, Braunbeck T, Grummt T, Hanslik L, Huppertsberg S, Jekel M. 2019. Relevance of nano- and microplastics for freshwater ecosystems: A critical review. *Trends Analyt Chem* 110:375–392.

US Environmental Protection Agency. 2016. OCSPP 850.1020: Gammarid amphipod acute toxicity test: Ecological effects test guidelines. EPA 712-C-16-012. Washington, DC.

van Handel E. 1985. Rapid determination of total glycogen and sugars in mosquitoes. *J Am Mosq Control Assoc* 1:299–301.

Werner I, Geist J, Okihiro M, Rosenkranz P, Hinton DE. 2002. Effects of dietary exposure to the pyrethroid pesticide esfenvalerate on medaka (*Oryzias latipes*). *Mar Environ Res* 54:609–614.

Yardy L, Callaghan A. 2020. What the fluff is this? *Gammarus pulex* prefer food sources without plastic microfibers. *Sci Total Environ* 715:136815.

Zhai Y, Brun NR, Bundschuh M, Schrama M, Hin E, Vijver MG, Hunting ER. 2018. Microbially-mediated indirect effects of silver nanoparticles on aquatic invertebrates. *Aquat Sci* 80:12968.

