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Ingestion of bivalve droppings by benthic invertebrates may lead to the transfer of nanomaterials in the aquatic food chain

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Abstract

Background: Manufactured nanomaterials (MNMs) are released into the environment in increasing quantities. Consequently, MNMs also reach the aquatic environment, where they can interact with different organisms. Previous studies have already shown that filter-feeding bivalves can ingest nanomaterials from the surrounding water leading to higher concentration of the material. Furthermore, they have been shown to be vectors for environmental chemicals and pathogens to other organisms, as their feces/pseudofeces (F/pF) play a crucial role as a food source for other species. We exposed bivalves (*Corbicula* sp.) to MNMs and performed experiments to investigate the possible transport of MNMs by their feces to the benthic amphipod *Hyalella azteca*. Silver (Ag) and gold (Au) nanoparticles (NPs) as well as fluorescent polystyrene nanoparticles were used in this study. They allowed the investigation of the metal content of the bivalves' feces and the amphipods feeding on it, as well as the localization of the fluorescent particles in the body of the animals.

Results: Examination of the feces by fluorescence microscope and determination of the total metal content by inductively coupled plasma mass spectrometry (ICP-MS) showed a high accumulation of the exposed MNMs in the F/pF. The examination of fecal matter, using transmission electron microscopy confirmed the nanoparticulate character of the metals in the examined fecal matter. After exposure of amphipods to the MNMs containing fecal matter, the fluorescent MNMs were localized in the animals gut. The chronic exposure of juvenile amphipods over 21 days to feces enriched with Au MNMs caused significant effects on the growth of the amphipods. The transfer of both metals (Ag and Au) from the fecal matter to the amphipods was confirmed after total metal measurements.

Conclusion: Probably, for the first time, it has been shown that when exposed to MNMs bivalves can transfer these particles to other benthic species. Transfer is via released F/pF upon which the benthic species feed and thus could ingest the particles. The high concentrations of MNMs in the fecal matter raises concerns about the potential accumulation and transfer of the materials and associated ecotoxicological effects in invertebrates such as benthic amphipods.

Keywords: Bioaccumulation, Nanomaterials, Invertebrates, *Hyalella azteca*, *Corbicula fluminea*

Background

Manufactured nanomaterials (MNMs) have been the focus of a wide range of studies to elucidate their environmental and ecotoxicological impact [1–10]. Further investigations dealt with the fate and potential accumulation of the particles in the aquatic environment [11–14]. Agglomeration of nanoparticles generally leads to a gravitational settling of the particles. Therefore, benthic

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species seem to be more specifically exposed to MNMs and nanoparticles (NPs) [15–17].

Filter-feeding bivalves such as *Corbicula fluminea* filter high volumes of water for feed intake and respiration. Ingested organic matter (e.g., algae or microbes) are digested in the gut, where the ingested matter is mostly chemically and structurally altered by the digestion processes. The feces (F) are ejected as pellets through the exhalant siphon [18]. Pseudo-feces (pF) are a mix of particles that were filtered out by the bivalves' gills and packed into mucus prior to be ejected periodically through the inhalant siphon or via the ventral margin of the mantle without being digested. Nichols et al. [19] described that *C. fluminea* released pF within 5 min after being exposed to suspended matter, e.g., algae. The aggregation of single particles within the pF may lead to a high accumulation of previously suspended particles [18]. The production of pF seems to be triggered by a threshold concentration of the particles in the surrounding water, as described by Sprung and Rose [20].

Sylvester et al. [21] described a significant increase of invertebrate biomass in river areas that were populated by the filter feeding bivalve *Limnoperna fortune*, whereas no comparable trend was observed in non-populated areas of the same river. The observations were explained by an enhanced availability of food due to the release of F/pF that caused a strong transfer of organic matter from the pelagic zone to the sediment [21].

An increasing abundance of benthic invertebrates has also been observed in the Great Lakes following the colonization with invasive bivalves [22–27] which was explained by the release of F/pF providing an additional supply of organic matter [28–30]. These findings are in agreement with the results from laboratory studies by Basen et al. [29], where gammarids were fed either with pelagic autothrophs or F/pF of *C. fluminea* which were fed with these autotrophs. The F/pF-fed animals showed a higher survival rate and growth [31]. Comparable results were described by González and Burkart [30]. Nichols et al. [19] further explained that amphipods find valuable habitats within colonies of bivalves, where they live on the sediments resulting from the F/pF organic biodeposits. Hargrave [32] described how deposit feeding animals like the benthic amphipod *Hyalella azteca* ingest sediment containing organic matter, including the F/pF of filter-feeding bivalves. According to Lopez and Levinton [33], *H. azteca* can ingest approximately 13 mg sediment per mg body weight per day. They further describe that also gastropods, other bivalves, annelids, arthropods and even vertebrates like the fish *Liza dumerili* also feed on sediments containing organic biodeposits [33].

Apart from the previously described non-disruptive effects of filter-feeding bivalves on the aquatic ecosystem, there are also some studies describing negative effects on the invertebrate fauna. Watkins et al. [34] reported that a decrease in abundance of the amphipod *Diporeia* sp. by around 90% was observed in Lake Ontario and Lake Michigan following the increase of the dreissenid populations. No decline of *Diporeia* sp. was observed only in Lake Superior, the only Great Lake without dreissenids [34]. They hypothesised that the pF released by the dreissenids may contain pathogens that are lethal for *Diporeia* sp. [34]. They referred to the studies of Dermott et al. [35] and Nalepa et al. [36] which came to similar conclusions in their investigations on the disappearance of *Diporeia* sp. The negative impact of filter-feeding bivalves and their F/pF on the benthic fauna was discussed by further authors [37–40].

During their investigations on the trophodynamic of PCBs, Morrison et al. [41] were able to show that the introduction of *Dreissena polymorpha* in the western Lake Erie led to a shift in the diet of *Gammarus* sp. from less contaminated detritus and phytoplankton to the higher contaminated F/pF of the bivalves. This resulted in an increased body burden of PCBs not only in the amphipods but also in crayfish, white and yellow perch, and black crappie that fed on the gammarids [41]. The transfer of xenobiotic compounds, taken up by the bivalves to higher trophic levels via organic biodeposits has also been observed for microcystin (the most common cyanobacteria toxin in fresh waters) [42]. Similarly, Bruner et al. [43] showed in a study on gammarids that the F/pF of *D. polymorpha* can serve as a source of exposure of organic contaminants like, e.g., polychlorinated biphenyls (PCBs) or polycyclic aromatic hydrocarbons (PAHs) after being exposed to these compounds [43]. The uptake of the released F/pF by *Gammarus fasciatus* led to an accumulation of the contaminants in the amphipod tissue [43]. Similar observations were concluded under marine conditions. Gilek et al. [44] showed that in the Baltic Sea the blue mussel (*Mytilus edulis*) contributes with their F/pF to the deposition of hydrophobic organic compounds and thus increased their bioavailability to benthic organisms.

With respect to the increased bioavailability and trophic transfer of xenobiotics enriched in F/pF of filter feeding bivalves, the impact of these organisms and their droppings on the fate of MNMs in the aquatic environment warrants further investigations.

Wegner et al. [45] exposed blue mussels to polystyrene nanoparticles (nPS) in concentrations ranging from 0.1 to 0.3 g/L. They observed an increase of F/pF production with increasing exposure concentrations and measured a concentration decrease in the exposure medium of 1.17 mg nPS/L per hour during the course of the study [45]. Although the released F/pF were not analyzed

for the nPS content, it is likely that the suspended nPS were ingested, concentrated, and finally eliminated by the release of F/pF. Similarly, Kuehr et al. [46] observed an enrichment of suspended TiO_2NPs in the freshwater bivalve *C. flumine*a with an accumulation factor of more than 9000 that was followed by a complete elimination of the TiO_2 load within 24 h of depuration. This was explained by the rapid and effective release of F/pF containing the previously enriched TiO_2NPs [46]. Therefore, filter feeding bivalves may play an important role in the exposure of benthic species that feed on the F/pF as described above. This may further result in the transfer of MNMs by the F/pF (loaded with a high burden of MNMs) to other invertebrates and finally to higher trophic levels including fish that feed on benthic species [47–51].

In this study, the freshwater bivalve *C. fluminea* was exposed to different types of MNMs to produce bivalve F/pF with a high load of NPs to investigate their role in the exposure of benthic species. We used fluorescence-labeled nPS to represent nanoplastic particles resulting from the degradation and erosion of plastic waste and microplastic present in the aquatic environment [52–58]. Furthermore, AgNPs representing one of the most widely commercially used NPs that release ions and AuNPs as a nearly chemically inert counterpart were used. The released F/pF were used to feed the benthic freshwater amphipod *H. azteca* during a chronic exposure test. The ingestion of the MNMs by the amphipods and potential chronic effects of the MNMs present in the F/pF were investigated. *H. azteca* has previously been used in a wide range of bioavailability and bioaccumulation tests using neutral and ionic organic compounds, metals and nanomaterials [59–67] and has recently been proposed as a test organism for the regulatory bioaccumulation assessment of MNMs [68]. The results of this study help to further elucidate the role of filter feeding bivalves and their F/pF in the transfer of MNMs along the aquatic food chain.

Materials and methods

Corbicula fluminea

Freshwater bivalves *C. fluminea* used in this study were taken from the husbandry of Fraunhofer IME, Schmallenberg. They were collected from the river Niers near Wachtendonk (47669, Germany; N 51° 24′ 25.2″, E 6° 20′ 16.5″) and kept in 1.5 m^3 glass microcosms. The culture procedure is further described in Kuehr et al. [46]. An acclimatization phase lasting 2 weeks was carried out before the animals were exposed for the production of NP containing F/pF. Only animals with a shell length (anterior–posterior) of 1.5 (±0.5) cm were used. All animals were separated before test starts in a 20 L glass aquarium for 4 days without feeding to allow defecation.

Hyalella azteca

The amphipods were taken from the stock culture of Fraunhofer IME, Schmallenberg. The stock culture was kept in 2 L flasks filled with reconstituted water containing bromide and stocked with 30 adult animals each [64]. The culturing procedure is further described by Kühr et al. [5]. The strain was originally obtained from "Freds Haustierzoo" (Cologne, Germany). Once a week juveniles were separated from the parental animals and cultured in separate flasks for approximately 8 weeks until they reached a sufficient size for the bioavailability tests [59]. Only healthy animals free from observable diseases and abnormalities were used for the studies. Male and female individuals were not separated before use in our studies. Before test start, the animals used in the bioavailability tests were isolated from the culture medium and kept under fasting conditions for 4 days in clean water (with daily water exchange, for water specification see Additional file 1: Table S1). This was to ensure that they immediately ingested the experimental diets applied during the bioavailability tests.

Nanoparticles

The fluorescence-labeled nano plastic products made of polysyterene (nPS) were purchased from Thermo Scientific™ as Fluoro-Max™. The nPS spheres with a nominal diameter of 47 nm were dyed internally to prevent leaching in aqueous media and provide a dye free surface. The green light emitting dye was not further specified by the supplier. The nPS were suspended in ultrapure water as stock suspension at a concentration of 10 g nPS/L.

Silver NPs (NM 300 K) were provided by the Fraunhofer Institute for Molecular Biology and Applied Ecology IME. NM 300 K is a reference material from the European Commission's Joint Research Centre and has been in the scope of the OECD Working party on Manufactured Nanomaterials (WPMN) Sponsorship Program. Physico-chemical properties and information on the characterization are summarized in JCR Reports [69]. The stock suspension of the AgNPs with a diameter of 15 nm was stabilized by a dispersing agent (NM 300 DIS), containing 4% (w/v) of polyoxyethylene, glycerol, trioleate, and polyoxyethylene(20)-sorbitan-monolaureate (Tween 20) each. The nominal Ag concentration was 10.16% (w/w) [69].

AuNPs with a nominal size of 60 nm were purchased from BBI Solution as stock suspension in ultrapure water with a concentration of 57 mg Au/L (0.01% AuCl).

The working suspension of NM 300 K was produced as described by Kuehr et al. [46]. The stock suspensions

of the AuNPs and nPS were manually shaken for 1 min before being sonicated for 10 min with a pulse/pause ratio of 0.2/0.8 using an ultrasonic homogenizer (Bandeline Sonoplus HD2200 ultrasonic homogenizer, 200W, Bandelin Cup Horn BB6). The homogenized stock suspensions were used as working suspensions.

Characterization of the nanoparticles

AgNPs and AuNPs were examined in ultrapure water and copper reduced tap water, by Dynamic light scattering (DLS) to estimate their hydrodynamic diameter using a zetasizer (Zetasizer, Nano Series, Malvern Industries Ltd.). The DLS measurements were carried out as described by Zeumer et al. [14]. In addition the electrophoretic mobility was measured to calculate the zeta potential of the NPs in ultrapure water and copper reduced water. Measurements were carried out three times with each measurement consisting of 10 to 100 runs at 25 °C and an equilibration time of 120 s. Disposable folded capillary cells (DTS1070, Malvern Panalytical) were used for the measurements of the electrophoretic mobility. The calculation of the zeta potential was carried out by the zetasizer software (Zetasizer, Nano Series, Zetasizer Ver. 7.11, Malvern Industries Ltd). Further characterizations were carried out using transmission electron microscopy (TEM) on the stock material of AgNPs and AuNPs as described by Kuehr et al. [46] using the TEM grid preparation method of Uusimaeki et al. [70].

Generation of bivalve F/pF containing nPS

Twenty *C. fluminea* were exposed to nPS under semi-static conditions in the absence of food in a 10 L glass aquarium containing 2 L of copper reduced tap water with a nominal concentration of 5 mg/L of 47 nm nPS (for specification of the copper reduced tap water see Additional file 1: Table S1). The exposure media were prepared by adding 1 mL of the nPS working suspensions to 2 L copper reduced tap water and stirring for 1 min using a magnet stirrer. Feces were collected every 12 h by means of a Pasteur pipette prior to the daily water replacement. The F/pF material collected within 24 h was pooled in one sample and stored at 4 °C in 20 mL glass vials. The duration of the exposure phase was 120 h. Aeration was not provided to avoid resuspension of F/pF. After 24 h of exposure, 9 bivalves were sampled as triplicates, each consisting of 3 bivalves. Viscera were dissected and examined by fluorescence microscopy. At the end of the exposure phase, the remaining animals (11 bivalves) were rinsed with clean water and transferred to a new aquarium containing clean water for depuration. Animals were collected after 12 h of depuration, rinsed with clean water and dissected for fluorescence microscopy.

Generation of bivalve F/pF containing Ag- and AuNPs

AgNPs were chosen as further test materials, because their particles have previously been shown to be taken up by *H. azteca* [5, 66, 67]. AgNPs which may release silver ions, were compared in this study with AuNPs which are insoluble in the aquatic media. This allowed the investigation of the transfer of NPs without the potential accumulation of an ionic fraction. To generate NP enriched F/pF, *C. fluminea* were exposed to AgNPs (NM 300 K) or AuNPs (BBI Solutions, 60 nm) (Fig. 1). Test media were generated by adding the respective working suspension to a clean aquarium filled with copper reduced tap water followed by continuous stirring for 1 min using a glass rod before the basket containing the bivalves were transferred to their respective tanks. For each treatment, 75 bivalves were placed in a stainless steel basket which was submerged in a 20 L glass aquaria (Additional file 1: Fig. S1) filled with 18 L of medium containing AgNPs with a nominal concentration of 10 µg Ag/L. For the generation of F/pF enriched with AuNPs, 10 L of medium with a nominal concentration of 1 mg Au/L were added to a 15 L aquarium. The exposure concentrations used were shown in previous studies to have no negative impact on the filtration behavior of *C. fluminea* and/or the release of F/pF [20, 46].

Animals were exposed over a period of 144 h. A control group of the same size was kept in 18 L copper reduced tab water in a 20 L aquarium to generate non-enriched control F/pF for the depuration phases of the bioavailability test with the amphipods, as well as for the control treatment of the chronic exposure study with *H. azteca*. The animals were checked at least daily and dead animals were removed immediately. The exposure media were completely replaced every 24 or 48 h for AgNPs and AuNPs, respectively. Media samples (10 mL) were taken in duplicate from the fresh exposure media and were acidified with nitric acid (69%, suprapure grade, Roth) and stored at 4 °C until measurements. The animals were fed every 48 h with suspended, fine milled stinging nettle leaves resulting in a final concentration of 5 mg dry mass/L. F/pF released by the caged animals settled to the bottom of the aquaria and were collected by means of a Pasteur pipette. The released F/pF from the AgNP, AuNP and control treatment (F/pF_{Ag}, F/pF_{Au} and F/pF_{C}) were used to load glass fiber filters as described by Kuehr et al. [67] to be used in feeding experiments with *H. azteca* (Additional file 1: Fig. S2). The suspended F/pF were rinsed three times with 100 mL of copper reduced tap water during filter preparation to remove free ions

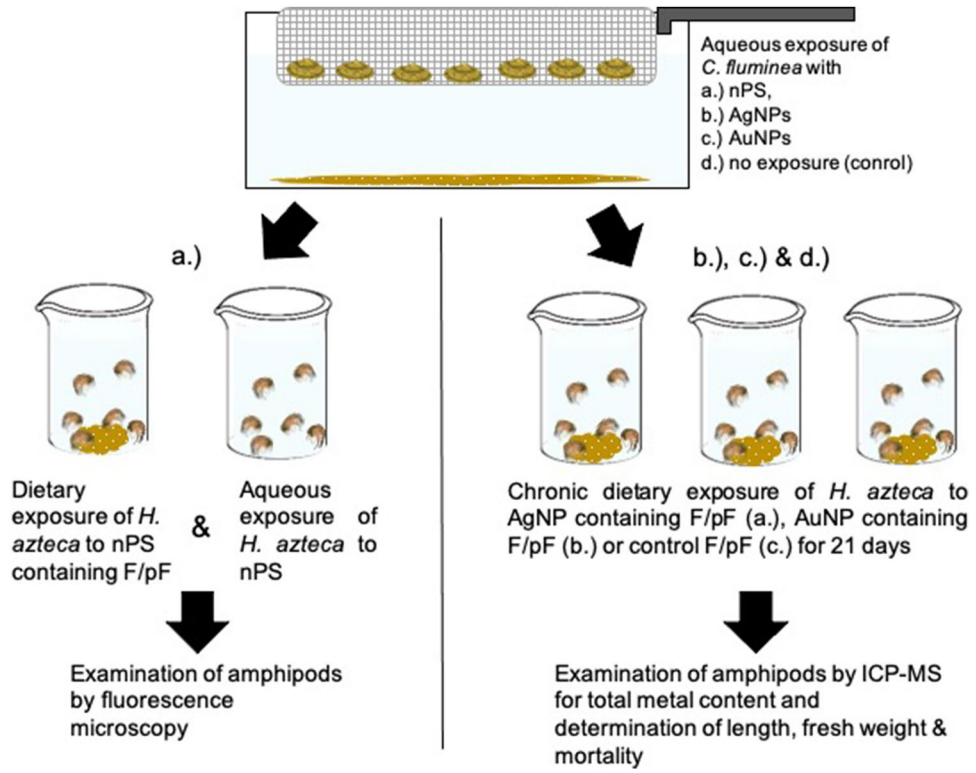

Fig. 1 Scheme of the general exposure and study design

or unbound NPs from the loaded filters. Prepared F/pF feeding filters were stored at − 20 °C before usage.

Before using the F/pF filters, 10 randomly picked filters of each treatment were dried by lyophilization at − 52 °C and 0.47 mbar (Alpha 1-2 LDplus, Christ) for 24 h. Four filters per treatment were used to determinate their dry mass and were further processed for the determination of the Ag or Au content as described below. The loading (dried F/pF) of the remaining 6 filters were removed from the filter surface using disposable scalpels (Braun, Cutfix®), pooled and used for examinations by Transmission Electron Microscopy (TEM) as described for lyophilized NP containing plankton by Zeumer et al. [14].

Bioavailability of nPS to *Hyalella azteca*

Two bioavailability experiments were carried out with *H. azteca* to investigate the uptake path of the nPS having a nominal diameter of 47 nm (Fig. 1). In the first test 20 amphipods were kept in a 600 mL glass beaker and exposed to nPS dispersed in the test medium. The media were prepared by adding 250 µL of the nPS working suspension to 500 mL copper reduced tap water and stirred for 1 min using a magnet stirrer in 600 mL glass beakers, resulting in a nominal concentration of 5 mg nPS/L. Feeding occurred ad libitum with test item free DECOTABs containing TetraMin® flakes which were prepared according to Kampfraath et al. [71]. After 24 h of exposure 10 aphipods were sampled and rinsed with clean water before examination by fluorescence microscopy. The remaining 10 individuals were also rinsed with clean water and transferred to a new beaker filled with clean water for a 24 h depuration phase. After 12 h of depuration the amphipods were rinsed and transferred to a fresh beaker containing clean water again and were finally sampled after 24 h of depuration. Collected amphipods were rinsed with clean water and examined by fluorescence microscopy.

In the second experiment 20 amphipods were exposed to nPS enriched F/pF which were used as an experimental diet using 600 mL beakers filled with 500 mL clean copper reduced tap water. The F/pF that were previously stored at 4 °C in 20 mL glass vials were added to the test beaker without further processing using a glass pipette. After 12 h of exposure the amphipods were transferred to a fresh beaker containing uncontaminated water and fresh nPS enriched F/pF to avoid a second exposure path via the water due to free nPS released from the amphipods feces or the F/pF diet. Samplings and water exchange occurred as described for the aqueous exposure. For the 24 h depuration phase the amphipods were

fed nPS free control F/pF. At the end of the depuration phase the animals were rinsed with clean water and examined by fluorescence microscopy.

Examination of bivalves, F/pF and amphipods by fluorescence microscopy

The samples from bivalves, their F/pF and amphipods from the different nPS exposure treatments were examined by fluorescent microscopy. This was done using a DMI600B (Leica) Microscope and the L5 filter cube with an excitation range of 480 ± 20 nm and a range of 527 ± 30 nm for the recorded emission. The settings of exposure time, gain, and saturation were based on the highest level of these parameters, where no autofluorescence of control samples of the same tissue from control animals or control F/pF was visible. Hence all F/pF and bivalve samples were examined with exposure times of 450 ms, gain of 7.4 and a saturation value of 30. *H. azteca* sampled from the exposure with nPS containing F/pF were examined using exposure times of 150 ms, gain of 2.2 and a saturation value of 30.

Chronic exposure test with *H. azteca* and NPs loaded F/pF

To investigate the bioavailability of NPs present in the F/pF of filter feeding bivalves that have been previously exposed to the NPs to benthic amphipods, a chronic exposure test was carried out using *H. azteca* as described by Kühr et al. [5] (Fig. 1). For both treatments (F/pF_{Ag}, F/pF_{Au}) and the control group (F/pF_C) five test groups (replicates), each with 20 juvenile amphipods (7–14 days), were kept in 600 mL glass beakers filled with 500 mL copper reduced tap water. The animals were only fed with F/pF feeding filters ad libitum to guarantee the exposure. The test lasted for 21 days under semi-static conditions with water replacement every 7 days. During every water replacement the living animals of each beaker were counted while transferring to clean beakers and water samples ($n = 2$) were taken to determine the Ag and Au contents in the water. For that purpose, the samples were acidified with nitric acid (69%, suprapure grade, Roth) and stored at 4 °C until measurement. A check of the water quality by photometric measurements of ammonia, nitrite and nitrate (NANOCOLOR® 500D, Machery-Nagel) was carried out weekly. The pH and oxygen levels were measured three times a week. After 21 days, the animals were sampled, rinsed with clean water and all surviving animals of each replicate were photographed as group to determine the mean length of the amphipods by image analysis using the software ImageJ® (Wane Rasband, National Institute of Health) as described by Kühr et al. [5]. The weight of the animals of each replicate was determined after blotting the animals dry. The animals were stored at − 20 °C until further processing for the determination of metal content.

Determination of total metal concentrations in F/pF and *H. azteca*

The samples of *H. azteca* and four F/pF filters of each treatment and control group were digested using aqua regia and microwaves as described by Kuehr et al. [66]. The remainder of the fiber glass filters were rinsed with nitric acid (10%) and the nitric acid was pooled with the aqua regia solution of the corresponding sample.

The media samples from the exposure of *C. fluminea* for production of enriched F/pF and from the chronic exposure study with *H. azteca* were digested analogously. To validate the digestion process for Ag a certified reference material standard (Oyster tissue NIST® SRM® 1566b, Merck, Darmstadt, Germany) was treated in the same to validate the digestion process. In the case of Au the AuNP BBI Solution as stock suspension (with a concentration of 57 mg Au/L), certified Au element standard for ICP-MS (Au Certipur®, Merck, Darmstadt, Germany) and the above mentioned reference material (after spiking with the AuNP certified element standard) were treated equally for validation. Total metal concentrations in all digested samples were measured using ICP-MS as described by Kuehr et al. [5, 66], resulting in values of 0.096 µg Ag/L and 0.006 µg Au/L for the limits of quantification (LOQ), respectively.

Data analysis

The percentages relative of the survival rates of *H. azteca* during the chronic exposure test were arcsine transformed using Excel® (Mircosoft). The transformed data were subjected to an analysis of variance (ANOVA) using the data analysis software Origin (OriginLab Corporation; OriginPro 2017G). Mean values of the Ag and Au concentrations in the different treatments were calculated for the experimental periods. The total Ag and Au concentrations in the test animals were divided by the total concentrations of the respective metal in the exposed F/pF to derive accumulation factors for the metals exposed via the respective F/pF. Transfer factors for the metals from the respective NPs that were exposed to the bivalves by the water were derived by dividing the total metal concentrations of the respective metal in the F/pF by the total metal concentrations in the corresponding exposure medium.

Results

Characterization of the nanoparticles

The hydrodynamic diameter of the particles were measured by DLS in ultrapure water and copper reduced tap water. With the exception of nPS, the Z-average of the NPs were higher in copper reduced tap water (46.6, 51.2 and 143.5 nm for nPS, AgNP and AuNP, respectively) compared to the values measured in ultrapure

water (53.3, 26.7 and 60.69 nm for nPS, AgNP and AuNP, respectively).

The Ag- and AuNP from stock suspensions were examined by TEM and the resulting diameters (14.2 and 62.7 nm, Fig. 2) were closer to the hydrodynamic diameters determined in ultrapure water and were in agreement with the nominal diameters.

The zeta potential of the metal NPs were more negative in the copper reduced tap water (−22.8 and −26.6 mV for AgNP and AuNP, respectively) than in ultrapure water (−5.1 and −6.58 mV). nPS showed a more negative Zeta potential in ultrapure water (−57.7 mV) than in copper reduced tap water (−31.9 mV).

Generation of F/pF loaded with nPS

During exposure of *C. fluminea* to nPS no mortality was observed. The animals' siphones were visible almost permanent during the whole exposure phase, the shells were slightly opened and the foot presented. An increased release of F/pF was observed starting after around 2 h of exposure. The F/pF released during the exposure phase were of a yellowish color comparable to the color of the nPS stock suspension. After transfer of the bivalves, without nPS, into a clean system without nPS for depuration, the valve opening time as well as the release of F/pF decreased after around 6 h.

Strong fluorescence was visible by fluorescence microscopy in the F/pF and nearly all soft tissue compartments of the exposed bivalves after 24 h of exposure with nPS (Fig. 3). Using the same fluorescence microscope settings for all samples of the same type (F/pF, bivalve soft tissue), samples from 24 h of exposure and 12 h of depuration could be compared allowing to semi-quantitatively assessment of the nPS burden of the samples (Fig. 3): the samples of F/pF and viscera after 12 h of depuration showed a decreased fluorescence compared to the samples from the exposure phase.

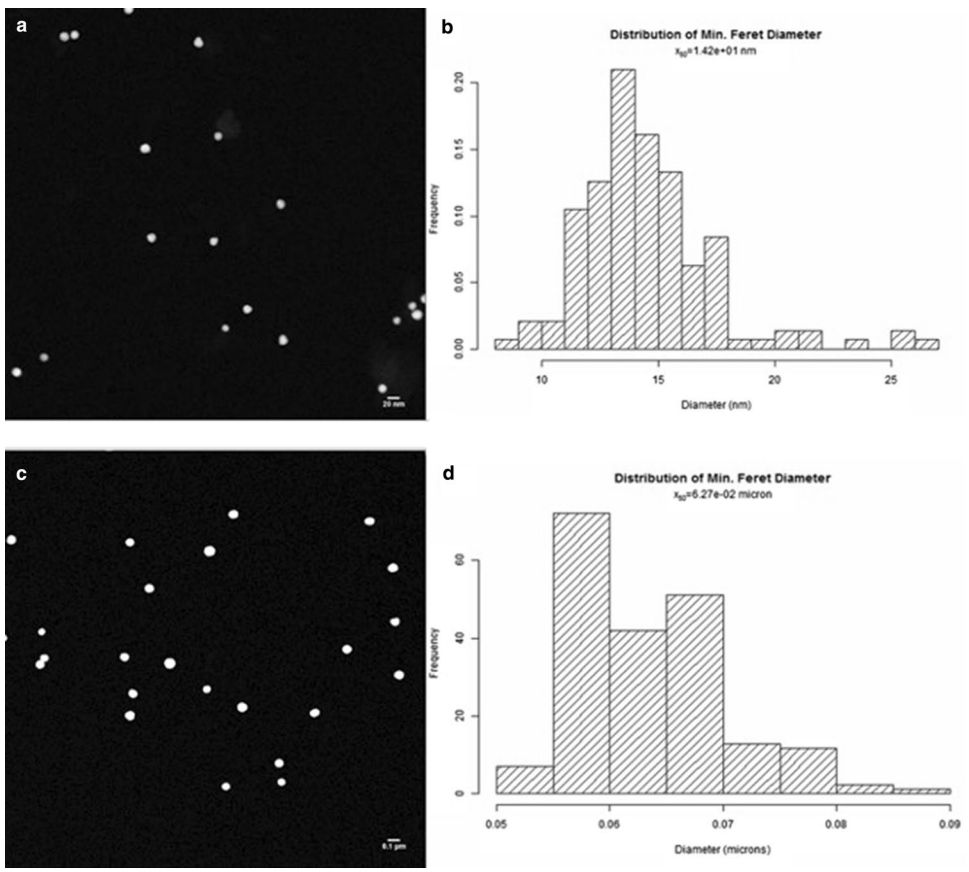

Fig. 2 TEM images (high angular annular dark field, HAADF) of NPs and histograms of the particle size distribution. **a** AgNPs, indicated magnification 35 kx, scale bar shows 20 nm; **b** AgNPs, histogram based on 143 particles; **c** AuNPs, indicated magnification 30 kx, scale bar shows 0.1 µm; **d** AuNPs, histogram based on 172 particles

Tests on bioavailability of nPS in *Hyalella azteca*

No altered behavior was observed in *Hyalella* during exposure to nPS via the water or via the diet (nPS enriched F/pF). Examinations by fluorescence microscopy revealed that fluorescence of the animal samples from the aqueous exposure collected at the end of the exposure phase was mainly visible at the rims and cleaves of the carapace, but in particular at the limbs and antennae (Fig. 4a). The samples collected during the depuration phase showed only a slight decrease of fluorescence. However, the fluorescence hotspots were again mainly localized at the rims and cleaves of the limbs (Fig. 4c).

In contrast, samples collected at the end of the dietary exposure study showed localized fluorescence hotspots mainly in the gut area resembling the structure of fecal pellets (Fig. 4b, red mark) and some spots at the limbs and antennae. Samples taken after 24 h of depuration showed no clear fluorescence in addition to the autofluorescence (Fig. 4d).

Exposure of *C. fluminea* with Ag- and AuNP for the production of NP enriched F/pF

Total metal concentrations in the exposure media and F/pF from the Ag- and AuNP exposure approach with *C. fluminea* were measured using ICP-MS.

The mean concentrations in the exposure media were calculated to be 1.08 ± 0.17 mg Au/L in the AuNP treatment and 10.75 ± 0.83 µg Ag/L in the AgNP treatment.

The released F/pF of the AuNP treatment were of purple color (see Additional file 1: Fig. S2) and were measured to have a burden of $24{,}162 \pm 2799$ g Au/kg (dry mass), resulting in a transfer factor for Au from the exposure of the bivalves by the medium into the released F/pF of around 22,372.

The released F/pF of the AgNP treatment were of yellow–brown color, comparable to the F/pF of the control group and had a burden of 250.65 ± 15.90 mg Ag/kg (dry mass). The calculated transfer factor of Ag was calculated to be 23,316. Examinations of the F/pF using TEM showed the presence of Au- and AgNP in the respective F/pF (Fig. 5). The F/pF of the control group showed a natural burden of 2.91 ± 1.74 mg Au/kg and 6.00 ± 3.92 mg Ag/kg in the dry mass.

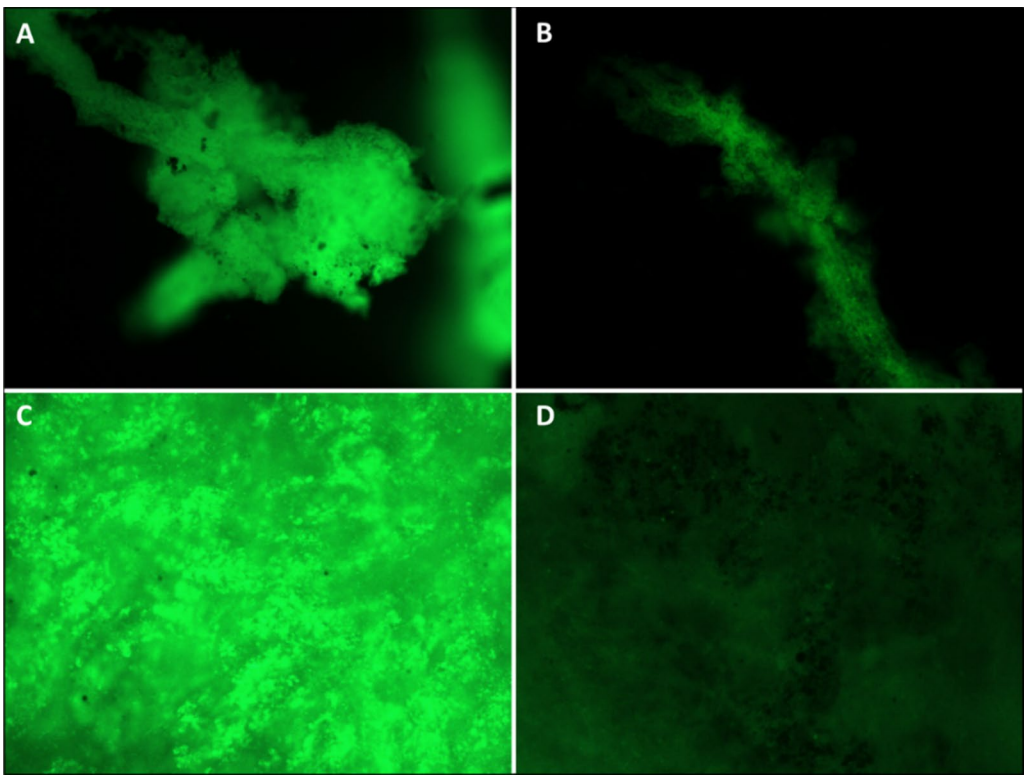

Fig. 3 Samples from the nPS bioavalibility test with *C. fluminea* observed under FM via fluorescence channel. **a** F/pF after 24 h of nPS exposure; **b** F/pF after 12 h of depuration; **c** viscera after 24 h of nPS exposure; **d** viscera after 12 h of depuration

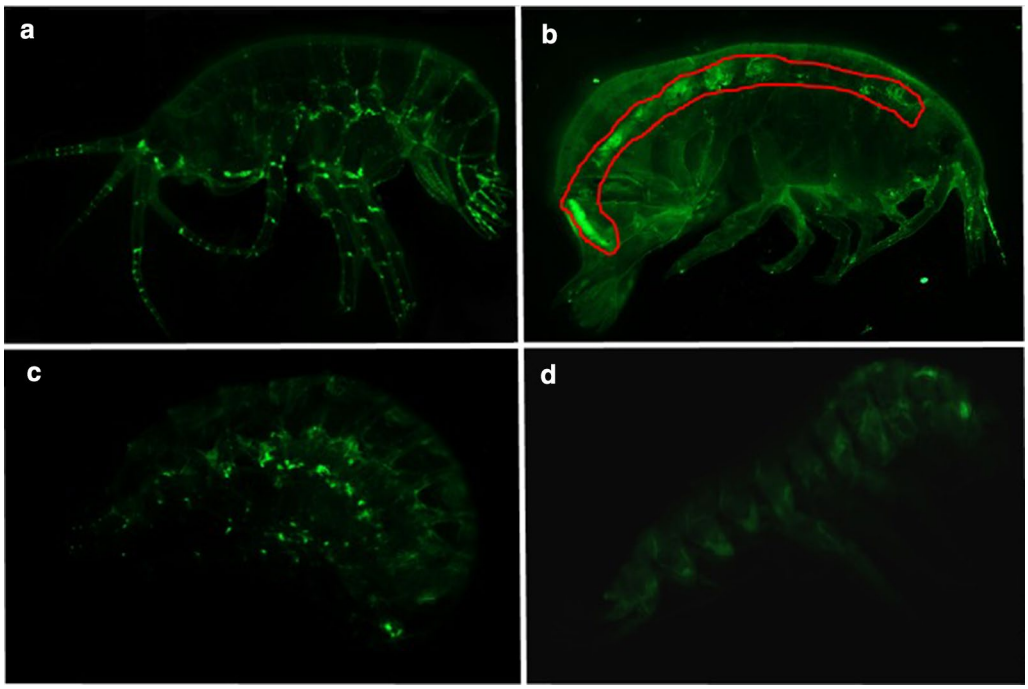

Fig. 4 FM pictures (flourescence channel) of *H. azteca* sampled from the nPS bioavailability tests. **a** After 24 h of aqueous exposure, **b** after 24 h of dietary exposure (the red mark shows the gut area), **c** after 24 h of depuration after aqueous exposure, **d** after 24 h of depuration after dietary exposure. The amphipods had a length of around 30 mm

Corbicula fluminea exposed to AgNPs showed a decreased valve opening time, filtration behavior and release of F/pF compared to the control or AuNP group (by visual observation). A mortality of 64% was observed in the AuNP group at the end of the exposure phase, no mortality was observed in the control or AgNP groups.

Chronic exposure tests with *H. azteca* and F/pF

During the whole chronic exposure test the water quality was acceptable for all parameters. pH values ranged from 7.60 to 8.03 and oxygen saturation was also good with values ranging from 6.27 mg/L and 80.9% to 8.81 mg/L and 111.5% over all treatments. Ammonia concentrations were all below 1.3 mg/L across all measurements. Nitrite and nitrate concentrations were below 0.245 and 9 mg/L, respectively, over the whole test period. More details are listed in the Additional file 1: Tables S2–S7.

Water samples ($n=3$) were taken weekly from each treatment before water exchange and measured for the total metal content. Based on the measurements, mean Ag and Au metal concentrations of 0.98 and 8.01 µg/L were calculated for the media of the F/pF_{Ag} and F/pF_{Au} treatments, respectively. Mean values for Ag and Au concentrations measured in the medium of the control treatment were 0.01 and 0.07 µg/L, respectively.

Total metal concentrations were measured in amphipod samples collected after 21 days of exposure (Fig. 6). The total Ag concentration in the animals from the F/pF_{Ag} treatment was 9.59 ± 1.11 mg Ag/kg and thus nearly 5 times higher compared to the control group (1.94 ± 0.66 mg Ag/kg) and resulting in an accumulation factor of 0.038 with respect to the total Ag concentration in the F/pF diet.

No measurable Au concentration was found in the control group. However, the animals from the F/pF_{Au} treatment showed a body burden of 60.54 ± 32.11 mg Au/kg. Based on the total Au concentration of the F/pF an accumulation factor for Au of 0.003 was calculated.

No significant differences (overall significance level < 0.05) were found in the mortality rate among all treatments after 21 days of exposure, with values of $28 \pm 10.8\%$ for the F/pF_{Au} treatment, $17 \pm 14.4\%$ for the F/pF_{Ag} treatment and $23 \pm 11.2\%$ for the control treatment (Fig. 6). However, the F/pF_{Au} treatment resulted in significant sublethal effects. The animals' mean length and individual fresh weight were significantly reduced when compared to the control or F/pF_{Ag} treatment (Fig. 6). The mean length of the animals from the F/pF_{Au} treatment were around one third smaller (2.04 ± 0.04 mm) than those from the F/pF_{Ag} treatment (3.08 ± 0.22 mm) or the control treatment

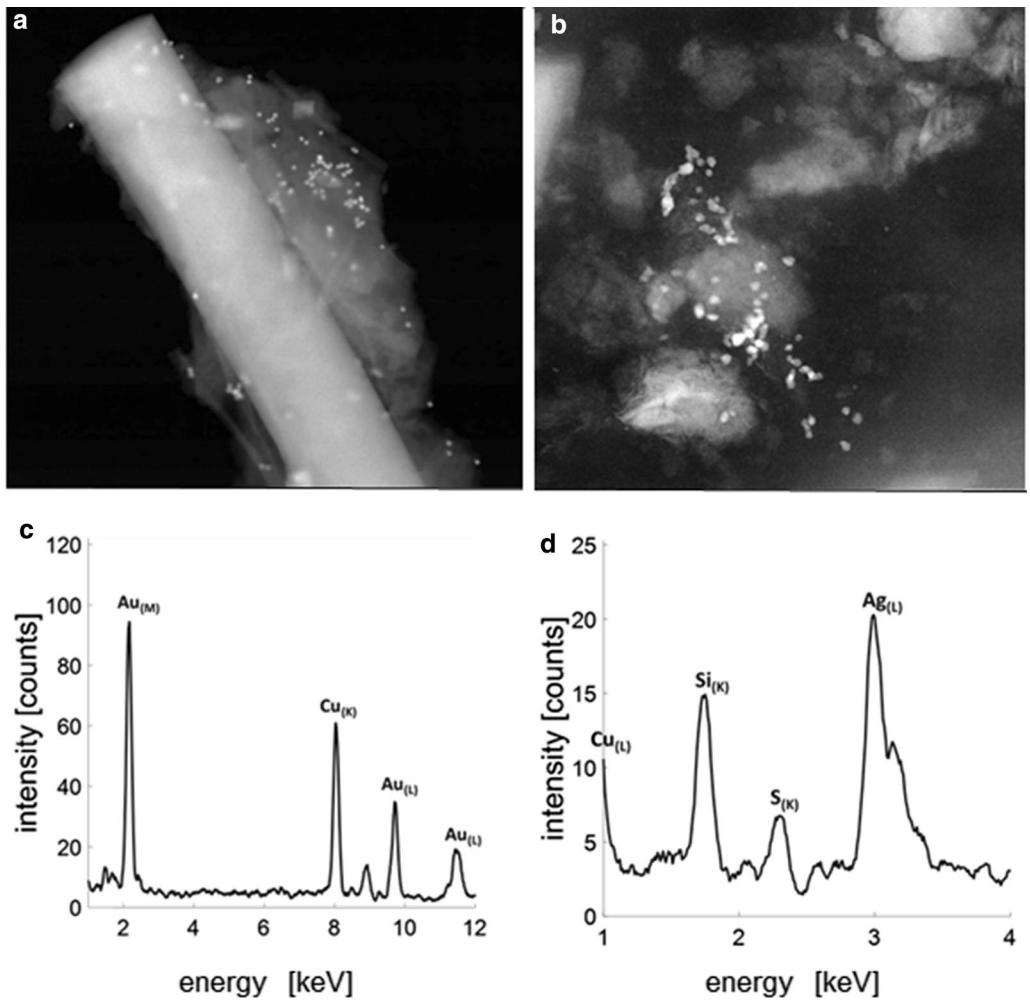

Fig. 5 TEM (high angular annular dark field, HAADF) images and the corresponding energy dispersive x-ray (EDX) spectrum of NPs enriched in the F/pF obtained from previously exposed *C. fluminea*. **a** AuNPs, magnification 15 kx; **b** AgNPs, magnification 110 kx; **c** AuNP, EDX signals; **d** AgNPs, EDX signals. The Si signal is related to a contamination occurring during the TEM analysis

(3.18 ± 0.20 mm). The difference between the F/pF_{Ag} and control treatment was not significant. Details on the statistical procedures are described in Additional file 1.

The individual fresh weight of the animals from the F/pF_{Au} treatment (0.281 ± 0.050 mg) was around 50% of the individual fresh weight determined for the animals of the F/pF_{Ag} (0.560 ± 0.083 mg) and the control treatments (0.623 ± 0.101 mg).

Discussion

Fate of MNMS exposed to filter feeding bivalves

FM examinations of *C. fluminea* tissue and F/pF samples from the nPS exposure approach showed that bivalves are able to filter nPS from the medium and concentrate and transfer them into F/pF. Similarly, the measurements of the total metal concentrations in the F/pF collected from bivalves exposed to Ag- or AuNPs revealed that the metals were strongly accumulated in the fecal matter. However, the main part of the ingested NPs was quickly eliminated by the release of fecal matter containing an increased amount of NPs. However, the fast depuration of the previously ingested particle indicates that no bioavailability of the tested NPs to *C. fluminea* occurred under the applied conditions. The fast reduction of the visceral tissue loading during the depuration phase (as seen by the fluorescence intensity) may be the result of the simple ingestion of MNMs that can be easily transferred relocated to the F/pF matter and thus efficiently eliminated with the excretion of NP-contaminated F/pF. The remaining low fluorescence in the samples of visceral tissue following depuration probably results from

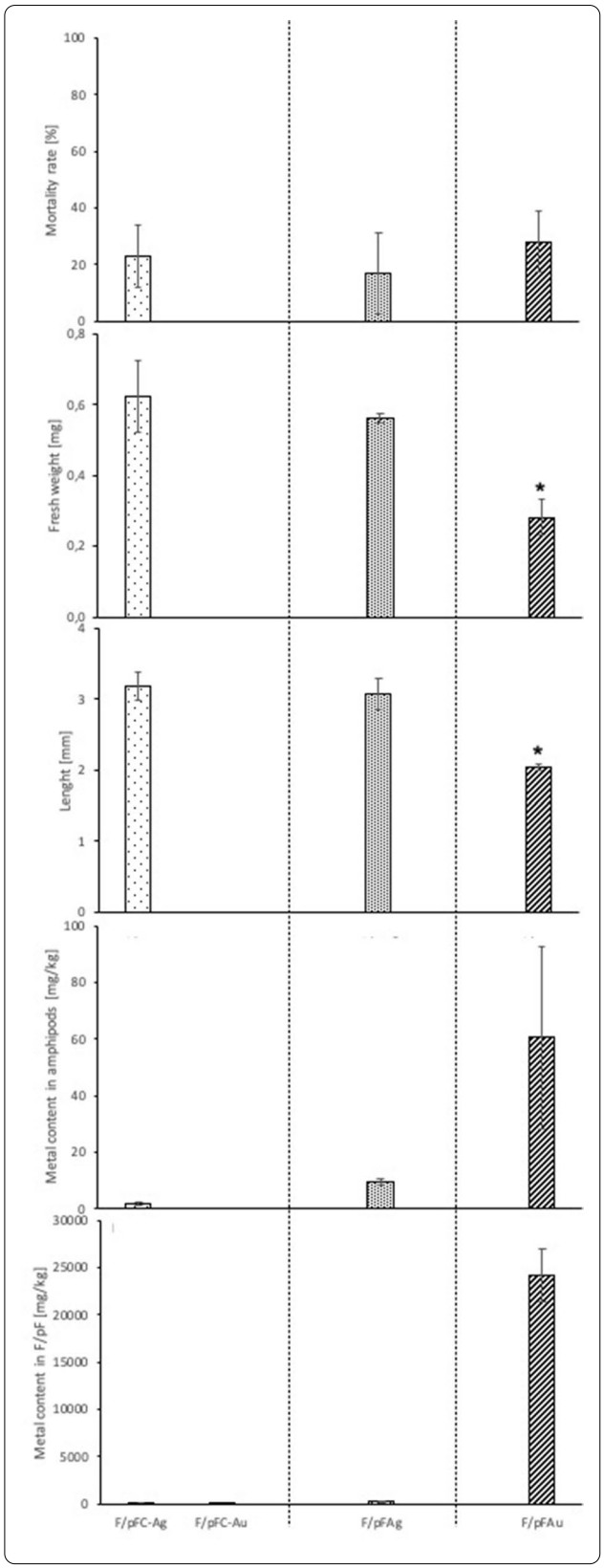

Fig. 6 Results of chronic exposure test with *H. azteca* fed F/pF enriched with AgNP or AuNPs. Measured Ag and Au mean content in generated F/pF (mg/L) ± SD from *C. fluminea* ($n = 4$) as well as the respective total metal body burden of amphipods ($n = 5$, replicates consisting of 11 to 19 amphipods) after 21 days of dietary exposure with AgNP or AuNP containing F/pF (mg/kg) ± SD. F/pFC-Ag: Ag content in F/pF of control group, F/pFC-Au: Au content in F/pF of control group. Mean length of the amphipods in mm, mean individual fresh weight of the amphipods in mg and mean mortality rate in %, each ± SD and $n = 5$ replicates (consisting of 11 to 19 amphipods). *$p < 0.05$

nPS attached to the tissues' surface. However, a previous study using fluorescence labelled nanoplastics showed that the dye may leach from the particles and accumulate in the lipid fraction of the test organism. This may lead to artifacts and misinterpretations concerning the presence of the labelled material when examinations are based only on fluorescence microscopy [72].

Even if the supplier of the used nPS used explains that the fluorescence dye does not leach into water, this aspect should be evaluated and may explain the observation of the residual fluorescence after the depuration phase. This is in contradiction to the elsewhere observed fast and efficiently elimination of MNMs by bivalves by others [16, 45, 46].

The examinations of the F/pF from the Ag- and AuNP exposure test using TEM and EDX confirmed that the measured concentrations of Ag and Au are caused by nanoparticular matter and thus by the exposed NPs which have been enriched in the fecal matter. The signals from the EDX examinations show a peak for sulphur in the Ag loaded F/pF suggesting that the AgNPs present in the F/pF were sulfidized. The shapes of the NPs examined under the TEM showed aggregates of few AgNPs that were connected by nano-bridges as observed by Metreveli et al. [73] during examinations of AgNPs after 100 min of sulfidation using NaHS (see Additional file 1: Fig. S3). However, even if the release of Ag^+ ions were reduced due to the sulfidized surface, previous studies have shown that Ag from sulfidized AgNPs could still be available to aquatic species like *H. azteca* [5, 66, 67]. Thus, concerns regarding the transfer of manufactured MNMs between filter feeding bivalves and other aquatic invertebrates that feed on their F/pF and potential ecotoxicological effects induced by the ingested MNMs seems to be legitimate.

Effects of MNMs exposed to filter feeding bivalves

If bivalves are negatively influenced by hazardous environmental conditions that may cause toxic effects, like high metal ion concentrations, bivalves like *C. fluminea* rapidly close their valves and reduce filtration activity to a minimum [74–80]. In contrast to that, we observed a

high filtration activity in the AuNP treatment that may be the result of the particulate character of the test items. Similar observations have been reported by Kuehr et al. [46] for bioaccumulation studies with *C. fluminea* and TiO_2NPs. In addition, in this case a very fast and effective elimination of TiO_2NPs during the depuration phase was observed.

Due to the very limited solubility of the AuNPs and thus a lack of ions, avoiding behavior by valve closing and the reduction of filtration activity may potentially not be induced. The lacking avoidance behavior observed during AuNP exposure might be the reason for the increased mortality caused by physiological mechanisms. This can only be speculated, if the induction of such a high filtration activity due to the presence of high amounts of particulate matter inevitably causes a higher demand for energy. Furthermore, the excretion of high volumes of nutrient rich F/pF matter required for the elimination of the ingested NPs, may have led to a strong loss of energy and nutrients for the bivalves. However, this needs further investigations to elucidate the impact of the filtration activity and the excretion of high F/pF volumes on the energy and nutrient level in the organisms that may have caused the observed mortality in the bivalves during the AuNP exposure.

Another explanation for the mortality could be linked to the observations by Fouqueray et al. [81]. They observed a strong inhibition of digestive enzymes such as amylase and trypsin in *Daphnia magna* after exposure to TiO_2NPs, which are also sparingly soluble in the exposure media and thus comparable to AuNPs [81]. However, such NPs may still induce the formation of reactive oxygen species causing harmful effects [82–85].

In addition, Baudrimont et al. [86] observed oxidative stress with a negative impact on the mitochondrial respiration and reduced ATP production in *Gammarus fossarium* after dietary AuNP exposure. Arini et al. [11] described a strong up-regulation of genes (up to 7.7 times) that were involved in the regulation of the immune system and apoptosis after exposure of *C. fluminea* to AuNP concentrations in the range of 1 to 24 mg Au/L. The effects were even stronger than those observed for treatments using ionic Au [11]. Further studies are required to elucidate which factors are responsible for the high mortality of *C. fluminea* observed during the AuNP exposure. However, the used concentration level used in this study is magnitudes above realistic environmental concentrations and was chosen to guarantee measurable body burdens in the amphiods after potential ingestion by the amphipods.

Availability of MNMs exposed via F/pF to *H. azteca*

Amphipod samples collected during the dietary approach using MNMs containing F/pF showed fluorescence hot spots, in particular in the gut region. No fluorescence hot spots were visible in other regions/tissue or the animals' carapace. Exceptions were the limbs and antennae, which both are in contact with the contaminated food (nPS-containing F/pF) during food intake.

Once ingested, particles smaller than 500 nm are incorporated into cells predominantly by receptor mediated endocytosis pathways rather than by phagocytosis [87–89]. Clathrin-mediated endocythosis is supposed to be the main uptake mechanism for AuNPs [90–92]. As described by Harush-Frenkel et al. [93] negatively charged NPs show a significant reduced endocytosis rate and cannot use clathrin-mediated endocytosis, making it less likely that the particles are incorporated or accumulated by cells. Furthermore, the used NPs used showed negative zeta potentials ranging from −22.8 mV (AgNPs) to −31.9 mV (nPS) making these particles less bioavailable [93]. However, we have no information on the surface charge of the NPs after passing through the bivalves and being incorporated into the F/pF matrix [94]. It can be assumed that particles are bio-transformed by the addition of proteins and the formation of an eco-corona. In addition, the low accumulation factor of 0.003 for Au could be the result of the very high exposure concentration (24,162 ± 2799 mg Au/kg) and a limited uptake capacity.

In contrast to the samples from the dietary exposure study, individuals collected during the aqueous exposure of *H. azteca* with nPS showed fluorescence hot spots only at the outside of the carapace. The nPS seems to be attached to the surface of the amphipods only and no evidence was found for incorporation of the NPs. However, in the case of metal NPs, particles attached to the surface of amphipods may release metal ions leading to toxic effects.

However, these observations are in accordance with the results of aqueous and dietary exposure of Au- and TiO_2NP (also not ion-releasing MNMs) which were tested for their bioaccumulation potential in *H. azteca* described by Kuehr et al. [66]. They also observed a higher enrichment of the respective metals after dietary exposure and no measureable metal concentrations after aqueous exposure [66].

These observations, together with the lack of nPS present in the gut after aqueous exposure in this study, highlights the role of bivalves and their F/pF as vectors for transfer of MNMs to other benthic invertebrates and thus into the aquatic food chain.

Nevertheless, an effective elimination of the exposed nPS was observed during the depuration phase, resulting

in no visible fluorescence by nPS in the amphipods sampled after 24 h of depuration following dietary exposure. Comparable to the bivalves, the fast and effective elimination seems to be based on defecation. Furthermore, no resorption of NPs from the gut lumen to adjacent tissues was observed which is in agreement with former histological examinations using correlative microscopy and TEM with EDX on *H. azteca* after chronic exposure to AgNP-containing sewage sludge [67].

The potential risk of an uncontrolled co-exposure via MNMs leached from the F/pF or the amphipods feces into the medium, as well as by coprophagy seems to be very small or even negligible as we did not find any nPS (as seen as fluorescence) attached to the animals surface and after the aqueous nPS exposure. Within the experiments of Kuehr et al. [67] the dietary exposure path was the only scenario leading to an ingestion of AgNPs by *H. azteca*. In addition, they also have found no indications of any attachment of AgNPs to the animal's surface [67]. These findings also reveal the negligible role of the aqueous exposure and thus co-exposure during the experiments of this study, at least for the particle fraction. Further investigations should also investigate the role of dissolved fractions in the media during the exposure of amphipods to F/pF from bivalves.

Effects of MNMs exposed via F/pF to *H. azteca*

The presumed low bioavailability of the used NPs may explain the absence of effects in the chronic exposure test with *H. azteca* when comparing the F/pF_{Ag} and the control group. Toxic effects would have been expected due to the relatively high Ag concentration in the F/pF (250.65 ± 15.90 mg Ag/kg) and amphipods (9.95 ± 1.11 mg Ag/kg) of the F/pF_{Ag} treatment and the known sensitivity of *H. azteca* to Ag and other metals [94]. The low toxicity might be the result of either a sulfidized surface of the AgNPs causing lower toxicity or the result of proteins from the F/pF that may have formed an eco-corona as described by Nasser and Lynch [95] and discussed by Mehennaoui et al. [96]. This may be an explanation for decreased toxicity as observed in tests with AgNPs and with *Gammarus fossarum*.

The significant effects observed in the F/pF_{Au} treatment on length and fresh weight of the amphipods cannot be explained by the experimental conditions which were characterized by a good oxygen supply, moderate pH conditions and low concentrations of ammonia, nitrite and nitrate. The effects might be the result of a negatively altered nutrient supply caused by reduced enzyme activity as mentioned above and oxidative stress and inflammatory responses caused by the presence of high NP concentrations in the gut. However, further investigations are necessary to prove this assumption.

Conclusions

For the first time it has been proven that MNMs can be transferred via the F/pF from bivalves to other animals. AuNPs as well as nPS, both used as inert and non-ion-releasing tracers, have been shown to be transferred to the amphipod *H. azteca* using NP contaminated F/pF. Thus, the results of this study revealed, that NPs and not only their potentially released ions haven been transferred to the amphipod. Furthermore, in the case of nanoplastics, represented by nPS, the availability of the particles for the amphipod was highly increased when applied as contaminated F/pF in comparison to no ingestion during aqueous exposure. The transfer of AuNPs by the F/pF and the resulting uptake by exposed amphipods caused significant effects during chronic exposure. Due to the virtually inert characteristic and persistence of nanoplastics, as erosion products of microplastics, similar effects are expected. Furthermore, the transfer and ingestion of such particles by F/pF may alter the bioavailability of organic compounds for example UV-filters, present in the plastics. Thus, further investigation regarding the potential trojan horse effect, caused by nano or microplastics transferred to the benthic species by F/pF are recommended. This effect may alter the bioavailability of adsorbed pollutants and thus further investigations are of higher interest. Further investigations should be carried out to elucidate the impact of the surface charge of transferred NPs on their bioavailability, as well as the particles impact on the bioavailability, transfer and accumulation of other pollutants along the aquatic food chain. In addition, the observed effects following AuNP exposure and potential effects of nanoplastics need to be further examined and the results should be considered in the risk assessment process of NPs.

Abbreviations

Ag^+: Silver (I) ion; AgNPs: Silver nanoparticles; AuNPS: Gold nanoparticles; ANOVA: Analysis of variance; F/pF: Feces/pseudo-feces; ICP-MS: Inductively coupled plasma mass spectrometry; MNM: Manufactured nanomaterials; nPS: Nanostructural polystyrene; TEM: Transmission electron microscopy; TWA: Time weighted average concentration.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12302-021-00473-3.

Additional file 1: Figure S1. Exposure scenario for the production of NP enriched F/pF. **Figure S2.** F/pF loaded feeding filters. Left: control F/pF; middle: AuNP containing F/pF_{AunP}; right: AgNP containing F/pF_{AgNP}. **Table S1.** Chemical and physical parameters of the copper reduced tap water used for the acclimatization and test phases. **Table S2.** Values from

temperature, pH and oxygen monitoring of the F/p$F_{control}$ treatment during the chronic exposure test. **Table S3.** Values from temperature, pH and oxygen monitoring of the F/pF_{AuNP} treatment during the chronic exposure test. **Table S4.** Values from temperature, pH and oxygen monitoring of the F/pF_{AgNP} treatment during the chronic exposure test with *H. azteca*. **Table S5.** Examination of water burdens of nitrate, nitrite and ammonium in the F/p$F_{control}$ treatment during the chronic exposure test with *H. azteca*. **Table S6.** Examination of water burdens of nitrate, nitrite and ammonium in the F/pF_{AuNPl} treatment during the chronic exposure test with *H. azteca*. **Table S7.** Examination of water burdens of nitrate, nitrite and ammonium in the F/pF_{AgNP} treatment during the chronic exposure test with *H. azteca*. **Figure S3.** Left: TEM HAADF image of AgNPs from the F/pF_{AgNP} showing aggregates of AgNPs fused by presumable Ag_sS-nanobridges. Right: STEM-HAADF image of aggregates of sulfidized AgNPs connected with Ag_sS-nanobridges taken from Metreveli et al. [73]

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Authors' contributions
SK: design and conducting of exposure studies; total metal analysis; sample examination using fluorescence microscopy; collection, statistical analysis and visualization of the data; writing—original draft; writing—review and editing. ND: conducting of exposure studies; sample examination using fluorescence microscopy. RK: conducting characterization of NMs using TEM and EDX; resources (NM characterization); writing—review and editing. CS: funding acquisition; design of study, resources; writing—review and editing; supervision. All authors read and approved the final manuscript.

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Availability of data and materials
Additional data to this article can be found in Additional file 1 related to this manuscript.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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