



Testing the bioaccumulation potential of manufactured nanomaterials in the freshwater amphipod *Hyalalella azteca*

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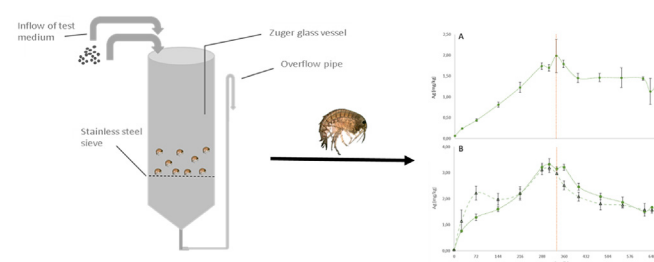
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HIGHLIGHTS

- The amphipod *Hyalalella azteca* was used for bioaccumulation assessment of nanomaterials.
- Biomagnification and bio-concentration factors were gained in separate tests.
- The importance of ions for the accumulation of metals from nanoparticles was shown.
- This method may allow a waiver of bioaccumulation fish studies for risk assessment.

GRAPHICAL ABSTRACT



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ABSTRACT

Standardized experimental approaches for the quantification of the bioaccumulation potential of nanomaterials in general and in (benthic) invertebrates in particular are currently lacking. We examined the suitability of the benthic freshwater amphipod *Hyalalella azteca* for the examination of the bioaccumulation potential of nanomaterials. A flow-through test system that allows the generation of bioconcentration and biomagnification factors was applied. The feasibility of the system was confirmed in a 2-lab comparison study. By carrying out bioconcentration and biomagnification studies with gold, titanium dioxide and silver nanoparticles as well as dissolved silver (AgNO_3) we were able to assess the bioaccumulation potential of different types of nanomaterials and their exposure pathways. For this, the animals were examined for their total metal body burden using inductively coupled mass spectroscopy (ICP-MS) and for the presence of nanoparticulate burdens using single-particle ICP-MS. The role of released ions was highlighted as being very important for the bioavailability and bioaccumulation of metals from nanoparticles for both examined uptake paths examined (bioconcentration and biomagnification). In 2018 a tiered testing strategy for engineered nanomaterials was proposed by Handy et al. that may allow a waiver of bioaccumulation fish studies using inter alia invertebrates. Data gained in studies carried out with invertebrates like the developed *Hyalalella azteca* test may be included in this proposed tiered testing strategy.

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1. Background

Nanomaterials (NMs) are present in nearly every sector of the industry and, due to their broad range of properties (Yang et al., 2003; Hainfeld et al., 2006; Maier and Korting, 2005; Martin et al., 2015; Rahman et al., 2011; Rodrigues et al., 2017; DeLoid et al., 2018; Piccinno et al., 2012), can be found in various consumer products such as textiles, medicine, cosmetics, printer toners, car tires and even agri-foods. For example metals and metal oxides like gold and titanium dioxide may be highly efficient catalysts when used as NPs (Chithrani et al., 2006; Peer et al., 2007; Kim et al., 2009; Peng et al., 2005; Jang et al., 2001; Zhou et al., 2010; Mikami et al., 2013). Silver nanoparticles (AgNPs) are mainly used due to their antimicrobial properties. However, NMs can be released into the environment during manufacturing, usage and disposal and thus pose a potential environmental risk. Due to the high production volume of NMs, they are subject to bioaccumulation assessment as required by the European Chemicals Registration REACH, the Japanese Chemical Substance Control Act “Kashinho” or others, e.g. the Turkish REACH like law KKDIK (Kaydi, Değerlendirilmesi, İzni, Kısıtlanması) (Ministry of Environment and Urbanization MoEU of Turkey, 2017), the High Production Volume Challenge Program of the USA (USEPA, 2014), or Toxic Chemicals Control Act of Korea (Korea Ministry of Government Legislation, 1997). The bioconcentration factor (BCF) expressing the potential of a test substance to be accumulated from the surrounding medium is the ultimate decisive bioaccumulation criterion as part of the regulatory chemical safety assessment. The BCF is commonly determined by fish-flow-through tests according to OECD test guideline 305 (Organisation for Economic Co-operation and Development OECD, 2012). The test was developed for water-soluble and primary lipophilic test items. Aggregation and sedimentation of NM make it very challenging to maintain aqueous concentrations at a constant level during flow-through tests. Thus, the establishment of suitable experimental conditions for determining the bioaccumulation potential of NMs or NPs is difficult (Aschberger et al., 2011; Hankin et al., 2011). In addition, some NMs show a complex behavior, such as AgNPs releasing dissolved constituents. As shown by Zeumer et al. (2020), the bioavailability and accumulation of such NPs exposed to fish during a flow-through bioconcentration study according to OECD guideline 305, seem to depend on the bioaccumulation of the released ions rather than on the uptake of the used NPs (Organisation for Economic Co-operation and Development OECD, 2012; Zeumer et al., 2020). Considering the tendency of NMs to aggregate and thus sediment, benthic organisms may in fact represent a worst case scenario for bioaccumulation testing as compared to fish. In 2018, Handy et al. proposed a tiered testing strategy for engineered NMs that may allow a waiver of further bioaccumulation studies using vertebrates (Handy et al., 2018). For the second tier of the assessment work flow, data gained from studies using invertebrates needs to be taken into account. So far, bioaccumulation studies with NMs of different quality are available using invertebrate organisms of different phyla such as marine bivalves, Daphnia and earthworms, making the comparison of the results difficult. A standardized experimental approach to quantify bioaccumulation of NMs in invertebrates in general and benthic species in particular is still missing (Petersen et al., 2019).

In 2020, Kuehr et al. described a new test system using the filter feeding fresh water bivalve *Corbicula fluminea* to gain bioaccumulation data for NMs (Kuehr et al., 2020a). However, this test provides bioaccumulation factors (BAF) that are not that specific as the common regulatory endpoints, which are normally BCF or BMF (biomagnification factor), to describe the bioaccumulation potential of chemical compounds.

Another promising approach for bioaccumulation testing of NMs may be the bioaccumulation test using the benthic fresh water amphipod *Hyaella azteca* that was described by Schlechtriem et al. (2019). *H. azteca* is a well-established test organism for ecotoxicological studies (Canada. Environment Canada, 2013; USEPA, 2000; Borgmann, 2002) and is sensitive to environmental chemicals and metals in the environment (Othman and Pascoe, 2001; Wood et al., 2002; Blaser et al., 2008). The amphipod can be easily cultured in the laboratory, is available all year round and shows a high reproduction rate and fast growth. *H. azteca* has already been successfully used in studies on the bioavailability of NMs present in the water, sediment or sewage sludge (Kuehr et al., 2018; Poynton et al., 2019). Therefore, the benthic species might also be a suitable test organism for the determination of the bioaccumulation potential of NMs.

In this study we investigated the bioaccumulation of different NMs including AgNPs (NM 300 K), titanium dioxide nanoparticles (NM 105; TiO₂NPs), and AuNPs in *H. azteca* following aqueous and dietary exposure. The body burden of the animals was measured for the total content of Ag, Au and Ti using inductively coupled plasma mass spectrometry (ICP-MS). The presence of nanoparticles in the test media as well as in the whole animals were determined by single particle ICP-MS (spICP-MS) and BCF and BMF values for the different types of NMs were calculated. Differences in the results of studies carried out with male or female animals were examined. The feasibility of the testing procedure applied in this study was investigated as part of a 2-lab comparison approach.

2. Materials and methods

2.1. *Hyaella azteca*

The amphipods used in this study were collected from the stock culture of Fraunhofer IME, Schmallenberg. The culturing procedure was carried out according to Kuehr et al. (2018). Only healthy animals free from observable diseases and abnormalities were used. Male and female individuals were separated before use in the different experiments. Males were identified by the presence of a large gnathopod and females by the presence of eggs in the marsupial plate as described by Schlechtriem et al. (2019).

2.2. Handling and preparation of the test items and stock suspensions

The bioaccumulation studies were carried out using NM 300 K as well dispersed and ion-releasing NPs. AgNO₃ was tested as a dissolved form of the same element. NM 105 (TiO₂) was used as test material representing a non-ion releasing NM with a high tendency to agglomerate (Rasmussen et al., 2014). AuNPs were tested as well dispersed, non-ion releasing NPs. NM 300 K and NM 105 were provided from the Fraunhofer Institute for Molecular Biology and Applied Ecology IME and are representative test and reference materials from the European Commission's Joint Research Centre and in the scope of the OECD Working Party on Manufactured Nanomaterials (WPMN) Sponsorship Program. Information on the characterization and physico-chemical properties are summarized in JRC Reports (Rasmussen et al., 2014; Klein et al., 2011). The NM 300 K stock suspension was stabilized by a dispersing agent (NM 300 DIS), containing 4% (w/v) of polyoxyethylene, glycerol, tri-oleate, and polyoxyethylene (20)-sorbitan-monolaureate (Tween 20) each and 10.16% (w/w) AgNPs with an average size of 15 nm (Klein et al., 2011). The working suspension of NM 300 K and NM 105 (present as nano powder) were produced as described by Kuehr et al. (2020a). AgNO₃ (purchased from Carl Roth, purity of >99.9%) was dissolved and diluted with ultra-high quality water

(UHQ) for preparation of the stock solution (For results of the particle characterization see section 3.1 or Table S1 of the supplementary information). The AuNPs (purchased from BBI Solutions), with a nominal size of 60 nm were supplied as a stock suspension in ultra pure water at a concentration of approximately 57 mg Au/L (0.01% AuCl). The AuNP working suspension was produced analogously to the NM 300 K working suspension.

2.3. Experimental feed preparation

The method for the production of agar-agar cubes (DECOTABs) described by Kampfraath et al. (2012) was modified to produce nanomaterial enriched DECOTABs as experimental diet that allows the controlled exposure of NMs via the magnification pathway at stable concentrations. The use of enriched DECOTABs showed a negligible risk of NP dispersion or leaching of ions that could have led to a secondary and uncontrolled uptake phase.

For each nanomaterial individual DECOTABs were produced. For this, 500 mg agar-agar (Roth) were dissolved in 23 mL boiling UHQ under constant stirring using a magnetic stirrer. 500 μ L of the respective NP stock solutions (ca. 50 mg NPs/L) were added and heated and stirred for 1 min before 1500 mg TetraMin® flakes were added under constant stirring. The suspension was stirred for 2 min and transferred into silicon ice trays to form cubes of 1 mL volume. The trays were stored at 4 °C until DECOTABs were completely hardened. Five DECOTABs of each treatment were taken for metal content analysis by ICP-MS to check the homogeneity of the experimental feed. Further samples were taken and frozen with liquid nitrogen and stored at -20 °C for the determination of single particle concentrations by spICP-MS and the characterization of the NMs by transmission electron microscopy (TEM). The DECOTABs applied during the studies were stored at 4 °C until use.

2.4. Performance of bioconcentration studies

The flow-through studies for bioaccumulation testing were carried out in a modified test system as described by Kuehr et al. (2020a), to ensure a homogenous exposure of the dispersed NPs (Fig. 1). The test system consists of a Zuger glass jar with a volume of 8 L. A stainless steel sieve fixed in the lower part of the jar prevents loss of the amphipods from the system through the outlet pipe at the bottom of the Zuger glass jar. A stainless steel mesh was placed into the test system providing a place of refuge for the test animals.

The working suspension of NM 105 was applied directly to the test vessel to avoid sedimentary processes in the mixing vessel. The stock solutions of the other test items were first transferred into a mixing vessel using a peristaltic pump (IPC High Precision Multi-channel Dispenser, ISMATEC®). They were further diluted with dilution water supplied by a membrane pump (gamma/X, ProMinent®) to produce the test medium which was transferred to the Zuger glass jar by TYGON® tubes (E-3603, TYGON®) with a flow rate of 3 L/h. The dilution water was aerated and heated to 25 °C prior to being used in the test. During the flow through tests, animals were fed ad libitum with agar-agar cubes (DECOTABs) manufactured as described above but without the addition of NPs.

Before the start of the bioconcentration test, the system was allowed to equilibrate for at least 48 h until stable media concentrations with a variation of $\leq 20\%$ were reached for at least three sampling times (separated by at least 3 h).

During the test, water properties like pH-value, temperature and dissolved oxygen (mg/L and saturation in %) were monitored daily. Measurements of ammonia, nitrite and nitrate were carried out at the start and end of the uptake and end of depuration phase using a test kit for photometric measurements (NANOCOLOR®

500D, Machery-Nagel).

The animals were exposed to the different test suspensions at concentrations ranging from 0.085 to 6.83 μ g/L for up to 336 h before being transferred into a clean system for depuration where they were kept in dilution water without the NMs under conditions that were otherwise identical to the uptake phase. The animals were washed with dilution water before being transferred into the clean system to avoid the transfer of test item from the exposure medium. Test conditions of the bioconcentration studies are summarized in Table S2.

Medium samples and triplicate tissue samples each consisting of 20 individuals each, were taken from the test system at different time points during the uptake and depuration phases. For the sampling time points see Figs. 4–6. The medium samples were acidified by adding 200 μ L of nitric acid (69%, suprapure grade, Roth) and stored at 4 °C. Media samples for single particle-ICP-MS (spICP-MS) were not acidified but measured directly. Animal samples were washed with dilution water, blotted with lint free paper, weighed (AUW220D, SHIMADZU) and stored at -20 °C. Tissue samples for spICP-MS were frozen using liquid nitrogen before being stored at -20 °C.

The tests with AgNO₃ were carried out three times: using only male (AgNO_{3-M}) and only female individuals (AgNO_{3-F}) in order to investigate potential effects of the animal's sex on the bioaccumulation of metals or ions released from NPs during the tests. In addition, a third, extended test was carried out with females testing a higher exposure concentration to investigate potential concentration dependence of the bioaccumulation (AgNO_{3-FH}). Additional samples (triplicates of 10 animals) were taken at the end of the uptake and the additional depuration phase of the third test to determine the total Ag content associated with the protein fraction of the animals.

The tests with NM 300 K were additionally carried out using males in two different systems, the Zuger glass system (NM 300K_{Zg}) and a 25-L glass aquarium (NM 300K_{Aq}). This was done to identify potential differences induced by the test systems regarding exposure conditions and potential effects on the NP's bioaccumulation behavior. The same stock solutions of NM 300 K were used, to allow comparison of the results. For measured exposure concentrations in the bioconcentration tests see Tables 2–4.

An additional test with NM 300 K (NM 300K_{cm}) was carried out in aquaria using a physiological culture media with reconstituted UHQ prepared according to Borgmann (1996) (Table 1) to evaluate the potential effects of the ion content of the test medium on the bioavailability and bioaccumulation of metals from NPs. This study was carried out as part of a 2-lab comparison approach to investigate the robustness of the testing procedure.

2.5. Performance of biomagnification studies

The Zuger glass system described above was also used for the biomagnification tests. The design of the test system incorporating the stainless steel sieve allows removal of feces, and potentially leached ions and NMs from the feces or experimental feed (DECOTABs) from the test chamber with the water flow to avoid co-exposure of the amphipods via the aqueous pathway. For each test item two biomagnification tests were carried out using two different concentrations to determine the potential concentration dependent effects on the uptake and elimination of metals from the NPs. Test conditions of the biomagnification studies are summarized in Table S3, for measured exposure concentrations in the diet see Table 5. The animals were fed (addition of fresh DECOTABs containing NPs) in the morning after removing the old feed.

Water samples were taken during the uptake period to determine any potential contamination of the test water. Duration of the

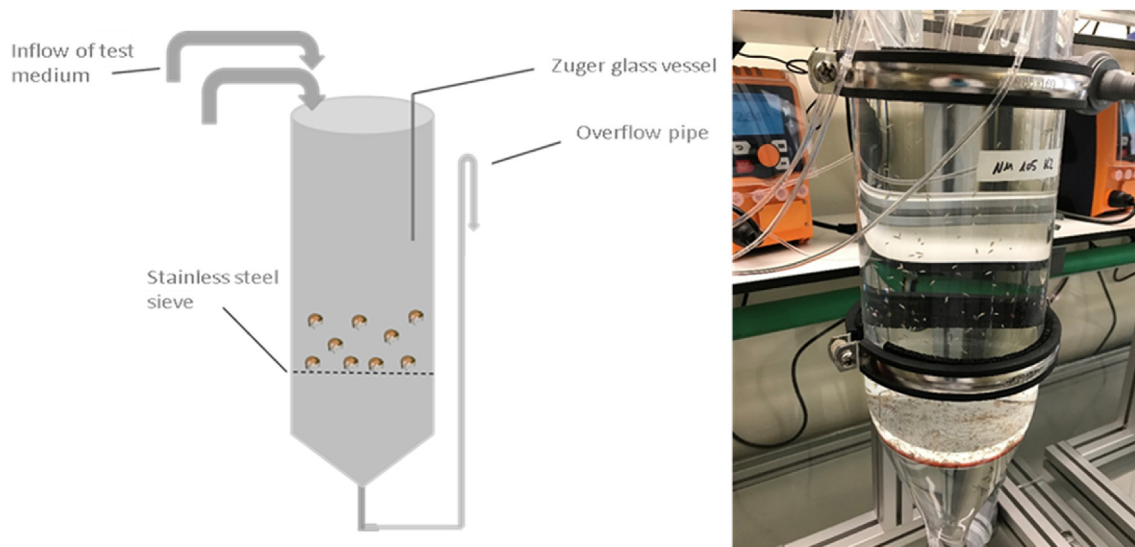


Fig. 1. Overview of the flow-through system for testing nanomaterials.

Table 1

Composition of the culture medium according to Borgmann (1996).

Compound	Molecular formula	Concentration [mol/L]
Calcium chloride dihydrate	$\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$	0.004
Sodium bromide	NaBr	0.0004
Potassium chloride	KCl	0.0002
Potassium hydrogen carbonate	NaHCO_3	0.004
Magnesium sulfate	MgSO_4	0.001

Table 2

Characteristics of the bioconcentration tests with AgNO_3 and calculated BCF values.

Study	Test item	Sex	TWA [$\mu\text{g Ag/L}$]	BCF
$\text{AgNO}_3\text{-FL}$	AgNO_3	female	0.64	5100
$\text{AgNO}_3\text{-ML}$	AgNO_3	male	0.67	4900
$\text{AgNO}_3\text{-FH}$	AgNO_3	female	1.64	4700

uptake and depuration phase as well as the schedule of the animal samplings were adjusted depending on the experiences from the bioconcentration tests. For sampling time points see Fig. 7. A depuration phase was only carried out in the tests with the high exposure concentration to gain information on the elimination of the previously accumulated metals/NPs.

2.6. Characterization of the nanoparticles

The stock material and processed nanomaterials were characterized using TEM in combination with energy dispersive x-ray (EDX) analyses to assess elemental composition of the NM and thus

Table 3

Characteristics of the bioconcentration studies with NM 300 K and calculated BCF values.

Study	Test item	Test system	Exposure Medium	TWA [$\mu\text{g Ag/L}$]	BCF
NM 300K _{Cm}	NM 300 K/AgNP	aquarium	culture medium	3.00	604
NM 300K _{Aq}	NM 300 K/AgNP	aquarium	dilution water	6.73	480
NM 300K _{Zg}	NM 300 K/AgNP	zuger glass system	dilution water	6.83	453

Table 4

Characteristics of the bioconcentration studies with NM 105 and AuNPs and calculated BCF values.

Study	Test item	Exposure Medium	TWA [$\mu\text{g/L}$]	BCF
NM 105 _L	NM 105/TiO ₂ NP	dilution water	1.49	—
NM 105 _H	NM 105/TiO ₂ NP	dilution water	3.3	—
AuNP _L	AuNP	dilution water	0.085	424
AuNP _H	AuNP	dilution water	0.704	166

Table 5

Characteristics of the biomagnification studies and calculated BMF values. *Au concentration in the AuNP_L treatment was 3.9 ng/kg.

Study	Test item	Concentration [mg/kg]	BMF
NM 300K _L	NM 300 K/AgNP	0.07	0.25
NM 300K _H	NM 300 K/AgNP	0.751	0.93
NM 105 _L	NM 105/TiO ₂ NP	3.67	0.12
NM 105 _H	NM 105/TiO ₂ NP	19.43	0.02
AuNP _L	AuNP	<0.00*	—
AuNP _H	AuNP	0.889	0.02–0.03

to confirm their identity. The feed stock dispersion of NM 300 K was diluted 1:10⁶ in UHQ prior to TEM analysis. 20 mg of NM 105 was suspended in 100 mL 0.2% Novachem and sonicated for 10 min before dilution 1:20. 1 mL of the diluted suspensions was directly centrifuged (1 h, ~14 000×g) on TEM grids. The AuNP suspension

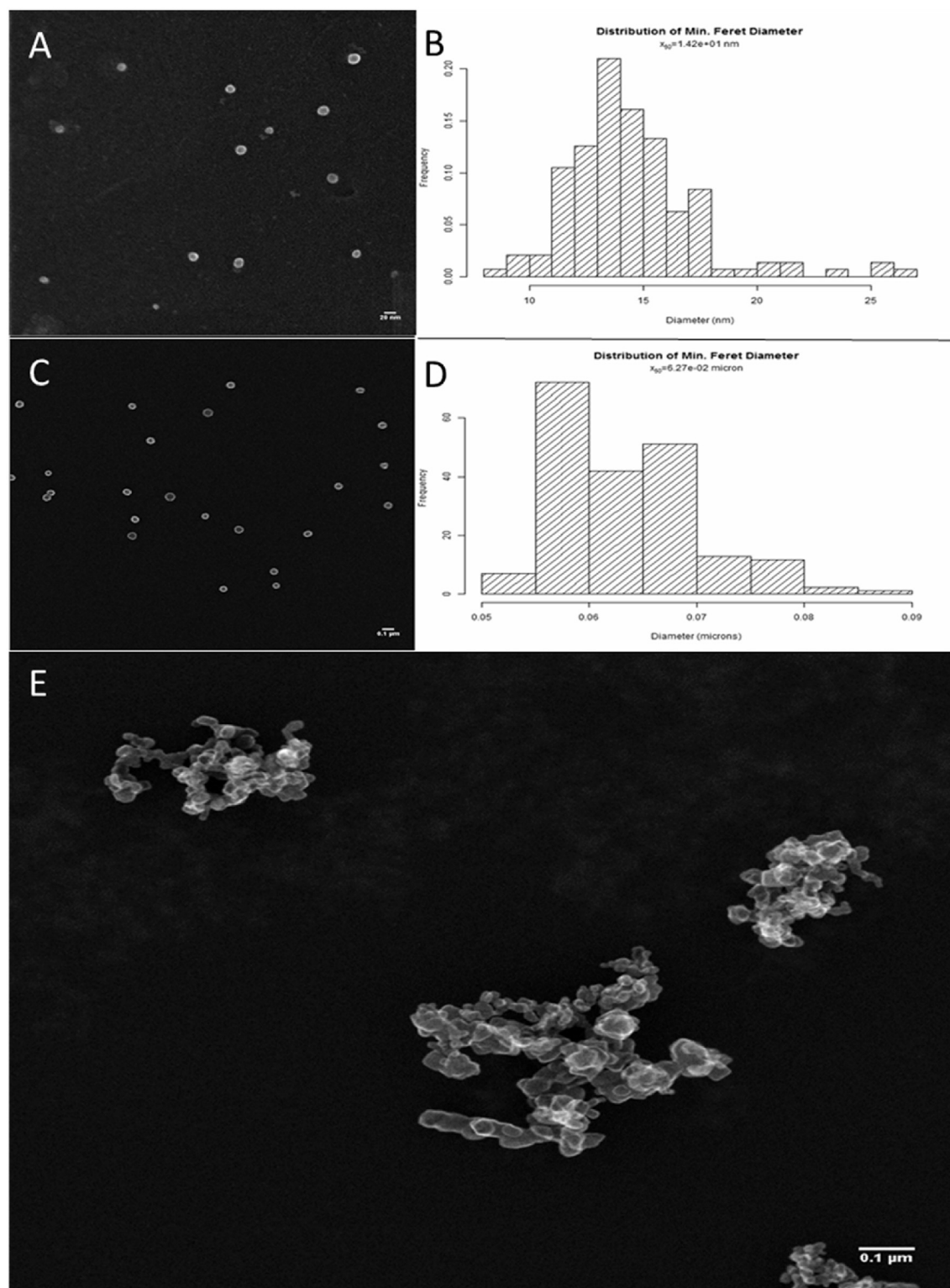


Fig. 2. TEM images of NPs and histograms of the size distribution. A: NM 300 K, magnification 35 kx; B: NM 300 K, histogram based on 143 particles; C: AuNPs, magnification 30 kx; D: AuNPs, histogram based on 172 particles; E: NM 105, magnification 80 kx.

was centrifuged directly without any dilution. Samples of the experimental diet enriched with NPs (described above) were dried by lyophilization at -52°C and 0.47 mbar (Alpha 1–2 LDplus, Christ) for 24 h and ground to a fine powder using a mortar. 30 mg of freeze dried material were added to 1 mL of 0.2% FL-70 (Thermo Fisher

Scientific) and sonicated for 1 min in a Vial Tweeter (Hielscher Ultrasonics GmbH). The resulting dispersion was diluted 1:1 in deionized water and directly centrifuged onto TEM grids. As the NPs carried a negative surface charge, the TEM grids were functionalized with Poly-L-Lysine (PLL, 0.1% (w/v) in H_2O , Sigma

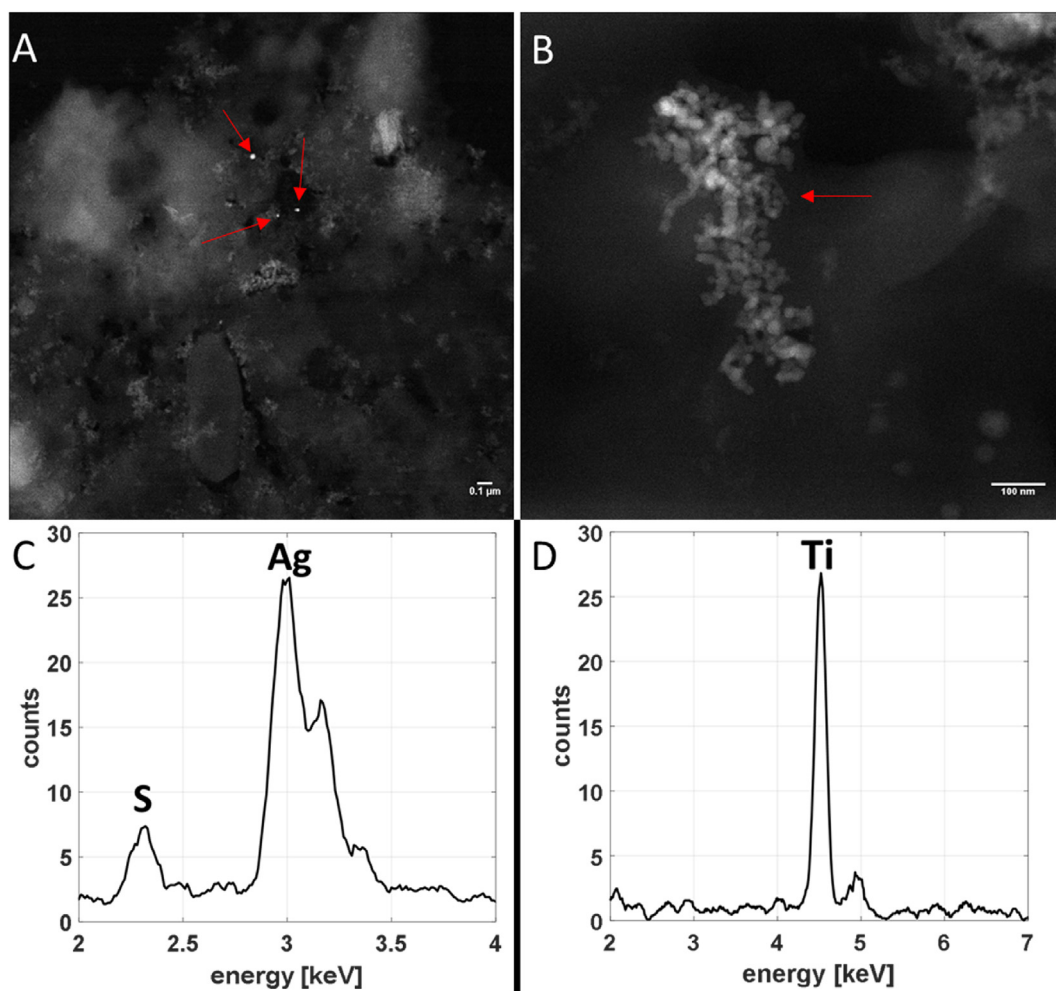


Fig. 3. TEM HAADF images and the corresponding EDX signals of NPs enriched in the experimental feed. A: NM 300 K, magnification 30 kx; B: NM 105, magnification 100 kx; C: NM 300 K, EDX signals; D: NM 105, EDX signals.

Aldrich) to enhance NP deposition on the TEM grids. The preparation of the TEM grids is described in more detail in [Uusimäki et al. \(2019\)](#). A dedicated scanning transmission electron microscope (STEM, HD2700Cs, Hitachi), operated at an acceleration voltage of 200 kV was used to investigate the TEM grid. For image formation the secondary electron (SE) or the high-angle annular dark field (HAADF) signal was used. Elemental analyses were conducted using an EDX system (EDAX) and the spectra were recorded and processed using Digital Micrograph (v.1.85, Gatan Inc.).

The stock materials were additionally characterized for their hydrodynamic diameter using dynamic light scattering (DLS) with a zetasizer (Zetasizer Nano Series, Malvern). Each stock material was measured in UHQ and additionally in the dilution water (low in copper tap water) and culture medium ([Borgmann, 1996](#)) (Table 1) to investigate potential effects of the medium. The dispersion medium was filtered using syringe filters with a 0.2 μm pore-size (Minisart® NML, 0.2 μm) before dispersing the particles. The dispersions were freshly made, shaken by hand for 1 min and sonicated for 10 min with a pulsation pause ratio of 0.2/0.8 using an ultrasonic homogenizer (Bandeline Sonoplus HD2200 ultrasonic homogenizer, 200 Watt, Bandelin Cup Horn BB6) before analysis. Concentrations were chosen to obtain count rates of 150 kcps or slightly higher values. The measurements were performed using disposable polystyrene cuvettes with an optical path of 1 cm. Each

sample was measured after an equilibration time of 180 s in 3 runs of 10 single measurements for 10 s each at 25 °C. Z-Average and percentage of different peak intensities were calculated by the Zetasizer software.

2.7. Determination of total metal concentrations

The test media samples (20 mL) which were acidified after sampling and stored at 4 °C were measured directly without further processing. Total concentrations of silver, gold and titanium (as titanium dioxide equivalent) in the aqueous test media were determined by ICP-MS (Agilent 7700 ICP-Q-MS, Agilent Technologies). The instrument calibration and method verification were carried out as described by [Kuehr et al. \(2018\)](#) using certified element and multi-element standards (Merck and Sigma Aldrich) as well as reference water (TM 25.4; Environment Canada). A rhodium standard solution (Merck KGaA; CertiPUR) was applied as internal standard for compensation of instrumental fluctuations. At least three measurements were recorded for each standard and sample and the mean concentration was determined by the ICP-MS software.

The tissues samples and samples of the experimental diets were digested using microwaves. A MLS turboWave® was used for the digestion of Ag and Au samples (30 min at 220 °C and 40 bar). 8 mL of aqua regia (3:1, nitric acid: hydrochloric acid) were added to the

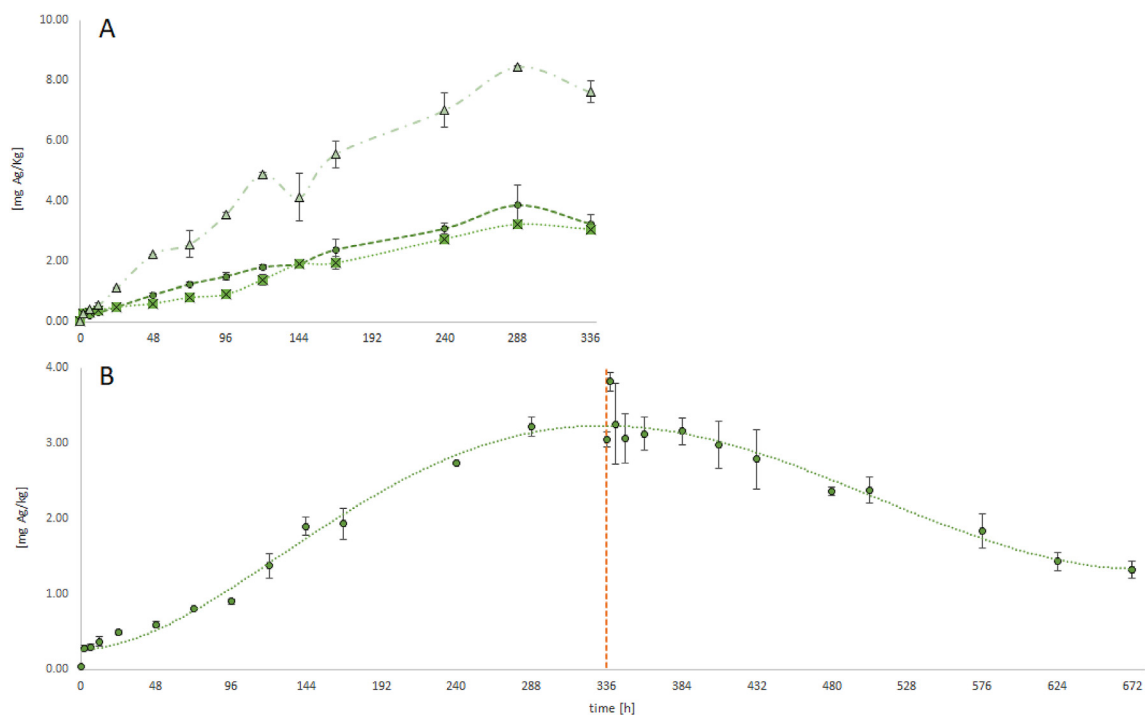


Fig. 4. A: Total Ag tissue concentrations tests during uptake phase of AgNO_3 bioaccumulation, triangles: $\text{AgNO}_3\text{-FH}$, circles: $\text{AgNO}_3\text{-FL}$, crosses: $\text{AgNO}_3\text{-ML}$; B: Total Ag tissue concentrations during uptake and depuration phase of the extended $\text{AgNO}_3\text{-FL}$ bioaccumulation test. The red line represents the end of the uptake phase during the extended $\text{AgNO}_3\text{-FL}$ bioaccumulation test. NM 300 K.. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

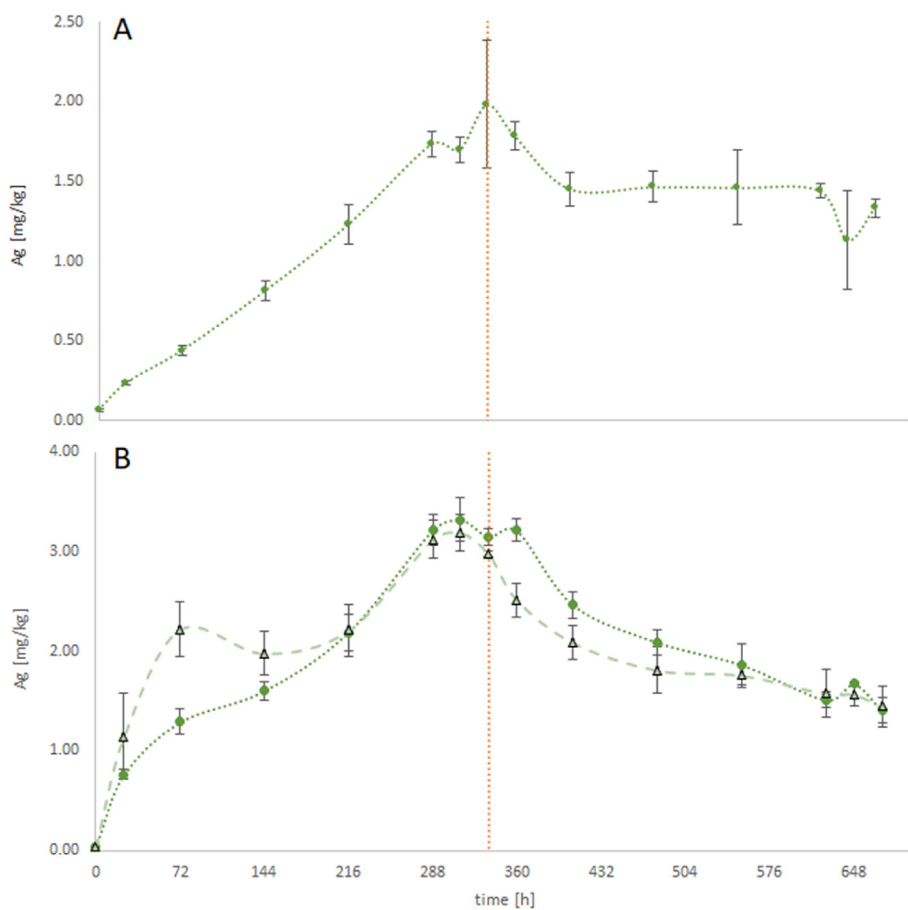


Fig. 5. Total Ag tissue concentrations in NM 300 K bioconcentration tests. A: NM 300Kcm; B: NM 300KZg (triangles) and NM 300KAg (circles). NM 105.

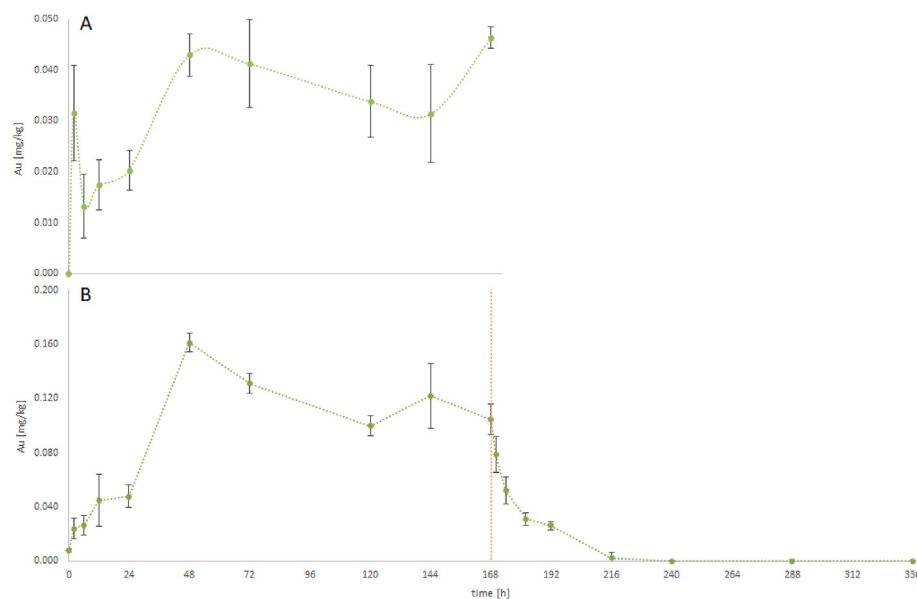


Fig. 6. Total Au tissue concentrations in the bioaccumulation test with AuNP; A: AuNP_L; B: AuNP_H.

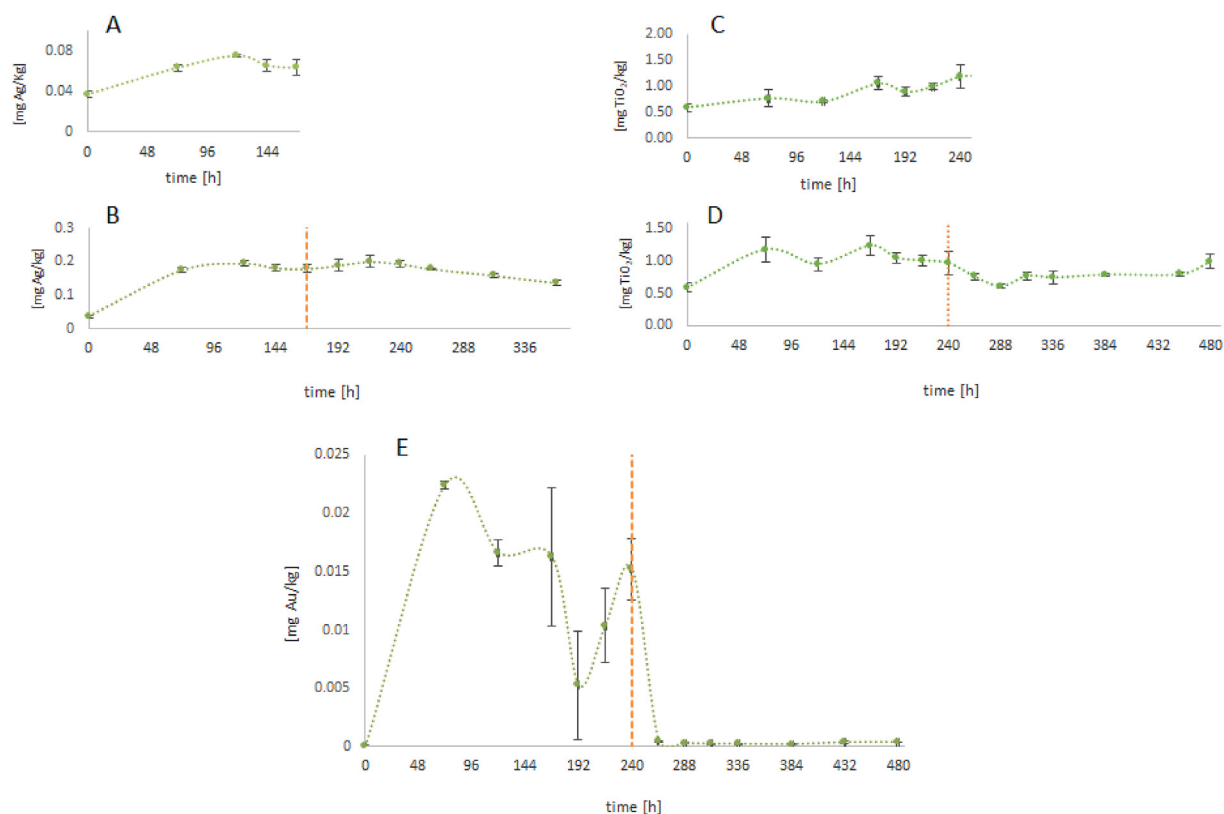


Fig. 7. Total Ag, TiO₂ and Au tissue concentrations during the biomagnification studies. Red vertical line shows the end of the uptake phase. A: NM 300K_L, B: NM 300K_H, C: NM 105_L, D: NM 105_H, E: AuNP_H. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

samples before vortex stirring (VORTEX GENIE 2, SiTM Scientific Industries) and the digestion process. The Ti samples were digested using nitric acid by means of a MLS Ultra Clave with the method described by Kuehr et al. (2020a). All digested samples were made up to 15 mL with nitric acid (10%) before measurements.

Animal samples which were taken at the end of the uptake and depuration phase of the third bioconcentration test on NM 300 K

were digested to determine the total Ag content associated with the protein fraction of the animals. Digestion was carried out using the enzyme proteinase K (Sigma Aldrich) according to the method described by Schmidt et al. (2011) and Loeschner et al. (2013). The dried animals (10 per replicate) were transferred into a 50 mL glass beaker and gently ground using a glass rod. The glass rod was rinsed with 10 mL of the digestion solution (45 mg proteinase K in

1 L buffer solution + 0.5% SDS + 50 mM NH_4HCO_3 , pH adjusted to 8.0–8.2) which was collected in the glass beaker containing the crushed tissue samples. The samples were incubated in the 10 mL digestion solution for 3 h at 50 °C and 100 rpm. As shown by Kuehr et al. (2020a) this process has no or only a negligible impact on the dissolution and size distribution of the present NPs. The digestion solution was filtered using 0.45 μm syringe filters (Minisart® NML, 0.45 μm). 3 mL of the filtrate were digested using 5 mL aqua regia in a microwave by the method described above. All microwave digested samples were filtered using 0.45 μm syringe filters (Minisart® NML, 0.45 μm) before measurements. This allowed comparison of the content of Ag in the protein fraction of the animals with the total amount of Ag from the whole samples (including AgNPs attached to the carapace etc.).

2.8. Examination of exposure media, experimental feed and animals tissue using single particle-ICP-MS

All samples (medium, experimental feed and animals) collected for the analysis of single particles using an ICP-QQQ-MS (Agilent 8900, Agilent Technologies) were enzymatically processed and measured using the method described by Kuehr et al. (2020a). Due to the fact, that the sample matrix of the DECOTABs and the carapace of the amphipods were not liquefied during enzymatically treatment, this method was only used to obtain qualitative results. The results of the particle size measurements are summarized in Table S1.

2.9. Data analysis

All data were subjected to an analysis of variance using the data analysis software OriginPro 2017 (OriginLab Corporation). Time weighted average concentrations (TWA) of Ag, Au and TiO_2 in test media were calculated according to OECD TG 211 (Organization for Economic Co-operation and Development OECD, 2012). Total concentrations of Ag, Au or TiO_2 in the tissue samples collected under steady-state conditions were divided by the calculated TWA of Ag, Au or TiO_2 measured in the exposure medium to gain BCF values. In the same way, BMF values were derived by dividing the mean concentration of Ag, Au or TiO_2 measured in the tissue samples under steady-state conditions by the respective concentrations measured in the experimental diets (DECOTABs).

3. Results

3.1. Characterization of the used NPs

The diameters of the stock materials (NM 300 K and AuNPs) derived from TEM measurements were in agreement with their nominal diameters (14.2 and 62.7 nm for the AgNPs and AuNPs, respectively). For NM 105 no diameters were determined because of particle agglomeration that occurred during the transfer of the NPs to the TEM grids due to the lack of a stabilizing agent (Fig. 2). The DLS measurements of the stock materials resulted in values of 26.7 nm (NM 300 K), 351.1 nm (NM 105) and 60.7 nm (AuNPs) for the hydrodynamic diameter (when measured in UHQ water). For all NPs higher hydrodynamic diameters were measured when suspended in dilution water that was used in the tests. For the AgNPs and AuNPs the measured values of 51.2 and 143.5 nm were two-times higher, whereas the value measured for the TiO_2 NPs was more than 5 times higher in the dilution water (1861 nm). The hydrodynamic diameter of the NM 300 K NPs additionally measured in the culture medium used in one of the bioconcentration tests was also around two times higher (50.3 nm) compared to the value measured in UHQ water.

The examination of the NP enriched experimental diets (DECOTABs) using TEM and EDX showed that the AgNPs and TiO_2 were still present (Fig. 3). In contrast, AuNPs were not detected. During the EDX measurement of dietary AgNPs a distinct signal for the element sulfur was also identified pointing to the presence of sulphidized particles.

3.2. Bioconcentration studies

3.2.1. AgNO_3

Three bioconcentration tests with AgNO_3 were carried out to identify the impact of the exposure concentration and the sex of the test animals on the bioaccumulation of the metal.

The time weighted average concentrations (TWA) of total Ag in the test on bioaccumulation in male and female animals were 0.64 for females ($\text{AgNO}_{3\text{-FL}}$) and 0.67 $\mu\text{g/L}$ for males ($\text{AgNO}_{3\text{-ML}}$). In the related test on the concentration dependent bioaccumulation of Ag carried out with female animals a 2.5 times higher TWA of 1.64 $\mu\text{g/L}$ ($\text{AgNO}_{3\text{-FH}}$) was measured (Table 2).

The steady state tissue concentrations were calculated as an average of the values measured in the samples taken between 240 and 336 h of exposure. Male and female animals exposed to the AgNO_3 treatments revealed tissue concentrations of 3.40 ± 0.34 mg Ag/kg and 3.14 ± 0.09 mg Ag/kg, respectively. The steady state tissue concentration measured in the $\text{AgNO}_{3\text{-FH}}$ treatment, in animals taken between 288 and 336 h of exposure (not between 240 and 336 h), was 7.70 ± 0.59 mg Ag/kg. Considering the measured tissue and water concentrations, BCF_{SS} values of 4900 ($\text{AgNO}_{3\text{-ML}}$), 5100 ($\text{AgNO}_{3\text{-FL}}$) and 4700 ($\text{AgNO}_{3\text{-FH}}$) were calculated (Table 2). The course of the tissue concentrations measured during the uptake and depuration phases are presented in Fig. 4.

3.2.2. NM 300 K

Three bioconcentration tests were carried out to identify the impact of the design of the test system and the composition of the test medium on the bioaccumulation of NM 300 K.

The media samples collected during the uptake phase of the NM 300K_{cm} test lasting 336 h, which was carried out in aquaria using a physiological culture medium had a total Ag TWA concentration of 3.00 $\mu\text{g/L}$ (Table 3). In this study a fast increase of the total Ag body burden was observed (Fig. 5) which slowed down after 288 h of exposure and was nearly constant at a level of approximately 1.81 ± 0.13 mg Ag/kg until the end of the uptake phase (336 h). Using this averaged total Ag concentration as the steady state concentration, a BCF_{SS} value of 604 could be calculated for the NM 300K_{cm} test (Table 3). During the depuration phase the total Ag body burden decreased within 72 h to a level of approximately 1.5 mg Ag/kg and remained stable on this high level until the end of the study.

Media samples collected during the uptake phase of the other tests performed in dilution water and carried out in different test systems (aquarium vs. Zuger glass) were measured and TWA values of 6.73 (NM 300K_{Aq}), and 6.83 $\mu\text{g/L}$ (NM 300K_{Zg}) were determined (Table 3). Media samples taken from the aquarium and the Zuger glass system were analyzed using spICP-MS. Median particle sizes for Ag particles were determined to be 15 nm in both systems and thus identical to the size of the stock material.

The Ag body burden of the animals increased during the uptake phase and reached stable concentrations of 3.22 ± 0.07 (NM 300K_{Aq}) and 3.09 ± 0.09 mg Ag/kg (NM 300K_{Zg}) after 288 h of exposure (Fig. 5). Based on the calculated tissue concentrations at steady state (288–336 h) and the calculated TWA, BCF_{SS} values of 480 and 453 were determined for the test using the aquarium (NM 300K_{Aq}) and the Zuger glass system (NM 300K_{Zg}), respectively (Table 3). In both tests, a similar decrease of the Ag tissue

concentration was observed during the depuration phase leading to a final tissue concentration of approximately 1.5 mg Ag/kg.

Additional samples were collected at the end of the uptake and end of the depuration phase from the NM 300K_{Aq} test to determine the Ag burden of the protein fraction of the animals. At the end of the uptake and depuration phases concentrations of approximately 1.87 and 1.58 $\mu\text{g Ag/kg}$, respectively, were measured amounting to 59.3 and 112% of the total Ag measured for the complete samples that were taken at the same time.

Particles extracted from animals collected at the end of the uptake phase of the NM 300K_{Aq} test were measured using spICP-MS and had a median particle size of 22.6 ± 0.6 nm.

3.2.3. NM 105

In the bioconcentration test with NM 105, TWA values of 1.49 (NM 105_L) and 3.30 $\mu\text{g TiO}_2/\text{L}$ (NM 105_H) were determined for the test media applied during the exposure phase lasting 168 h (Table 4). Media samples were analyzed using spICP-MS. The median particle size for TiO_2 particles present in the test medium was determined to be 56.3 ± 6.2 nm which is similar to the median size of the particles in the working suspension of 55.7 ± 1.3 nm. Animals collected from both treatments showed that no significant increase of tissue concentrations during the test occurred. For both treatments the measured concentrations of total Ti in the acid digested animals, as equivalent to the TiO_2 body burden, were mostly lower than the limit of quantification (LOQ, 1.390 $\mu\text{g Ti/L}$) and the limit of detection (LOD, 0.463 $\mu\text{g Ti/L}$). Particles extracted from animals sampled at the end of the exposure were measured to have a median particle size of 107.0 ± 3.9 nm.

3.2.4. AuNPs

TWA concentrations of Au measured in the bioconcentration study with AuNP were 0.085 (AuNP_L) and 0.704 $\mu\text{g Au/L}$ (AuNP_H) and thus 10 times lower than the target exposure concentrations (Table 4). A high variation in media concentrations in the course of the uptake phase was observed. Median particle sizes for AuNPs present in the test medium were determined to be 56.9 ± 1.0 nm by spICP-MS. The median size of the stock material was measured to be 56.2 ± 0.5 nm.

A strong adsorption of Au to the inner surface of the glass system and tubes was observed. However, no real bioaccumulation of Au was detected by the measurement of the total Au concentrations in the animals collected during the uptake phase (Fig. 6). In both treatments the highest Au body burden was reached within 48 h of exposure. In the AuNP_L treatment the tissue concentration remained more or less stable until the end of the uptake phase (168 h). The average total concentration of Au of 0.036 ± 0.006 mg Au/kg (48 h–168 h) was used as the steady state tissue concentration resulting in a calculated BCF_{SS} of 424 (Table 4). An average steady state tissue concentration of 0.117 ± 0.015 mg Au/kg was reached after 120 h for the AuNP_H treatment, resulting in a BCF_{SS} of 166. During the depuration phase of the AuNP_H test lasting 168 h a rapid decrease of the total Au body burden was observed. Within 48 h of depuration the total Au body burden reached the initial background concentration measured in the control animals at 0 h (Fig. 6). Particles extracted from animals sampled at the end of the uptake phase were measured to have a median particle size of 56.9 ± 0.7 nm.

3.3. Biomagnification studies

Biomagnification tests were carried out using NP enriched DECOTABs. Each NP was tested at two concentrations (Table 5). All experimental diets were accepted by the amphipods. High amounts of feces were released during the studies and removed from the test

system by the water flow. No measurable concentrations of dissolved test item were detected in the test media samples collected during the biomagnification studies.

3.3.1. NM 300 K

Total Ag concentrations of 0.07 ± 0.00 and 0.751 ± 0.01 mg Ag/kg were measured in the NM 300 K enriched DECOTABs prepared for the low (AgNP_L) and high (AgNP_H) treatments (Table 5). For the control DECOTABs, that were fed during the depuration phase only, a total Ag concentration of 0.01 ± 0.00 mg Ag/kg was measured. Samples of the applied DECOTABs were digested using proteinase K to allow the examination of the embedded AgNPs using spICP-MS. The median particle size of Ag particles present in the experimental diet was determined to be 14.1 ± 0.8 nm.

In the AgNP_L treatment of the biomagnification study an increasing total Ag body burden was observed in animals collected during the first 72 h of the uptake phase followed by a plateau. This was stable until the end of the uptake phase (144 h) with an average tissue concentration of 0.07 ± 0.01 mg Ag/kg (Fig. 7). A BMF_{SS} value of 0.93 was calculated. In the AgNP_H treatment a stable body burden was also reached after 72 h of exposure (Fig. 7) which remained stable until the end of the uptake phase (144 h). With an average tissue concentration of 0.18 ± 0.01 mg Ag/kg a BMF_{SS} value of 0.25 was calculated (Table 5). Particles extracted from animals collected at the end of the uptake phase were analyzed to have a median particle size of 17.1 ± 1.3 nm.

During the first 96 h of the depuration phase nearly no elimination of Ag was observed. At the end of the depuration phase lasting 192 h, in the AgNP_H treatment the tissue concentration reached a level of 0.14 mg Ag/kg representing 75% of the previously determined steady state tissue concentration.

3.3.2. NM 105

Total TiO_2 concentrations of 3.67 ± 0.05 (TiO_2NP_L) and 19.43 ± 0.60 mg TiO_2/kg (TiO_2NP_H) were measured in the two experimental diets (Table 5). A background concentration of total TiO_2 of 2.17 ± 0.34 mg TiO_2/kg was determined for the control DECOTABs that were applied during the depuration phase of the study with TiO_2NP_H .

As described above, NM 105 particles in the experimental diets were highly agglomerated as shown by TEM (Fig. 2). Particles extracted from the experimental feed were examined by spICP-MS and a median particle size of 55.9 ± 8.4 nm was determined.

In the TiO_2NP_L treatment a slight increase of the total TiO_2 concentration in the animal tissue was observed during the uptake phase lasting 240 h (Fig. 7). The initial concentration of the animals (0 h) was 0.59 ± 0.07 mg TiO_2/kg . An average body burden of 1.03 ± 0.46 mg TiO_2/kg was observed at the end of the uptake phase (168–240 h) resulting in an BMF_{SS} value of 0.12 considering the observed increase of the total TiO_2 concentration in the animals of around 0.44 mg TiO_2/kg .

In the TiO_2NP_H treatment a relatively stable body burden was reached after 72 h of the uptake phase (Fig. 7). The total TiO_2 tissue concentration increased by 0.476 mg TiO_2/kg to an average concentration level of 1.07 ± 0.03 mg TiO_2/kg , resulting in a BMF_{SS} value of 0.02 (Table 5).

The median size of the particles in animals collected at the end of the uptake phase and determined by spICP-MS was 62.2 ± 10.7 nm.

In the TiO_2NP_H treatment, during the depuration phase (240 h) a nearly stable tissue concentration of 0.74 ± 0.06 mg TiO_2/kg was reached after 24 h of elimination and remained on this level until the end of the study.

3.3.3. AuNPs

The total Au concentrations measured in the experimental diet were 0.003 ng Au/kg in the control feed (fed during depuration phase), and 3.9 ± 0.004 ng Au/kg and 0.889 ± 0.096 mg Au/kg in the low (AuNP_L) and high treatment (AuNP_H), respectively (Table 5).

AuNPs concentration in two enriched experimental diets were too low to be detected by TEM. However, AuNPs could be extracted from the experimental diets and the animals collected at the end of the uptake phase and measured by spICP-MS. Median particle sizes of 57.0 ± 1.4 nm for feed and 51.2 ± 0.4 nm for animals were determined.

Tissue concentrations of animals collected during the test with the AuNP_L treatment were all below the limit of quantification for Au (0.002 µg/L, in the digested samples). However, measurable Au tissue concentrations were present in animals collected during the uptake phase of the AuNP_H treatment. The course of the measured Au body burden is presented in Fig. 7. After washing the animals with dilution water and their transfer into a new test system for the depuration phase, the initial Au body burden was nearly reached after 24 h (Fig. 7). Using the highest Au body burden (around 0.0225 mg Au/kg) measured after 48 h of exposure and the tissue concentration present at the end of the uptake phase (0.0153 ± 0.003 mg Au/kg) a magnification factor between 0.03 of 0.02 could be calculated based on the Au concentration in the diet of 0.889 mg Au/kg.

4. Discussion

Bioconcentration tests on NMs with aquatic species are difficult to carry out due to the agglomeration and sedimentation of NMs in aquatic systems. Kuehr et al. (2020a) described a test system that allows the continuous and homogenous exposure of bivalves to NMs via the water. However, the filter feeding process of the test species only allows the determination of BAF values which represent a less specific endpoint that is less accepted from the regulatory point of view compared to the commonly used BCF (and BMF) values. Thus the bivalvia test system using a Zuger glass unit as test vessel was adapted to be used with another benthic test species, the freshwater amphipod *H. azteca*, which provides the opportunity to perform biomagnification and bioconcentration tests. Continuous and homogenous exposure concentrations could be maintained in the test system and stable water quality parameters were ensured. The use of DECOTABs was shown to be an easy and clean way to feed the animals ad libitum during the studies without causing a notable risk due to artifacts polluting the test system or the potential adsorption of the test item to the surface of the feed in BMF and BCF studies, respectively. The feces excreted by the animals were efficiently removed by the water flow keeping the test vessel as clean as possible which is a distinct advantage of the Zuger glass column system in comparison to a cube shaped aquarium.

Bioconcentration tests with AgNO₃ were carried out in addition to the studies with AgNPs to allow a comparison of the bioaccumulation potential of ionic and particulate Ag. The tests with AgNO₃ showed that Ag⁺ exposed via the water is available for *H. azteca*. Uptake and elimination kinetics of the ions measured as total Ag were determined. The calculated BCF_{SS} values indicate a high bioaccumulation potential. The trend of the Ag concentrations in *H. azteca* during the elimination phase of the AgNO_{3-FL} test showed that the Ag body burden after 14 days of depuration was still at a level of 50% of the steady state concentration. It is not clear if this observation was caused by a very low elimination rate, the binding of Ag⁺ by metal binding proteins like metallothioneins or the sequestration of Ag as solids (concretions) in granules as described below.

The bioconcentration of AgNO₃ was tested in two independent

bioconcentration studies with male and female amphipods to investigate potential gender-specific differences. The comparison of both groups exposed to comparable test concentrations showed that the uptake kinetics of total silver as well as the calculated BCF_{SS} are comparable for both sexes. Therefore, both male and female *H. azteca* seem to be suitable for testing metal or metal oxide based NPs, but further investigations with more different types of NMs are required. Nevertheless, test animals need to be separated and only male or female animals should be used per test to avoid elimination of accumulated test item through the release of juveniles.

The uptake of metals in crustaceans may be at least partly regulated (Viarengo, 1989; Rainbow, 1995; Rainbow, 1997). Due to the underlying mechanism, it is anticipated that the metal uptake in crustaceans is dependent on the metal concentration in the water. Therefore, it is necessary that at least two concentrations were tested. Comparing the BCF_{SS} values calculated for the high and low treatment, a negative concentration dependency for the bioaccumulation of Ag⁺ was observed, with the animals exposed to the higher test concentration showing a lower BCF_{SS} value.

All bioconcentration tests using the AgNP NM 300 K carried out as part of this study showed that *H. azteca* is suitable to be used as test organism for bioconcentration tests with stable dispersed NPs. The measurement of the uptake and elimination of the metal in the amphipods as well as the calculation of BCF_{SS} values were possible. The use of tap water as test medium for bioconcentration studies requires a tolerable water quality. In places where this is not available the use of reconstituted water or culture media suitable for the culture of *H. azteca* is required. The result of the bioconcentration test with AgNPs using culture medium shows that the presence of increased levels of chloride, bromide, carbonate and sulfate in comparison to tapwater do not inhibit the uptake of Ag from NM 300 K e.g. by passivation of the NPs or sequestering potentially releases Ag⁺ as chlorides, bromides or carbonates. The higher BCF_{SS} value obtained from the test using culture medium (NM 300K_{CM}) in comparison to the tests using dilution water (NM 300K_{Zg} and NM 300K_{Aq}) might be explained by the negative correlation of the BCF_{SS} and the exposure concentration as previously described for the exposure with AgNO₃ and not by the composition of the exposure medium. Comparable observations of negative correlation between the bioaccumulation of metals and their exposure level have been described several times elsewhere, also for crustaceans (DeForest et al., 2007; Verschoor et al., 2012; Lebrun et al., 2014). This finding was explained by mechanisms like sequestration, binding of the metals to proteins or their excretion as well as by a saturation of the uptake capacities at high exposure concentrations (DeForest et al., 2007; Rainbow, 2007).

As described for the bioconcentration studies with AgNO₃, also the trend of the Ag concentrations in *H. azteca* during the elimination phase of the AgNP test showed that the Ag body burden after 14 days of depuration was still at a level of around 50% of the previously measured steady state concentration. The measurement of the total Ag concentration in the dissolved protein fraction of the Hyalella samples taken at the end of the uptake and depuration phase clearly indicates that the remaining tissue concentrations even after 336 h of depuration were potentially associated with proteins like metallothioneins as discussed below.

Even if high tissue concentrations of total Ag were observed during the course of the bioconcentration studies, the question remains how the AgNPs were taken up. The results from the examinations of *H. azteca* tissue using spICP-MS indicate that particulate Ag was likewise ingested (Table S1). However, it cannot be excluded that Ag particles remained attached to the animal's body, even after rinsing the animals with clean water and blotting dry after sampling. The median particle sizes of AgNPs that were

detected and measured in the exposure medium using spICP-MS showed that the AgNPs had nearly the same median size as measured for the AgNPs taken from the stock material. Particles found in the dissolved protein fraction extracted from the test animals seemed to be bigger in comparison to the particles in the exposure medium. This may be explained by agglomeration or an artefact of the spICP-MS measurement caused by detection of Ag associated with inorganic or organic Ag complexes. Another explanation for the bigger particles measured in the protein extracts might be the formation of solid concretion granules. Baccaro et al. (2018) found AgNPs in earthworms (*Eisenia fetida*) that were bigger than the AgNPs used for exposure. They hypothesized that the measured AgNPs were granules actively formed by the earthworms to sequester Ag^+ as also observed in intestinal fish cells exposed to AgNO_3 (Minghetti and Schirmer, 2016). Methods like TEM and EDX are required to further elucidate the mechanisms involved.

BCF_{ss} values were calculated for the accumulation of total Ag following exposure to AgNPs. The impact of the test system used for the bioconcentration studies on the resulting BCF_{ss} was investigated using conical and cubic shaped test vessels. The results from the flow-through tests using the same stock solution (NM 300K_{Zg} and NM 300K_{Aq}) showed that both test systems can lead to comparable outcomes even though the Zuger glass system is limiting the risks associated with the accumulation of NPs within the system which may result in an inhomogeneous exposure scenario. However, this kind of artefact should be of higher relevance for the testing of NMs that show a higher tendency to sediment in the aquatic environment than NM 300 K. The following tests with NM 105 and AuNPs were carried out using the Zuger glass system.

Bioconcentration studies with TiO_2 resulted in low tissue concentrations and thus a clear indication of limited bioavailability and accumulation of the test item. Even in the test with the higher exposure concentration only a slight increase of the total tissue concentration in comparison to the control animals (0 h, all data below LOQ) could be observed. This might be explained by the lack or limited (bio)availability of the TiO_2 particles and the lack of potentially released ions as observed for NM 300 K. Particles in the working suspension and exposure medium had a calculated median particle size of 55.7 and 56.3 nm, respectively, and thus seemed not to be highly agglomerated. In contrast, particles measured in animals collected during the bioconcentration studies with NM 105 using sp-ICP-MS showed a calculated median particle size of >100 nm and seemed to be agglomerated. Even though the amount of TiO_2 particles attached to or ingested by the amphipods did not affect the measurable body burden at a significant level, the low amount was sufficient to be detected by the very sensitive method of spICP-MS. Particles measured by the spICP-MS were obviously attached to the carapax or ingested by *H. azteca* but obviously not incorporated into their tissues as indicated by the rapid and complete elimination of TiO_2 from the organisms during the depuration phase.

Similar observations were made in the bioconcentration studies with AuNPs. Also in this case the measured body burden was obviously the result of the simple ingestion of the NPs without further bioaccumulation, as again indicated by the rapid and complete elimination of Au/AuNPs from the organisms during the depuration phase. Further investigations are required to determine the dependence of a constant exposure of AuNPs and other NMs from the flow rate of the exposure medium.

Biomagnification tests with AgNPs, AuNPs, and TiO_2 were carried out as part of this study. DECOTABs as control feed were shown to be suitable for the feed borne exposure of NMs without causing the risk of NMs or ions from the diet leaching into the surrounding medium. As shown by the investigations using TEM,

the NMs embedded in the diet were still present in a particulate form. The low Au concentration in the lower concentrated experimental feed of the AuNP treatment might be explained by the loss of particles during the diet preparation process due to adsorption of the AuNPs to the glass surface of the mixing beakers. The Au concentration in the higher concentrated feed could be measured by ICP-MS. The presence of AuNPs was confirmed using sp-ICP-MS. However, the concentration was obviously too low to allow the detection of the AuNPs using TEM.

The production of NM enriched DECOTABs may also lead to agglomeration as seen by TEM for NM 105 or transformation processes like sulfidation as revealed by EDX for NM 300 K. Ti is chemically stable in the oxidized compound and should thus not be influenced by the sulfur present in the diet. Whereas, the detected S signal during the EDX measurement of NM 300 K enriched DECOTABs seem to be the result of sulfidation processes leading to Ag_2S as indicated by the ratio of the signals from Ag and S (~2:1).

However, Ag from the presumably sulfidized AgNPs has been demonstrated to be still bioavailable as shown by the increasing total Ag body burden during the course of the uptake phase of the biomagnification test with NM 300 K. This is in accordance with the observations made by Kampe et al. (2018) where the terrestrial isopod *Porcellio scaber* was able to accumulate Ag from sulfidized AgNPs (NM 300 K) present in sediment enriched with sewage treatment plant sludge (Kampe et al., 2018). Kuehr et al. (2018) and (2020) also observed the bioavailability and accumulation of Ag from presumably sulfidized wastewater and sewage sludge borne NM 300 K AgNPs by *H. azteca*.

Similar to the observations made during the bioconcentration tests with AgNP, a delayed and incomplete elimination of Ag from the animals during the depuration phase of the biomagnification test with NM 300K_H was observed. This may be explained by ions taken up during the exposure that were associated with structural proteins in tissues and mucus or with functional proteins present in the haemolymph or cells. Khan et al. (2017) observed that up to 92% of the total Ag content measured in fish during a study to investigate the intestinal uptake of Ag^+ in rainbow trout, was bound to the intestine mucus layer, whereas only small amounts of Ag were found in the blood of the fish. Functional proteins like metallothioneins (MTs) are present in nearly all species and the expression is triggered by the presence of free Ag^+ and several other metal ions. The presence and role of these proteins as part of a detoxifying strategy has been described for several species including crustaceans e.g. by Roesijadi (1992), Ahearn et al. (2004) or Sterling et al. (2007). MTs that are present in intestine cells are described to bind metals taken up by epithelial transport mechanism from the lumen of the gastro intestinal tract (Roesijadi, 1992). In invertebrate groups like crustaceans, the digestive gland combines the functional role of liver and intestines of vertebrates and is involved in intestinal and hepatic functions. It also has intracellular digestion and phagocytosis abilities (Gardiner, 1972). Thus, metals ingested with feed are expected to bind first in the cells of the digestive gland and are then transferred to other organs (Engel and Brouwer, 1984). The binding to the MTs may be seen as a barrier to rapid transfer of metals to other organs and tissues due to the reduced and retarded transfer of metals across the intestinal epithelium (Roesijadi, 1992). MTs (loaded) with the associated metals may accumulate in the lysosomes and thus represent a sink for (heavy-) metals like Ag, but may also be transported to the nucleus and intermembrane space of mitochondria (Ye et al., 2001). Mitochondria, endoplasmatic reticulum and lysosomes are sites of a second detoxifying strategy that may lead to the reduction of soluble cytoplasmic metals by sequestrations of the metals as concretions using sulfate, phosphate and carbonate (Sterling et al., 2007; Al-Mohanna and Nott, 1985; Chavez-Crooker et al., 2003;

Mandal et al., 2005, 2006). The formation of concretions has been examined in a wide range of invertebrates and also plays a key role in the accumulation of metals (Sterling et al., 2007). These processes are very effective and the metals remain within the organism until necrosis or apoptosis for the cells or active excretion of concretions as granules via the lumen of the digestive gland occur (Nott and Nicolaidou, 1990).

Consequently, the amphipods represent a potentially higher contaminated food source of fish and other predators and may contribute to the transfer of NMs to higher trophic levels. In the biomagnification tests with NM 105 a high initial background concentration of TiO_2 was present in the control animals collected at 0 h which may be explained by the TiO_2 contamination of the diet used for the husbandry of the test animals and which was equivalent to the control diet (DECOTABs) used for the biomagnification study with NM 105 having a Ti/TiO_2 concentration of 2.167 mg TiO_2/kg . However, it needs to be borne in mind that ICP-MS measurements can only quantify the content of Ti but not TiO_2 . Therefore, we cannot be sure that the measured concentrations of Ti measured in the diets and animal's tissues consisted of TiO_2 only. The low increase of the body burden observed during the biomagnification tests with NM 105 indicates a limited bioavailability of the TiO_2 NPs. Results from the experimental feed using TEM showed that the TiO_2 particles present in the experimental diet were highly agglomerated. However, it can only be speculated whether or not the size and form of the particles/agglomerates in the diet lead to the limited uptake and accumulation of TiO_2 observed in the biomagnification test with NM 105. Following the uptake phase, a concentration, which was slightly higher than the initial background concentration of Ti was reached quickly during the depuration phase and was a little higher than the initial background concentration of Ti. This was probably due to a fast elimination of the previously ingested but non-internalized TiO_2 particles/agglomerates. The slightly increased concentration may be the result of the Ti contaminated control feed (2.17 ± 0.34 mg TiO_2/kg). Comparable observations were made during the biomagnification studies with AuNPs. In this case the initial background concentration was also reached within 24 h of depuration again pointing to a fast elimination of the previously ingested NPs.

The fast and effective elimination observed for NM 105 and AuNPs is comparable to the observations made during the bioaccumulation tests with NM 105 using freshwater bivalves (Kuehr et al., 2020a). The strong metal binding as seen in the *Hyaella* bioconcentration and biomagnification tests with AgNPs, leads to a slow elimination of the previously accumulated Ag, as well as the ten times higher BCF_{55} values calculated for Ag from AgNO_3 in comparison to Ag from NM 300 K. This highlights the role of ions in the uptake, incorporation and bioaccumulation of metals from metal based NMs. This is in accordance with the results gained in studies using fish (Zeumer et al., 2020), mussels (Kuehr et al., 2020a), benthic amphipods (Kuehr et al., 2018; Kuehr et al., 2020b), and terrestrial isopods (Kampe et al., 2018). However, the bioaccumulation of ions following their release from NPs, as well as the physical attachment of NPs to the animal's surface do not represent mechanisms leading to a real incorporation of NPs. As shown in this study by spICP-MS measurements, NM 105, NM 300 K and the AuNPs were ingested and accumulated in the animals following aqueous or dietary exposure. However, the true bioaccumulation of the particles in the animal tissues could not be demonstrated. Further techniques and methods like histological examinations using TEM (e.g. as described by Kuehr et al. (2020b)b, fluorescence NPs or radiolabelled NPs followed by autoradiography (as described by Rath et al. (2020) for ^{14}C -labeled organic compounds exposed to *H. azteca*) are required to allow conclusions on the real incorporation and distribution or localization of NPs in *H. azteca*.

5. Conclusion

This study has shown that bioconcentration and biomagnification studies with *H. azteca* in an adapted test system are suitable for testing the bioaccumulation potential of metal and metal oxide NPs. The test concept presented allows testing of NMs under controlled test conditions and provides results of high value which might be considered within a tiered approach for bioaccumulation assessment of NPs as presented by Handy et al. (2018). Bioaccumulation tests of NPs with different properties are required to further assess the scope of the applicability domain of the test system for NM testing. Furthermore, investigations and method developments allowing more stable NM suspensions for testing of more difficult NMs are needed.

Credit author statement

Sebastian Kühr: Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing, Ralf Kaegi: Investigation, Resources, Writing - review & editing, Dirk Maletzki: Investigation, Christian Schlechtriem: Conceptualization, Resources, Writing - review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References

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| Ag^+ | Silver (I) ion |
| BCF | Bioconcentration factor |
| BMF | Biomagnification factor |
| EDX | Energy dispersive X-ray spectroscopy |
| DLS | Dynamic light scattering |
| ICP-MS | Inductively coupled plasma mass spectrometry |
| ICP-OES | Inductively coupled plasma optical emission spectrometry |
| MT | Metallothionein |

NM	Nanomaterial
NP	Nanoparticle
OECD	Organization for Economic Co-Operation and Development
spICP-MS	single particle inductively coupled plasma mass spectrometry
STP	Sewage treatment plant
TEM	Transmission electron microscopy
TWA	Time weighted average concentration
UHQ	Ultra high quality water

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