REGULATORY BIOACCUMULATION ASSESSMENT OF NANOMATERIALS. DEVELOPMENT OF NEW CONCEPTS AND TESTING PROCEDURES

Dissertation

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„Wenn ein unordentlicher Schreibtisch einen unordentlichen Geist repräsentiert, was sagt dann ein leerer Schreibtisch über den Menschen, der ihn benutzt aus?“

Albert Einstein

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer, nicht angegebener Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten oder Konzepte sind unter Angabe der Quelle gekennzeichnet.

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Zusammenfassung

Die besonderen Eigenschaften und das breite Spektrum an Anwendungsmöglichkeiten von Nanomaterialien haben dazu geführt, dass sie aus unserem Alltag nicht mehr wegzudenken sind. Die Verwendung von Nanomaterialien (NMs) in mittlerweile fast allen Industriezweigen hat die Nanotechnologie zu einem großen und immens wachsenden Wirtschaftszweig werden lassen. Das Volumen an NMs, die jährliche produziert werden, und in vielen Produkten Verwendung finden, liegt bei mehreren Millionen Tonnen. NMs können über das Abwasser in die aquatische Umwelt gelangen, wo sie von den dort leben Organismen aufgenommen, akkumuliert und über die Nahrungskette angereichert werden können.

Daher unterliegen NMs wie anderen chemische Industrieprodukte einer Umweltrisikobewertung inklusive der Beurteilung ihres Potenzials sich in der Umwelt anzureichern. Die aktuell dafür verwendeten Testsysteme wie auch die Bewertungsschemata wurden jedoch nicht für die Testung von NMs entwickelt. Zudem würde für die Testung aller NMs mit den üblichen Methoden eine enorme Menge an Fischen benötigt und sich somit neben der finanziellen und logistischen Herausforderung auch eine ethische Problematik ergeben.

In den Studien dieser Arbeit wurden daher Testsysteme entwickelt, welche die Bestimmung des Bioakkumulationspotenzials von NMs mittels wirbelloser Tiere wie Muscheln und Amphipoden ermöglicht. Hierzu wurde eigens eine Testanlage entwickelt, die eine konstante und stabile Exposition mit NMs ermöglicht. Es wurden zudem Studien mit Fischen durchgeführt, um die im neuen Testsystem mit wirbellosen Tieren erzielten Ergebnisse vergleichen zu können. Mittels Einzelpartikel-ICP-MS und angepasster Methoden der korrelativen Mikroskopie konnte am Beispiel von Silbernanopertikeln (AgNPs) gezeigt werden, dass die Akkumulation von Metallen im Gewebe der Tiere nach Exposition über das Futter und das Wasser primär über die Aufnahme von Ionen erfolgt, welche von den NMs freigesetzt werden. Laborstudien mit der Körbchenmuschel *Corbicula fluminea* und der Süßwasseramphipode *Hyalella azteca* haben gezeigt, dass benthischen Invertebraten vermutlich eine Schlüsselrolle im Transfer von NMs in der Nahrungskette zukommt.

Unter Berücksichtigung der entwickelten Nicht-Wirbeltiertests wurde ein gestuftes Bewertungskonzept für die Beurteilung des Bioakkumulationspotenzials von NMs entwickelt, welches die Ergebnisse aus Bioakkumulationsstudien mit *H. azteca* integriert. Regulatorische Endpunkte für die Bewertung der Bioakkumulation von NMs wurden entwickelt, welche den Aufnahmeweg von NMs in den Testorganismus berücksichtigen und somit eine eindeutige Bewertung der NMs als „bioakkumulierend“ oder „nicht-bioakkumulierend“ ermöglichen. Mittels der entwickelten Testmethoden kann zukünftig möglicherweise auf einen Teil der heute noch benötigten Fischtests verzichtet werden, ohne die Qualität der regulatorischen Bewertung zu beeinträchtigen.

Summary

The special properties and the wide range of possible applications of nanomaterials (NMs) make them an essential part of our everyday life. The use of nanomaterials in almost all branches of industry has made nanotechnology a large and immensely growing market. The volume of NMs produced annually and used in many products is about several million tons. However, this also means that large quantities of these NMs find their way into the environment. NMs can reach the environment via wastewater, where they can be ingested, enriched and passed on through the food chain by aquatic organisms.

Therefore, NMs, like other chemical industrial products, are subject to regulation and risk assessment including investigations on their potential to accumulate in the environment. However, the test systems and assessment schemes currently used for this purpose were not developed for NMs. In addition, testing with the established method requires an enormous amount of fish and thus poses an ethical problem and a financial and logistical challenge.

In the studies of this thesis, therefore, test systems were developed which allow the determination of the bioaccumulation potential of NM using invertebrates such as filtering bivalves or amphipods. For this purpose, a test system was developed which allows a constant and stable exposure. In addition, studies with fish were conducted to compare the results with those obtained with invertebrates exposed to NMs in the new test system.

However, using single particle ICP-MS and adapted methods of correlative microscopy, it could be shown by the example of silver nanoparticles (AgNPs) that the accumulation of metals after dietary and aqueous exposure can be primarily explained by the uptake of ions released by the NMs. The results of the laboratory studies showed that invertebrates are not only well suited for bioaccumulation studies, they also provided a clear indication on the role of benthic invertebrates regarding the transfer of NMs in the (aquatic) food chain.

A new assessment scheme for the evaluation of the bioaccumulation potential of NMs was developed integrating the new *H. azteca* bioaccumulation test. Modified regulatory endpoints are suggested considering the uptake pathways of NMs and allowing a clear definition of the NMs as “bioaccumulative” or “non-bioaccumulative”. By means of the developed methods and the assessment scheme it might be possible in the future to reduce the amount of fish without impairing the quality of the regulatory risk assessment.

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Author contributions

Prof. Dr. Christian Schlechtriem provided the resources, laboratories and technical support in his department at Fraunhofer IME to carry out the experiments described in **Chapters 2** to **6**. He contributed to the conceptualization and coordination of the studies, as well to the development of the assessment scheme of **Chapter 7**. He was involved in the reviewing and editing of the manuscripts of chapters **2** to **7**.

The experiments of **Chapters 3** to **6** were conducted by myself with support of students from the RWTH Aachen University and WWU- University of Münster.

Lara Hermsen completed her Master´s Thesis in the department Bioaccumulation and Animal Metabolism at the Fraunhofer IME and was partly supervised by me. She helped to carry out the sample preperations and total metal measurements from the experiments of **Chapters 3**, **5** and **6**.

Noemi Diehle also completed her Master´s Thesis in the department Bioaccumulation and Animal Metabolism and was supervised by Prof. Dr. Christian Schlechtriem and me. She contributed to the implementation of the exposure tests of **Chapter 6**.

Technicians (staff of FhG-IME) supported the measurements using spICP-MS, ICP-MS and ICP-OES. The individual contributions are listed in the acknowledgements of the respective chapters / publications.

Dr. Burkhard Knopf, Boris Meisterjahn and Nicola Schröder from the Fraunhofer IME provided technical assistance for the sample preparations and measurements of total and single-particle metal concentrations by sp-/ ICP-MS and ICP-OES for the studies of **Chapters 2** to **6**.

Dr. Ralf Kaegi from EAWAG in Switzerland carried out the sample preparation and examination using TEM and EDX for samples and nano stock materials of the **Chapters 2**, **3**, **5** and **6**.

Dr. Jessica Klehm and her team (Matthias Menzel & Claudia Stehr) from the Fraunhofer IMWS carried out the method evaluation of the sample preparation for the investigations with the methods of correlative microscopy and carried out the investigations of samples from **Chapter 4**.

Verena Kosfeld, Ph.D. student at Fraunhofer IME, contributed to the literature research of the chapters „Amphipods“ and „Branchiopoda“ of **Chapter 7**.

Dirk Maletzki and his team from the German Environmental Agency carried out the *Hyalella azteca* bioconcentration test with NM 300K using culture medium in aquarium (treatment NM $300K_{Cm}$) of **Chapter 2**.

Dr. Doris Völker and Dr. Kathrin Schwirn from the German Environmental Agency contributed on the reviewing and editing of the original submitted manuscript related to **Chapter 3**.

My contribution to the different chapters is shortly summarized below and described in detail at the beginning of each chapter.

Chapter 2 consists of a publication of Zeumer et al. (2020) for which I am a Co-author. I contributed to the implementation of the wastewater treatment plant simulation unit for the production of AgNP containing effluent. Further, I contributed to the collection and exposure of zooplankton and the implementation of the fish studies and preparation of the fish samples. I also carried out the examination of the hydrodynamic diameter of the AgNP applied in the study. I further contributed to the writing, review and editing of the manuscript.

For **Chapters 3** to **7** I was responsible for the collection, handling, statistical analysis and visualization of the data. In **Chapters 3** to **6** I made the conceptualization of the studies and performed the biological tests, performed the sample preparations for the analytical methods and carried out the measurements with contributions of L. Hermsen and support by the technical staff of FhG-IME (see acknowledgements of the respective chapters). I was not involved in the preparation of the samples for the investigations using TEM, EDX and correlative microscopy and the method development related to these analyses. However, I carried out the characterizations of the nanomaterials of **Chapter 2** to **6** for their hydrodynamic diameters and zeta potentials. I wrote the original drafts (with exception of **Chapter 2**) and revised and edited the final versions of all respective manuscripts and **Chapter 6**. The assessment scheme of **Chapter 7** was developed by myself and Prof. Dr. Christian Schlechtriem.

The results of the experiments have been presented as poster presentations and talks on several national and internal conferences. The **Chapters 2** to **7** are published in international peer-reviewed journals.

Chapter 1: General Introduction

1.1 Introduction and definition of nanomaterials

“There is plenty of room at the bottom.“ (Feynmann 1959, [1]). This sentence from the speech given by Richard Feynmann at the annual meeting of the American Society in 1960 is often mentioned as the birth of the concept of nanotechnology. From the first idea to manipulate materials in the dimension of molecules or even atoms [1] until today, more than 60 years have passed and this concept and its applications can no longer be ignored in our everyday life.

But why are these applications, the “nanomaterials” (NMs) integrated into our everyday life and what makes them so special and important? So important, that in 1996 three chemists (R. F. Curl, R. E. Smalley and H. W. Kroto) were awarded the Nobel Prize for their discovery of fullerenes, a special modification of the chemical element carbon [2]. Fullerenes are NMs which molecular structure are comparable to the pattern of the pentagons and hexagons of a soccer ball and which sizes are the range of a few nanometers [nm] or below.

Their small size is what defines the NMs. The European Commission defines them as “a natural or manufactured material containing disseminated or aggregated particles with a particle size distribution with > 50% cases between 1 and 100 nm in one dimension, at least” [3]. A nm is 10^{-9} meter or one billionth of a meter [4]. NMs can be further distinguished by the number of dimensions in which their size is below the threshold of 100 nm. NMs with only one dimension below 100 nm are often called nanolayers or nanoplates. If two dimensions of a NM are below 100 nm the material is often defined as nanowire, nanorod or nanotube. Nanoparticles (NPs) are NMs that show sizes below 100 nm in all 3 dimensions [5], [6].

The small size of NMs causes their special characteristics, which makes them so interesting for manifold applications. Due to their small size, they have a relatively large specific surface, which gives them new and/ or enhanced properties in comparison to their bulk material counterparts [7], [8]. According to Navarro et al. (2008) silver nanoparticles with a particle size of 10 nm have a surface of around 10 m^2/ g, 10,000 times higher than the surface of a compact silver cube of the same mass [9]. Wani et al. (2010) measured a specific surface area of 34.5 m^2/ g for silver nanoparticles with a size of 5 nm and described in 2011 that silver nanoparticles with diameter of 3 nm may have a specific surface area of up to 190 m^2/ g [10], [11]. Due to this high surface area, nanomaterials have a significantly higher number of sites that are chemically and physically reactive, which leads to the advanced and/ or changed properties of the nanomaterials regarding their chemical reactivity, electric conductivity, magnetic and optic properties [7], [12]–[14]. According to Poole and Owens (2003) roughly 5.8% (7,500 atoms) of all atoms (around 1.29 x 10^5) in a gallium arsenide nano cube with a size of around 14 nm are present at the cubes surface [15]. They further explain, that if the size of the nano cube decreases to 2.83 nm, the percentage of the atoms at the cubes surface increases to

around 25.8% (300 atoms) of all 1,165 remaining atoms in the nano cube [15]. Especially the high amount of surface atoms contribute to the special reactivity and properties of NMs.

Based on these new properties of the material, which is in transition from atom and molecule to bulk material, new technical developments and innovations open up with a high potential to save resources. In 2001, due to this promising outlook and the presumable enormous benefits of nanotechnology, the USA started the project “National Nanotechnology Initiative” to improve the research and development within that field of science and technology [16]. Three years later, in 2004, the European commission established the “European Strategy for nanotechnology” with similar aims and the hope that potential innovations may become the key to several existing problems of the society [17]. Nanotechnology was supposed to lead to new applications and solutions in the fields of medicine, information and communication, energy production and storage, material science, production, safety and much more [17].

The “Project on Emerging Nanotechnology” provides data on the number of products that are available on the market and based on nanotechnology [18]. The constant increase of the number of these products demonstrates the growth of nanotechnology and its value for the global economy. In 2006 only 212 products were listed and the number increased to 1,833 products that are available in 2020 [18]. The European commission estimated the global market production volume of nanomaterials to be around 11.5 million tons [20]. Inshakova and Inshakova (2017) reported, that the global market value of the “nano” products of the year 2015 estimated to be around 4.1 billion, whereas the global market of the “nano” products was expected to reach a value of 11.3 billion US $ in 2020 [19]. Further they summarized, that the global “nano” market will reach a value of up to 55 billion US $ in 2022. According to them, the european market generated a value of around 2.5 billion US $ in 2015 and is expected to generate a more than three times higher value in 2022 [19]. This trend of growing production still seems to continue. Hou et al. (2017) forecast a production volume of 1,600 tons just for CuONPs in 2025, while the production volume in 2014 was still at 580 tons [21].

These high production volumes will inevitably lead to an exposure of the environment by the release of these materials [22]–[24]. NMs can be released into the environment throughout their whole product life cycle [25]. A small amount enters the environment during the production of the NMs or their processing for nano functionalized products [23]. The greater part is released into the environment during use or disposal of the products. Especially for AgNP containing products it is well describe how they are released, e.g. by washing textiles with nano functionalized fabrics for disinfection purposes [26] or by erosion of NP containing exterior paints [27]. By this, the NMs can reach the waste water. For AgNPs it was estimated that 50% of the NPs that reach the environment, previously went through waste water treatment plants (WWTP) and reach aquatic environments directly by their effluents or by leachates from agriculture fields that have been fertilized with (incinerated) sewage sludge [28]. The other main

exposure of AgNPs occurs by washing out from waste at landfills [29]. Once entered into the environment the NMs can be further distributed by wind, rainwater or the flow of surface waters [30].

Even if NMs are not necessarily man-made [31], the unintentional exposure of the environment can cause effects with unknown extent. NMs with natural background can be found in soil (as product of chemical and physical erosion), atmospheric dust (e.g. as small fractions of sand from desert winds), volcanic dust/ ashes and the smoke of forest fires [32]–[35]. The latter can produce carbon based NPs. According to Murr et al. (2004) such carbon based NPs have been detected in 10,000 years old ice from core drillings [36].

With such a long time of exposure one can of course assume that the biota may have adapted to NM exposure evolutionarily. Nevertheless, manufactured NMs (MNMs) are materials that are specifically designed to be very reactive, catalytic etc. The most produced and used NPs are made of TiO_2, Ag and Au and thus are either very persistent and chemical inert, causing potential long term risks, e.g. by their catalytic properties or are the source of toxic ions like Ag^+ [37]. Therefore, MNMs may pose a serious environmental risk, when released into the environment.

Thus, already in 2004, as part of its communication on the “European Strategy for nanotechnology”, the European Commission pointed out the need to consider and investigate the possible risks posed by MNMs. It was proposed to “address any potential public health, safety, environmental and consumer risks upfront by generating the data needed for risk assessment, integrating risk assessment into every step of the lifecycle of nanotechnology-based products, and adapting existing methodologies and, as necessary, developing novel ones; […]” [17].

1.2 State of the art

Nearly two decades after the Communication of the European Commission in 2004, it is worth to have a look on the state of the art, to summarize regulatory processes for MNMs and to describe the challenges involved, especially with regard to the assessment of the environmental impact of MNMs.

Due to the general lack of information about the rapid increasing number of chemicals on the national and global markets, national and international regulatory authorities declared regulations [38] like the European Chemicals Regulation REACH (REACH) [39] that regulates the registration, evaluation and authorization of chemical substances used in the EU. Manufacturers of chemicals are obligated to conduct a chemical safety assessment, which means, that they should provide data that indicate the risk of their products to the environment and humans. In addition, information on the chemical and physical properties, an assessment on persistence, bioaccumulation potential and toxicity is required (PBT assessment) [39].

As a follow up to the “European Strategy for nanotechnology” of 2004 the European Commission published the “Action plan for Europe 2005 – 2009” in 2005. Within that communication it was underlined, that all forms of a chemical substance and thus also MNMs are included under the REACH regulation significantly stimulating research on MNMs [40].

Before 2004 most research related to nanomaterials was mainly focused on the application or syntheses of MNMs [6]. Publications about their toxicity or environmental relevance started to appear already between 2000 to 2004. In November 2020 a literature search using the scientific literature database Scopus® was carried out. With the keywords “nanomaterial” or “nanoparticle” in combination with “toxicity” a cumulated number of nearly 37,000 results was obtained [41]. By using the keywords “nanomaterial” or “nanoparticle” in combination with “bioaccumulation”, “bioconcentration” or “biomagnification” the cumulative number of results was below 5,000 being equivalent to the number of new published works found by using the keywords including “toxicity” for the year 2020 only.

The broad range of studies on the toxicity of MNMs also provided the basis for several literature reviews summarizing the effects of MNMs in the environment and developments of new analytical strategies and methods [5]–[7], [32], [42]–[47]. Risks and effects caused by MNM exposure have been widely described. The observed effects were mostly related to oxidative stress, e.g. by the creation of reactive oxygen species on the MNMs surface [48]–[51], genotoxicity and mutagenicity [52]–[56], immunotoxicity that e.g. induce inflammatory response [56]–[58], behavioral changes [59], [60] and interactions with metal metabolisms and homoestasis due to released (heavy) metals that may cause e.g. mitochondrial and respiratory dysfunctions and/ or even lead to mortality [61]–[69].

As mentioned above, significantly fewer publications are available that deal with the bioaccumulation of MNMs. However, for both, toxicity and bioaccumulation, the same trend is visible: the number of new publications per year starts to increase around the years 2004/ 2005 and is still increasing in the years 2019/ 2020 [41].

Nevertheless, the test guidelines used for the standard tests carried out as part of the environmental risk assessment, e.g. as part of the REACH regulation, were developed without considering the specific properties of MNMs, including their fate and behavior in the environment [70], [71]. When reaching the environment, most MNMs are underlying chemical and physical transformations [72]–[75]. These formations include chemical reactions with atoms on the surface such as oxidation or sulfidazion but also the formation of aggregates/agglomerates. These transformations inevitably cause altered effects and properties, as well as altered (bio)availability of MNMs. Thus the development of more suitable test methods was suggested [70], [76].

The transformation of MNMs and their fate after reaching the environment are well studied [29], [72], [75], [77]–[81] and test designs that consider more realistically exposure scenarios

were applied e.g. by using waste water borne MNMs during toxicity tests [60], [64], [67], [69], [82], [83]. But, especially after aggregation/ agglomeration, most NMs tend to sediment more or less quickly in aquatic systems and the vertical transfer generates inhomogeneous concentrations and exposure conditions [84]–[90]. However, especially for the estimation of the bioaccumulation potential of MNMs, constant exposure conditions are required.

The bioaccumulation potential of a test compound can be estimated by laboratory studies investigating the bioconcentration, biomagnification or the bioaccumulation of the respective substance. Bioconcentration is defined as the accumulation of a substance following uptake from the surrounding medium. The bioconcentration factor (BCF) is calculated as the ratio between the body burden of a substance in the test organism and the exposure concentration in the surrounding medium (water). For the assessment of the bioaccumulation potential under most regulations like REACH, the BCF is the key endpoint [91]. Biomagnification, in contrast to the bioconcentration, is measured by the biomagnification factor (BMF) and describes the ratio between the body burden of a xenobiotic compound that is resulting from dietary exposure and the concentration of the substance in the diet. The bioaccumulation factor (BAF) results from the ratio of the body burden and the exposure concentration, is independent from the exposure or uptake pathway, and thus less specific than the other endpoints.

Usually, the BCF value, which is the standard endpoint used for most regulatory bioaccumulation assessments, is calculated from data that are gained from fish tests carried out under flow-through conditions according to OECD TG 305 [92]. Nevertheless, several problems and challenges related to the application of MNMs may occur.

First of all, the fish bioaccumulation test according to OECD TG 305 was developed for water-soluble lipophilic compounds and does not consider the characteristics of MNMs. The classical test systems with simple aquaria could lead to inhomogeneous, non-continuous exposure conditions due to the sedimentation of MNMs within the experimental tanks. That would be in contradiction to the validity criteria of the test guideline, which requires data that must be generated under constant exposure conditions [39].

Considering the potentially lower risk of exposure of pelagic species due to the vertical shift of MNM concentrations in aquatic systems due to the gravitational sedimentation, the usage of benthic or filter feeding species should be considered as test organisms representing a worst case scenario [85]–[87], [93], [94].

Furthermore, the high number of MNMs that potentially needs to be individually tested as part of the environmental risk assessment is leading to a high number of studies. de Wolf et al. (2007) estimated a demand of around 327,000 fish for the assessment of 5,500 chemical compounds, when considering the minimum of 108 fish that were needed for one test, and only for the regulation under REACH [95]. The further demand for fish for the much higher number of

MNMs that potentially needs to be evaluated for their bioaccumulation potential is several times higher and thus poses an ethical problem and a financial and logistical challenge and collides with the requirements of the 3R-principles (Reduce, Refine, Replace) [96]–[98]. Although there are ideas for alternative approaches to determine the bioaccumulation potential of MNMs, these still include fish and, at their best, achieve only a slight reduction in the number of fish tests required [99].

A solution for those problems may be the usage of adapted exposure systems and the utilization of (benthic) invertebrates. This would be in accordance with the REACH regulation, as specified in Annexes VIII to XIII. According to this regulation, test methods may be adapted if the established test systems do not allow suitable exposure conditions. In that case, other taxonomic groups than fish are allowed to generate data and endpoints that could contribute to the bioaccumulation assessment [39], [100]. The usage of invertebrates may also solve the ethical problem by fulfilling the demands of the 3R-principles and by being in agreement with the European Council Directive 86/609/EEC on the protection of animals during experiments [101]. According to the directive, animals are defined as all living vertebrates, with exception of the human, however, invertebrates could be used during bioaccumulation tests without any restrictions [101], [102].

Nevertheless, alternative test species must still be suitable for bioaccumulation testing and their suitability must be demonstrated. A key factor for the ingestion of MNMs by aquatic organisms is their way of breathing for respiration. Due to the small size of the respiratory system in invertebrates, it is usually associated with a relatively large respiratory surface and the animals show high ventilation or filtration rates. In the case of filter feeding (benhtic) invertebrates, the uptake of MNMs from the surrounding water can occur. Benthic invertebrates have an important role within aquatic ecosystems, e.g. for the decomposition of biological material and the nutrient transport [103]–[106], or as an important source of feed for other aquatic species, and even terrestrial vertebrates [107]–[110]. In this way, aquatic invertebrates could also promote the transfer of previously accumulated MNMs along the food chain [111] and may lead to the exposure of species, which otherwise would probably not be exposed or only at a much lower concentration level of MNMs.

Further benefits of using invertebrate studies for the regulatory bioaccumulation assessment could be the potentially lower demand of resources. The test could be carried out in smaller scale test units resulting in a lower amount of MNM containing test media required for testing. Tests may also require shorter exposure periods and thus need less time to supervise and manage the studies.

1.3 Outline

The aim of this PhD project was to develop new concepts and testing procedures which will help to improve the regulatory bioaccumulation assessment of MNMs. The fate of MNMs in aquatic systems, their bioaccumulation behavior and transfer along the aquatic food chain were investigated.

The underlying hypotheses of this work are…

… that biomagnification studies using fish for bioaccumulation assessment on (metal/ metal oxide based) MNMs, as previously proposed, are less suitable.

… that the metal bioaccumulation from MNMs is mainly based on the soluble species of the metals/ their ions.

… that aquatic invertebrates play an important role for the transport of MNMs along the aquatic food chain.

… that aquatic invertebrates represent a worst case scenario for testing MNMs in aquatic systems regarding their interaction with MNMs.

… that aquatic invertebrates represents suitable substitutes for fish (vertebrates) in alternative test methods that could be integrated into regulatory risk assessment.

The following chapters describe a range of laboratory studies, which were carried out as part of the PhD project to evaluate the hypotheses described above.

First, in **Chapter 2**, the uptake and bioavailability of Ag from AgNPs were investigated in an aqueous exposure study with rainbow trout (*Oncorhynchus mykiss*) using AgNP contaminated WWTP effluents and AgNP supplemented dilution water. In a further dietary exposure study pre-exposed zooplankton was applied. The gained data allow to draw conclusions on the bioaccumulation potential of AgNPs in fish. The comparison of WWTP effluent (transformed/ sulphidized AgNPs) and dilution water spiked withAgNPs applied as pristine particles as exposure medium, allows to draw conclusions on the role of potentially released Ag^+ ions (Ag^+) on the measured body burdens.

Chapter 3 describes a test system and method, which was developed as part of this study to match the special challenges of testing MNMs in aquatic environments. The test system allows the performance of flow-through studies under controlled conditions that are required for regulatory purposes. In addition, the suitability of the filter feeding freshwater bivalve *Corbicula fluminea* for bioaccumulation studies with MNMs is demonstrated. The obtained data allow to draw first conclusions on the toxicokinetics of ingested MNMs in aquatic organisms and the role of ions in the total-metal-based bioaccumulation of MNMs.

In **Chapter 4** a bioaccumulation study with the benthic amphipod *H. azteca* is described. The previously introduced test system is used to test the suitability of the fresh water invertebrate, which has been commonly used for ecotoxicological studies for the estimation of the bioaccumulation potential of MNMs for regulatory purposes. The test concept for testing the bioaccumulation potential of soluble organic compounds [112] is adapted for the testing of MNMs with the benthic amphipod *Hyalella azteca.* To gain BCF and BMF data for three different MNMs, each representing different character-based groups of MNMs, aqueous and dietary exposure studies are carried out.

In **Chapter 5** methods of correlative microscopy including transmission electron microscopy are applied to examine samples gained from MNM exposure studies with *H. azteca*. Further, the role of ions in the uptake and accumulation of Ag from AgNPs is investigated and potential underlying physiological processes are described.

Chapter 6 describes a study on the transfer of MNMs by feeding (pseudo) feces from MNM exposed *C. fluminea* to *H. azteca* and potential chronic effects in the amphipod. The new methods described in **Chapters 3** and **4** are coupled in this study allowing investigations on the potential ecological interaction of MNMs including their interspecies transfer and potential remobilization by filter feeding mussels to other species.

The results of a literature search are summarized in a review publication that is presented in **Chapter 7**. This chapter deals with the suitability of different aquatic invertebrate species for bioaccumulation assessment, describes their biology and summarizes available bioaccumulation data. Based on the data obtained in the PhD project (Chapter 2-4), an assessment scheme including alternative and adapted endpoints for regulatory bioaccumulation assessment of MNMs is proposed in **Chapter 7**.

Finally, in **Chapter 8**, I summarize and discuss all results of the previous chapters with regard to the aims and hypotheses of this work. Additionally, an outlook is given on the potential future application of the methods developed as part of this work.

Chapter 2: Bioavailability of silver from wastewater and planktonic food borne silver nanoparticles in the rainbow trout *Oncorhynchus mykiss*

Chapter 2 consists of the following publication:

Bioavailability of silver from wastewater and planktonic food borne silver nanoparticles in the rainbow trout *Oncorhynchus mykiss*

Richard Zeumer, Lara Hermsen, Ralf Kaegi, Sebastian Kühr, Burkhard Knopf, Christian Schlechtriem

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The published supporting information directly follows the article.

Authors' contribution Chapter 2:

Richard Zeumer

Design and conducting of fish studies, WWTP simulation & plankton exposure; total metal analysis, collection, statistical analysis & visualization of the data, writing – original draft, writing – review & editing

Lara Hermsen

Conduction of fish studies and total metal analysis

Ralf Kaegi

Conducting characterization of NMs using TEM and EDX; Recourses (NM characterization); writing – original draft (method part – NM characterization using TEM and EDX)

Sebastian Kuehr

Design and conducting of fish studies, WWTP simulation & plankton exposure; NM characterization for hydrodynamic diameter; writing – original draft; writing – review & editing

Burkhard Knopf

Resources (total metal analysis)

Christian Schlechtriem

Funding acquisition; resources; design and refinement of experimental layout; writing – review & editing; study supervision

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Bioavailability of silver from wastewater and planktonic food borne silver nanoparticles in the rainbow trout *Oncorhynchus mykiss*

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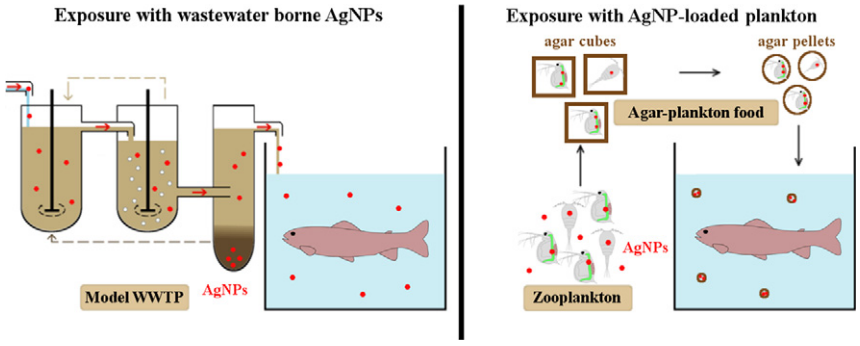
HIGHLIGHTS

- WWTP effluents were used to test the bioavailability of AgNP for fish.
- Bioavailability of Ag from AgNP in *O. mykiss* is reduced after wastewater treatment.
- Gills play a crucial role regarding the uptake of Ag into fish tissue.
- No transfer of Ag through the intestinal walls into other tissues could be detected.

GRAPHICAL ABSTRACT



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ABSTRACT

Silver nanoparticles (AgNPs) are present in a wide field of applications and consumer products and are likely to be released into the environment, mainly via urban and industrial sewage due to their extensive use. Even though AgNPs are mostly retained within the sludge of wastewater treatment plants (WWTPs), a small amount of mainly sulfidized particles still enters the aquatic environment, where they can be taken up by various aquatic organisms and transferred along the food chain. In this study, uptake and bioavailability of Ag from AgNPs following aqueous and dietary exposure were investigated in the rainbow trout *Oncorhynchus mykiss*. AgNPs in the effluent of model WWTPs and in tap water were used to perform aqueous exposure studies. No significant Ag uptake into the gills and carcass of the analyzed fish could be found for wastewater-borne AgNPs. However, when added to tap water at a concentration of 12.4 μg L^{-1}, a maximum total Ag tissue concentrations of around 100 μg kg^{-1} and 50 μg kg^{-1} in gills and carcass were measured, respectively. For the dietary exposure studies, freshwater zooplankton was exposed to AgNPs, and used for the preparation of food pellets with a total Ag concentration of 121.5 μg kg^{-1}. During the feeding study with rainbow trout significant total Ag concentrations up to 34.3 μg kg^{-1} could be found in the digestive tract. However, only a limited transfer of Ag through the intestinal walls into the carcass could be detected. AgNPs in plankton and WWTP effluent were characterized by transmission electron microscopy (TEM) in combination with energy dispersive X-ray spectroscopy (EDX) and found to be sulfidized. This

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transformation most presumably has led to their limited bioavailability for fish. The results emphasize the importance of realistic test conditions for the risk assessment of AgNPs by the use of environmental matrices.

1. Introduction

In the last decades, nanotechnology applications have entered nearly all fields of industry and production, creating new materials and concepts to improve processes in industries like water purification, agriculture, nanomedicine and energy storage (Roco et al., 2011). From the 1800 products which contained manufactured nanomaterials (MNMs) in 2018, silver nanoparticles (AgNPs) could be found in 443 of them (PEN, 2013). AgNPs are used in a wide field of products leading to an annual worldwide production volume of >10,000 t/year in 2010 (Piccinno et al., 2012). Due to their antimicrobial properties, AgNPs are predominantly used in textiles, cosmetics and coatings (Piccinno et al., 2012; Vance et al., 2015). During production, use and disposal of these products, MNMs can be released into the environment, e.g. by washing AgNP-containing clothes (Voelker et al., 2015). One of the major entry pathways of AgNPs into the environment is the release via wastewater treatment plant (WWTP) effluents (Kaegi et al., 2010; Sun et al., 2016). During sewage treatment, >90% of AgNPs adsorb to wastewater biosolids and have been shown to be transformed to Ag_2S within 2 h in a pilot WWTP (Kaegi et al., 2011). Although only a small fraction of the mostly transformed AgNPs leaves the WWTP, considerable AgNP concentrations of 0.7–11.1 ng L^{-1} could still be found in WWTP effluents (Li et al., 2016). Their discharge into receiving lake and river waters can lead to environmental concentrations which may have a significant impact on the aquatic life (Keller et al., 2013; Mueller and Nowack, 2008; Rozan et al., 1995). Gottschalk et al. (2009) predicted that the environmental concentrations of AgNPs in European surface waters are 0.76 ng L^{-1}. Peters et al. (2018) confirmed these findings by detecting average AgNP concentrations of 0.80 ng L^{-1} in Dutch surface waters. In general, the toxicity of metallic MNMs is driven by the release of ions, which is accompanied by physical damages (agglomeration, accumulation, attachment to organismic surface) caused by the physicochemical properties of the nanoparticles themselves (Asghari et al., 2012; Beer et al., 2012; Navarro et al., 2008). In fish, pristine AgNPs mostly affect the vitality of the organism via the gill tissue. Here, the nanoparticles agglomerate and adsorb to the tissue, leading to severe injuries, oxidative stress and may also release ions into the body via Na^+/K^+-ATPase ion channels in the gills and the blood circulatory system (Scown et al., 2010; Wood et al., 2004). However, in natural environments, several processes like sulfidation, dissolution, aggregation, adsorption and sedimentation influence the fate of AgNPs and may thus alter their effect on aquatic organisms (Furtado et al., 2016; Liu and Hurt, 2010). Even though sulfidized Ag species like Ag_2S show a reduced release of ions and are mostly resistant against oxidation and dissolution, they might still be bioavailable for aquatic organisms and thus potentially accumulate along the food chain (Azimzada et al., 2017; Choi et al., 2009; Kaegi et al., 2011; Kalman et al., 2015; Rozan et al., 2000). Kühr et al. (2018) assessed the chronic effects and bioavailability of AgNPs in the effluent from model WWTPs in the freshwater amphipod *Hyalella azteca*. Compared to pristine AgNPs supplied to tap water, wastewater borne AgNPs led to a reduced toxicity and bioaccumulation, which might be attributed to sulfidation or detoxification of the AgNPs by organic ligands like proteins or humic acids (Cedervall et al., 2007). Muth-Köhne et al. (2013) found an increased toxicity of AgNPs from WWTP effluents on fish embryos compared to pristine AgNPs and $AgNO_3$. Nevertheless, Bruneau et al. (2016) found no difference regarding the immunotoxicity of Ag^+ ions and AgNPs from wastewater effluents on the rainbow trout *Oncorhynchus mykiss*. Information on the bioavailability and fate of suspended MNMs in the aquatic environment is still lacking but is fundamental for a detailed risk assessment. Once taken up or accumulated in the tissues of aquatic organisms, MNMs can be transferred across the food chain, an issue, which has been subject to little research so far (Cedervall et al., 2012; Chae and An, 2016; Skjolding et al., 2014; Wang and Wang, 2014; Zhu et al., 2010). Chae and An (2016) discovered that silver nanowires exhibit a significantly higher trophic transfer from algae, over daphnids to the zebrafish *Danio rerio* when the particles were smaller. This indicated that the size of the particles might be one of the main parameters facilitating the transfer along the food chain. Wang and Wang (2014) investigated the uptake of AgNPs by brine shrimp *Artemia salina* and the trophic transfer of AgNPs by feeding the AgNP-loaded shrimp to marine medaka *Oryzias melastigma*. Despite the low trophic transfer factors (TTF, Ag concentration in fish divided by Ag concentration in brine shrimp) of 0.009–0.011, a reduced growth and inhibition of Na^+/K^+-ATPase and superoxide dismutase activity could be detected, emphasizing the potential risk of AgNPs or Ag^+ released from them transferred across the aquatic food. Nevertheless, a comparison of the bioavailability of potentially transformed, wastewater-borne or planktonic food-borne AgNPs in fish is still missing. In a recent study, Vogt et al. (2019) analyzed the concentration of silver–containing nanoparticles in a prealpine lake that receives the discharge of a municipal WWTP. They did not find considerable AgNP levels neither in the WWTP effluent, in any of the measured lake water samples, nor in tissues from lake fish. However, Ag concentrations of 0.98 ± 0.62 μg g^{-1} were detected in the sediment of the lake in close proximity of the WWTP discharge point and also distributed throughout the lake, indicating a continuous sedimentation of low amounts of wastewater-borne Ag, that accumulates at the bottom of the lake (Vogt et al., 2019). From here, the sedimented Ag could be re-mobilized into the aquatic food chain by benthic organisms and a more detailed analysis of the bioavailability and trophic transfer of Ag in aquatic food webs is thus needed.

In this study, we investigated the uptake and bioavailability of Ag from AgNPs in the rainbow trout *Oncorhynchus mykiss* that are exposed via the water and their diet. Several model WWTPs were conducted according to OECD TG 303A to investigate the bioavailability of Ag from AgNPs in WWTP effluent compared to pristine particles suspended in tap water. To assess the bioavailability of MNMs contained in feed, fresh zooplankton was exposed to AgNPs. The AgNP-loaded plankton was embedded in agar cubes, cut into pellets and fed to *O. mykiss*. Following aqueous and dietary exposure, tissue samples of *O. mykiss* were analyzed for total Ag contents by quantitative inductively coupled plasma - mass spectrometry (ICP-MS). AgNPs in stock dispersion, WWTP effluents and AgNPs loaded in zooplankton were characterized by transmission electron microscopy (TEM) in combination with energy dispersive X-ray spectroscopy (EDX).

2. Material and methods

2.1. Ag nanomaterial characterization and stock dispersions

The aqueous silver nanomaterial dispersion NM-300K was used for the aqueous and dietary exposure studies and stipulated by the OECD repository of representative manufactured nanomaterials (Totaro et al., 2016). The nanoparticles have a main particle size of 15 nm with a narrow size distribution (99% <20 nm) according to TEM analyses and are dispersed by an aqueous matrix of 4 wt% each of polyoxyethylene glycerol trioleate and polyoxyethylene (20) sorbitan monolaurate (Tween® 20) (Klein et al., 2011). The nominal silver

concentration (10.16% w/w) and the particle number were shown to be stable for at least 12 months according to ICP-OES and UV-VIS analyses (Klein et al., 2011). The hydrodynamic diameter (DZ) of NM-300K was characterized in ultrapure water, copper ion-reduced tap water, pond water and WWTP influent by dynamic light scattering (DLS) using a zetasizer (Zetasizer Nano Series, Malvern Instruments Ltd., UK) (see Supporting Information).

The AgNP stock dispersion used for the production of WWTP effluents was prepared by adding 8 mL of ultrapure water to glass vials containing 2 mL NM-300K dispersion, resulting in a total Ag concentration of 20 g L^{-1}. The vials were thoroughly shaken by hand for 1 min and sonicated for 15 min in a sonication bath (160 W, Sonorex Super RK 510, Bandelin electronic GmbH & Co. KG, Germany). Subsequently, 15 mL of the dispersions were added to a polyethylene container with 6 L of ultrapure water to achieve a nominal Ag concentration of 0.05 g L^{-1} in the stock solution.

AgNP stock solutions for the preparation of the spiked test media applied in the aqueous exposure study were prepared by adding 300 μL NM-300K dispersion to a 50 mL polypropylene vial with 30 mL ultrapure water. The resulting dispersions (1 g Ag L^{-1}) were thoroughly shaken by hand and sonicated for 10 min (2 min effective, 200 W, pulsation pause ratio of 0.2/0.8) by indirect probe sonication (Cup Horn BB6, Bandelin electronic GmbH & Co. KG, Germany).

2.2. Model WWTPs

Two lab-scale WWTP units (behrotest® Laboratory Sewage Plant KLD 4 N, behr Labor Technik GmbH, Düsseldorf, Germany) were conducted according to OECD Guideline 303A (OECD, 2001) to simulate the transformation of nanomaterials during sewage treatment. A single WWTP unit (total volume 10 L) consists of a non-aerated reactor, an aerated reactor and a secondary clarifier to simulate denitrification, nitrification and sedimentation processes of a full-scale WWTP (see Supporting Information, Fig. S1). The units were conducted as previously described (Kampe et al., 2018; Kühr et al., 2018) and initially fed with activated sludge (2.5 g dry mass L^{-1}) from a municipal WWTP in Schmallenberg, Germany (51°09′N 8°16′E). The sludge inoculum was sieved (≤2 mm) on the first day of the study. The dry matter of the sludge was determined with a moisture analyzer (HB43-S, Mettler Toledo, USA) and did not exceed 3.0 g dry mass L^{-1} during the study. Both units were operated in a temperature controlled room (20–25 °C) and continually fed with artificial wastewater (30 mg L^{-1} urea, 28 mg L^{-1} K_2HPO_4, 7 mg L^{-1} NaCl, 4 mg L^{-1} $CaCl_2 2H_2O$, 2 mg L^{-1} $MgSO_4 7H_2O$, meat extract, peptone) according to OECD Guideline 303A (OECD, 2001). In contrast to the guideline, the concentrations of peptone and meat extract in the artificial wastewater were raised to 150 and 180 mg L^{-1}, respectively, in order to avoid the formation of bulking sludge. The stock solutions of artificial wastewater and the AgNP dispersions (both prepared 10-fold concentrated) were freshly prepared every 3–4 days and stored at 4 °C. Via a tube system (PLP 33; SP04/3.5 K, behr Labor-Technik, Germany), wastewater and AgNP dispersions were diluted 1:10 with tap water and pumped into the denitrification reactor with a continuous flow of 750 mL h^{-1} (total influent volume of 288 L during the study), leading to a retention time of 6 h within the WWTP units. One WWTP unit was running as control and one unit was exposed to AgNPs with a concentration of 5 mg L^{-1} via the influent. An adaptation phase without the addition of AgNPs lasting six days was carried out until processes within the WWTP units reached stable conditions which were characterized by a dissolved organic carbon (DOC, measured with TOC-V CPH Total Carbon Analyzer, Shimadzu, Japan) elimination rate >80% and constant concentrations of ammonium, nitrite and nitrate in the effluent (OECD, 2001). After this phase, AgNPs were added to the WWTP influent for a total of 6 days. After four days, significant Ag concentrations could be found in the effluent. From this day on, the effluent was collected for 40 h and stored as one composite sample per treatment (one effluent sample containing AgNPs, one with control effluent) in a 30 L polyethylene (PE) container (Züchner GmbH, Köln, Germany) in the dark at 4 °C for 4 days. During the WWTP study, concentrations of ammonium, nitrite and nitrate in the effluents were measured photometrically (NANOCOLOR® 500D, Macherey-Nagel, Germany) at least once per week. The pH values in the denitrification (7.66 ± 0.06 and 7.59 ± 0.05) and the nitrification reactor (7.34 ± 0.17 and 7.26 ± 0.11) of the WWTPs without and with AgNPs, respectively, were measured daily. The oxygen concentration in the nitrification reactor was monitored and kept in the range between 2 and 4.5 mg L^{-1}.

2.3. Exposure studies with O. mykiss

2.3.1. Fish maintenance

Juvenile rainbow trout (*Oncorhynchus mykiss*) were obtained from Fischzucht Störk (Bad Saulgau, Germany) and maintained in a flow-through system in 200 to 250 L tanks with constant aeration at 14 ± 2 °C. Fish were kept in copper ion-reduced tap water (for physicochemical properties see Supporting Information, Table S2) under a 16:8 light-dark cycle until the start of the study. The fish were fed with a commercially available food for fish breeding (Inicio Plus®, BioMar, Denmark).

2.3.2. Aqueous exposure study

The aqueous exposure study with *O. mykiss* was performed according to OECD guideline 305 (OECD, 2012a). Juvenile rainbow trout (40 fishes per tank, 40 L medium, 1 tank per treatment) were exposed over 14 days (uptake phase) to (i) effluent from model WWTP with AgNP-treated influent (see Section 2.2), (ii) effluent from control WWTP manually spiked with pristine AgNPs and (iii) dilution water spiked with pristine AgNPs. Copper ion-reduced tap water was used as dilution water (see Supporting Information, Table S2). All tested WWTP effluents were 10-fold diluted with dilution water to simulate the dilution effect of the receiving waters of a full-scale WWTP (for physicochemical properties of undiluted WWTP effluents see Supporting Information, Table S2). Based on the measured total Ag concentrations of 14.7 μg L^{-1} in the diluted WWTP effluent, the nominal test concentrations in all treatments was set to 15 μg L^{-1}. Following the uptake phase, the test organisms were transferred to new tanks containing dilution water without any test item. The depuration phase lasted 14 days. A control group was kept in dilution water over the entire experimental period of 28 days. The study was performed in a semistatic approach (change of media at 48 h intervals) and the fish were fed daily with commercially available fish food (Inicio Plus®, pellet size 1.1 mm, BioMar, Denmark) at a rate corresponding to 1.5% body weight per day, according to the OECD guideline 305 (OECD, 2012a). During the entire study the fish were kept under a 16:8 light-dark cycle and all tanks were aerated continuously (oxygen saturation >60%). The vitality of the organisms, the water conditions [concentrations of ammonium (0.1–4.7 mg L^{-1}), nitrate (18–22 mg L^{-1}) and nitrite (0.0–1.4 mg L^{-1}), pH (7.4–8.5) and water temperature (14 ± 2 °C)] were monitored periodically. Prior to the test start, an adaptation period of 14 days was performed to verify that the test animals were in good condition. Before the start of the test, feeding was suspended for 24 h. At test start, two times 40 animals were randomly distributed to the experimental tanks. The mean wet weight of 20 fish, which were randomly selected from each tank was measured (4.6 ± 1.3 g). During the complete test period, groups of 4 animals were taken periodically from each treatment (day 0, 2, 6, 10, 12, 14, 15, 17, 20, 23, 27) and euthanized with 5 g L^{-1} Chlorobutanol (Merck, Germany) followed by the dislocation of the neck according to the directive of the EU on animal welfare (EU, 2010). Subsequently, the digestive tract, gills (from day 6 on) and carcass were dissected on ice, snap frozen in liquid nitrogen and stored at −20 °C until further processing. On day 0, the digestive tract was rinsed with ultrapure water to exclude possible contaminations by the fish food.

2.3.3. Preparation of plankton food

Stoten_135695Fresh zooplankton was chosen as feed component within the dietary exposure study to simulate the natural freshwater food chain. The plankton was collected in a natural pond at the fish farm "Fischzucht Riegger" in Ettenheim, Germany (48°26′N 7°83′E) in a rural environment close to the Black Forrest. The collection was done by a rope winching device, which pulled a plankton net (200 μm) in parallel lines through the water. The condensed zooplankton was transferred to 30 L PE containers filled with 25 L pond water (see Fig. 1). Subsequently, 7.5 μL NM-300K were added to the containers to achieve a nominal Ag concentration of 30 μg L^{-1}. The exposure concentration of 30 μg L^{-1} was chosen to ensure a sufficient uptake of silver by the plankton organisms. Exposure concentrations in the range of 4–64 μg L^{-1} were used by Vincent et al. (2017), who exposed natural plankton communities with differently coated AgNPs. Additional containers without the addition of AgNPs were used as a control. After gentle stirring of the test containers, live zooplankton was exposed for 5 h with hourly measurements of the oxygen concentration. Subsequently, the plankton was drained by pouring the previously exposed samples through a plankton net (mesh size 65 μm, Conatex Lehrmittel, Germany), transferred into 6 L freezer bags (QuickPack, Germany) and frozen at −20 °C.

Control and AgNP-loaded plankton were embedded into agar pellets, to achieve an experimental diet suitable for fish that still preserves the natural plankton structure (see Fig. 1). The homogenous distribution of the test substance and the sufficient stability of the agar pellets avoiding the release of the test substance into the surrounding water were confirmed in a preliminary study, where the agar pellets were added to 500 mL dilution water and kept under static conditions for 7 days in the absence of fish. No significant total Ag concentrations (below background of 0.04 μg L^{-1}) were found in the surrounding medium (see Supporting Information, Table S3). Agar pellets (5 replicates, 200 mg each) were analyzed for total Ag content. The results show that total Ag concentrations (121.5 ± 0.1 μg kg^{-1}) were homogenously distributed in the experimental diet. For the preparation of the agar pellets, the frozen plankton was lyophilized (Alpha 1–2 LDplus, Christ, Germany). Afterwards, 250 mg agar (Agar-Agar, Kobe I, Carl Roth GmbH, Germany) were dissolved in 15 mL ultrapure water under heating in a microwave, mixed with 600 mg dried plankton and 800 mg ground fish food pellets (Inicio Plus®, Biomar, Denmark) and poured finally into small cube moulds (1 cm^3 each cube, silicon mould, Belmalia, Germany). The cubes were dried overnight and pressed through a nylon net (mesh size 3 mm) to achieve small pellets of a suitable size to be eaten by juvenile fish. A pre-test with rainbow trout was carried out to exclude toxic effects induced by the AgNP loaded plankton food pellets.

2.3.4. Characterization of plankton and food pellets

The species composition of the collected zooplankton was analyzed using an identification key (Streble et al., 2002). Subsamples of the frozen plankton plates were carefully defrosted and species were determined microscopically. The zooplankton consisted of 65% *Copepoda* (44% *Cyclopoida*, 20% *Calanoida*, 1% Nauplius larva) and 35% *Branchiopoda* (24% *Bosmina longirostris*, 11% *Daphnia* spec.). All of the identified species and taxa were also detected when analyzing the food pellets clearly showing that the integrity of the plankton material was maintained throughout the feed preparation process.

The nutritional value of the previously described plankton food pellets was determined by quantitative analysis of the lipid according to Smedes (1999). For lipid extraction, pellets (50 mg) were transferred to glass vials, mixed with 200 μL extraction solution I (55% cyclohexane/44% isopropyl alcohol (v/v)) and homogenized (Potter homogenizer, B. Braun, Germany). Subsequently, 2.75 mL ultrapure water were added, the solution was centrifuged (12 min, 543 ×*g*, Mega Star 1.6R, VWR, USA) and the organic phase was transferred to glass vessels (previously stored over night at 65 °C and weighed). The remaining aqueous phase was mixed with 2.5 mL extraction solution II (87% cyclohexane/ 13% isopropyl alcohol (v/v)), centrifuged again (same conditions) and the resulting organic phase was combined with the organic phase from the first extraction step. The extract was dried with nitrogen gas, stored over night at 65 °C and weighed to calculate the lipid content.

The total lipid of the plankton food pellets (dry weight) was 3.0 ± 0.4%.

2.3.5. Dietary exposure study with O. mykiss

The bioavailability of AgNPs contained in fish feed by *O. mykiss* was assessed by performing a dietary exposure study following the principles of OECD guideline 305 (OECD, 2012a). Since the lipid content of the plankton pellets (3.0 ± 0.4%) was 5-fold lower than in commercially available fish food (17.5 ± 0.2%, Inicio plus®, Biomar, Denmark), the daily ration was raised from 1.5% (as recommended by OECD guideline 305) to 7.8% of fish body wet weight. The feeding was performed once per day and the pellets were eaten immediately. Feces and potential feed residuals were removed 1 h after feeding by vacuuming through a glass tube. Juvenile rainbow trout (45 fish per tank, 75 L medium, 1 tank per treatment) were exposed over 14 days (uptake phase) to AgNP via the diet. A control group was fed with unfortified plankton pellets under otherwise identical experimental conditions. After the uptake phase, the test organisms in all tanks were fed with control pellets for another 14 days (depuration phase). The study was performed under flow-through conditions with a flow rate of 15.6 L h^{-1} equivalent to a 5-fold water renewal per day. The experimental conditions within the test system such as oxygen saturation (>60%), concentrations of

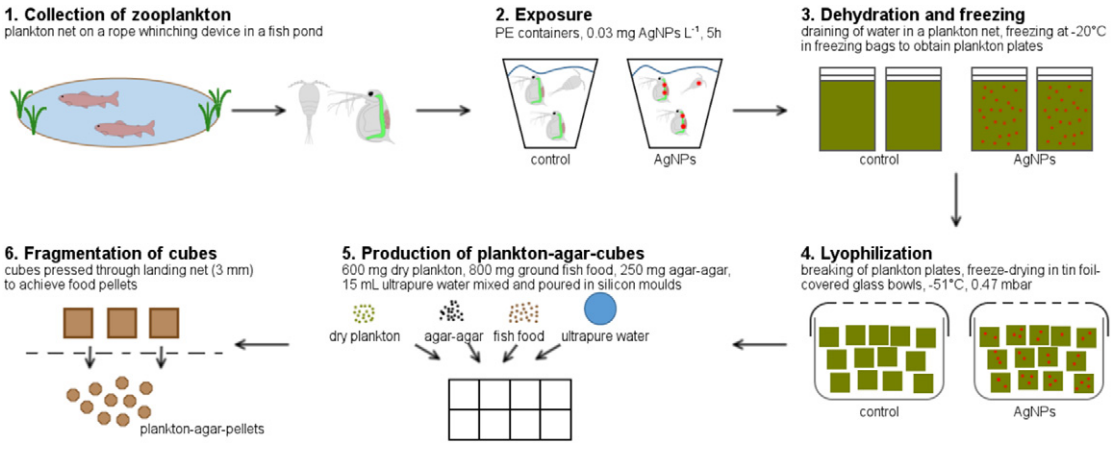

Fig. 1. Scheme of the production of plankton-agar-pellets. Zooplankton was collected in a fish pond and exposed to AgNPs in PE containers, 5 h. After freezing and freeze-drying, plankton was embedded in agar matrix enriched with commercial fish food. Natural structure of plankton organisms was maintained during processing.

nitrate (8–10 mg L^{-1}), nitrite (<0.01 mg L^{-1}) and ammonium (0.01–0.03 mg L^{-1}), pH (7.1–7.8) and water temperature (14 ± 2 °C) were monitored periodically. During the test, groups of 5 fish were taken periodically (day 0, 7, 14, 15, 17, 19, 22, 25, 28) from each treatment and euthanized as described in Section 2.3.2. Similar to the method described in Section 2.3.2, the digestive tract and carcass (all body parts apart from the digestive tract) of the fish were dissected on ice, snap frozen in liquid nitrogen and stored at −20 °C until further processing. Additionally, groups of 3 fish were taken from each treatment (on day 0, and on day 28) to analyze the lipid content of the fish (Smedes, 1999).

2.4. Transmission electron microscopy

The AgNPs from WWTP effluents used in this study were extracted by cloud-point extraction and characterized by scanning transmission electron microscopy (STEM) in combination with high-angle annular dark-field (HAADF) and energy-dispersive x-ray (EDX) detectors as part of a parallel study carried out by Hartmann et al. (2019). AgNPs in the NM-300K stock dispersion and the AgNP-loaded, lyophilized zooplankton were characterized by TEM and the elemental composition of selected NP was assessed using an EDX system attached to the microscope. The stock dispersions (NM-300K) were diluted $1{:}10^6$ in deionized water and 1 mL of this was directly centrifuged (1 h, ~14,000 ×*g*) on TEM grids. For the plankton spiked with NM-300K, 30 mg of freeze dried material was added to 1 mL of 0.2% FL-70 (Thermo Fisher Scientific, USA) and sonicated for 1 min in a Vial Tweeter (Hielscher Ultrasonics GmbH, Germany). The resulting dispersion was diluted 1:1 in deionized water and directly centrifuged on TEM grids using the same conditions as for NM-300K. As the AgNP carried a negative surface charge, the TEM grids were functionalized with Poly-L-Lysine (PLL, 0.1% (w/v) in H_2O, Sigma Aldrich) to enhance AgNP deposition on the TEM grids. The preparation of the TEM grids is described in more detail in Uusimaekia et al. (2019). The TEM grid was investigated using a dedicated scanning transmission electron microscope (STEM, HD2700Cs, Hitachi, Japan), operated at an acceleration voltage of 200 kV. For image formation the HAADF signal was used. Elemental analyses were conducted using an EDX system (EDAX, USA) and the spectra were recorded and processed using Digital Micrograph (v.1.85, Gatan Inc., USA).

2.5. Determination of Ag concentrations

2.5.1. Preparation of aqueous samples

During generation of WWTP effluents and the course of the aqueous exposure study, aliquots of the media were sampled periodically for determination of total silver concentrations. For WWTP studies, aliquots were taken daily in duplicate from the effluent following the addition of AgNPs to the influent of the WWTPs. Effluent samples were taken for 4 days and analyzed for total Ag content until stable Ag concentrations were found in the effluent. Over the course of the aqueous exposure study with *O. mykiss*, water samples were collected from fresh (0 h) and aged media (24 h and 48 h old) at least twice per week and within the dietary exposure studies at the beginning and the end of uptake and depuration phase to exclude that leaching of test item into the surrounding medium occurred. For total silver analysis, 5 mL of the aqueous sample were transferred to 15 mL vials and mixed with 5 mL *aqua regia* and 5 mL ultrapure water. Nitric acid (69%, Suprapur®, Carl Roth, Germany) and hydrochloric acid (30%, Suprapur®, Baker, The Netherlands) were mixed 1:3 for the preparation of *aqua regia*. Water was purified using an ELGA Pure Lab Ultra water purification system (>18 MΩcm).

2.5.2. Preparation of tissue samples

Carcass samples, taken during the exposure studies with rainbow trout, were ground manually in a zirconium oxide mortar under constant cooling by liquid nitrogen followed by lyophilisation (Alpha 1–2 LDplus, Christ, Germany). Digestive tract and gill samples were not ground since they were small enough to be processed completely. Subsamples of 200 mg ground carcass as well as digestive tract and gill samples were mixed with 5 mL of nitric acid (69%) and digested in a microwave (UltraClave II, MLS GmbH, Germany, 25 min heating up to 220 °C, 30 min on 220 °C, 95 min cooling, max pressure 80 bar). Further, a certified reference material standard (Oyster tissue NIST® SRM® 1566b, Merck, Darmstadt, Germany) was treated equally to validate the digestion process. Plankton and food samples were prepared identically but were mixed with 8 mL *aqua regia* instead of nitric acid. After sample digestion, samples were filled up to 15 mL with ultrapure water.

2.5.3. Quantitative silver analysis by ICP-MS

The measurement of total silver concentrations in the previously prepared media and tissue samples was performed by using inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700, Agilent Technologies, Waldbronn, Germany) set on isotope ^{107}Ag in collision gas mode with helium. In order to detect and compensate for instrument drifts, a rhodium standard (Merck KGaA; Certipur®) was used. The matrix adjusted calibration standards were prepared with commercially available silver ICP standard solutions (Merck Certipur® 1000 mg L^{-1} Ag in 3% (v/v) nitric acid, Merck, Darmstadt, Germany). The calibration function as well as the limit of detection (LOD) were calculated by the ICP-MS software (Agilent MassHunter workstation) using a linear regression. The limit of quantification (LOQ) was calculated as three times the LOD. In all measurement series, all samples were measured in triplicate (internal triplicate measurement). Further, certified aqueous reference materials (TMDA 70.2, Environment Canada) and quality control samples were prepared independently from the calibration samples (e.g. from multielement standard Merck IV, Merck, Darmstadt, Germany) and measured in the same measurement series for validation.

2.6. Statistical analysis

All statistical analyses were performed with OriginPro 2017 (OriginLab Corp., Northampton, MA, USA). The α-level for all tests was set to 0.05 (Zar, 2009). All data sets with at least 4 replicates were checked for outliers by using the Grubb's test (SQS 2013 Version 1.00 by J. Klein and G. Wachter). Time weighted average concentrations (TWA) of Ag in test media of the different treatments were calculated according to OECD 211 (OECD, 2012b) for the aqueous exposure studies with *O. mykiss*.

3. Results

3.1. Total Ag concentrations in test media and plankton food

The total Ag concentrations measured in the test media and in the plankton and food samples are shown in Table 1. For the studies with the model WWTPs, AgNP stock dispersions of 50 mg L^{-1} were prepared and diluted 10-fold before being added to the WWTP via the influent, as described previously (see Section 2.3.). An Ag concentration of 202.6 μg L^{-1} was measured in the effluent, which was collected 4 days after the initiation of AgNP dosing to the influent. After a storage time of 4 days, the effluents were 10-fold diluted before they were used in the aqueous exposure studies, resulting in a time weighted average Ag concentration of 14.8 μg L^{-1}. Control effluents and dilution water which were manually supplemented with pristine AgNPs showed total Ag concentrations of 16.3 μg L^{-1} and 12.4 μg L^{-1}, respectively.

Live zooplankton was exposed to AgNP dispersions of 29.8 ± 0.3 μg L^{-1} (Table 1). The lyophilized plankton showed a homogenous Ag concentration of 1639.6 ± 43.3 μg $kg^{-1}$and was used to produce agar-plankton pellets with a total Ag concentration of 121.5 ± 0.1 μg kg^{-1}. The food pellets contained agar, plankton and commercial fish food. Since it turned out that the commercial food itself contained

Table 1
Composition of test media and food. Aqueous samples were measured in duplicate; plankton and food samples were measured in quintuplicate. TWA = time weighted average Ag concentration. LOQ < 7 ng L^{-1}.

Study	Matrix	TWA
Aqueous exposure study with *O. mykiss*	Effluents from MNM treated WWTPs	14.8 μg L^{-1}
	WWTP effluent supplemented with MNMs	16.3 μg L^{-1}
	Dilution water supplemented with MNMs	12.4 μg L^{-1}
	Control effluent without MNMs	0.0 μg L^{-1}

Study	Matrix	Total Ag	St. Dev.
Preparation of plankton food	Exposure medium	29.8 μg L^{-1}	0.3 μg L^{-1}
	Lyophilized plankton	1639.6 μg kg^{-1}	43.3 μg kg^{-1}
Dietary exposure study with O. *mykiss*	Plankton food pellets	121.5 μg kg^{-1}	0.1 μg kg^{-1}
	Control plankton food pellets	14.7 μg kg^{-1}	1.0 μg kg^{-1}
	Commercial fish food	37.3 μg kg^{-1}	10.9 μg kg^{-1}

a total Ag concentration of 37.3 ± 10.9 μg kg^{-1}, Ag was also found in the control plankton food pellets (14.7 ± 1.0 μg kg^{-1}) which were fed to the fish during the second half of the study and to the control group during the entire study.

During the dietary exposure study, water samples taken from the experimental tank were measured for total Ag concentrations to check for AgNPs or Ag^{+} species that might have leached from the fortified food pellets. However, no significant Ag concentrations (>LOQ) were found.

3.2. DLS and TEM results

The results of the DLS measurements of AgNPs in different media (ultrapure water, dilution water, pond water, WWTP influent) are shown in the Supporting Information, Table S1. The DZ of the particles was in the range of 30–60 nm when high AgNP concentrations (100 or 1000 μg L^{-1}) were dispersed in the media. When low Ag concentrations (1 and 10 μg L^{-1}) were used, the DZ increased to very high values (max. 1017 nm). These exorbitant values were most presumably caused by the low quality of the measurement at low concentrations as it is shown by the high attenuator values (9–11).

The AgNPs in the stock dispersion were well dispersed on the TEM grids (Fig. 2A). A particle size distribution was derived from multiple images using the NanoDefine-Particle sizer (Wagner, 2016) and revealed a mean particle size of 18.3 nm with a standard deviation (1σ) of 3.1 nm (Fig. 2A, inset). Elemental analyses of individual particles showed a dominant signal of Ag (Lα), next to Cu (Kα) which was related to signals resulting from the Cu grid (Fig. 2B).

STEM analyses of AgNPs from WWTP effluents showed that the particle size was not changed during the passage of the WWTP, while the EDX revealed an association of wastewater-borne AgNPs with sulfur, indicating their sulfidation (see Hartmann et al., 2019).

By using high-angle annular dark field (HAADF) images of the plankton samples, AgNPs appeared as bright spots incorporated in a light grey fuzzy matrix representing the planktonic matrix (Fig. 2C). The low Ag concentration in the plankton and thus the rather low particle number concentration of the AgNPs did not allow the establishment of a particle size distribution of the AgNPs. However, individual AgNPs that were detected in the planktonic matrix were always of a very comparable size to the particles observed in the NM-300K stock dispersion. Elemental analyses of individual AgNPs, however, always revealed a considerable sulfur (S) signal intensity, next to the Ag signal, suggesting that the AgNPs were sulfidized to a large extent (Fig. 2D).

3.3. Aqueous exposure of O. mykiss

During the aqueous exposure study no mortalities or diseases were observed. The mean wet weight of the rainbow trout in the control group increased during the study by 51% (30% during uptake phase, 16% during depuration phase) from 4.7 ± 1.0 g on day 0 to 7.1 ± 2.2 g on day 28. The evolution of total Ag concentrations measured over the uptake and elimination period in carcass, gills and digestive tract are shown in Fig. 3. At test start, fish were sampled to assess the background Ag concentration in the investigated tissues. For carcass and gut the concentrations were found to be 2.4 ± 0.4 μg kg^{-1} and 11.6 ± 1.5 μg kg^{-1}, respectively (Fig. 3A). The initial total Ag concentrations measured in gill tissues were below LOQ.

The carcass of the fish (whole fish without gut and gills) showed no significant uptake of Ag following exposure to AgNPs from WWTP effluents. The highest total Ag concentration in this treatment of 4.0 ± 1.1 μg kg^{-1} was found on day 14. Similarly, the exposure to AgNPs supplemented to control effluents resulted in a non-significant increase of the carcass burden to a level not higher than 7.5 ± 1.3 μg kg^{-1} (day 14) within the uptake phase of the study. In contrast, significant Ag concentrations of 9.1 ± 1.0 μg kg^{-1} and 10.7 ± 6.5 μg kg^{-1} were detected during the course of the depuration phase on day 17 and 20, respectively. Afterwards, the total Ag concentrations decreased to 3.8 ± 1.1 μg kg^{-1} on day 28. The highest Ag concentrations in the carcass of the tested fishes were found after exposure to AgNPs supplemented to dilution water. During the uptake phase, a significant Ag uptake could be detected on days 10, 12 and 14 with 18.7 ± 1.6 μg kg^{-1}, 23.9 ± 3.9 μg kg^{-1} and 40.0 ± 3.6 μg kg^{-1}, respectively. At the beginning of the depuration phase, this tendency continued until day 17 (45.7 ± 6.6 μg kg^{-1}), followed by a steady decrease of Ag concentrations until day 28 (18.2 ± 0.9 μg kg^{-1}). A complete depuration of previously accumulated Ag (decrease to the initial value measured on day 0) was not observed until the end of the test period. The total Ag concentration in the carcass of the control group fish slightly decreased from day 0 (2.4 ± 0.4 μg kg^{-1}) to day 28 (1.0 ± 0.0 μg kg^{-1}).

The gill tissue was analyzed separately (Fig. 3B). After exposure to AgNPs present in or added to WWTP effluent, the gills of both treatments exhibited Ag concentrations of 13.4 ± 7.4 μg kg^{-1} (day 12) and 10.6 ± 3.0 μg kg^{-1} (day 14) towards the end of the uptake phase, followed by a decrease to 1.6 ± 0.5 μg kg^{-1} and 2.2 ± 0.7 μg kg^{-1} after 13 days of depuration, respectively. In contrast, Ag concentrations in the gill tissues of fish exposed to AgNPs supplemented to dilution water were 10.5 ± 2.7 μg kg^{-1} on day 6, increased significantly to 90.9 ± 17.5 μg kg^{-1} on day 12 and remained stable at that level until day 20 (98.9 ± 14.4 μg kg^{-1}) after 6 days of depuration. The tissue concentrations decreased to 56.9 ± 14.5 μg kg^{-1} until the end of the depuration phase and did not reach the initial tissue concentration measured on day 0. The Ag concentrations in gills of the control group animals stayed on a very low level (2.4 ± 1.5 μg kg^{-1}, 0.2 ± 0.2 μg kg^{-1} and 0.3 ± 0.1 μg kg^{-1} on days 6, 14 and 28, respectively).

In the gut tissue (Fig. 3C), significantly elevated Ag levels were detected during the uptake phase reaching total Ag concentrations of 27.4 ± 10.6 μg kg^{-1}, 46.7 ± 17.0 μg kg^{-1} and 69.7 ± 27.8 μg kg^{-1} following exposure to AgNPs from WWTP effluents, AgNPs supplemented to control effluents and AgNPs supplemented to dilution water, respectively. During the depuration phase the Ag concentration decreased in all matrices (Ag concentrations on day 28 were 10.8 ± 1.7 μg kg^{-1}, 11.3 ± 2.1 μg kg^{-1}, 30.1 ± 4.8 μg kg^{-1} for WWTP effluent, supplemented effluent and supplemented dilution water, respectively). The measured Ag concentrations in the digestive tract of the organisms were generally higher than the measured Ag concentrations in the carcass. The control group animals displayed constantly low Ag concentrations (8.2 ± 2.8 μg kg^{-1}, 7.1 ± 3.2 μg $kg^{-1}$and 8.7 ± 1.9 μg kg^{-1} on days 6, 14

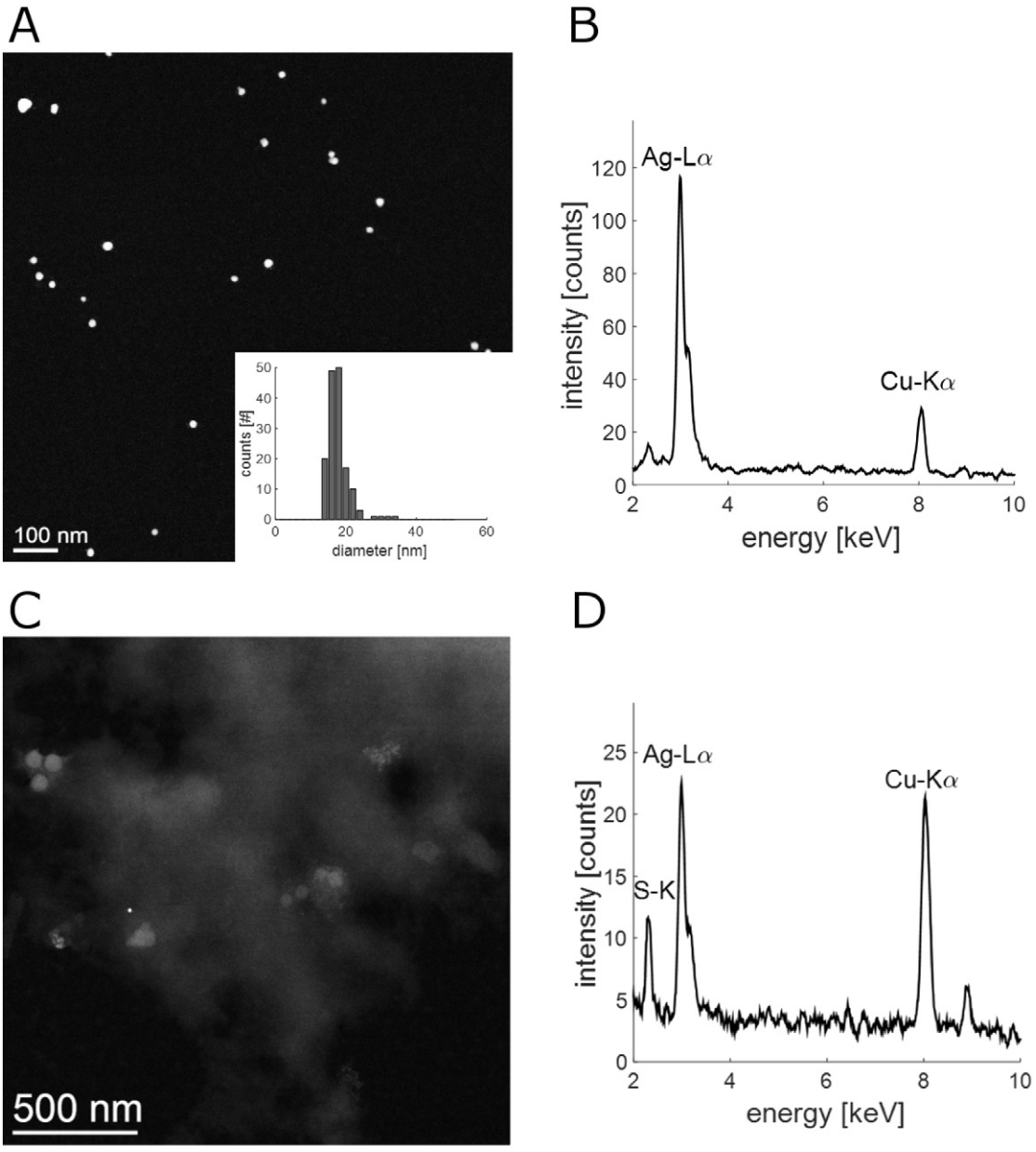

Fig. 2. High-angle annular dark field (HAADF) image with an energy dispersive X-ray (EDX) spectrum of AgNP from the stock dispersion (A, B) and freeze-dried zooplankton containing AgNPs with the corresponding EDX spectrum (C, D). The particle size distribution of AgNPs ($n = 153$) derived from several HAADF images is shown as an inset in (A).

and 28, respectively) in the digestive tract until the end of the test period.

3.4. Dietary exposure of O. mykiss

The wet weight of the rainbow trout in the control group increased during the dietary exposure study by 150% (91% during uptake phase, 31% during depuration phase) from 2.0 ± 0.5 g on day 0 to 5.1 ± 0.3 g on day 28. On day 0, the total lipid content of the test fish was 5.0 ± 0.7% and decreased during the study to 2.9 ± 1.1%. During the study, no behavioural changes, mortalities or physical damage were observed.

The total Ag concentrations in digestive tract and carcass of the fish collected during the dietary exposure study are shown in Fig. 4. On day 0, 7 and 14 Ag concentrations of 8.18 ± 1.0 μg kg^{-1}, 27.9 ± 17.7 μg kg^{-1} and 34.3 ± 13.8 μg kg^{-1} were measured in the digestive tract, respectively. AgNP-free food pellets were fed from day 14 onwards. Thus, the Ag concentrations decreased to 4.8 ± 1.7 μg kg^{-1} until day 17 and stayed at this level until the last sampling on day 28 (3.3 ± 0.8 μg kg^{-1}). The Ag concentrations in the digestive tract of the control group, stayed on a low level until the end of the study with 1.2 ± 1.5 μg kg^{-1}, 1.1 ± 1.4 μg kg^{-1}, 3.3 ± 3.7 μg kg^{-1} on days 7, 14, 28, respectively. Throughout the entire study, no significant uptake of Ag was observed in the carcass of the control fish. On day 0, an Ag concentration of 2.9 ± 0.2 μg kg^{-1} was measured. The background Ag concentration level decreased to 1.0 ± 0.2 μg kg^{-1} on day 7 and stayed on this level until the end of the test with 1.5 ± 0.6 μg kg^{-1} measured on day 28. In contrast, the Ag concentration in the digestive tract stayed relatively constant during exposure to AgNP-containing food pellets (2.9 ± 0.2 μg kg^{-1}, 2.2 ± 0.4 μg $kg^{-1}$and 3.0 ± 0.7 μg kg^{-1} on days 0, 7 and 14, respectively) and even kept that level until day 28 (2.2 ± 1.1 μg kg^{-1}).

4. Discussion

4.1. Aqueous exposure of O. mykiss

Significant amounts of Ag were found in the carcass but especially the gills of the rainbow trout after exposure to AgNPs that were added to copper-ion reduced tap water. However, when exposed to AgNPs in WWTP effluent (added prior or after the passage through the WWTP), Ag levels in carcass and gills remained at a low level below 10 μg kg^{-1}. This difference might be due to the transformations that AgNPs undergo in WWTP effluents. Many studies have shown that AgNPs adsorb to biosolids, are sulfidized to a large extent and transformed to Ag_2S (Fletcher et al., 2019; Georgantzopoulou et al., 2018; Kaegi et al., 2013, 2011; Kim et al., 2010; Levard et al., 2012; Ma et al., 2014). Hartmann et al. (2019) performed multi-generation studies with *Daphnia magna*, where the animals were exposed to the same WWTP effluents which were also used in the present study. Likewise, they confirmed that AgNPs in WWTP effluents were probably sulfidized according to EDX analyses. This transformation process takes <2 h (Kaegi et al., 2011), which might explain the similar bioavailability of Ag from AgNPs present in or supplemented to the WWTP effluent matrix. Fletcher et al. (2019) analyzed the release of Ag^+ from AgNPs at various levels of sulfidation and found that even if sulfide concentrations in the media are low, sulfidation limits the release of Ag^+ ions. However, it is not yet clear to what extent Ag^+ ions and the particles themselves, contribute to the toxicity and bioaccumulation potential of Ag from AgNPs in aquatic organisms (Behra et al., 2013). The results of our studies may highlight a primary role of Ag^+ uptake from AgNPs in fish. Moreover, they are in accordance with the results of Kühr et al. (2018) where a limited bioavailability of Ag from AgNPs for *Hyalella azteca* was observed when the AgNPs were spiked into WWTP or in the effluent of control WWTP and were thus presumably sulfidized.

In our study with rainbow trout, AgNPs were most likely adsorbed to the gill epithelia where Ag^+ was potentially released. Ionic silver is known to disturb the ionoregulation in the gills by competing with other cations for anionic binding sites and this is able to block the Na^+/K^+-ATPase activity and cause respiratory stress (Bianchini et al., 2002; Bilberg et al., 2010; Janes and Playle, 1995). Dissolved Ag species can be transported to other organs via the circulatory system, especially the liver, the central organ of detoxification. As a result, Wood et al. (1996) found the highest Ag tissue concentrations in the liver of adult rainbow trout after exposure to $AgNO_3$. Similar observations were made by Scown et al. (2010) and Joo et al. (2013) after exposure of rainbow trout to AgNPs. In our test design, the liver was not analyzed individually but as part of the carcass. While the Ag concentrations in the gills reached a maximum of around 100 μg kg^{-1} at the end of the exposure phase with AgNP supplemented dilution water, the Ag levels in the carcass developed at a lower level but were still rising after 14 days of exposure. The difference in tissue concentrations might be explained by metallothioneins (MTs), cytosolic proteins, that could be found in a wide range of organisms especially in gill tissue (Kaegi and Schaeffer, 1988; Kaji et al., 1993). By binding free metal ions, MT shows a detoxifying effect (Schlenk et al., 1999). The MT gene expression is upregulated by various metals like Cd, Au, Pt and Ag and the MT reaction has been shown to correlate well with increasing body burdens of the metals (Lansdown et al., 2001, 1999, 1997). Due to its high amount of cysteine (approx. 30%), MT shows a high binding affinity and capacity of 12 mol Ag per mol MT (Lansdown, 2002; Nielson et al., 1985). Chowdhury et al. (2005) showed that an exposure to Cd resulted in an 8.2-fold increased MT level in the gills and 400 and 15 times higher Cd level in gills and carcass if compared to control, respectively. Heerden et al. (2004) described, that the upregulation of MT expression in gills of *O. mykiss* could be used as a more sensitive biomarker for metal exposure than the mere accumulation of the metals in the tissue. Regarding this, the delayed or limited uptake and accumulation of Ag in the carcass, compared to the gills, may be the result of MTs in the gill tissue trapping Ag^+ released from AgNPs. The MTs in the gills may bind a high proportion of Ag^+ before it can reach other tissues like the carcass. When the loading capacity of the MTs for Ag is reached in the gill tissue additional Ag^+ may reach the carcass unimpeded. This may explain that during the 14d exposure to AgNPs no saturation of Ag tissue

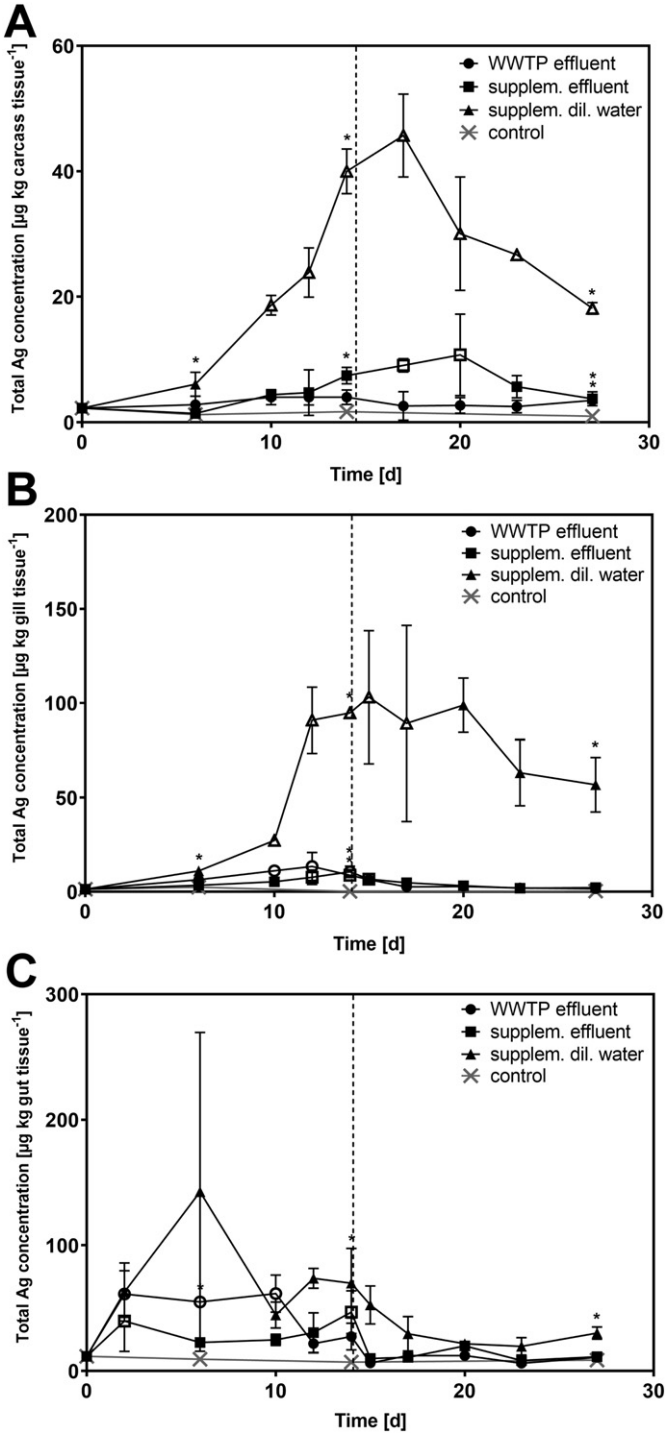

Fig. 3. Total Ag concentrations in carcass (A), gill tissue (B) and gut (C) of *Oncorhynchus mykiss* during 14d exposure to AgNPs present in or supplemented to WWTP effluent, and added to water, followed by 14d exposure to dilution water without test item. Ag concentrations are presented as μg/kg wet weight. Data points are mean ± standard deviation of four organisms. Asterisks and empty symbols show statistically significant differences ($p \leq 0.05$) relatively to the respective control group value and the respective start value on day 0, respectively, according to one-way ANOVA followed by Dunnett's post-hoc test. Dotted lines represent change of media (with and without test item, respectively) after 14d.

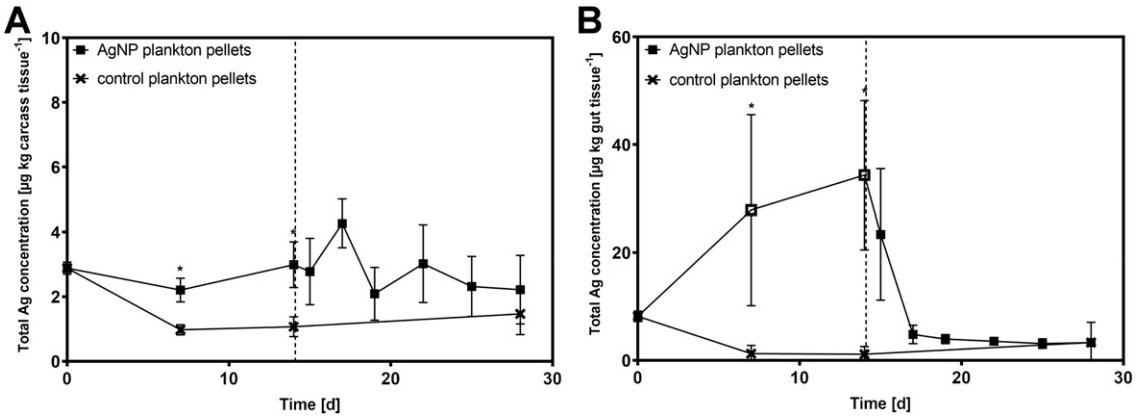

Fig. 4. Total Ag concentration in carcass (A) and digestive tract (B) of *Oncorhynchus mykiss* during 14d dietary exposure to plankton pellets with/without AgNPs followed by 14d exposure to control plankton pellets. Ag concentrations are presented as µg/kg wet weight. Data points are mean ± standard deviation of five organisms. Asterisks and empty symbols and show statistically significant differences ($p \leq 0.05$) relatively the respective control group value and the respective start value on day 0, respectively, according to *t*-test. Dotted lines represent change of food (with and without test item, respectively) after 14d.

concentrations in the carcass could be observed, due to the slow transfer and distribution of dissolved Ag species from the gills via the circulatory system. However, even after the end of the exposure phase, Ag concentrations in the rainbow trout carcass were still increasing, which might be attributed to the further release of Ag^+ ions from AgNPs, that were previously adsorbed to the gills or the intestinal walls.

MTs may also be the reason for the slow and incomplete elimination of Ag in the gills and carcass during the depuration phase. Norey et al. (1990) observed a retention of Cd after intraperitoneal injection to rainbow trout. They found that 98% of the retained metal was localized in the tissue of liver, kidney and gills and no significant elimination of the metal was observed during the following 98 days of depuration. They also showed that the Cd was bound to the MT that they isolated from the tissues. MTs are also discussed as an explanation for an incomplete elimination of Ag from the fresh water mussels *Corbicula fluminea* that were exposed to $AgNO_3$ for 144 h (Sebastian Kühr, pers. com), showing comparable kinetics of Ag tissue concentrations during the depuration phase as observed in this study.

In this study, a gill total Ag concentration of around 100 µg kg^{-1} was observed after 14d exposure to AgNPs supplemented to water at a level of 12.4 µg L^{-1}. In contrast, Scown et al. (2010) found an Ag tissue concentration in the gills of juvenile rainbow trout of approx. 300 µg kg^{-1} after 10d exposure to AgNPs at 10 µg L^{-1} (Scown et al., 2010). This might be explained by the fact, that Scown et al. (2010) did not feed the rainbow trout during exposure. AgNPs have a tendency to adsorb to natural organic matter like food or feces and might therefore be predominantly taken up by ingestion rather than being adsorped to the gills. This might also explain the ambiguous results we observed for total Ag concentrations in gut tissue. When exposed to AgNPs in tap water, the Ag concentration in the intestinal tract varied significantly during the exposure phase, which might be attributed to the ingestion of agglomerated AgNPs or particles adsorbed to the food pellets. Nevertheless, these variations in gut tissue concentrations had obviously no effect on the Ag concentrations in the carcass, which indicates a limited or no transfer of Ag species through the intestinal wall.

4.2. Dietary exposure of O. mykiss

A comparison of the dietary uptake and bioavailability of wastewater-borne and planktonic food-borne AgNPs in fish is still missing. Hence, the dietary uptake pathway was also assessed in this study. Zooplankton collected from a natural pond and exposed to AgNPs was chosen as experimental diet for the exposure studies with rainbow trout to simulate a natural aquatic food chain. Based on the total Ag analysis, it could be shown that the exposure of plankton to AgNPs led to considerable Ag concentrations in the zooplankton. However, it remained unclear, whether the AgNPs were ingested by the planktonic organisms or if they were attached to the carapace of the plankton organisms. Total silver concentrations in the experimental diet were comparable to concentrations as found in planktonic organisms exposed to silver from environmental sources (Eisler, 1997). The AgNP-loaded plankton was not ground to maintain the original structure of the plankton matrix within the food pellets. Freshwater zooplankton from fertilized earthen ponds contain around 73–79% and 10–14% of proteins and lipids, respectively (Mitra et al., 2007; Schlechtriem et al., 2003). Due to the agar matrix and the accompanying high water content of the food pellets, the lipid values were considerably lower with the final food pellets containing 3.0% lipids in the fresh material. Although the total lipid content of the experimental diet was quite low, the wet weight of the fish doubled (2.0 to 4.3 g) during the study, indicating that the quality of the plankton pellets which were supplemented with ground commercial fish food provided a sufficient amount of nutrients to maintain growth in the experimental fish. Interestingly, the commercial fish food, which was used for the maintenance of the fish prior to the study and also to enrich the plankton food matrix, showed a considerable total Ag concentration of 37.3 µg kg^{-1}. Since this fish food was also part of the control plankton pellets, they also showed a total Ag concentration of 14.7 µg kg^{-1}. Moreover, Ag species released from the commercial fish food before test start may have been bound to the mucus layer and thus may have contributed to the measured Ag concentrations of 11.6 and 8.18 µg kg^{-1} in the intestine tissue on the first day of the aqueous and the dietary exposure studies. This effect was already described by Khan et al. (2017) who analyzed the intestinal uptake of Ag in rainbow trout. It was shown, that most of the Ag was bound to the mucus layer of the intestine, while only 8 to 15% were transferred to the blood compartment (Khan et al., 2017). During the uptake phase intestinal Ag levels increased to 34.3 µg kg^{-1} due to the exposure to the AgNP-loaded plankton food followed by a significant decrease of Ag concentrations to around 3 µg kg^{-1} during the depuration phase. The control group was exposed to control plankton pellets throughout the test period leading to a decrease in intestinal Ag concentrations from 8.2 µg kg^{-1} to 1.2 µg kg^{-1} within the first 7 days. Taking into account the decrease of intestinal Ag concentrations after exposure to AgNP-loaded plankton food, the Ag species of both fish foods (commercial fish food applied before test start and AgNPs in the plankton pellets applied in the study) were eliminated from the digestive tract very quickly. The decrease of Ag concentrations in the carcass of the control group from 2.9 to 1.0 µg kg^{-1} within the first 7 days was related to the lower Ag concentrations in the control plankton pellets than the commercial fish food (14.7 µg kg^{-1} and 37.3 µg kg^{-1}, respectively). In contrast, the Ag concentration in the carcass remained constant when fish were exposed to the AgNP-containing plankton pellets

although they contain a considerably higher Ag concentration (121.5 μg kg^{-1}) than the commercial fish food, indicating a limited bioavailability of the AgNPs from the experimental diet.

Elemental analyses of the AgNPs in the lyophilized zooplankton by EDX showed that the particles kept their original size and shape but were mainly sulfidized. It can be only speculated at which stage of the feed preparation process, during exposure or during the freezing and lyophilization process, the sulfidation of the particles happened. Sulfidation might have been the main reason why only a limited Ag transfer from AgNPs-loaded plankton through the intestinal walls could be detected as it was already shown after exposure to AgNP-loaded WWTP effluents. Nevertheless, the fact that a limited bioavailability of Ag was observed for sulfidized AgNPs that were exposed via the food, underlines our assumption that the gills are the major uptake route of Ag from AgNPs and that the intestinal tract of *O. mykiss* only plays a negligible role.

4.3. Environmental implications

Our studies investigated the bioavailability of Ag from AgNPs in WWTP effluents or loaded live zooplankton. Our results indicate, that Ag from AgNPs is hardly bioavailable for fish under the given conditions. As described before, the uptake of Ag from the surrounding medium and its distribution in fish tissues is mainly mediated by the adsorption of the AgNPs to the gill lamellae, where Ag^+ ions are released that may enter the circulatory system of the organism. The results of our aqueous exposure study indicate, that this only occurs when fish are exposed to pristine AgNPs, that are likely to release Ag^+ ions. Presumably, due to the transformation of Ag from AgNPs to the insoluble Ag_2S within WWTP effluents (Georgantzopoulou et al., 2018; Hartmann et al., 2019) no significant uptake could be shown for AgNPs added to or present in WWTP effluent. Likewise, only a limited uptake of Ag into the carcass of the fish could be shown, when the fish were exposed to AgNP-loaded plankton. Within the freshwater plankton matrix, the Ag at the AgNP surface was also transformed to Ag_2S as it could be shown by EDX analyses, leading to a limited bioavailability in the digestive tract as previously shown for this Ag species when exposed via the surrounding medium. Our results indicate a low bioavailability of Ag from AgNPs from natural matrices, regardless if exposed via WWTP effluent or via loaded zooplankton, and thus explain why fish collected from a prealpine lake (Vogt et al., 2019) did not contain considerable AgNP levels although clear concentrations of Ag were detected in the sediment of the lake. However, a potential impact of AgNPs on the aquatic environment still cannot be entirely excluded. Despite the limited uptake of wastewater-borne AgNPs by the gills, the exposure can still have an impact on this organ on the molecular level by decreasing the epithelial integrity and increasing the oxidative stress (Georgantzopoulou et al., 2018). Bruneau et al. (2016) stated, that AgNPs from WWTP effluents have an impact on the immune system and cause inflammation, despite the fact that they are 2.3 times less bioavailable to rainbow trout than $AgNO_3$. Hence, even though our results indicate that the uptake and distribution of AgNPs from WWTP effluents and zooplankton within the aquatic food chain might not be a major concern, the chronic impact of these Ag species on the aquatic environment should not be underestimated. This is especially true, when taking into account, that other aquatic organisms like e.g. zooplankton might be even more receptive to AgNP toxicity, which could thus indirectly affect the fish population.

5. Conclusion

Our studies provide clear indications that the bioavailability of AgNPs (NM-300K) in fish is reduced following wastewater treatment with the gills still playing a crucial role regarding the uptake of Ag into fish tissue. However, no transfer of Ag through the intestinal walls into other tissues could be detected.

Further studies are required to elucidate the impact of further Ag species (Ag^+ ions, nanoparticles, Ag_2S) on Ag uptake via the gills and the digestive tract. As shown in this study, the distribution of tested Ag species within the tissues and organs of fish should be analyzed to be able to draw conclusions on how synthetic AgNPs may influence the aquatic food chain.

Declaration of competing interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.135695.

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Supporting Information

Bioavailability of silver from wastewater and planktonic food borne silver nanoparticles in the rainbow trout *Oncorhynchus mykiss*

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Number of Figures: 1

Number of Tables: 3

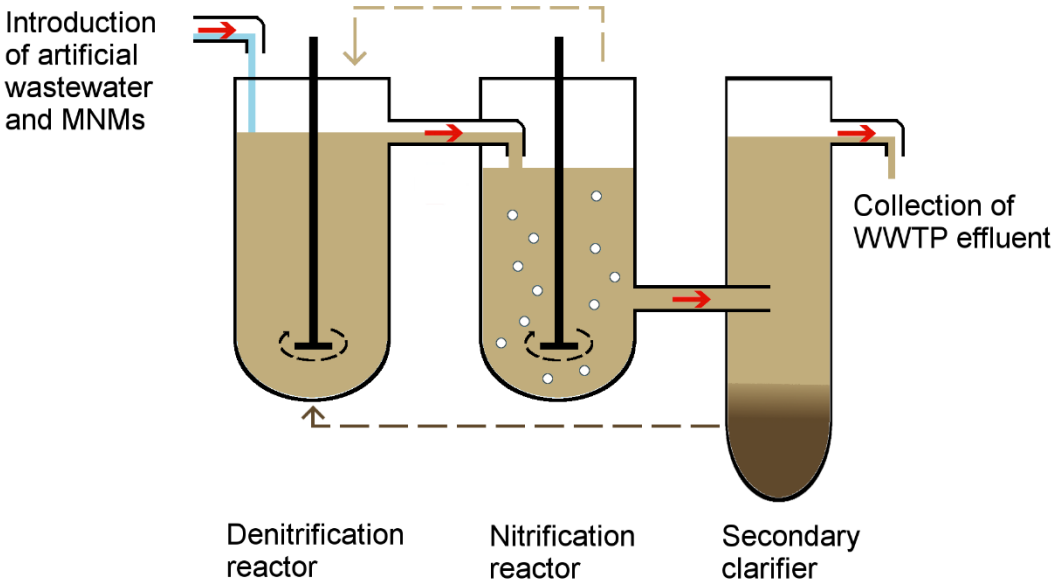

Figure S1 - Schematic illustration of the model WWTP. The three vessels simulate denitrification, nitrification and settling processes of the biological removal step. Red arrows = flow direction of MNMs through the WWTP; Light brown areas and arrows = liquid WWTP matrix; dark brown area and arrow = sedimented sewage sludge.

Dynamic light scattering (DLS)

For the characterization of the behavior of AgNPs in different media, DLS measurements were performed. The AgNP material was dispersed and measured in ultrapure water, copper ion-reduced tap water, pond water and WWTP influent to detect potential effects of the media on the DZ of the AgNPs. The media were filtrated using syringe filters with a 0.2 µm pore-size (Minisart® NML, Sartorius, Germany) before dispersing the particles. The dispersions were freshly made, hand shaken for 1 min and sonicated for 10 min (2 min effective, 200 W, pulsation pause ratio of 0.2/0.8) by indirect probe sonication (Cup Horn BB6, Bandelin electronic GmbH & Co. KG, Germany) before analysis. Concentrations were chosen to obtain count rates ≥150 kcps. 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 at 25 °C. The Z-Average and percentage of different peak intensities were calculated by the Zetasizer software.

Table S1 – DLS measurement results of AgNPs in ultra pure (UP) water, dilution water (Cu ion-reduced tap water), water from a representative pond and WWTP influent. PDI – poly dispersity index, n.d. – not determined.

Matrix	**pH**	**Alkalinity [mmol L^{-1}]**	**Ag conc. [µg L^{-1}]**	**Count rate [kcps]**	**Z-Average (d. nm)**	**PDI**	**Intercept**	**Attenuator**
UP water	5.4	n.d.	1000	471,1	58,9	0,189	0,833	6
			100	175,2	38,39	0,535	0,856	7
			10	118,3	596,2	0,678	1,07	9
			1	119,8	1017	0,942	1,16	11
Dilution water	8.3	2.1	1000	157,2	57,66	0,313	0,876	5
			100	194,2	101,1	0,166	0,882	7
			10	318,4	733,5	0,678	1,06	10
Pond water	7.4	1.2	1000	499,9	46,72	0,466	0,831	6
			100	188,5	86,62	0,145	0,833	7
			10	327,8	343,8	0,405	0,949	10
WWTP influent	7.5	6.9	1000	375,2	32,96	0,593	0,834	6
			100	177,5	71,91	0,23	0,88	7
			10	172,4	142,7	0,182	0,914	9

Table S2 – Physicochemical properties of dilution water (Cu ion-reduced tap water) and undiluted WWTP effluents with and without AgNP supplement. n.d. – not determined.

	Cu ion-reduced tap water	**WWTP effluent, control (AgNP)**	**WWTP effluent, with AgNPs**
NO_2^-	< 0.01 mg L^{-1}	7.92 mg L^{-1}	7.28 mg L^{-1}
NO_3^-	9 mg L^{-1}	12 mg L^{-1}	10 mg L^{-1}
NH_4^+	< 0.01 mg L^{-1}	0.20 mg L^{-1}	0.30 mg L^{-1}
TOC	0.9 mg L^{-1}	4.8 mg L^{-1}	4.8 mg L^{-1}
Hardness	1.3 mmol L^{-1}	n.d.	n.d.
Alkalinity	1.4 mmol L^{-1}	n.d.	n.d.
Salinity	258 µS cm^{-1}	n.d.	n.d.

Table S3 – Preliminary study results on the release of Ag from the agar pellets to the surrounding medium. Control and AgNP-containing agar pellets, 2.7 g each, were placed in 500 mL dilution water (static system) for a total of 7 days. Samples were taken in triplicates on days 3 and 7. Digestion background = 0.04 µg L^{-1}. LOQ = 0.005 µg L^{-1}.

Day	Sample	Total Ag in surrounding medium [µg L^{-1}]
3	Control plankton food pellets	0.04 ± 0.01
	AgNP plankton food pellets	0.03 ± 0.00
7	Control plankton food pellets	0.01 ± 0.00
	AgNP plankton food pellets	0.01 ± 0.01

Chapter 3: Testing the bioaccumulation of manufactured nanomaterials in the freshwater bivalve *Corbicula fluminea* using a new test method

Chapter 3 consists of the following publication:

Testing the bioaccumulation of manufactured nanomaterials in the freshwater bivalve *Corbicula fluminea* using a new test method

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The published supporting information directly follows the article.

Authors' contribution Chapter 3:

Sebastian Kuehr

Design and conducting of bivalve studies; development of the test system; total metal analysis; collection, statistical analysis & visualization of the data; writing – original draft; writing – review & editing

Boris Meisterjahn

Resources (spICP analysis); writing – original draft (method part – spICP-MS); writing – review & editing

Nicola Schröder

Conducting spICP-MS analysis; development of the sample preparation methods

Burkhard Knopf

Resources (total metal analysis)

Doris Völker

Writing – review & editing

Kathrin Schwirn

Writing – review & editing

Christian Schlechtriem

Funding acquisition; resources; design of study, development of the test system; writing – review & editing; study supervision

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Testing the bioaccumulation of manufactured nanomaterials in the freshwater bivalve *Corbicula fluminea* using a new test method†

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Increasing amounts of manufactured nanomaterials (MNMs) are produced for their industrial use and released to the environment by the usage or disposal of the products. As depending on their annual production rate, substances are subjected to PBT assessment, the availability of reliable methods to evaluate these endpoints for (corresponding) nanoforms/MNMs becomes relevant. The classical method to elucidate the bioaccumulation potential of chemicals has been the flow-through study with fish, which has limitations as regards meeting the requirements of MNMs. Most MNMs tend to sediment in the aquatic environment. Thus, maintenance of stable exposure conditions for bioaccumulation testing with fish is nearly impossible to achieve when using MNMs. *Corbicula fluminea*, a freshwater filter-feeding bivalve distributed worldwide, has been previously shown to ingest and accumulate MNMs present in the water phase. To investigate the suitability of *C. fluminea* for bioaccumulation testing we developed a new flow-through system to expose mussels under constant exposure conditions. Two nanoparticles (NPs), the AgNP NM 300K and the TiO_2NP NM 105, were applied. In addition, *C. fluminea* was exposed to $AgNO_3$ as a source of dissolved Ag^+ to compare the bioaccumulation of Ag in dissolved and nanoparticulate forms. For each MNM exposure scenario we were able to determine steady-state bioaccumulation factors. BAF_{ss} values of 31 and 128 for two NM 300K concentrations (0.624 and 6.177 μg Ag per L) and 6150 and 9022 for TiO_2 (0.099 and 0.589 μg TiO_2 per L) showed the exposure dependence of the BAF_{ss} estimates. The progression of metal uptake and elimination in the soft tissue provided clear indications that the uptake and thus accumulation is mainly driven by the uptake of NPs and less of dissolved ions.

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Environmental significance

Filter-feeding organisms such as bivalves represent a major target for the bioaccumulation of nanomaterials in the aquatic environment. Therefore, bivalves should be considered as test organisms for the bioaccumulation assessment of nanomaterials. A new flow-through system to expose the freshwater bivalves under constant exposure conditions was developed. Bioaccumulation studies with the freshwater bivalve *C. fluminea* on two nanoparticles, the AgNP NM 300K and the TiO_2NP NM 105, demonstrated the suitability of the new test system. The results obtained with this test system can be used to generate useful endpoints required for regulatory purposes and could be included in a tiered bioaccumulation testing strategy for manufactured nanomaterials.

Introduction

The European Commission estimated the global amount of produced manufactured nanomaterials (MNMs) to be 11.5 million tons per year which corresponds to a market value of €20bn.[1] Further industrialization and economic expansion of industrial countries and economies will increase the production and usage of MNMs. MNMs are released to the environment during their production, their use and the disposal of MNM-containing products.[2–4] The increasing production of MNMs leads inevitably to a larger

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environmental burden and a reliable environmental risk assessment of MNMs is thus required.[5]

Due to the high annual production of corresponding bulk substances, several MNMs are subject to assessment of bioaccumulation such as required by the European Chemicals Registration REACH,[6] the Japanese Chemical Substance Control Act "Kashinho" or others, *e.g.* HPV, TCFCA or KKDIK.[7–10]

Under REACH, the bioconcentration factor (BCF) represents the most important endpoint for bioaccumulation assessment.[11] The BCF is mostly determined by fish flow-through studies in accordance with OECD test guideline 305 which was developed for water-soluble test items.[12,13] However, dispersed MNMs in aquatic systems represent meta-stable systems that are only kinetically stabilized and tend to agglomerate and sediment, leading to problems with respect to the maintenance of stable and continuous exposure conditions during classic flow-through studies with MNMs. Thus, the establishment of suitable experimental conditions is difficult and consequently there is a need to develop suitable test methods adapted to the specific need of testing MNMs. The REACH regulation was developed without considering the specific properties of MNMs, and thus the development of more suitable test methods was suggested.[14,15]

In 2018, Handy *et al.*[16] presented a tiered testing approach to meet the requirements of MNM bioaccumulation testing that also includes bioaccumulation tests with invertebrates which provide indications that may allow a waiver of further studies using fish as the test organism.

This is in accordance with the *Guidance on Information Requirements and Chemical Safety Assessment* of REACH where it is mentioned that taxonomic groups other than fish are allowed to gain data for the assessment of the B criteria if representing a relevant target organism.[17] Within this guidance the mussel bioconcentration test of the ASTM is given as an example.[11,18]

Marine and freshwater bivalves are widely used and established as bioindicators for pollution in aquatic systems, *e.g.* due to their ability to accumulate high concentrations of heavy metals in their tissue.[19–23] Moreover, bivalves are used for the determination of the bioavailability and effects of xenobiotics and genotoxic compounds.[22,24,25] Filter-feeding bivalves may ingest considerable amounts of particulate materials that are concentrated in their feces or pseudo-feces.[26,27] As described by Hull *et al.*,[28] these particulate materials could be MNMs that are dispersed in the water. The filter-feeding behavior of bivalves for respiratory and nutritional purposes and their benthic mode of life make bivalves a group of organisms predominantly exposed to MNMs.[29–34] Mesocosm studies of Ferry *et al.*[35] showed that bivalves represent an important sink for MNMs, and similar results were presented by Cleveland *et al.*[36] Because the feces and pseudo-feces of bivalves represent an important part of the diet of benthic invertebrates, they may play a key role regarding the transfer of MNMs into the aquatic food chain.[37,38] Bivalves may also represent a link between the aquatic and the terrestrial environment being part of the diet of water birds.[39,40]

The freshwater bivalve *Corbicula fluminea* is an invasive species that is, due to its ability to tolerate a wide range of environmental conditions, widely spread in Africa, North and South America, Europe and the Pacific Islands.[41–44] The high filtration rate of this species makes *C. fluminea* ideal for use in bioaccumulation test systems.[45] Due to the euryoecious characteristics of this species, *C. fluminea* can be used in test systems that are highly adjustable to meet the requirements of the wide spectrum of different MNMs.

The aim of this study was to develop a flow-through system that allows a continuous and constant exposure of MNMs to determine the bioavailability and bioaccumulation of MNMs in bivalves. Based on the above facts, *C. fluminea* was used as the test species. Silver nanoparticles (NM 300K) and the titanium dioxide NP NM 105 were selected as test items representing typical MNMs from the European Commission's Joint Research Centre repository.[46,47] AgNPs are mainly employed due to their antibacterial properties and belong to the most investigated MNMs.[36,48–52] The antibacterial effects are based on released silver ions (Ag^+) that can cause disruption of the respiratory chain of the cells, the deactivation of proteins and the disturbance of membrane transport processes.[53–55] To compare the fate and potential bioaccumulation of AgNPs and Ag^+, we also tested $AgNO_3$ as a source of dissolved Ag^+.

In contrast to AgNPs, TiO_2NPs are nearly chemically inert, representing one of the most commonly used MNMs[56] belonging to the group of non-ion-releasing MNMs under environmental conditions and showing condition-dependent dispersion stability.[57] TiO_2NPs are of great ecotoxicological interest due to their potential to alter the bioavailability and thus the toxicity of coexisting contaminants like heavy metals or organic compounds in aquatic organisms like fish and bivalves.[58–63]

For the evaluation of the bioaccumulation potential of the MNMs applied in this study, total metal concentrations were analysed in the whole animals and the distribution of accumulated material in the soft tissue was determined. In addition, particle concentrations and particle sizes were measured in the different tissue compartments of the animals to allow the differentiation between bioaccumulation of dissolved and particulate material. Similarly, dispersions applied in this study were characterized for their concentrations and particle size distribution. A promising technique for analyzing particle sizes and numbers at low concentrations is single-particle ICP-MS (spICP-MS).[64,65] The underlying concept is based on the measurement of diluted suspensions, enabling the introduction of individual particles into the ICP-MS plasma. For the analysis of bioaccumulated MNMs by spICP-MS, particles have to be extracted from the biological matrix under mild conditions to avoid dissolution of the particles (*e.g.* AgNPs) and changes in their state, *e.g.* in terms of

their size distribution. Therefore, mild extraction procedures such as enzymatic digestion are required.[66] The development of a workflow for the analysis of MNMs bioaccumulated in mussel tissue using spICP-MS was part of this study and is presented.

Methods

Corbicula fluminea

Freshwater bivalves *C. fluminea* used in this study were collected from the river Niers near Wachtendonk (47669, Germany) and kept in 1.5 m^3 glass microcosms. Up to 2000 animals were placed in 3 stainless steel baskets within a microcosm with regular water change (copper-reduced tap water) every three weeks. Water was aerated with two airstones per microcosm, and slight water circulation was provided by a circulation pump; the water temperature was around 12 ± 4 °C. Animals were fed with 0.5 L of a suspension containing 200 mg of fine-milled stinging nettle leaves[67] (BRENNNESSEL TEE N, Aurica®) per liter after each water change. Microcosms were checked for dead animals at least 3 times a week and carcasses were removed from the microcosms. An acclimatization phase of at least 2 weeks after harvesting of the bivalves from the river was required before the usage of the animals in the studies. For the studies, only individuals with a length (anterior–posterior) of 2.5 (±0.5) cm were used. The length of the animals was measured as described below.

Test system

A flow-through system was developed to allow continuous exposure of MNM containing test media at constant concentrations (Fig. 1). The central part of the new test system is a Zuger glass jar test vessel with a volume of 8 L. Within this vessel a V4A stainless steel rack allows placement of up to 170 bivalves (shell length in the range of 2–2.5 cm) on perforated shelves. Adjustable aeration as well as a stirrer (RZR 1, Heidolph) with adjustable spin rates from 35 to 2200 rpm can be used to ensure sufficient aeration and constant mixing of the test media. Stock solution (MNM suspension) and food suspension are added to a mixing vessel using peristaltic pumps (IPC High Precision Multichannel Dispenser, ISMATEC®) and further diluted with copper-reduced tap water supplied by a membrane pump (gamma/X, ProMinent®) to produce the test medium which is supplied into the Zuger glass jar using TYGON® tubes (E-3603, TYGON®). The whole system allows a flow rate from <0.5 L h^{-1} to >20 L h^{-1}. The test medium leaves the test system at the bottom of the Zuger glass jar by an overflow pipe.

Feed evaluation study

A feeding study lasting 192 h was carried out to identify a suitable experimental diet for bioaccumulation studies with the freshwater bivalve *C. fluminea*. Therefore, 5 potential diets were tested in the newly developed test system: *Spirulina* species (SP), ground stinging nettle (SN), ground plant-based fish food tablets (FFT) (www.ms-tierbedarf.de), a combination of ground

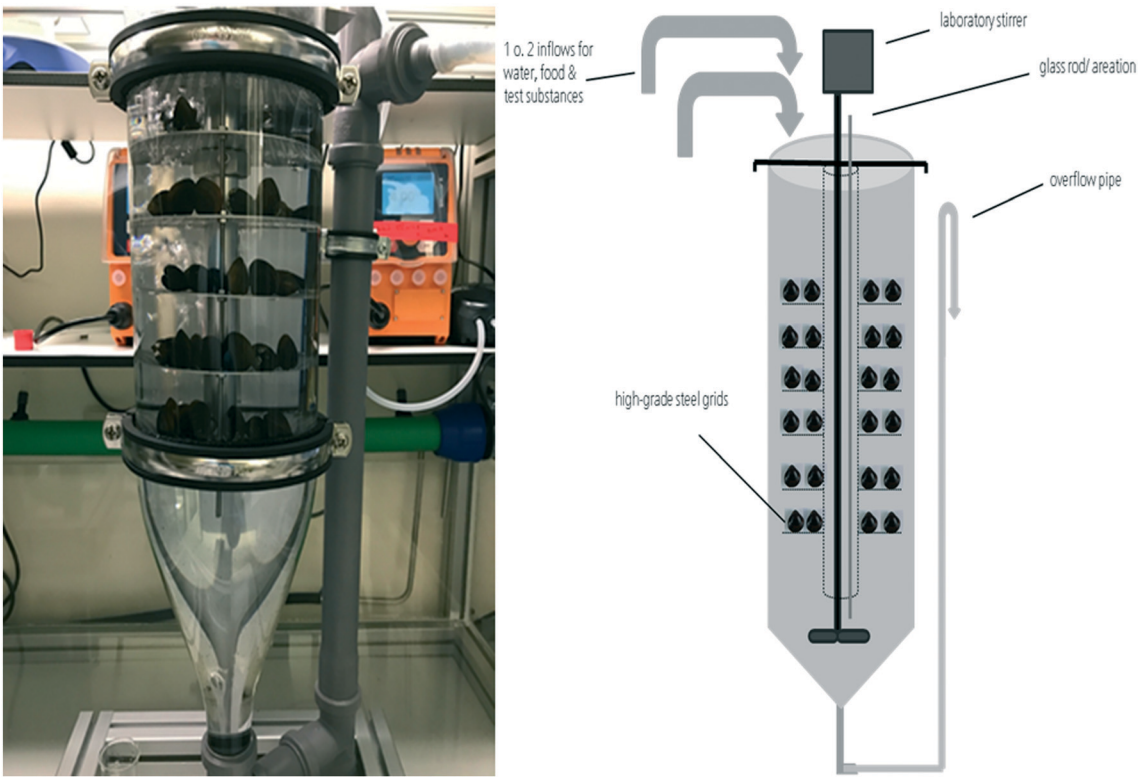

Fig. 1 Bivalvia flow-through system containing *C. fluminea*.

stinging nettle and fish food tablets (SN + FFT), and a no food treatment (/). The test system ran for 24 h before the test started to allow equilibration of experimental conditions.

For the feeding study, 5 flow-through systems (one unit per treatment) were stocked with 50 animals each, which were pre-conditioned in microcosms for several weeks. Prior to the test the animals were brushed and transferred into a fresh microcosm without any food source in order to promote defecation. After 24 h, the cleaning procedure was repeated and animals were transferred into a second microcosm for 24 h prior to being transferred into the test systems. Before this, the length of the animals was measured using a ruler and their valves were dried using paper towels to allow the accurate measurement of the animals' weight (AUW220D, SHIMADZU). During the dietary test, the test systems were supplied with test medium (copper-reduced tap water) at a flow rate of 4 L h^{-1} and each liter of test medium contained 16 mL of food suspension (equivalent to 400 mg dry mass per L). The temperature, dissolved oxygen, and the pH value in the test system were measured daily. Measurements of ammonia, nitrite and nitrate were carried out by photometric measurements (NANOCOLOR® 500D, Machery-Nagel) at the start and end of the test. During the dietary test the valve opening filtration activity was monitored by visual judgement. At the end of the dietary test, dead animals were counted. The valves of the live animals were cleaned and dried, and the length and weight of each animal was measured (AUW220D, SHIMADZU). The mortality, average weight and length of the animals were calculated for each treatment.

Bioaccumulation studies

Preparation of stock suspensions. Bioaccumulation studies were carried out with NM 300K as a test material representing well-dispersed and ion-releasing AgNPs. $AgNO_3$ was used as a non-nano structural and soluble species of Ag. NM 105 (TiO_2) was used as a test material representing a non-ion-releasing MNM with a tendency to agglomerate.[47] Both NMs 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 and were provided by the Fraunhofer Institute for Molecular Biology and Applied Ecology IME. Information on the characterisation and physico-chemical properties of the NPs is summarized in the JRC Reports for NM 300K and the titanium dioxide series.[46,47] Electron microscopy was performed on the feedstock materials (Fig. S10 and S11†); the acquired grain size distributions are in accordance with the data from the JRC Reports. The stock suspension of NM 300K contains 10.16% (w/w) AgNPs having an average particle size of 15 nm.[46] The NM 300K suspension was stabilized with a dispersing agent (NM-300 DIS) containing 4% (w/v) polyoxyethylene, glycerol, trioleate, and polyoxyethylene (20) sorbitan monolaurate (Tween 20) each.[68] For the production of the AgNP working suspension, 300 mg of NM 300K were diluted with 30 ml ultra high quality water (UHQ-water), hand-shaken for 1 min and sonicated for 10 min with a pulsation pause ratio of 0.2/0.8 using an ultrasonic homogenizer (Bandelin Sonopuls HD2200 ultrasonic homogenizer, 200 W, Bandelin Cup Horn BB6) to disperse the NM 300K and to carefully homogenize the suspension for the dilution with UHQ to achieve the final concentration of 834 μg Ag per L. During the sonication process, the suspension (contained in a 50 ml centrifugal tube, PP) was cooled with ice water in the cup horn. $AgNO_3$ was purchased from Carl Roth with a purity of >99.9% and diluted with UHQ-water for the preparation of the stock solution. NM 105 was present in the form of a powder and was also suspended in UHQ-water as described above for NM 300K.

Performance of bioaccumulation studies. In three bioaccumulation tests NM 300K, $AgNO_3$ and NM 105 were tested in two concentrations each with 170 mussels per test. As described for the dietary test, the shell of all bivalves was cleaned before the animals were transferred into the test vessels. Again, only individuals with a length (anterior–posterior) of 2.5 (±0.5) cm were used. During none of the studies, the stirrer which is part of the test system was applied. The NM 300K working suspension (834 μg Ag per L) was produced by dilution of the NM 300K stock suspension after sonication with UHQ-water. The $AgNO_3$ working solution (834 μg Ag per L) was produced by dissolving a defined amount of $AgNO_3$ in UHQ-water. The NM 105 stock suspension was diluted after sonication using UHQ-water. The diluted suspension (1 mg TiO_2 per L) was allowed to settle for 72 h in 2 L glass beakers at room temperature. After decanting, the suspension with the remaining stable dispersed fraction was used as the working suspension. Test media were composed of copper-reduced tap water, a suspension of ground stinging nettle tea (400 mg dry mass per L) and the respective MNM suspension or $AgNO_3$ solution and were applied at a flow rate of 4 L h^{-1}. In the case of NM 300K and $AgNO_3$ the working suspension/solution was pumped into the mixing vessel before being introduced into the test vessel. The NM 105 working suspension was introduced directly into the test vessel to avoid sedimentation processes in the mixing vessel. The working suspension was discharged into the test system at three different surface positions of the water body to ensure an even distribution of the TiO_2NPs in the test water which was constantly supplied into the test vessel at a flow rate of 4 L h^{-1}.

The test system was allowed to equilibrate for at least 48 h before the test start until constant flow through conditions and stable media concentrations (variation of concentration ≤20%) were reached for at least three sampling times, separated by at least 3 h. Measurements of ammonia, nitrite and nitrate were carried out at the start and end of the uptake and elimination phase to check the culture conditions in the test system during the study. Animals were exposed for 144 h ($AgNO_3$), 96 h (NM 300K), and 120 h (NM 105). Following the uptake phase, previously exposed animals were

placed in a new test vessel and maintained under the same experimental conditions but without the test item for 144 h ($AgNO_3$), 48 h (NM 300K), and 144 h (NM 105). Triplicate samples of three animals were taken from the test system at different sampling dates during the uptake and elimination phase as shown in Fig. 2. The shells of collected animals were cleaned. Disposable scalpels (Cutfix Fig. 10, B. Braun) were used to open the shells by gently pressing the blade between the shells at the position of the siphons. Before levering the shells, the blade was slid to the posterior and anterior end to sever the adductor muscles. The opened bivalves were cleaned by dipping and moving the animal in two separate wash boxes filled with UHQ-water. The soft tissue was then removed from the shells using the disposable scalpels, blotted with lint free laboratory paper and weighed. All tissue samples were immediately frozen using liquid nitrogen and stored at −20 °C until further processing for determination of total Ag or Ti concentrations. Prior to the collection of test animals, duplicate media samples were taken for the measurement of media concentrations. In addition, media samples were taken from different levels of the test system to check whether homogeneous concentrations of test item were applied. In the case of the Ag treatments, the samples (20 mL) were acidified by adding 200 μL of nitric acid (69%, suprapure grade, Roth) and stored at 4 °C. Media samples for single particle measurements were not acidified and measured directly. The appropriate duration of the uptake phase to ensure steady-state conditions was estimated for each test item in a pretest. Depuration phases following the uptake phase were continued for as long as enough bivalves were available. Due to the increased loss of animals exposed to the high concentrations of $AgNO_3$ and NM 300K, only a few samples could be obtained during the depuration periods.

Determination of total metal concentrations

Total silver and titanium concentrations in the aqueous test media were determined by inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700 ICP-Q-MS, Agilent Technologies, Waldbronn, Germany). The instrument calibration and method verification was carried out as described by Kühr *et al.*[69] using certified element and multi-element standards (Merck) and reference material (TM 25.4; Environment Canada). A rhodium standard solution (Merck KGaA; CertiPUR) was applied as an internal standard for compensation of instrumental fluctuations. For each standard and sample, at least three measurements were recorded and the mean concentration was determined using the ICP-MS software. The test media samples (20 mL) which were acidified after sampling were measured directly by ICP-MS. The tissue samples were digested using a microwave (MLS Ultra Clave) for the determination of total metal concentrations. First, 5 mL nitric acid (69%, suprapure grade) were added to the tissue samples followed by vortex stirring (VORTEX GENIE 2, Si™ Scientific Industries). Samples were transferred to the microwave and digested (max. temperature: 220 °C, max. pressure: 95 bar, energy: 1 kW h). The digested samples were diluted up to 15 mL with nitric acid (10%) and measured by ICP-MS.

Calculation of bioaccumulation factors

The data analysis software OriginPro 2017 (OriginLab Corporation) was used to subject all measured concentration data to an analysis of variance (ANOVA). Time-weighted average concentrations (TWA) of Ag and TiO_2 in test media of the different studies were calculated for the uptake phases.[70] Total Ag and TiO_2 concentrations in the test animals' soft tissue, sampled under steady-state conditions were divided by the TWA concentrations of the media to gain bioaccumulation

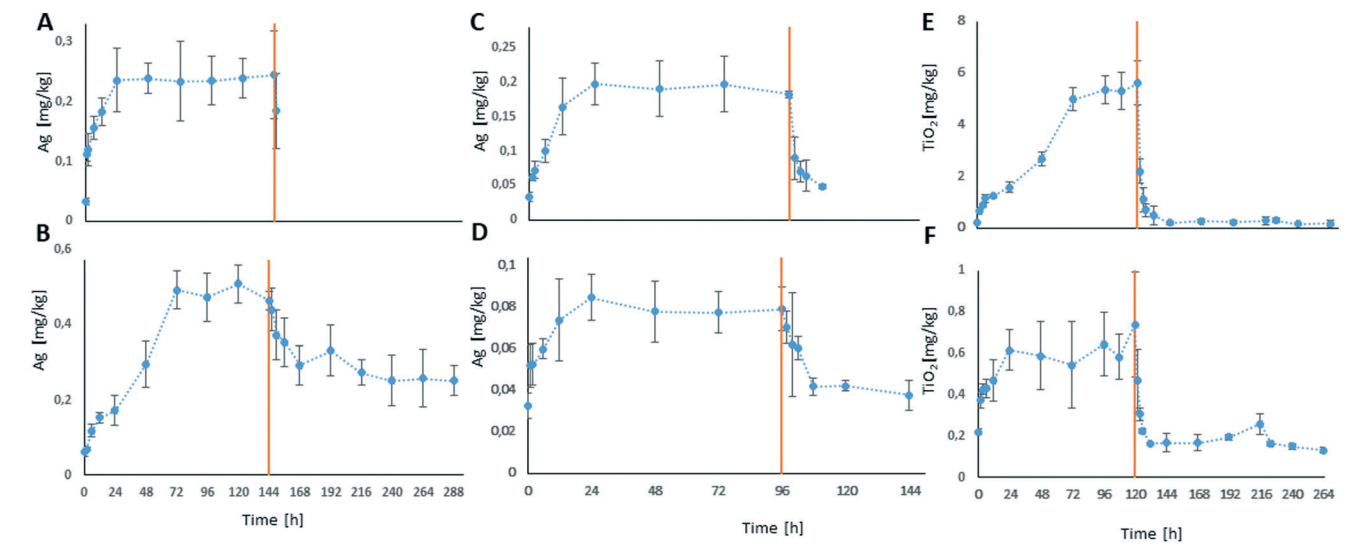

Fig. 2 Total Ag and TiO_2 concentrations in the mussel soft tissue during the bioaccumulation studies. Red vertical line shows the end of the uptake phase. (A) $AgNO_3$ high exposure concentration, (B) $AgNO_3$ low exposure concentration *n*, (C) NM 300K high exposure concentration, (D) NM 300K low exposure concentration, (E) NM 105 high exposure concentration, and (F) NM 105 low exposure concentration.

factors (BAF_{ss}) for the MNM treatments and steady-state bioconcentration factors (BCF_{ss}) for the $AgNO_3$ treatment.

Investigations on tissue distribution

Performance of bioaccumulation studies. Additional bioaccumulation studies were carried out with *C. fluminea* to further elucidate the uptake of the MNMs and $AgNO_3$ in the soft tissue. The distribution of the total metal and MNM content in the different compartments of the animals was investigated to evaluate whether the determined elemental concentrations in the soft tissue derived from nanoparticles or dissolved metal. The tests were carried out with $AgNO_3$, NM 300K and NM 105 under the same conditions as the main studies. The highest test concentrations tested in the bioaccumulation studies were applied to make the results comparable. The duration of the uptake phase was the same as in the main study to ensure that steady-state conditions were reached. The uptake phase was followed by a shortened elimination period. Animals for the analysis of compartment-specific differences were sampled at the end of the uptake period. To investigate the total metal concentrations as well as particle distribution in the different compartments, clean disposable scalpels (Cutfix Fig. 10, B. Braun) were used to separate the adductor muscle tissue (AM), the foot (F), the mantle including the siphons (M) and the remaining visceral mass (VM) (Fig. S8†). Five replicates consisting of five animals each were sampled to gain enough sample mass. In addition, triplicate samples, each consisting of 3 animals, were sampled for spICP-MS analysis during the uptake and elimination phase. Collected tissue samples were frozen immediately using liquid nitrogen and stored at −20 °C.

Calculation of distribution factors

The total metal concentrations were determined for the single compartments as described above and their contribution to the whole body burden was calculated.

Distribution factors (DFs) were calculated for each test item to clarify the differences between the single compartments of the animals using the following formula:

$$\mathrm{DF} = \frac{\text{mean concentration}_{\text{compartment}}}{\text{mean concentration}_{\text{whole soft tissue, calculated}}}$$

The mean concentration in the whole soft tissue was calculated by calculating the mean concentration of each single replicate based on the measured metal contents of the compartments of the respective replicate. All data were subjected to an outlier test (SQS 2013 Version 1.00 by J. Klein and G. Wachter). Replicates identified as outliers were excluded from further data analysis.

Determination of particle number concentrations and size distribution in test media and soft tissue

In addition to the determination of total metal concentrations in test media and in the mussel tissues, particle size distributions and particle number concentrations in the test media as well as in whole soft body and specific tissues were determined by spICP-MS. Samples of the test media were dispersed by ultrasonic treatment prior to appropriate dilution followed by analysis with spICP-MS. For the analysis of particles in mussel tissue a low perturbing sample preparation method was required that does not dissolve the particles and affects the particle properties as little as possible. Thus, tissue samples were digested using the enzyme proteinase K (Sigma Aldrich) according to the method described by Loeschner *et al.*[66] and Schmidt *et al.*[71] The defrosted tissues were incubated with 10 mL of the digestion solution per 400 mg (fresh weight) for 3 h at 50 °C and shaken at 100 rpm using an orbital shaker. The digestion solution was prepared by dissolving 45 mg proteinase K in 1 L buffer solution (0.5% SDS + 50 mM NH_4HCO_3, pH adjusted to 8.0–8.2). After complete dissolution of the tissue, which was confirmed by visual inspection, the solution was filtered using 0.45 μm syringe filters (Minisart® NML, 0.45 μm) and then measured using an ICP-QQQ-MS instrument (Agilent 8900, Agilent Technologies, Waldbronn, Germany). The possible influence of the digestion procedure on the particle size distributions was evaluated qualitatively. For this purpose, mussel tissue was spiked with an aliquot of the respective particle stock dispersion prior to digestion and the measured distribution was compared with that of the stock dispersion. Additionally, hydrolysates of mussel tissue not spiked/not exposed to the respective particles prior to digestion were spiked with an aliquot in order to assess the impact of the obtained solutions on the size distributions.

The dwell time in the single particle measurement mode of the ICP-MS was set to 100 μs and time resolved signals were recorded on the selected isotope for 60 s. Peak detection and integration was conducted automatically by the Agilent MassHunter software and converted into particle sizes. Dispersions of 60 nm gold nanoparticles (AuNPs 60 nm, BBI solutions, UK) were used for the determination of the nebulization efficiency and prepared freshly on the day of measurement. The samples were diluted in ultrapure water by a factor of 10^2–10^5 for measurement to reach a particle concentration of 200–2000 particle events per minute. According to Sannac, Tadjiki and Moldenhauer[72] and Mitrano *et al.*[73] this correlates to an element concentration in the range of ng L^{-1}. Ag was measured as the isotope ^{107}Ag. Titanium was measured as ^{48}Ti in the NH_3-reaction mode to minimize interferences with calcium. The threshold between background and particle signals was defined based on visual inspection of the measured signal distributions. The resulting histograms are shown in the form of histograms normalized to the most frequent particle size.

Results

Feed evaluation study

Animals used in the dietary test had an average weight of 68 (±0.79) g and average lengths of 26.84 (±1.23) mm (anterior–posterior) and 17.07 (±0.77) mm (ventral–dorsal).

Temperature in the single treatment units ranged from 17.7 to 19.0 °C over all test vessels, and pH values ranged from 7.5 to 7.9. Oxygen saturation ranged from 93% in the SP and FFT treatments to 97% in the "no food" treatment, which also showed the lowest water chemical parameters (0.2 mg NH_4^+ per L, 0.11 mg NO_2^- per L, 7 mg NO_3^- per L), whereas the SP treatment showed the highest water load (0.9 mg NH_4^+ per L, 0.37 mg NO_2^- per L, 8 mg NO_3^- per L). Mortality rates after 192 h ranged from 4% in the SN treatment to 10% in the SP treatment. In all treatments, filtration activity of the test animals was observed, whereas the animals in the no food treatment showed very low filtration activity. The strongest pollution of the test vessels caused by (pseudo) feces or biofilms appeared in the SP treatment, whereas nearly no pollution was detectable in the no food treatment. Ground stinging nettle was identified as a suitable diet. Compared to the other tested diets, the suspension of ground stinging nettle was leading to less pollution and a low buildup of biofilm and accumulation of organic matter.

Bioaccumulation studies

$AgNO_3$. Time-weighted average concentrations (TWAs) were calculated for media samples collected at different sampling points. A TWA of 0.684 μg Ag per L was calculated for the treatment with the lower Ag concentration. The TWA of the assay with the higher $AgNO_3$ concentration was calculated to be 7.791 μg Ag per L. Homogenous concentrations were present in the test vessels during the uptake phase (Fig. S1 and S2†).

Animals exposed to the higher Ag concentration showed a soft tissue concentration of 0.24 mg Ag per kg after 24 h of the uptake phase (initial 0.03 mg Ag per kg). The concentration remained stable until the end of the uptake phase (144 h) (Fig. 2A), resulting in a calculated BCF_{ss} of 30.5. The soft tissue concentration following exposure to the lower concentration increased from initially 0.03 mg Ag per kg to around 0.5 mg Ag per kg after 72 h of exposure and stayed at this level until the end of the uptake phase after 144 h (Fig. 2B). The body burden decreased to a soft tissue concentration of around 0.25 mg Ag per kg within 72 h of depuration (216 h after study start) and stayed at this level until the end of the depuration phase (144 h/288 h after study start). A BCF_{ss} of 710.7 was calculated for the lower concentration. In both treatments, a strong reduction of the bivalve's filtration activity was observed during the uptake phase.

NM 300K. The total Ag concentrations without differentiation of the particulate or dissolved Ag in the exposure media and soft tissue samples were measured as equivalent to NM 300K. The TWA of the total Ag concentrations in the test media of both treatments were 0.624 and 6.177 μg Ag per L. In both systems homogenous concentrations of Ag were measured in the course of the study (Fig. S3 and S4†).

All bivalves that were exposed to NM 300K during the 96 h uptake phase showed detectable Ag concentrations in their soft tissue. In both treatments a clear increase of the Ag concentration was observed over the duration of the uptake phase, as well as a clear decrease of the body burden during the depuration phases (Fig. 2C and D).

In animals exposed to the lower test concentration treatment the total Ag concentration in the soft tissue increased from initially 0.03 mg Ag per kg (0 h) to 0.8 mg Ag per kg at 24 h and stayed at this level until the end of the uptake phase (96 h, Fig. 2D). During the depuration phase of the lower concentration NM 300K treatment the total Ag concentration in the soft tissue decreased to 0.04 mg Ag per kg within 12 h of depuration and was stable until the end of the depuration phase (Fig. 2D). The calculated BAF_{ss} was 128.

During exposure to the higher test concentration the total Ag concentration in the soft tissue increased from initially 0.03 mg Ag per kg (0 h) to around 0.20 mg Ag per kg at 24 h and was stable until end of the uptake phase (96 h, Fig. 2C). Within 12 h of depuration the total Ag concentration decreased to 0.05 mg Ag per kg. The calculated BAF_{ss} was 31.

The filtration activities of the bivalves were reduced in both NM 300K treatments during the uptake phase, whereby the filtration activity at the lower test concentration assay was slightly higher than at the higher test concentration.

NM 105. No differences in the bivalves' filtration activity was observed during the whole bioaccumulation study with NM 105. The presented TiO_2 concentrations were calculated from the measured Ti concentrations. The TWA of the total TiO_2 concentrations in the exposure media of the two treatments containing NM 105 were 0.099 and 0.589 μg TiO_2 per L. During the uptake phase, homogeneous concentrations were given in the test systems (Fig. S5 and S6†). The initial total TiO_2 concentration in the bivalves' soft tissue increased during the uptake phases from 0.22 mg TiO_2 per kg to a plateau level of around 0.6 mg TiO_2 per kg after 24 h of the uptake phase and to around 5.4 mg TiO_2 per kg after 96 h of the uptake at the lower and higher concentration treatments, respectively (Fig. 2E and F). Plateau concentrations remained stable until the end of the uptake phase (120 h), resulting in BAF_{SS} values of 6150 (lower concentration) and 9022 (higher concentration). During the following depuration phase the TiO_2 body burden of the bivalves decreased rapidly. In the test with the lower concentration, a stable total TiO_2 concentration of 0.22 mg TiO_2 per kg was reached after 16 h of the depuration phase. A stable TiO_2 concentration of 0.16 to 0.17 mg TiO_2 per kg was reached after 24 h in the depuration phase of the test with the higher concentration.

During all bioaccumulation tests no notable mortality was observed.

Investigations on tissue distribution

Distribution of total metal concentrations in the soft tissues. Additional bioaccumulation studies with $AgNO_3$, NM 300K and NM 105 were performed. At the end of the uptake phase, mussels were collected to analyse the distribution of total metal concentrations in the soft tissue. Following exposure to NM 300K and $AgNO_3$, the mantle showed the

highest distribution factor (DF) with 1.20 and 1.36, respectively, followed by the viscera (1.09 and 1.04). The muscle tissue showed the lowest distribution factor for total Ag content 0.33 and 0.15 for NM 300K and $AgNO_3$, respectively. In contrast, the highest distribution factor for total TiO_2 content (1.45) was estimated for the visceral tissue. The DF values estimated for the other compartments were all lower but in a similar range from 0.40 in the foot tissue and 0.48 in the mantle to 0.50 in the muscle tissue (Fig. 3).

Particle size distribution in the test media. In order to estimate the particle size distribution in the test media and to check for possible changes/modifications of the applied MNMs, aliquots of the test media were sampled from the test systems prior to the collection of mussels for tissue analysis. Media samples were analysed by spICP-MS. As shown in Fig. 4A–D, no visible changes with regard to the size distribution of the applied MNMs could be observed in the test medium, indicating a high degree of dispersion stability for the time frame of the test and under test conditions. In addition to the MNM dispersions, the stock solution and media of the $AgNO_3$ test were analysed in the spICP-MS mode in order to check for possible precipitates. The results are shown in Fig. 4E and F. It is clearly visible that the "size distribution" obtained from the stock solution differs significantly from the histogram derived from analysis of test media (Fig. 4F). While the stock solution shows results that could be expected for a dissolved analyte, apparently silver nanoparticles are formed in the test medium. That these particles are indeed formed in the test medium can be supported by comparison of the respective transient signals (raw data, see Fig. S7A and B†), where a constant signal as expected for dissolved species was observed for the stock solution and a high number of particle-related spikes could be observed for the medium.

However, a few spikes are also still visible in the raw data for the stock solution and are interpreted as particles which together with the background signal form the size distribution shown in Fig. 4E. At this point, it is important to emphasize that the histograms shown use normalized scales and cannot be directly compared.

Validation of tissue digestion procedure. Mussel tissue had to be processed prior to analysis by spICP-MS. The impact of the digestion procedure on the size distribution of the nanoparticles was evaluated qualitatively. Mussel tissue was spiked with NM 300K and NM 105 prior to digestion and the measured size distribution was compared with the aqueous stock dispersion. Additionally, hydrolysates of mussels without prior exposure to the MNM were spiked after digestion with NM 300K in order to evaluate the impact of the resulting solution on the size distribution. No visible impact of the tissue hydrolysate (Fig. 5C), as well as the digestion procedure (Fig. 5B) on the size distribution was observed. Similar observations were made for the size distributions of particles extracted from the soft tissue of mussels which were exposed to NM 300K over an extended period of 48 h and 120 h (Fig. 7).

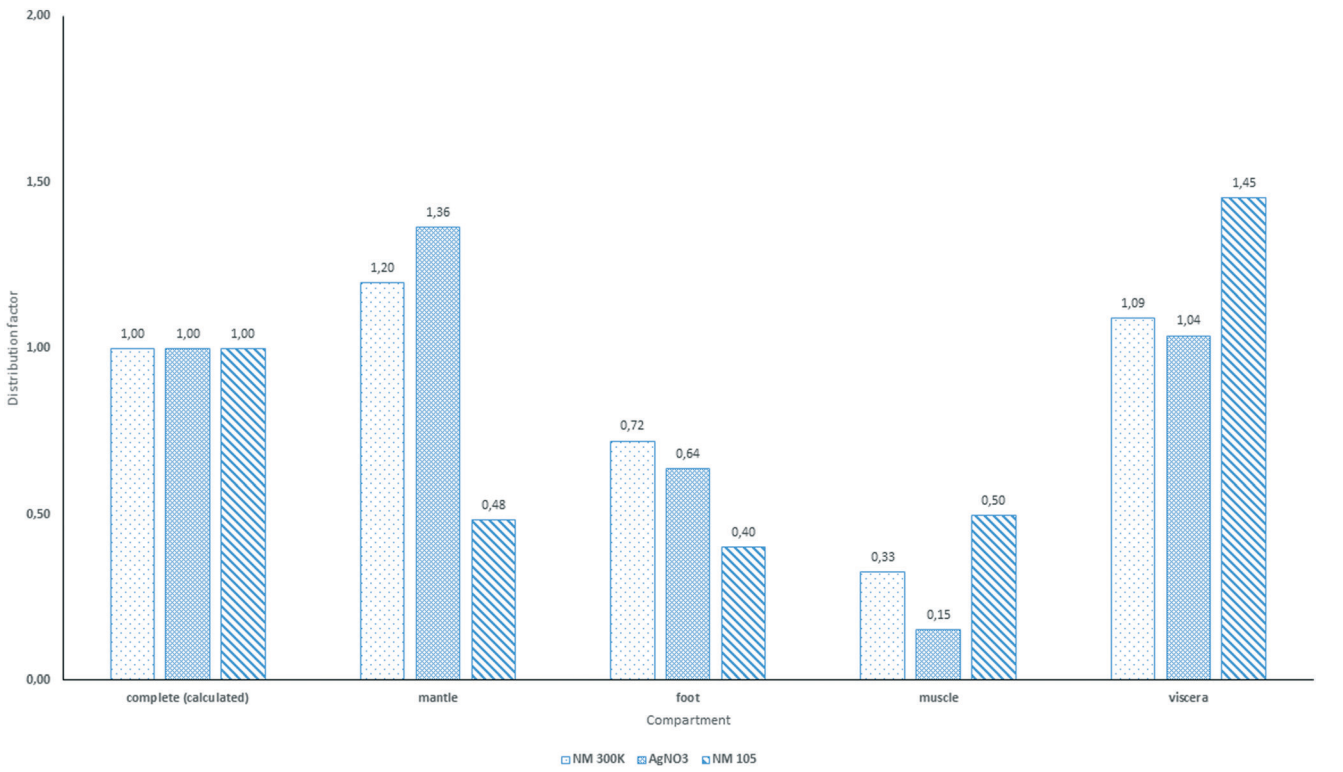

Fig. 3 Distribution factors for total concentration of Ag/TiO_2 under steady-state conditions, in relation to the calculated total concentration of Ag and TiO_2 in the whole soft body; n = 5.

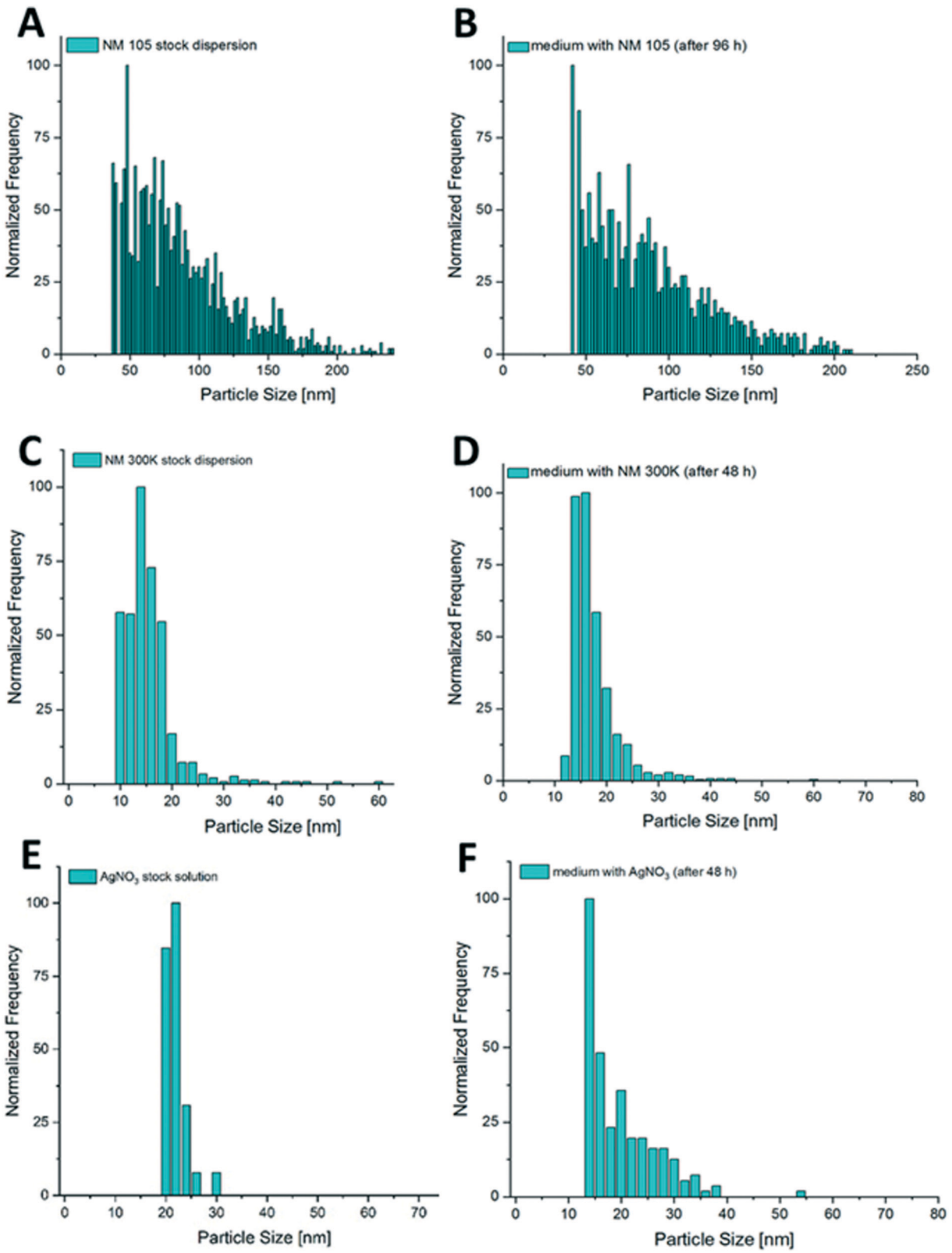

Fig. 4 Comparison of size distribution in media against stock dispersion. (A) NM 105 stock dispersion, (B) NM 105 in test medium at 96 h, (C) NM 300K stock dispersion, (D) NM 300K in test medium at 48 h, (E) $AgNO_3$ stock solution, and (F) $AgNO_3$ in test medium at 48 h.

The results of the respective experiment with NM 105 are shown in Fig. 6. For both hydrolysates spiked prior and after digestion a shift towards smaller sizes was observed. The effect was more pronounced for the mussel already spiked prior to the digestion.

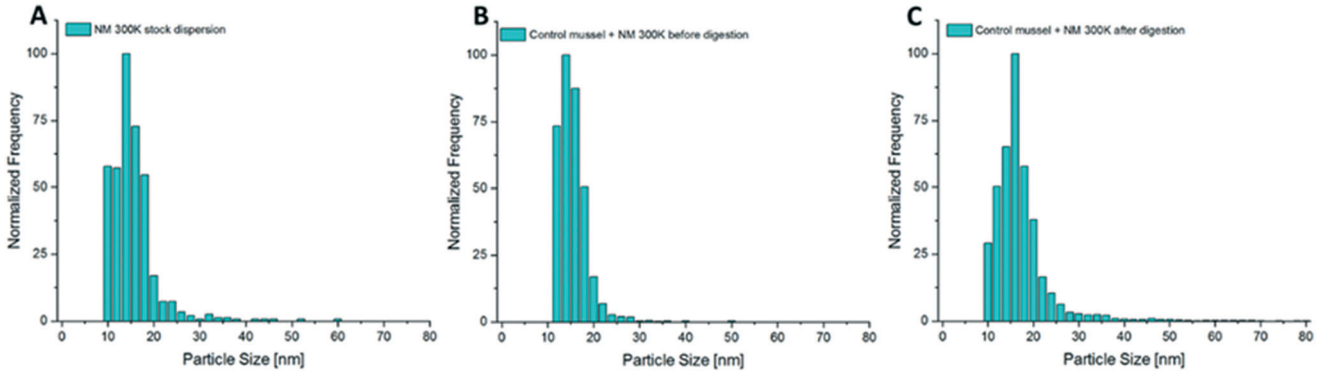

Fig. 5 Particle size distributions of NM 300K, AgNPs determined by spICP-MS analysis. (A): Analysis of stock dispersion in UHQ-water, (B) analysis of digested mussel tissue with NM 300K spiked prior to digestion, and (C) analysis of digested mussel tissue with NM 300K spiked after digestion of the tissue.

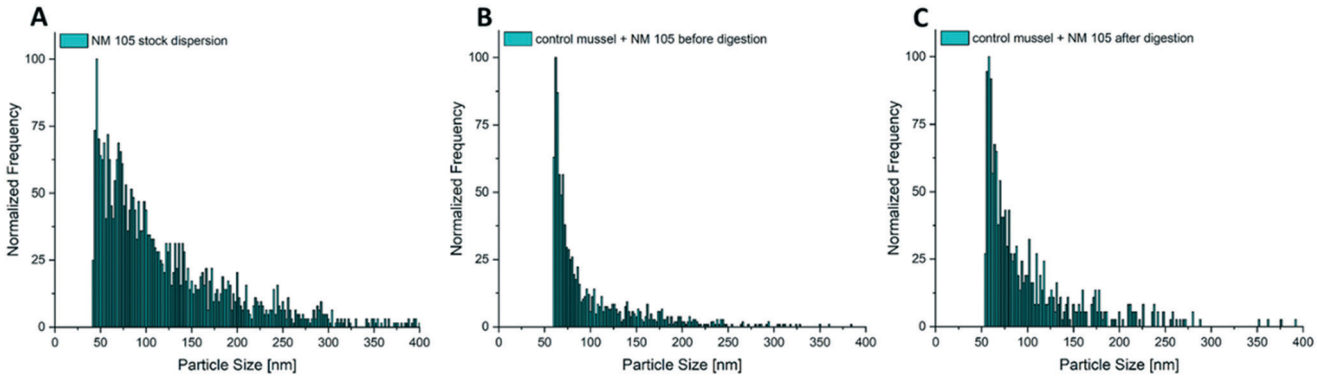

Fig. 6 Particle size distributions of NM 105, TiO_2NPs determined by spICP-MS analysis. (A) Analysis of stock dispersion in UHQ-water, (B) analysis of digested mussel tissue with NM 105 spiked prior to digestion, and (C) analysis of digested mussel tissue with NM 105 spiked after digestion of the tissue.

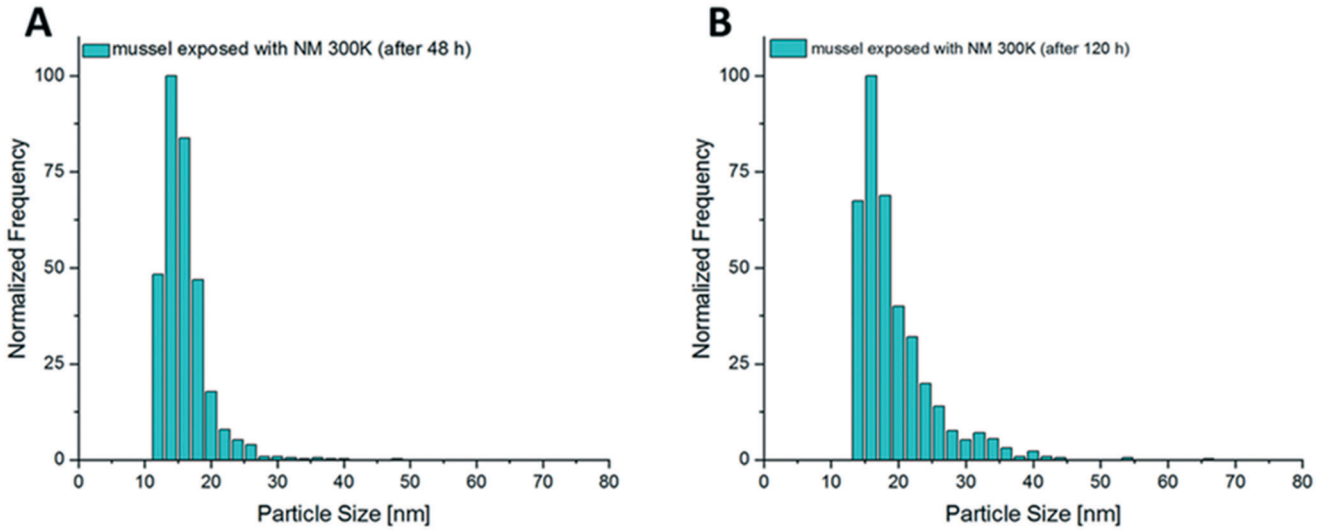

Fig. 7 (A) Size distribution of AgNPs from digested mussel tissue after 48 h of exposure and (B) size distribution of NM 300K in mussel tissue after 120 h of exposure time.

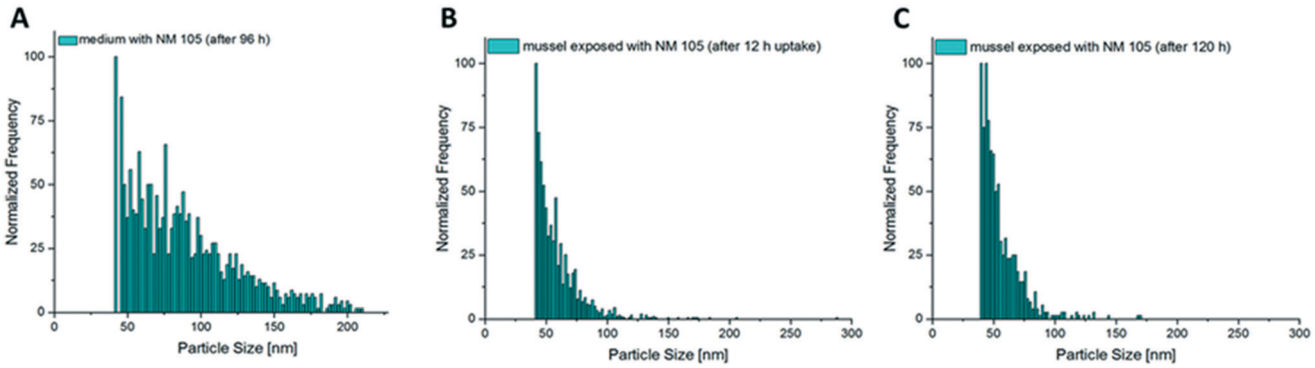

Fig. 8 Size distributions of NM 105 in (A) test medium, (B) tissue after 12 h, and (C) tissue after 120 h.

Fig. 8 shows the size distributions of NM 105 in mussel tissue extracts after different exposure times compared to the size distribution of NM 105 in the surrounding test medium. In contrast to the observations made for NM 300K (Fig. 5A and 7A), the distribution of NM 105 in mussel tissue extracts showed a loss of bigger particles compared to the size distribution of the stock dispersion and the test medium (Fig. 8A and B). Median particle size of the TiO_2NPs measured in the soft tissue was in the range of 42 to 49 nm, whereas the median particle size in the exposure media was measured to be in the range of 65 to 83 nm. The shift is more pronounced than observed for the digestion validation samples (Fig. 6). No difference was observed regarding the size distributions of NM 105 in extracts of mussel tissue collected after different exposure times.

Particle concentrations and distribution in the soft tissue

During the additional bioaccumulation study with $AgNO_3$, no significant increase of the measured Ag particles was observed during the 48 h uptake phase (Fig. 9A). The measured concentration of the presumed Ag particles in the different compartments was 5 times higher in the viscera when compared to the other compartments.

For NM 300K exposed animals, a clear increase of particle concentrations could be measured in the whole soft tissue of the test animals during the 48 h uptake phase. A trend of decreasing particle concentration in the soft tissue during the following depuration period lasting 24 h was observed (Fig. 9D). The determined median particle size of the AgNPs in the tissue was 14 nm, whereas the determined median particle size in the exposure media was 15 nm.

The tissue of the viscera showed the highest single particle concentration of all measured compartments, sampled after 48 h of exposure. Particle concentrations in the other compartments were significantly lower and not significantly different (Fig. 9C).

Also, in the additional bioaccumulation study with NM 105, an increase in the particle concentration was measured in the soft tissue of the test animals during the uptake period (Fig. 9F). A stable particle concentration was reached after 12 h of exposure and remained stable until the end of the uptake phase (96 h). Concentrations decreased significantly during the following 24 h of depuration (Fig. 9F). Median particle size of TiO_2 MNMs measured in the soft tissue was in the range of 42 to 49 nm, whereas the median particle size in the exposure media was measured to be in a range of 65 to 83 nm. The highest measured particle concentration of the compartment samples after 96 h of exposure was determined in foot tissue, whereas the particle concentrations in the mantle, muscle and viscera were up to four times lower and showed no significant differences (Fig. 9E).

Discussion

Bioaccumulation studies with MNMs are difficult to carry out due to their tendency to agglomerate and deposit in sediments. The bioaccumulation studies with the freshwater bivalve *C. fluminea* demonstrated the suitability of the new test system which meets the need to investigate bioaccumulation in filtering/sediment living organisms as organisms of special relevance for environmental risk assessment of MNMs. During all flow-through studies a continuous exposure of the bivalves with stable MNM concentrations could be achieved. Water quality parameters measured within the test system (dissolved oxygen, pH, ammonia, nitrate/nitrite) were constantly maintained in an acceptable range and provided optimal experimental conditions for the performance of the bioaccumulation studies. An experimental diet was required which ensures a sufficient nutrient supply during the extended experimental periods. Ground stinging nettle was identified as a suitable diet being rich in elements and minerals (*e.g.* ferric oxide, potassium, calcium, and silicium)[74] which are essential for the shell formation of bivalves. It is hypothesized that the small particles of the ground material were triggering the filtration activity of the mussels. However, the amount of food supplied to the test system should be generally reduced as far as possible to avoid sorption processes of the MNMs to the food while being high enough to allow sufficient nutrient uptake during the bioaccumulation studies.

Two MNMs were chosen based on notable differences in their major characteristics and tested for their

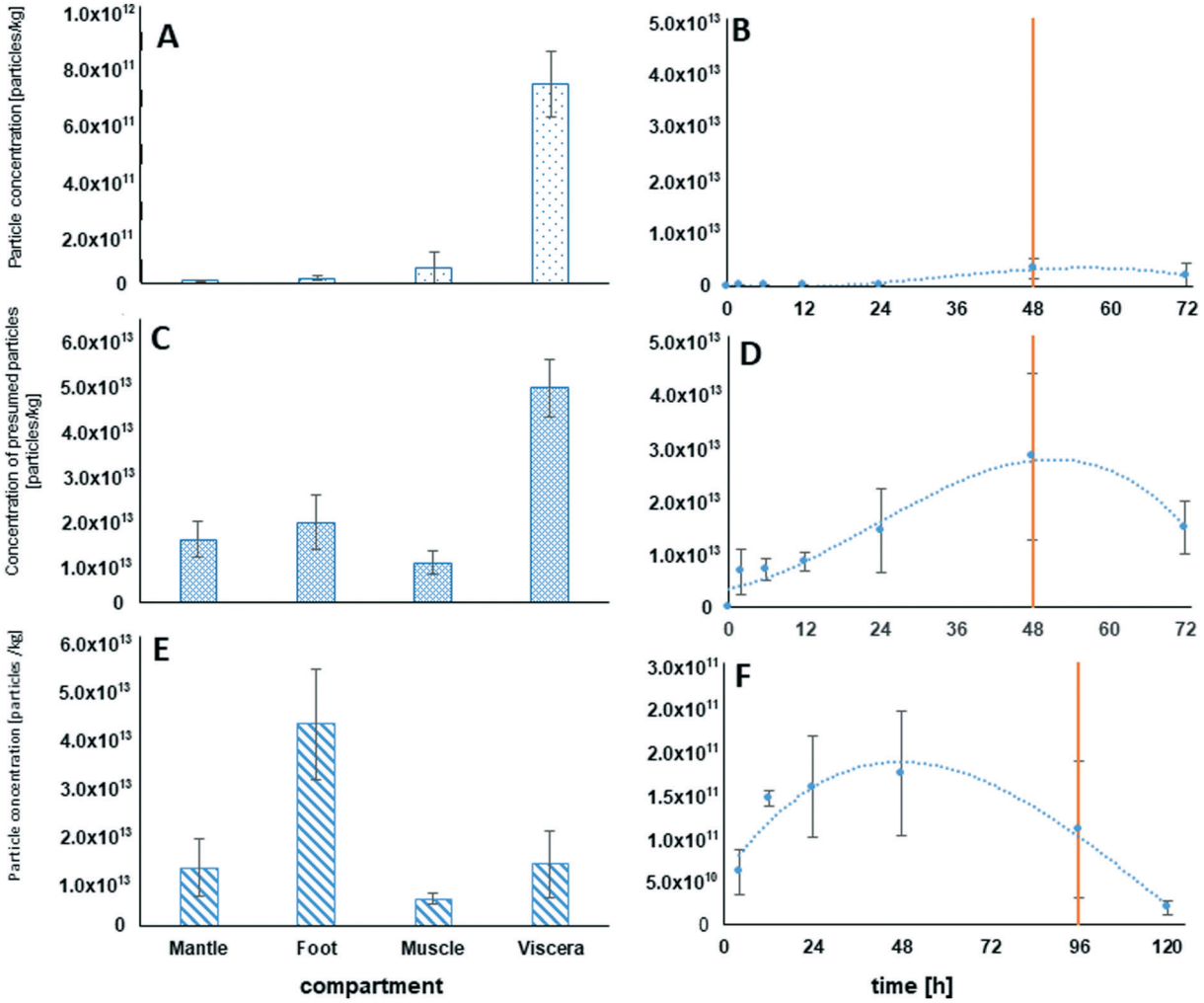

Fig. 9 Particle concentrations and distributions in the soft tissue of *C. fluminea*. (A) Concentration of presumed particles in different compartments at steady-state conditions (48 h) and in the whole soft body during the uptake phase and in the depuration time of the $AgNO_3$ treatment (B), (C) NM 300K particle concentration in different compartments at steady state conditions (48 h) and in the whole soft body during the uptake phase and in the depuration time (D), (E) NM 105 particle concentration in different compartments at steady state conditions (96 h) and in the whole soft body during the uptake phase and in the depuration time (F).

bioaccumulation potential. The silver nanoparticle NM 300K was tested as representative of MNMs that disperse well and release ions. In contrast the TiO_2 nanomaterial NM 105 is nearly chemically inert and is one of the most commonly used MNMs[56] representative of non-ion-releasing and condition-dependent sedimenting MNMs. $AgNO_3$ was used as an additional test item representing the same element as NM 300K but in a non-nanoparticulate form, allowing the possibility of comparing the bioavailability, accumulation and fate of dissolved and particulate Ag.

During $AgNO_3$ exposure the bioaccumulation of Ag resulted from the uptake of dissolved Ag^+ from the exposure media. Thus, the estimated endpoint was the BCF_{ss} and not BAF_{ss}. But the measurements using spICP-MS showed the presence of presumed particles that could be resulting from precipitation processes of Ag^+, *e.g.* Ag_2S, AgCl, or Ag_2CO_3. Due to the non-differentiable uptake of Ag in the case of the $AgNO_3$ exposure, the BAF should be used for bioaccumulation assessment and not the BCF. With a BAF_{ss} of 30.5, a significantly lower bioaccumulation of silver was observed following exposure to the higher test concentration compared to a BAF_{ss} of 710.7 for the lower concentration $AgNO_3$ treatment. This might be explained by reduced filtration activities of the mussels in response to increased metal concentrations which has been previously observed in bivalves and which were considered as a protection mechanism to avoid adverse effects by metal accumulation: for instance, a slight decrease of the filtration rate was observed in response to increased metal exposure, *e.g.* for the green mussel *Perna viridis*,[75] the zebra mussel *Dreissena polymorpha*[76] and *C. fluminea*.[77,78] A complete bivalve closing behavior in response to higher heavy metal concentrations in the water was described as a strategy to avoid toxic conditions.[79–84] Reduced filtration activities were also observed in this study during exposure to $AgNO_3$, however in varying intensities. Due to the visibly lower reduction of the

filtration rate in the lower concentration $AgNO_3$ treatment, the continuous uptake of small amounts of Ag^+ may have led to the higher BAF_{ss} value compared to that in the higher test concentration. Deviation in filtration activities between both treatments may also explain the differences in the time required to reach steady-state Ag concentrations in the soft tissue of *C. fluminea*. Therefore, the protective behaviour of bivalves needs to be considered in bioaccumulation studies of ionic or ion-releasing and other acute toxic substances as the reduction in filtration activity may lead to totally different results depending on the level of the concentrations used.

Pretests should be carried out to elucidate the fate of the test item in the test system and to estimate a suitable exposure concentration to avoid protective behavior as described for $AgNO_3$. At least two test concentrations should be applied to identify potential concentration-dependent effects.

During bioaccumulation studies with NM 300K, a visibly lower inhibition of the filtration activity was observed compared to the study with $AgNO_3$ at similar total Ag concentration levels. Due to this observation it is safe to assume that the protection behavior is mainly triggered by free Ag^+. The amount of dissolved Ag in the NM 300K test media was estimated in pretests to be between 1.6% to 21.5% within 24 h after production of the test media under static conditions. Considering the flow-through conditions applied in the bioaccumulation studies with NM 300K, the percentage of dissolved Ag^+ may have been even on a lower level but still sufficient to induce a weak valve closing behavior. Consequently, the potentially higher release of Ag^+ in the higher concentration NM 300K treatment compared to the lower concentration treatment has obviously led to a reduced filtration activity leading to different BAF_{ss} values of 31 and 128, respectively. During the depuration phase, a rapid and nearly complete elimination of Ag was observed which indicates that total Ag concentrations measured in the bivalve samples were supposed to be mainly represented by the particulate fraction. The ingested AgNPs obviously simply moved through the digestive tract. This was confirmed by the additional investigations using spICP-MS. Particle concentrations measured during the uptake and elimination phase in the soft tissue of the animals followed the total Ag concentration measured in the soft tissue during the bioaccumulation study. The majority of the particles were found in the viscera, including the gills and the digestive tract. All other compartments showed very low particle concentrations. This clearly indicates that there was no significant transport of AgNPs through the different compartment tissues or hemolymph and is thus pointing to the negligible bioavailability of AgNPs to *C. fluminea*. It can be only speculated as to which extent dissolved Ag^+ from the test material (NM 300K) was bio concentrated by the test organisms and contributed to the total Ag concentrations measured throughout the study. However, the distribution factors for accumulated Ag in the different compartments underpin the presumption that only Ag^+, even present at low concentrations, was really incorporated in the mussel tissue. Both exposure scenarios, $AgNO_3$ and NM 300K, led to comparable distribution factors for total silver (Fig. 3). Further investigations are required to elucidate the incorporation of AgNPs into the different compartments.

However, the results also show the limits of the analytical methods. For NM 300K there is a significant overlap with the background signals due to the small size of the material in the range of the instrumental size detection limit (~10 nm for Ag, given by the Agilent MassHunter Software). In addition the known presence of even smaller particles in the range of 5 nm and thus the resulting size distributions have to be treated carefully with respect to the lower sizes.[85] This significant overlap can be highlighted if the size distributions are compared with the distribution derived from the complete signal distribution, without application of a particle detection threshold (for the size distributions shown in Fig. S8B†). From Fig. S8B† it is clear that for NM 300K there is no clear gap between the particle-related signals and the background due to the small size of the material.

This observations are also well in line with reported particle detection limits (*e.g.* 16–20 nm in Lee *et al.*[86]).

The comparison of histograms derived from measurements of dissolved Ag (*e.g.* Fig. 4E) show similar distributions highlighting the significant overlap with the background. However, for the normalized histograms established for $AgNO_3$ stock solution it has to be noted that there were in fact nearly no particles in the sample and the derived "size distribution" was mainly based on much lower numbers of signal spikes than for the NM 300K-containing samples. Thus, if this silver nanomaterial has to be tested in the future it is recommended to either establish a procedure for removal of the dissolved background (*e.g.* by ion exchange resins, ultrafiltration or centrifugation) or measuring at higher time resolution (*e.g.* with shorter dwell times).[87]

Particle specific analysis of test media for $AgNO_3$ tests revealed the presence of nanosized silver containing particles that were not observed for the stock solution and are thus formed in the medium by precipitation.

The increase in the total Ti tissue concentration during the bioaccumulation studies can be explained by the uptake and potential accumulation of MNMs from the media. This was confirmed by single particle measurements in the mussel tissue showing a progression during the uptake and depuration phase that mirrored the course of the total Ti concentrations in the soft tissue. However, the measured concentrations were mainly caused by particles localized in the digestive tract and in the viscera as shown by the distribution factors for TiO_2 with the viscera showing the highest distribution factor.

The analysis of particle size distributions in test media and mussel tissue by spICP-MS following enzymatic digestion

was shown to be suitable for NM 105 TiO_2 particles. The particles have shown a broad size distribution clearly above the background signal. Even though there was a shift towards smaller particle sizes observed for the digestion procedure by analysis of spiked mussels, the size distributions measured for animals exposed in the test system, where the uptake was due to filtration, showed a more pronounced loss of bigger particles, indicating exclusion of these particles by the filtration mechanism. This finding also highlights the necessity of particle specific analysis methods, which could provide more insights into the relevant processes compared to standard total concentration analysis.

As for NM 300K, it is debatable whether the particles were only ingested or really incorporated into the tissue. However, the very effective and fast elimination that was observed during the depuration phase of both tested concentrations with TiO_2 points to the assumption that the MNMs were only ingested. Our observations are in accordance with the results of the work of Doyle *et al.* (2015),[88] where an elimination of more than 90% of TiO_2NPs previously ingested from *Mytilus edulis* within 12 h was observed.

The increased BAF_{ss} values (6150 and 9022) estimated for TiO_2NPs compared to NM 300K may be explained by two factors: first, by a potentially high filtration activity during the uptake phase, triggered by particulate matter. This effect should be stronger for NM 105 that were measured to be in the range of 63 to 83 nm in the exposure media, while particles of NM 300K were clearly smaller (15 to 17 nm). In addition, NM 105 shows a high tendency to agglomerate which may lead to a more effective uptake. It was shown that filter-feeding mussels take up bigger and agglomerated NMNs much more effectively than smaller und free particles.[28,89] Second, the higher BAF_{ss} values for the TiO_2NPs may be explained by the lack of dissolved toxic ions in the test media that could trigger a protective mechanism as assumed for the Ag tests.

The bioaccumulation studies with the freshwater bivalve *C. fluminea* demonstrated the feasibility of the new test system for testing MNMs in filtering organisms. During all studies, a continuous exposure with stable MNM concentrations was achieved. The elucidation of bioavailability, uptake and elimination as well as accumulation of the test items was possible on the level of total and particle concentrations for the whole soft body as well as the single tissue compartments. By this, the fate of MNMs within the body or different tissues could be further clarified. However, methods like transmission electron microscopy are required for the additional complementary evidence that MNMs are really incorporated into the tissue or penetrated into cells. Nevertheless, the results obtained with the described test system can be used to generate useful endpoints required for regulatory processes and could be included in a tiered bioaccumulation testing strategy for MNMs.[16] Even if some MNMs are not really bioaccumulated, the BAF_{ss} obtained provides a valuable indication of the ingestion of MNMs by bivalves, if combined with information on the elimination rate estimated following the ingestion of MNMs. A fast elimination (time back to start concentration $\leq$24 h) points to the ingestion but no incorporation of MNMs in the animal tissues. A slow elimination (time back to start concentration >24 h) provides clear indications of the incorporation of the MNMs. However, it cannot be excluded that the bioaccumulation occurred by incorporation of dissolved/ionic fractions or particulate matter which would require further elucidations using microscopy methods. Suitable criteria for the regulatory assessment of bioaccumulation based on BAF estimates need to be derived and verified.

Comparison of bioaccumulation of the nano forms with freely dissolved ions/forms of some elements or compounds is not possible if the ionic form tends to precipitate in the presence of ubiquitous elements or compounds like in the case of Ag and S. The same applies for elements with strong binding affinities to organic matter like proteins or humic acids.

Because bivalves and other filtering benthic organisms represent the main part of the biomass in freshwater systems, the benthic food chain is supposed to play a central role regarding the ecological impact of MNMs.[26,37,38,90] The steady-state concentration represents the maximum loading capacity of MNMs taken up from the surrounding medium by bivalves. Due to the high filtration rate of the bivalves the loading capacity for MNMs compared to the surrounding medium concentration may lead to an increased risk of secondary poisoning of predatory species even if no real bioaccumulation occurs. This was shown in the studies on TiO_2 where high body burden but no incorporation of the test material was observed. The results of the Bivalvia bioaccumulation test may thus provide important information regarding the transfer of MNMs into the aquatic food chain *via* predators or benthic invertebrates that feed on bivalve feces and/or pseudo-feces.[26,27,37–40] The high filtration rate of bivalves may cause feces/pseudo-feces with high concentrations of MNMs. Further investigations are required to elucidate the uptake of highly contaminated feces or pseudo-feces by benthic invertebrates that feed on fecal matter.

The suitability of the new test system for bioaccumulation studies with freshwater bivalves has been demonstrated in this study. The use of marine bivalves for bioaccumulation studies is described in two guidance documents of the American Society for Testing and Materials[91] and US EPA.[92] However, the systems described are assumed to be unsuitable for a constant exposure of MNMs. The new test system may thus also represent a potential alternative for testing MNMs in marine bivalves, however, specific media adjustments would be required. In addition to the bioaccumulation assessment of MNMs, the test system may also be suitable to investigate the uptake/bioaccumulation of microplastic in bivalves.[93–95]

Conclusions

The bioaccumulation studies with the freshwater bivalve *C. fluminea* demonstrated the suitability of the new test system.

During all studies a continuous exposure with stable MNM concentrations was achieved. The elucidation of bioavailability, uptake and elimination as well as accumulation of the test items was possible on the level of total and particle concentrations for the whole soft body as well as the single tissue compartments. In this way, the fate of MNMs within the body or different tissues could be further elucidated. However, methods like transmission electron microscopy are required as proof that MNMs are really incorporated into the tissue or penetrated into cells. The results obtained with this test system can be used to generate useful endpoints required for regulatory purposes and could be included in a tiered bioaccumulation testing strategy for MNMs.

List of abbreviations

Ag^+ Silver(I) ion
AgNPs Silver nanoparticles
ANOVA Analysis of variance
BAF_{ss} Bioaccumulation factor estimated at steady state
BCF_{ss} Bioconcentration factor estimated at steady state
DF Distribution factor
FFT Fish food tablet treatment in the dietary test
ICP-MS Inductively coupled plasma mass spectrometry
MNM Manufactured nanomaterial
SN Stinging nettle treatment in the dietary test
SP *Spirulina* species treatment in the dietary test
spICP Single-particle inductively coupled plasma mass spectrometry
TiO_2NP Titanium dioxide nanoparticle
TWA Time-weighted average (concentration)
UHQ-water Ultra high quality water

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

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Electronic Supplementary Material (ESI) for Environmental Science: Nano.

Supporting Information

Testing the bioaccumulation of manufactured nanomaterials in the freshwater bivalve *Corbicula fluminea* using a new test method

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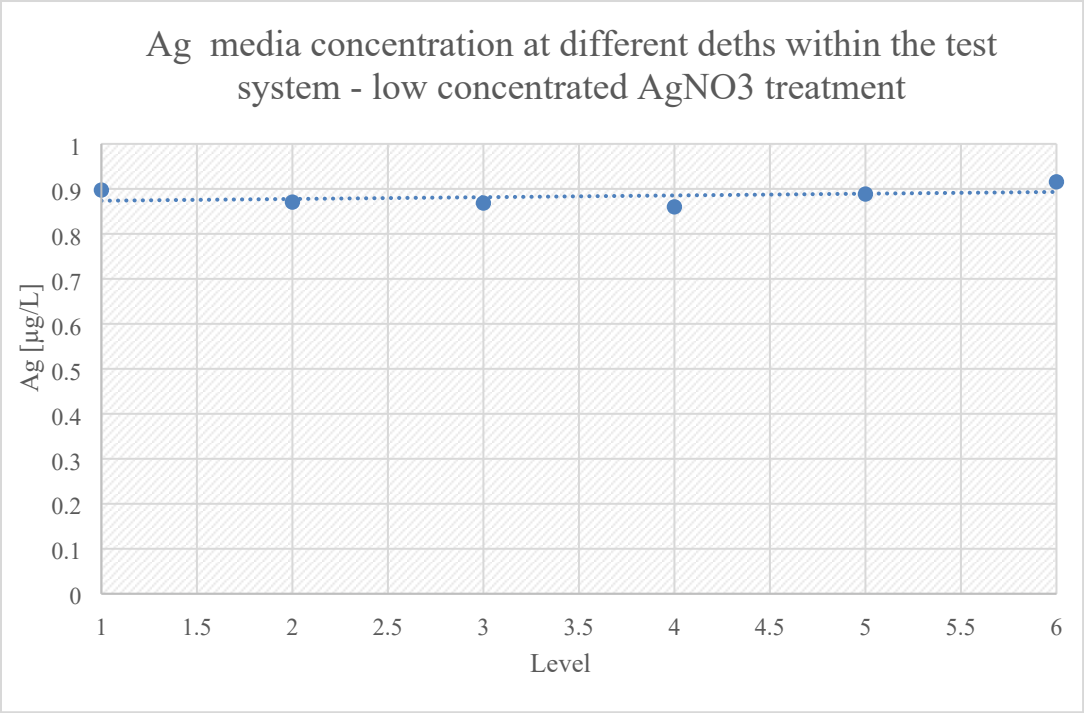
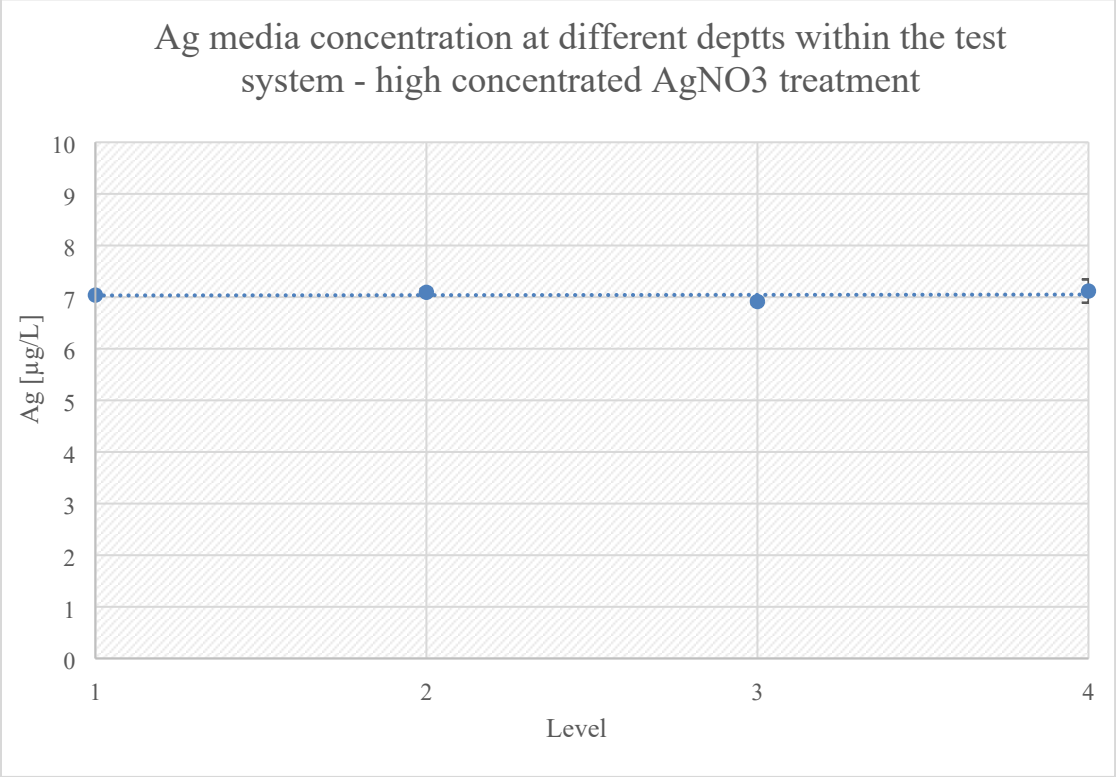


Fig. S1. Ag media concentration at different depths within the test system of the low concentrated AgNO3 treatment at 144h of uptake phase.



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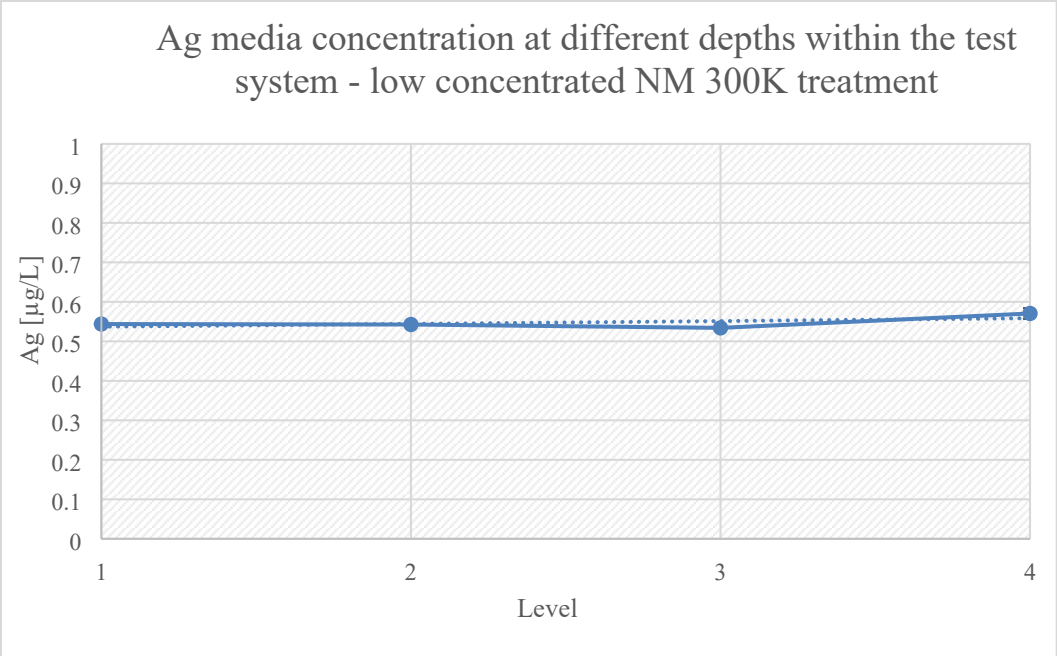

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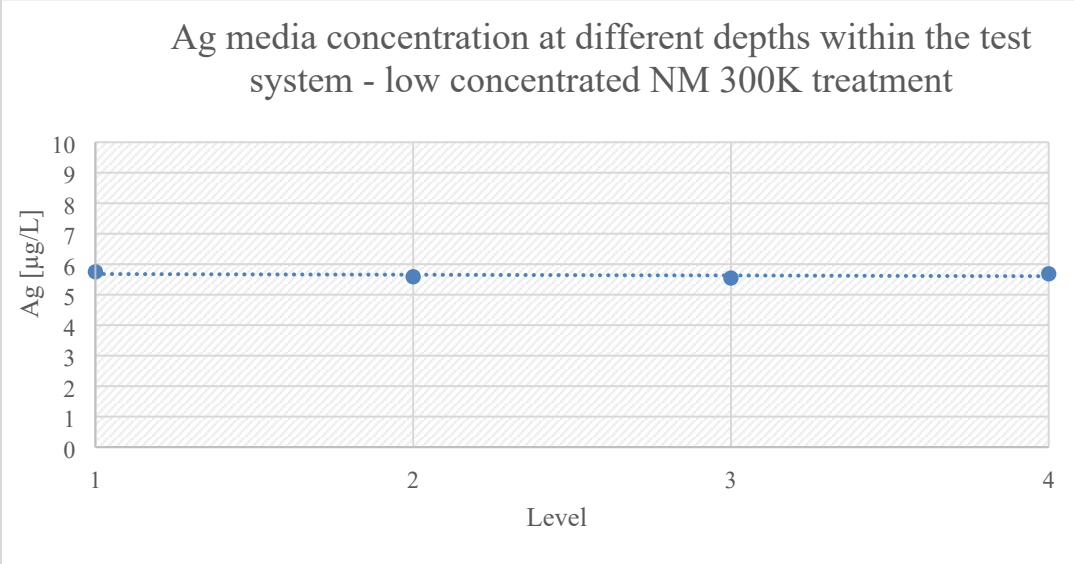

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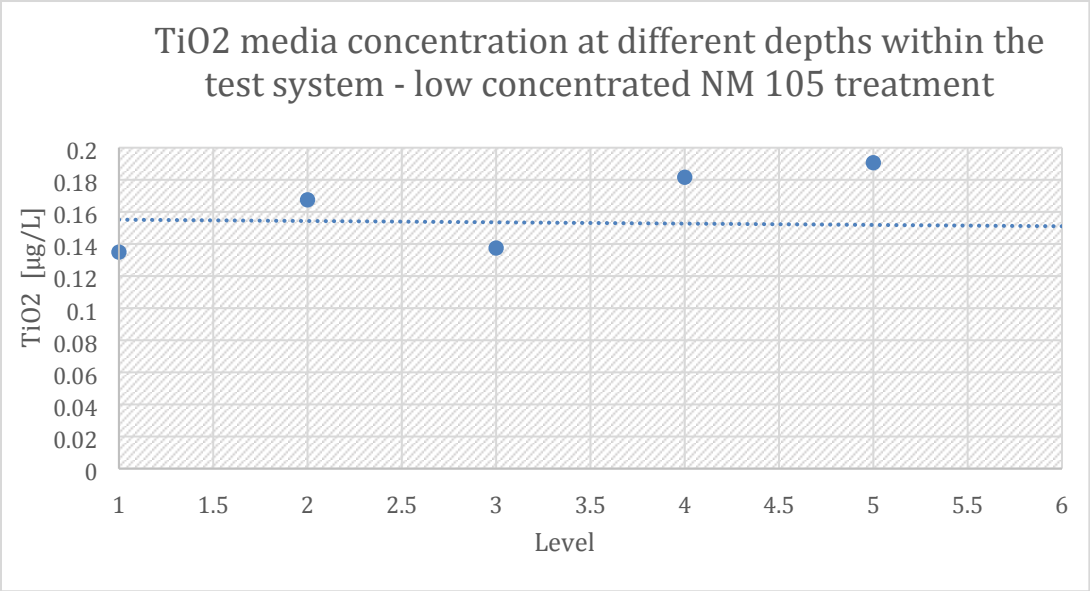

Fig. S5. TiO_2 media concentration at different depths within the test system of the low concentrated NM 105 treatment at 120h of uptake phase.

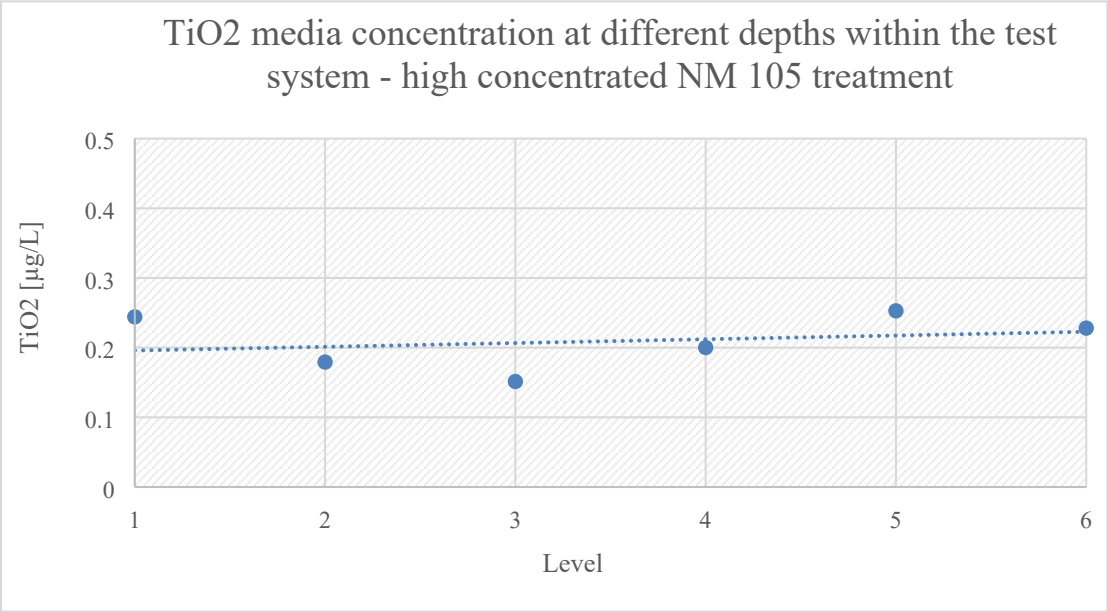

Fig. S6. TiO_2 media concentration at different depths within the test system of the low concentrated NM 105 treatment at 120h of uptake phase.

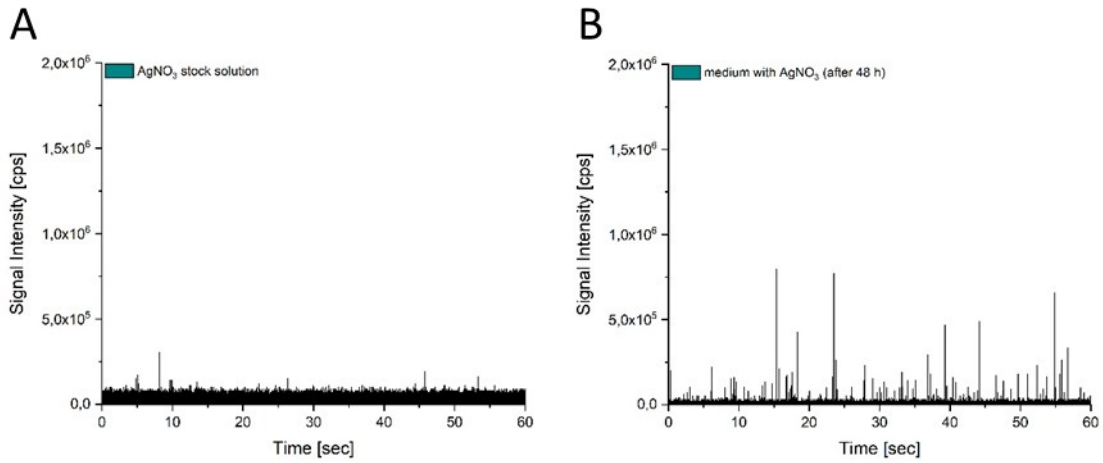

Fig. S7. Transient signals for dissolved Ag from freshly prepared $AgNO_3$ stock solution (A) and aged test medium containing $AgNO_3$ (B).

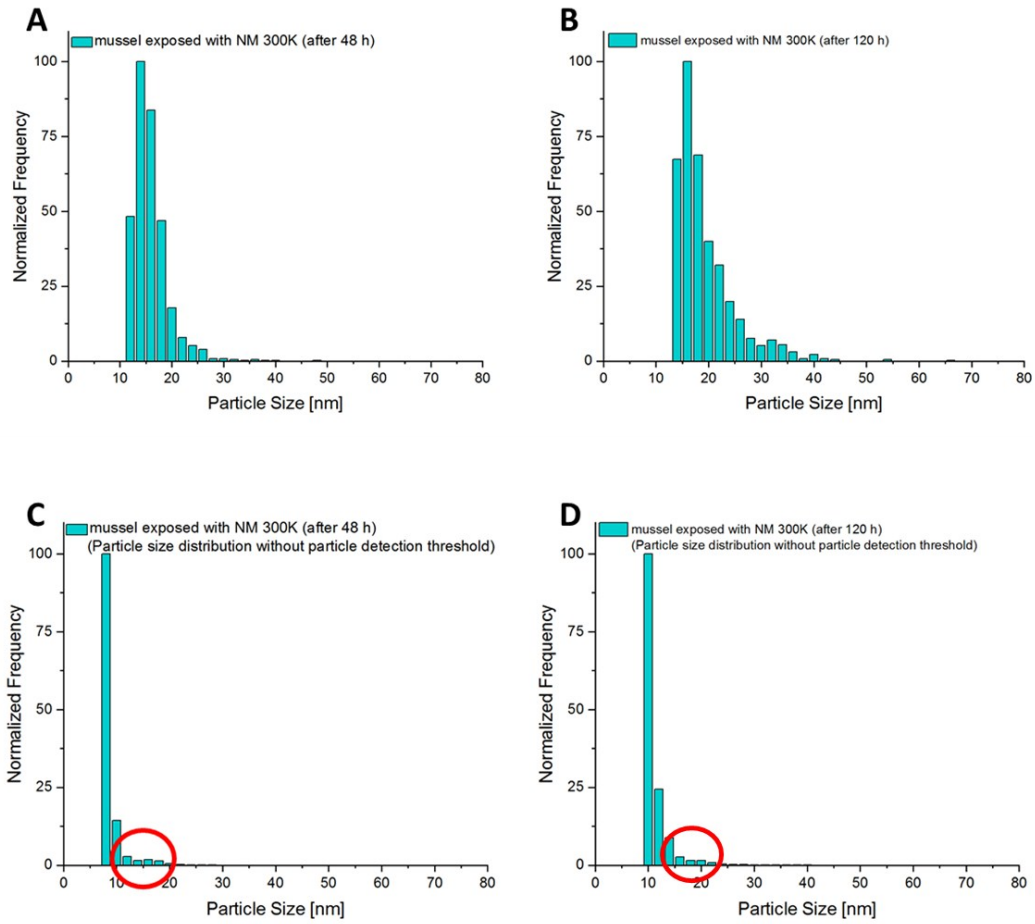

Fig. S8. A-B. Comparison of size distributions shown in Figure 5. C-D. Size distribution of A-B calculated without application of a particle detection threshold (with complete background signal). Red circles mark particle size range shown in A-B.

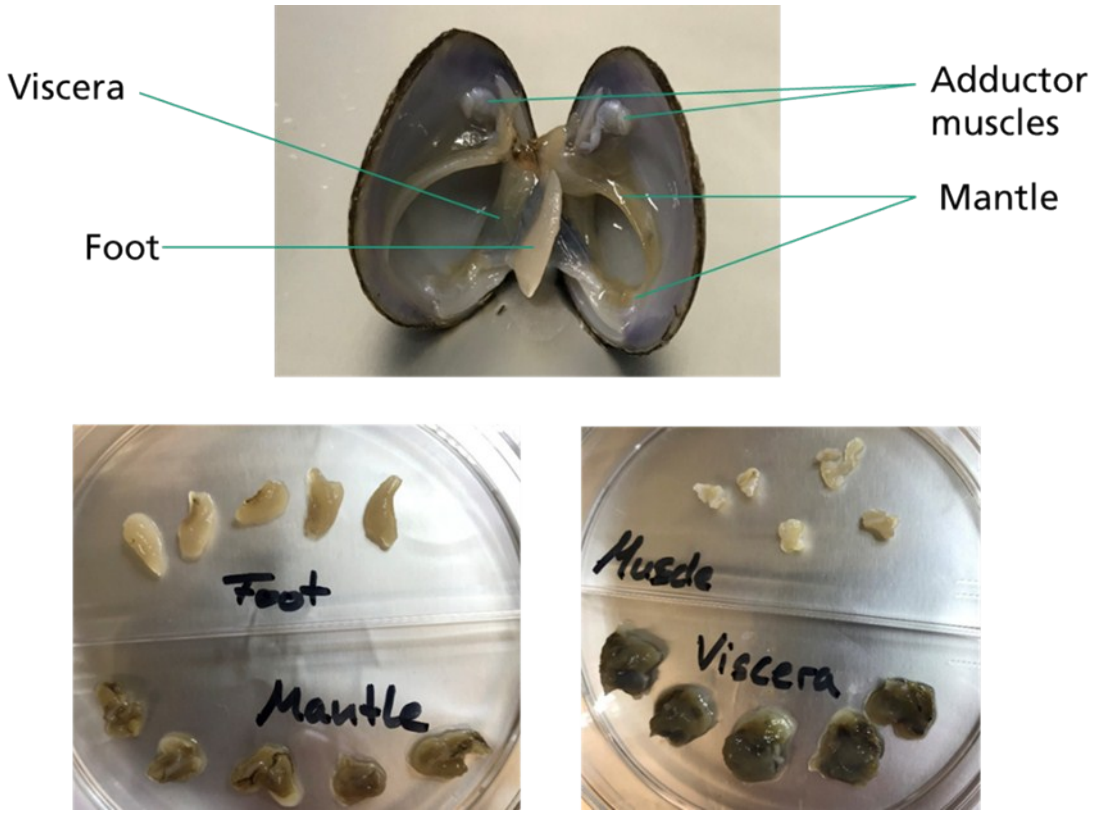

Fig. S9. Soft tissue compartments of *Corbicula fluminea* that were dissected for investigations on the tissue distribution.

Transmission electron microscopy

The NM-300K stock dispersion were characterized by TEM. The feed stock dispersion (NM-300K) was diluted 1:10^6 in deionized water 20mg of NM 105 were suspended in 100 mL 0.2% Novachem and sonicated for 10 min before diluted 1:20. 1 mL of the diluted suspensions was directly centrifuged (1h, ~14000 x g) on TEM grids. As the AgNPs carried a negative surface charge, the TEM grids were functionalized with Poly-L-Lysine (PLL, 0.1% (w/v) in H_2O, Sigma Aldrich) to enhance NP deposition on the TEM grids. The preparation of the TEM grids is described in more detail in Uusimaeki et al. (2019). A dedicated scanning transmission electron microscope (STEM, HD2700Cs, Hitachi, Japan), operated at an acceleration voltage of 200 kV was used to investigate the TEM grid. For image formation the HAADF signal was used.

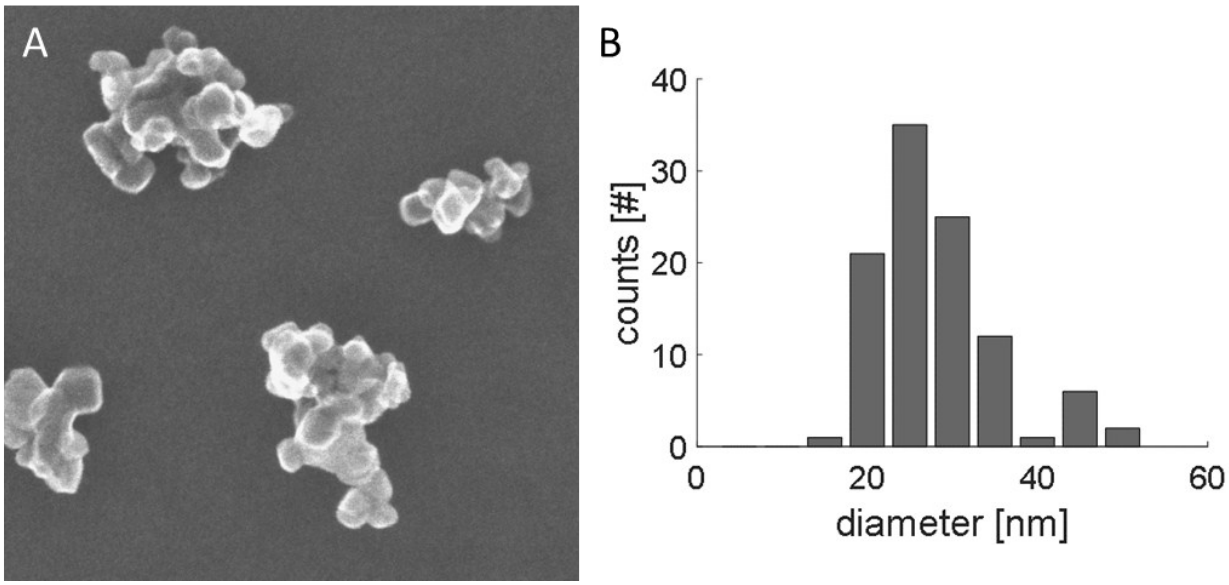

Fig. S10. TEM/ ZCM Image of feedstock material of NM 105 (A) and histogram of the grain size distribution of the NM 105 feed stock material (B).

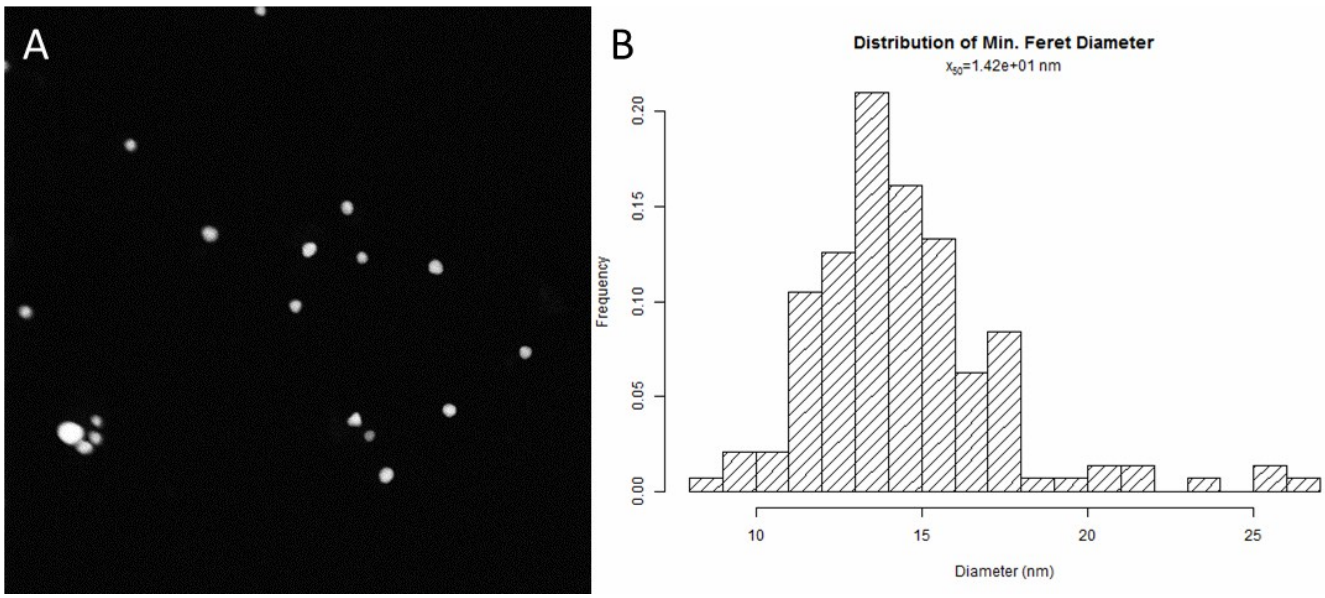

Fig. S11. TEM/ ZCM Image of feedstock material of NM 300K (A) and histogram of the grain size distribution of the NM 300K feed stock material (B).

Chapter 4: Testing the bioaccumulation potential of manufactured nanomaterials in the freshwater amphipod *Hyalella azteca*

Chapter 4 consists of the following publication:

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

Sebastian Kuehr, Ralf Kaegi, Dirk Maletzki, Christian Schlechtriem

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Authors' contribution Chapter 4:

Sebastian Kuehr

Design and conducting of amphipod studies; development of the test system; total metal analysis; collection, statistical analysis & visualization of the data; writing – original draft; writing – review & editing

Ralf Kaegi

Conducting characterization of NMs using TEM and EDX; Recourses (NM characterization); writing – review & editing

Dirk Maletzki

Conducting of amphipod studies

Christian Schlechtriem

Funding acquisition; design of studies, resources; writing – review & editing; supervision

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

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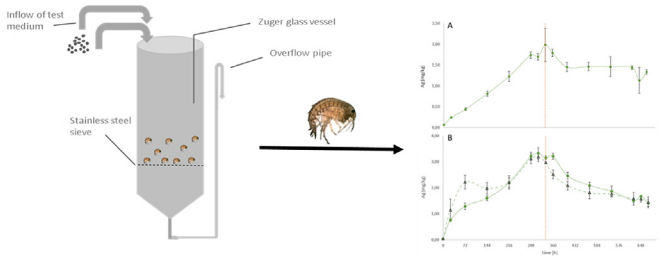
HIGHLIGHTS

- The amphipod *Hyallela azteca* was used for bioaccumulation assessment of nanomaterials.
- Biomagnification and bioconcentration 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 *Hyalella 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 *Hyalella 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, Kisitlanmasi) (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 (Organization 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 *Hyalella azteca* that was described by Schlechtriem et al. (2019). *H. azte*ca 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; TiO_2NPs), 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. Hyalella 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 biocaccumulation studies were carried out using NM 300 K as well dispersed and ion-releasing NPs. $AgNO_3$ was tested as a dissolved form of the same element. NM 105 (TiO_2) 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, trioleate, 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). $AgNO_3$ (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 path.

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 μL 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 Multichannel 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 ≤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 μg/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 μL 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 $AgNO_3$ were carried out three times: using only male ($AgNO_{3\text{-ML}}$) and only female individuals ($AgNO_{3\text{-FL}}$) 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 ($AgNO_{3\text{-FH}}$). 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

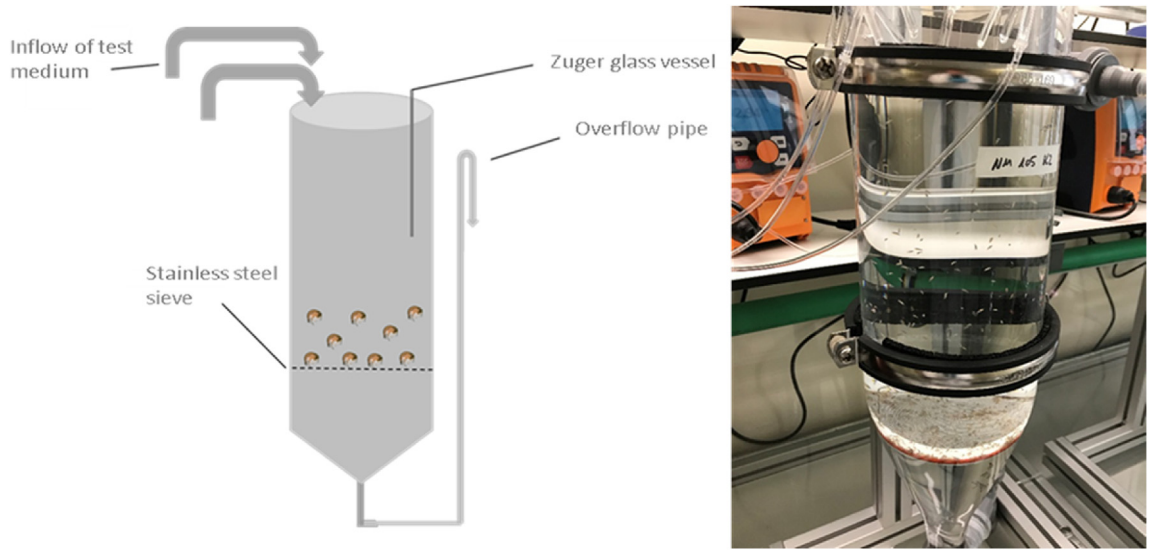

Fig. 1. Overview of the flow-through system for testing nanomaterials.

Table 1
Composition of the culture medium according to Borgmann (1996).

Compound	Molecular formula	Concentration [mol/L]
Calcium chloride dihydrate	$CaCl_2$ * 2 H_2O	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 [μg Ag/L]	BCF
$AgNO_{3\text{-FL}}$	$AgNO_3$	female	0.64	5100
$AgNO_{3\text{-ML}}$	$AgNO_3$	male	0.67	4900
$AgNO_{3\text{-FH}}$	$AgNO_3$	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 asses elemental composition of the NM and thus to confirm their identity. The feed stock dispersion of NM 300 K was diluted 1:10^6 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

Table 4
Characteristics of the bioconcentration studies with NM 105 and AuNPs and calculated BCF values.

Study	Test item	Exposure Medium	TWA [μg/L]	BCF
$NM\ 105_L$	NM 105/TiO_2NP	dilution water	1.49	–
$NM\ 105_H$	NM 105/TiO_2NP	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_2NP	3.67	0.12
$NM\ 105_H$	NM 105/TiO_2NP	19.43	0.02
$AuNP_L$	AuNP	<0.00*	–
$AuNP_H$	AuNP	0.889	0.02–0.03

Table 3
Characteristics of the bioconcentration studies with NM 300 K and calculated BCF values.

Study	Test item	Test system	Exposure Medium	TWA [μg 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

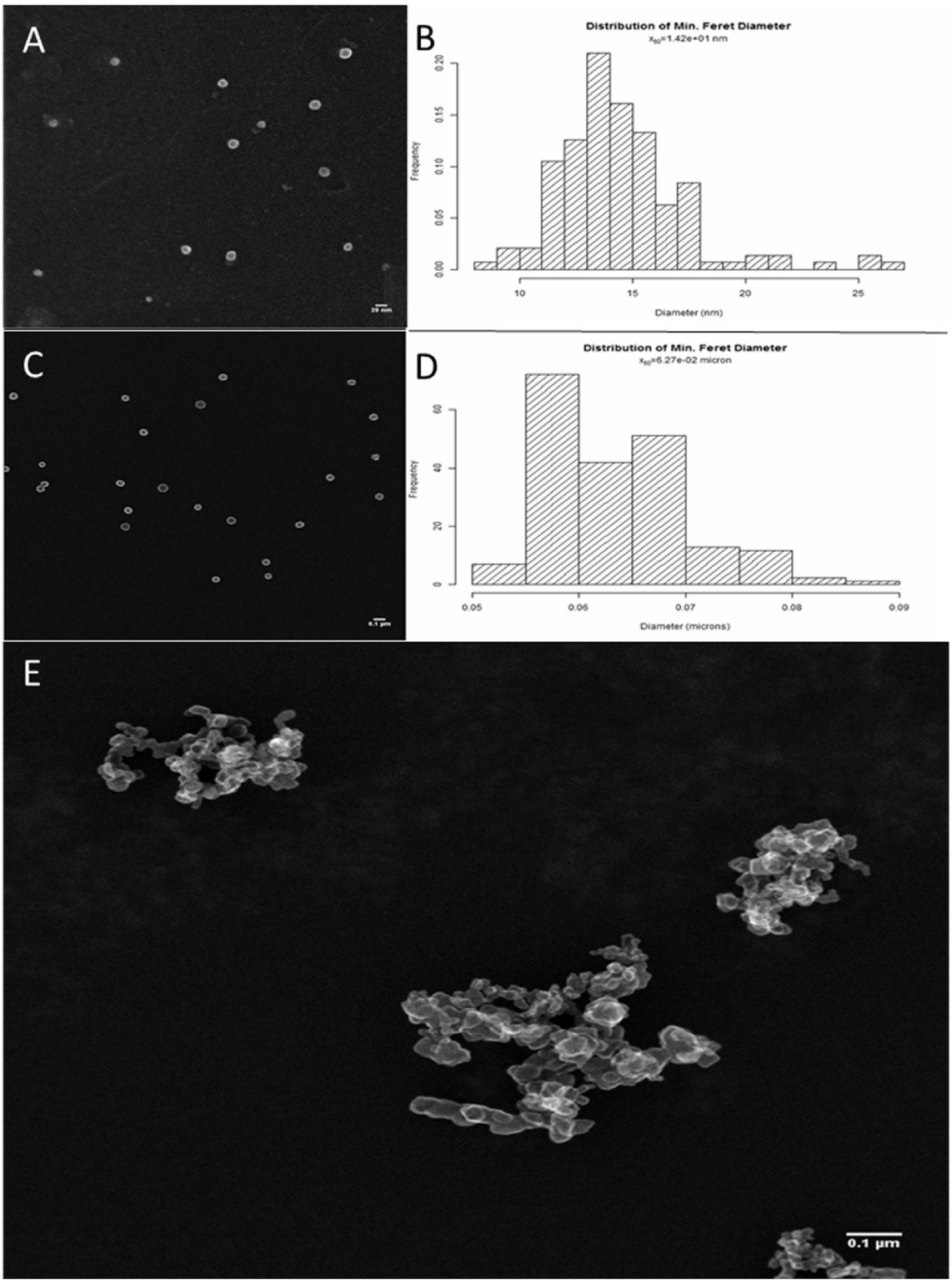

Fig. 2. TEM images of NPs and histograms of the size distribution. A: NM 300 K, magnification 35 kx; B: NM 300 K, histogram based on 143 particles; C: AuNPs, magnification 30 kx; D: AuNPs, histogram based on 172 particles; E: NM 105, magnification 80 kx.

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

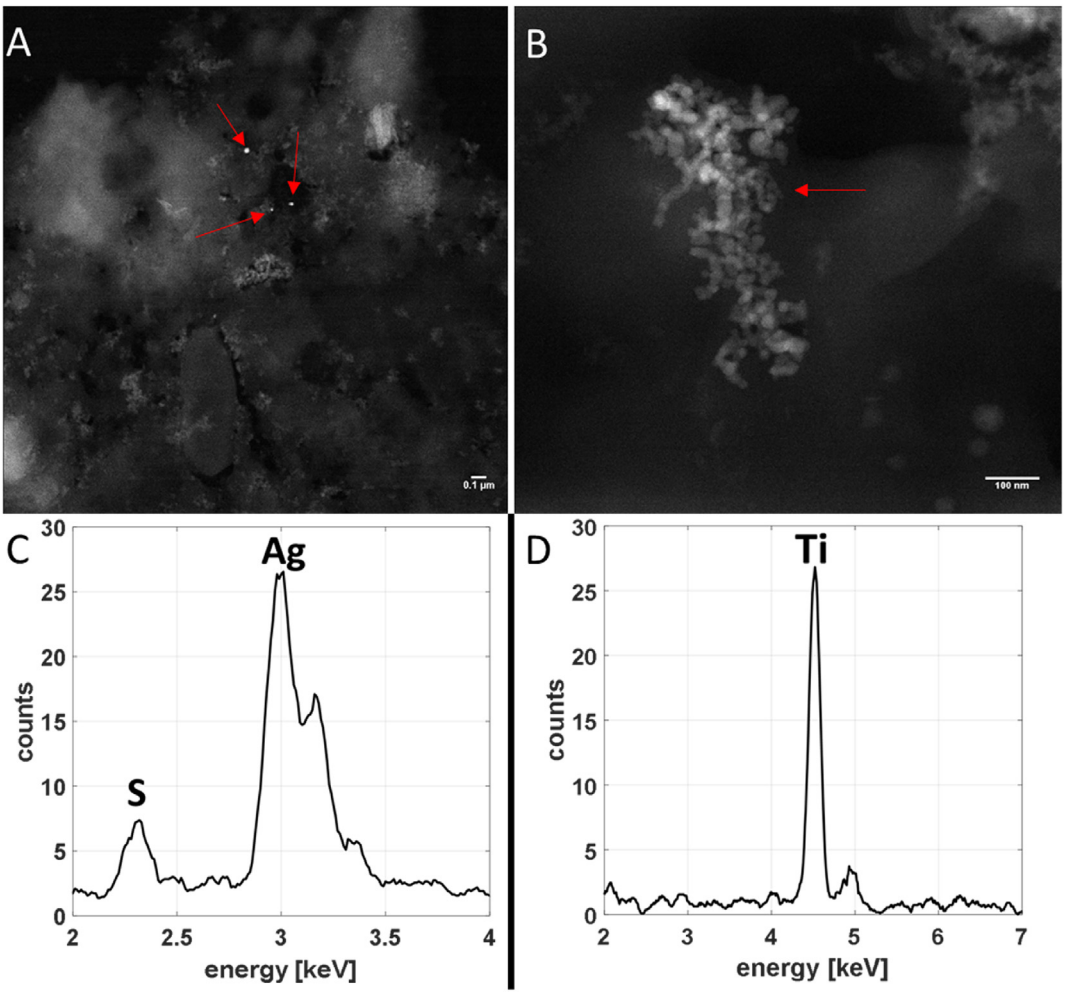

Fig. 3. TEM HAADF images and the corresponding EDX signals of NPs enriched in the experimental feed. A: NM 300 K, magnification 30 kx; B: NM 105, magnification 100 kx; C: NM 300 K, EDX signals; D: NM 105, EDX signals.

Aldrich) to enhance NP deposition on the TEM grids. The preparation of the TEM grids is described in more detail in Uusimaeki 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

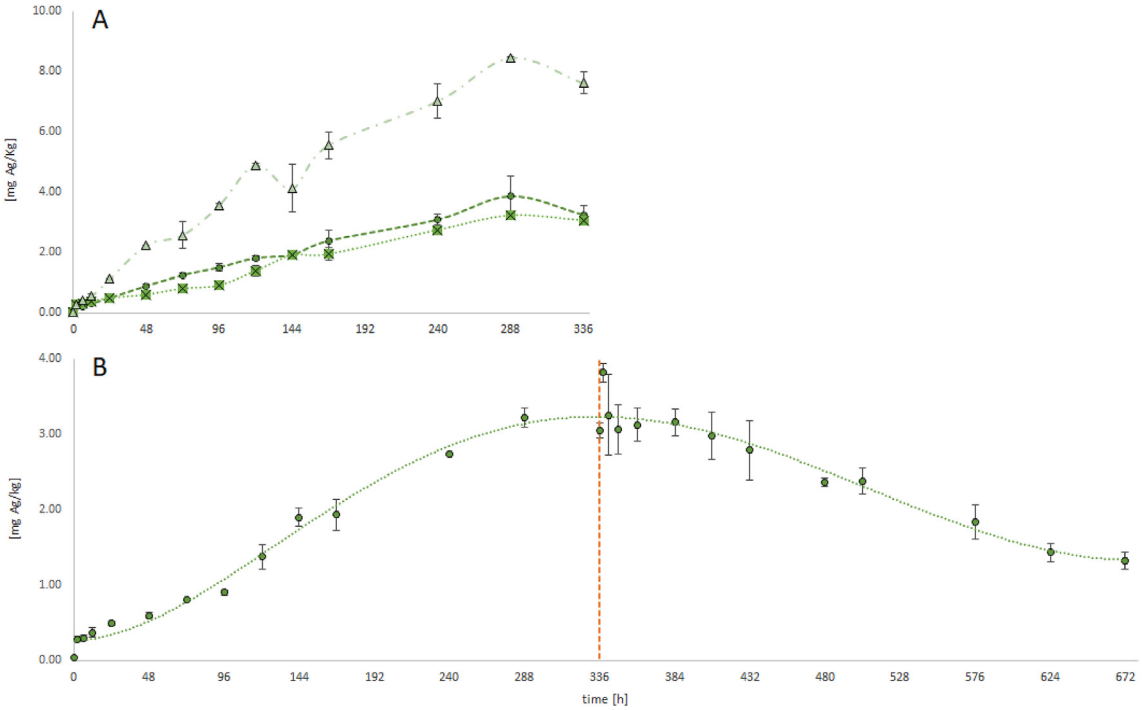

Fig. 4. A: Total Ag tissue concentrations tests during uptake phase of $AgNO_3$ bioaccumulation, triangles: $AgNO_{3\text{-}FH}$, circles: $AgNO_{3\text{-}FL}$, crosses: $AgNO_{3\text{-}ML}$; B: Total Ag tissue concentrations during uptake and depuration phase of the extended $AgNO_{3\text{-}FL}$ bioaccumulation test. The red line represents the end of the uptake phase during the extended $AgNO_{3\text{-}FL}$ 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.)

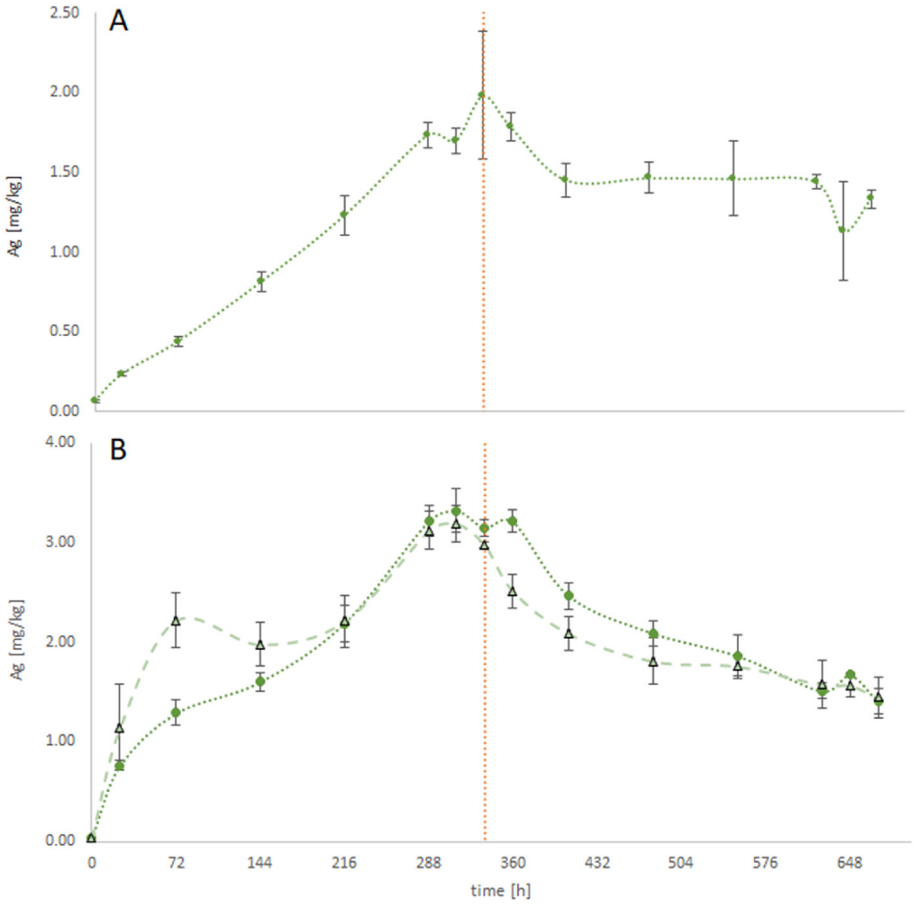

Fig. 5. Total Ag tissue concentrations in NM 300 K bioconcentration tests. A: NM $300K_{Cm}$; B: NM $300K_{Zg}$ (triangles) and NM $300K_{Aq}$ (circles). NM 105.

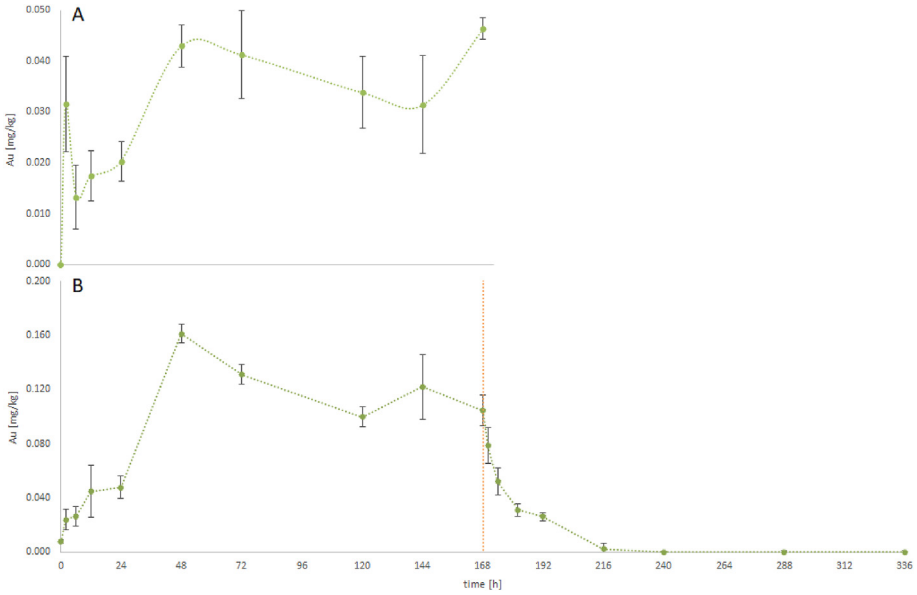

Fig. 6. Total Au tissue concentrations in the bioaccumulation test with AuNP; A: $AuNP_L$B: $AuNP_H$.

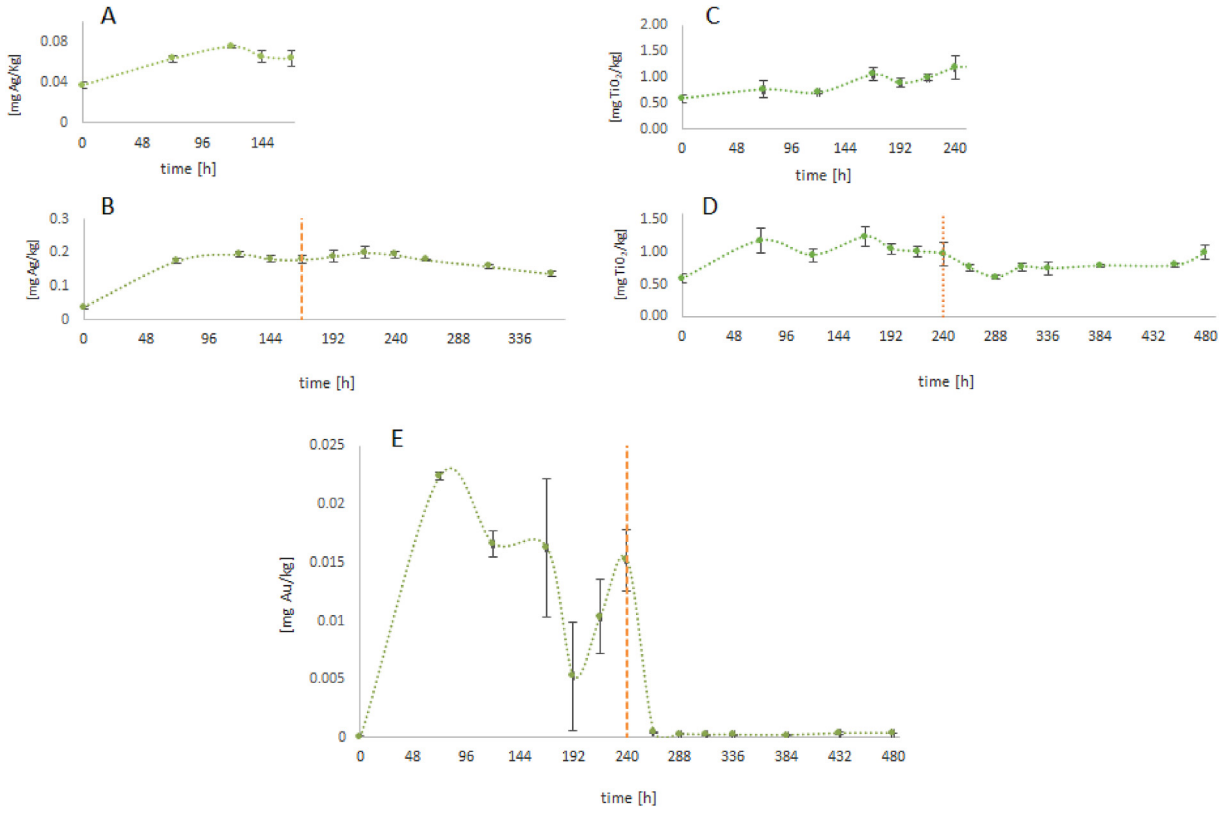

Fig. 7. Total Ag, TiO_2 and Au tissue concentrations during the biomagnification studies. Red vertical line shows the end of the uptake phase. A: NM $300K_L$, B: NM $300K_H$, C: NM 105_L, D: NM 105_H, E: $AuNP_H$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

samples before vortex stirring (VORTEX GENIE 2, Si™ 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_2NPs 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 ($AgNO_{3\text{-}FL}$) and 0.67 μ Ag/L for males ($AgNO_{3\text{-}ML}$). 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 μg Ag/L ($AgNO_{3\text{-}FH}$) 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 $AgNO_{3\text{-}FH}$ 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 ($AgNO_{3\text{-}ML}$), 5100 ($AgNO_{3\text{-}FL}$) and 4700 ($AgNO_{3\text{-}FH}$) 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 μg Ag/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 μg Ag/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 μg 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 μg TiO_2/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 μg Ti/L) and the limit of detection (LOD, 0.463 μg 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 μg 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 biomagnifcation 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 mgTiO_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 $AgNO_3$ 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 $AgNO_3$ 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 $AgNO_{3\text{-}FL}$ 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 $AgNO_3$ 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 $AgNO_3$ 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 $AgNO_3$, 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 used 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 lysosoms 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 lysosoms 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_2NPs. 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 Hyalella bioconcentration and biomagnification tests with AgNPs, leads to a slow elimination of the previously accumulated Ag, as well as the ten times higher BCF_{SS} 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 Raths 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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.127961.

References

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 nductively 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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Supporting Information

Testing the bioaccumulation potential of manufactured nanomaterials in the freshwater amphipod *Hyalella Azteca*

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Tab. S1. Particle sizes/ diameters of the test items measured by different methods and under different conditions. *Measured in culture medium: 50.3 nm.

Test item	Particle diameter by TEM [nm]	Hydrodynamic diameter by DLS: ultra pure water [nm]	Hydrodynamic diameter by DLS: dilution water [nm]	Calculated median particle size by sp-ICP-MS: from stock suspension [nm]	Calculated median particle size by sp-ICP-MS: from exposure medium [nm]	Calculated median particle size by sp-ICP-MS: extracted from animals after aquous exposure [nm]	Calculated median particle size by sp-ICP-MS: extracted from experimental diet [nm]	Calculated median particle size by sp-ICP-MS: extracted from animals after dietary exposure [nm]
NM 300K / AgNP	14.2	26.7	51.2*	15 ± 0.0	15 ± 0.0	22.6 ± 0.6	14.1 ± 0.8	17.1 ± 1.3
NM 105 / TiO_2NP	n.d.	351.1	1,861	56.3 ± 6.2	55.7 ± 1.3	107.0 ± 3.9	55.9 ± 8.4	62.2 ± 10.7
AuNP	62.7	60.7	143.5	56.2 ± 0.5	56.9 ± 1.0	56.9 ± 0.7	57.0 ± 1.4	51.2 ± 0.4

Tab. S2. Test conditions during the bioconcentration tests and determined BCF values.

Study	Test item	Test system	Sex	Exposure Medium	Duration of uptake phase [h]	Duration of depuration phase [h]	TWA [µg /L]	BCF
$AgNO_{3\text{-}F}$	$AgNO_3$	zuger glass system	female	dilution water	336	336	0.64	5,100
$AgNO_{3\text{-}M}$	$AgNO_3$	zuger glass system	male	dilution water	336	-	0.67	4,900
$AgNO_{3\text{-}H}$	$AgNO_3$	zuger glass system	female	dilution water	336	-	1.64	4,700
NM $300K_{CM}$	NM 300K / AgNP	aquarium	male	culture medium	336	336	3.00	604
NM $300K_{Aq}$	NM 300K / AgNP	aquarium	male	dilution water	336	336	6.73	480
NM $300K_{Zg}$	NM 300K / AgNP	zuger glass system	male	dilution water	336	336	6.83	453
NM 105_L	NM 105 / TiO_2NP	zuger glass system	male	dilution water	168	-	1.49	-
NM 105_H	NM 105 / TiO_2NP	zuger glass system	male	dilution water	168	168	3.3	-
$AuNP_L$	AuNP	zuger glass system	male	dilution water	168	-	0.085	424
$AuNP_H$	AuNP	zuger glass system	male	dilution water	168	168	0.704	166

Tabelle 1:Tab. S3. Test conditions during the biomagnification tests and determined BMF values.

Study	Test item	Test system	Sex	Exposure Medium	Duration of uptake phase [h]	Duration of depuration phase [h]	Concentration [mg/L]	BMF
$NM\ 300K_L$	NM 300K / AgNP	zuger glass system	male	dilution water	168	-	3.00	0.25
$NM\ 300K_H$	NM 300K / AgNP	zuger glass system	male	dilution water	168	192	6.83	0.93
$NM\ 105_L$	NM 105 / TiO_2NP	zuger glass system	male	dilution water	240	-	1.49	0.12
$NM\ 105_H$	NM 105 / TiO_2NP	zuger glass system	male	dilution water	240	240	3.3	0.02
$AuNP_L$	AuNP	zuger glass system	male	dilution water	240	-	0.085	-
$AuNP_H$	AuNP	zuger glass system	male	dilution water	240	240	0.704	0.02 - 0.03

Chapter 5: Unravelling the uptake pathway and accumulation of silver from manufactured silver nanoparticles in the freshwater amphiod *Hyalella azteca* using correlative microscopy

Chapter 5 consists of the following publication:

Unravelling the uptake pathway and accumulation of silver from manufactured silver nanoparticles in the freshwater amphiod *Hyalella azteca* using correlative microscopy

Sebastian Kuehr, Jessica Klehm, Claudia Stehr, Matthias Menzel, Christian Schlechtriem

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The published supporting information directly follows the article.

Two videos are included in the supplementary material and are available online:

Video S1: https://ars.els-cdn.com/content/image/1-s2.0-S2452074820300331-mmc2.mp4

Video S2: https://ars.els-cdn.com/content/image/1-s2.0-S2452074820300331-mmc3.mp4

Authors' contribution Chapter 5:

Sebastian Kuehr

Design and conducting of amphipod studies; total metal analysis; collection, statistical analysis & visualization of the data; writing – original draft; writing – review & editing

Jessica Klehm

Resources (correlative microscopy); Development of sample preparation method for correlative microscopy; conducting of sample preparation for correlative microscopy; conducting of sample examination for correlative microscopy writing – review & editing

Claudia Stehr

Development of sample preparation method for correlative microscopy; conducting of sample preparation for correlative microscopy;

Mathias Menzel

Conducting of sample examination for correlative microscopy, writing – original draft (method part about correlative microscopy)

Christian Schlechtriem

Funding acquisition; design of studies, resources; writing – review & editing; supervision

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Research paper

Unravelling the uptake pathway and accumulation of silver from manufactured silver nanoparticles in the freshwater amphipod *Hyalella azteca* using correlative microscopy

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ABSTRACT

Silver nanoparticles (AgNPs) are known to enter the aquatic environment via leachates from landfills or by the effluent or sludge of sewage treatment plants.

Even if the AgNPs are sulfidized, Ag from these particles was shown to still be available to several species like the benthic freshwater amphipod *Hyalella azteca*. However, it is still unknown if the primary uptake of Ag from these particles occurs mainly by Ag ions, or if the main uptake of Ag is by ingestion of AgNPs and if they can be found in the animal tissues or just in the gut content without any tissue transfer.

To elucidate the main uptake pathway and localization of AgNPs potentially taken up by *H. azteca*, we exposed the amphipods to Ag using AgNP containing sewage sludge or dissolved Ag from $AgNO_3$.

Further, we seperated the exposed animals into two groups. One had direct contact to the AgNPs enriched sludge, allowing them to feed on it, the second group seperated from the sludge by a strainer allowing only indirect contact by potentially released AgNPs or ions.

The animals exposed for 7 days were examined for their total and nano particulate Ag content using (single-particle) inductively coupled plasma mass spectrometry as well as using methods of correlative microscopy. Thus we were able to show, that low amounts of AgNPs were present exclusively in animals with direct contact to the AgNP enriched sludge and only in the region of the gut. No transfer of AgNPs from the gut into the animals tissue was observed by correlative microscopy. However, measurable Ag body burdens in animals from all treatments and groups indicated that ionic uptake is the main uptake pathway for (bio)accumulation of Ag from AgNPs.

1. Background

There is a growing trend for the use of nanoparticles (NPs) in different sectors like electronic industries, medical devices, clothes and more (European Commission, 2013; Future Markets Inc, 2017). The number of products containing NPs increased 25 fold from 2005 to 2010 leading to an increased impact on the environment (Bundschuh et al., 2018; PEN, 2013). A low amount (≤ 2%) of the manufactured NPs entering the environment is released during the production or further processing of the NPs (Gottschalk and Nowack, 2011). The major proportion of the NPs is entering the environment by waste management processes. In the case of silver NPs (AgNPs) 50% of the NPs reach the environment as solid waste like textiles, or packaging waste (Adam and Nowack, 2017). Once deposited at landfills the solid waste releases the AgNPs over time and they can reach the environment via leachates (Bundschuh et al., 2018). The other 50% of AgNPs reaching the environment are released from sewage treatment plants (STP) (Adam and Nowack, 2017), even if the main part is adsorbed by the sewage sludge (Kaegi et al., 2011; Schlich et al., 2013).

As widely described, AgNPs are mainly transformed to silver sulphide (Ag_2S) while passing through the waste water treatment plant (Burkhardt et al., 2010; Impellitteri et al., 2013; Kaegi et al., 2011; Kaegi et al., 2013; Kampe et al., 2018; Kraas et al., 2017; Levard et al., 2012; Lombi et al., 2013). In this form the Ag is hardly soluble and due to the reduced release of Ag^+ and the decreasing trend to generate reactive oxygen radicals at the sulfidized and thus passivated AgNPs

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surface, the AgNPs are less toxic than pristine AgNPs or dissolved Ag^+ ions (Ag^+) (Bianchini and Wood, 2008; Bianchini et al., 2002; Choi and Hu, 2008; Kim et al., 2009; Liu and Hurt, 2010; Reinsch et al., 2012; West, 1996). Nevertheless, different studies have shown that sulfidized AgNPs or Ag from these transformed particles are still bioavailable for plants (Kraas et al., 2017), nematodes (Starnes et al., 2016), terrestrial isopods (Kampe et al., 2018) as well as for fresh water amphipods like *Hyalella azteca* (Kühr et al., 2018). *H. azteca* is endemic to North and Central America and regularly used for ecotoxicity and bioaccumulation testing, primarily on metals (Alves et al., 2009a; Kühr et al., 2018; Nuutinen et al., 2003; Othman and Pascoe, 2001; Raths et al., 2020; Schlechtriem et al., 2019). Due to the fact, that sewage treatment plants (STPs) are the main pathway for NPs to enter the aquatic environment, chronic exposure studies with *H. azteca* were carried out on the accumulation of silver from AgNPs in sewage treatment plant effluents (Kühr et al., 2018). It was shown that accumulation of silver in the body of test animals is clearly dependent on the pretreatment of the AgNPs. Accumulation of silver ions (Ag^+) released from AgNPs is assumed to be the major pathway leading to the observed body burden (Kühr et al., 2018). However, it can only be speculated whether ingested AgNPs are also bioavailable and how and to what extent they contribute to the body burden, in contrast to Ag^+.

In this study, exposure tests with *Hyalella azteca* were carried out to elucidate the main uptake pathway, as well as the form and localization of Ag present in the animals. The exposure system used in a previous study (Kühr et al., 2018) was adjusted to allow investigations on the uptake of Ag by *H. azteca* either by direct or indirect contact to sewage sludge from a model STP containing Ag/AgNPs. In addition a comparison between animals exposed to $AgNO_3$ at comparable aqueous Ag concentrations as found in the studies with AgNPs was carried out. The exposed animals were examined for their total Ag body burden and accumulation factors were calculated taking account of the Ag concentrations in the water. Further animals were examined for the presence of AgNPs in the animal tissue. Mild methods were used for i) extraction of AgNPs from the animal tissue to allow investigations by single particle inductively coupled plasma mass spectroscopy (spICP-MS) and for ii) embedding of the animals for examinations by correlative microscopy.

2. Materials and methods

2.1. Handling of AgNP and preparation of the NP stock suspension

NM 300 K, a representative test and reference material from the European Commission's Joint Research Centre and in the scope of the OECD Working party on Manufactured Nanomaterials Sponsorship Program was used for the studies. The material was provided by the Fraunhofer Institute for Molecular Biology and Applied Ecology IME. Summarized information on the characterization and physico-chemical properties can be found in the JRC Report (Klein et al., 2011). The production of the AgNP working suspension to be applied to the model STP and to spike control sludge was carried out as described by (Kühr et al., 2018). The stock suspension was diluted with ultra high quality water (UHQ water), hand-shaken for 1 min and sonicated for 15 min (640 W) (Bandelin, Sonorex) to disperse the AgNPs and to carefully homogenize the suspension. $AgNO_3$ (purity of > 99.9%) was purchased as a salt from Carl Roth and dissolved and diluted using ultra-high quality water (UHQ water) to prepare the $AgNO_3$ stock solution.

2.2. Sewage sludge exposure and preparation of feeding filters

For our study we used the same sewage sludge as used by Kampe et al. (2018) from a lab scale STP simulation according to OECD TG 303A (Organisation for Economic Co-operation and Develpement (OECD), 2001). The sludge was examined by transmission electron microscopy (TEM) using energy dispersive x-ray spectroscopy (EDX). It was shown, that the AgNPs were metallic, still of comparable size as the pristine AgNPs from the NM 300 K stock suspension, but completely transformed into Ag_2S according to Kampe et al. (2018).

One batch of sludge from an STP run treated with AgNP (S_L), and pure sludge from a control STP run (S_0) were suspended in UHQ water for the preparation of food filters for the exposure tests (for further information see SI SA.). All sludge suspensions were homogenized using a disperser (IKA® T25 digital ULTRATURRAX®) and stirred for 3 h before being filtered through glass microfiber filters (Whatman® GF 6, 50 mm) by using a vacuum pump (Vacobox, KNF) as described by (Kühr et al., 2018) to achieve a loading of 50 to 80 mg (dry mass). The loaded filters were frozen and stored at −20 °C before being used.

2.3. Hyalella azteca

The freshwater water amphipod *H. azteca* was taken from the stock culture of Fraunhofer IME, Schmallenberg. The strain was originally obtained from "Freds Haustierzoo "(Cologne, Germany). The culturing procedure was carried out according to (Kühr et al., 2018) using reconstituted water containing bromide (Alves et al., 2009b). The stock culture was kept in 2 L flasks filled with reconstituted water containing bromide (Alves et al., 2009b), each containing 30 adult animals. Three times a week, 5 mg of ground fish feed (Tetramin®, Tetra) were added to each beaker to maintain optimal growth of the test animals. A small piece of gauze (3 × 3 cm) was added to provide a place of refuge. Juveniles were separated from the parent animals once a week to be cultured separately until used in tests. Only healthy amphipods free from observable diseases and abnormalities were used in these studies.

2.4. First exposure study (7d)

The first exposure study with NM 300 K was carried out with adult animals (at least 8 weeks old). In this test one Ag-treatment and a control treatment were compared (Table 1). Every treatment consisted of 5 test groups with 20 adult *H. azteca* each kept in 600 mL glass beakers filled with 500 mL copper reduced tap water (Fig. 1). In each beaker an additional group of 5 adult animals was separated from the major group by a stainless steel strainer. The group within the strainer was fed control sludge (S_0), while the major group outside the strainer was fed with sludge treated with AgNP (S_L). Animals were exposed for 7 days without water exchange. The sludge coated filters were added to the beakers 24 h before the animals were added to allow Ag^+ ions to be released into the media, even if the time was too short to enable the system to equilibrate. Water samples (n = 3, each 10 mL) for Ag measurement were taken at the start (before animals were added), at day 4 and at the end of the test (day 7) from the beaker near the strainer. The water samples were analysed directly after ultracentrifugation (for further information see SI SB). The filtrate was used

Table 1
Ag content in sludge [mg Ag/kg] and TWA concentrations of the test media [µg Ag/L]. Sludge samples were measured once (n = 5) for all treatments/tests. * sludge in strainer was S_0 sludge, ** $AgNO_3$ solution;

Study	Treatment	Matrix	TWA in medium [µg Ag/L]	Concentration in sludge [mg Ag/kg]
Exposure study I	S_0	Media	–	
		Sludge		1.17 ± 0.05
	S_L	Media	5.11	
		Sludge		7303 ± 873*
Exposure study II	S_0	Media	–	
		Sludge		1.17 ± 0.05
	S_L	Media	16.74	
		Sludge		7303 ± 873*
	$AgNO_3$	Media	5.41**	
		Sludge		1.17 ± 0.05

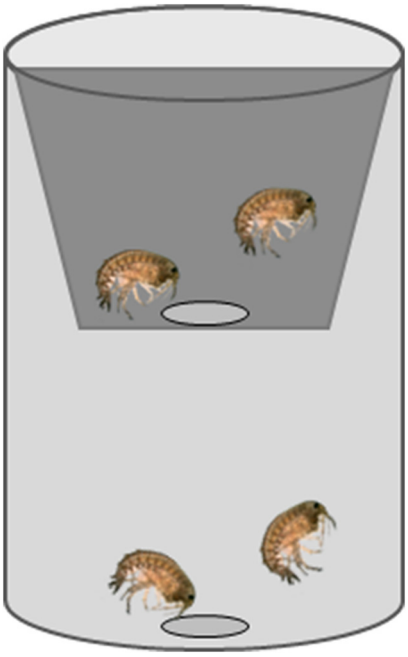

Fig. 1. Schematic overview of the exposure scenario including two groups of *H. azteca*, separated by a strainer to provide direct (bottom) or only indirect (top) contact to AgNPs present in sewage sludge loaded on glass fiber filter. The group within the strainer was fed control sludge.

to measure the concentration of Ag^+, while the filter residue was analysed for its Ag content as an indication of AgNPs in the water phase. The animals were collected at the end of the exposure phase, frozen with liquid nitrogen and stored at −80 °C until histological preparation for correlative microscopy and measurements using inductively coupled plasma mass spectrometry (ICP-MS) as described below. The measurement of the total Ag content of the water samples was carried out before and after ultrafiltration. Only the water samples for the measurement of total Ag content were acidified by adding 200 µL of nitric acid (69%, suprapure grade, Roth).

2.5. Second exposure study (7d)

A second exposure test was carried out under similar conditions as described before but including an additional treatment using control sludge only. In this treatment $AgNO_3$ solution was applied as the aqueous test medium mirroring the time weighted average concentration of Ag in the water measured during the first exposure test with sludge containing AgNPs (S_L) (nominal Ag concentration: 5.11 µg/L). Animals and water samples collected during the study were handled as described before. Further replicate beakers (n = 3) were used for examination using single-particle ICP-MS (spICP-MS). Animals were frozen using liquid nitrogen and stored at −80 °C. Water samples (10 mL, n = 3 for each treatment and sampling point) for total Ag measurement were taken at the start (before animals were added), at day 4 and at the end of the test (day 7) from inside the strainer and from outside near the bottom of the beaker. The water samples were prepared and measured as described above directly and after ultrafiltration. Water samples for analysis using spICP-MS (n = 3 for each treatment and sampling point) were taken at the test start from the fresh media and at the end of the test from the aged media, and were measured directly after sampling.

2.6. Determination of total silver concentrations in the aqueous test media, sewage sludge and Hyalella azteca samples

Collected samples of aqueous test media were prepared and analysed as described by Kühr et al. (2018), for further information see SI SB. To meausure the total Ag content of sewage sludge loaded on glass fiber filters, disposable scalpels (Braun, Cutfix®) were used to remove sludge samples from the filter surface (n = 5 for each treatment; one single filter = one replicate). The analysis of total Ag concentrations in sewage sludge and *Hyalella azteca* (n = 5 for each treatment; each replicate contained 15 to 18 pooled animals) collected during the studies were carried out according to Kühr et al. (2018) and (Wasmuth et al., 2016) (for further information see SI SC). The analysis of total Ag concentrations in the sludge was only carried out before exposure.

2.7. Examination of Hyalella tissue and aqueous test media by single particle ICP-MS

For the examination of *Hyalella* tissue by single particle ICP-MS (sp-ICP-MS) a gentle sample preparation method was applied that does not dissolve the particles and that affects the particle properties as little as possible. For this, tissue samples (n = 3 for each treatment) were digested using the enzyme proteinase K according to the method described by (Loeschner et al., 2013) and (Schmidt et al., 2011). The dried animals (10 pooled animals per replicate) were transferred to a 50 mL glass beaker and gently pressed using a glass rod to destroy the carapax and allowing the enzyme to enter the body and digest the proteins. 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 obtained from the glass beaker containing the crushed tissue samples to minimize the potential loss of any silver via glass rod. The samples were incubated in the 10 mL digestion solution for 3 h at 50 °C and 100 rpm. This process has no or only a negligible impact on the dissolution and size distribution of the NM 300 K NPs (Kuehr et al., 2020). The incubated digestion solution was filtered using 0.45 µm syringe filters (Minisart® NML, 0.45 µm) before analysis. The sp-ICP-MS analysis using an ICP-QQQ-MS (Agilent 8900, Agilent Technologies, Waldbronn, Germany) were carried out as described by (Kuehr et al., 2020) (for further information see SI SD). Due to poor analytical quality controls and incomplete digestion of the animal tissues, the quantitative values of the particle numbers can only be seen as a rough assessment, to elucidate the differences in particle concentrations in the different treatments (e.g $AgNO_3$ vs S_L-media or tissue). After measurement of particle concentrations in the digested fraction using sp-ICP-MS, the filtered solution was further digested by aqua regia to allow analysis of total Ag content. For digestion, 2 mL of aqua regia were added to 5 mL of the filtered solution prior to digestion in a microwave as described above (turboWave® Inert, MLS; max temperature 220 °C, max pressure 40 bar). Only half of the collected animals were processed and analysed as described above. The non-enzymatically digested part of the samples (n = 3, each consisting of 10 pooled animals) was also digested using aqua regia (8 mL) using the same microwave method. Both solutions obtained were measured for their total Ag content. This allowed comparison of the content of Ag in the protein fraction of the animals with the whole amount of Ag from the complete samples (including AgNPs attached to the carapace etc.). Aqueous media samples collected during the studies were measured following the same procedure but without enzymatic digestion.

2.8. Imaging of AgNPs in Hyalella azteca by correlative microscopy

Correlative microscopy, combining the power and advantages of different imaging systems, e.g., light microscopy, electron microscopy, X-ray, etc., has become an important tool for material and life science during the last years. Using correlative microscopy additional information can be obtained at the same sample site because different complementary information can be spatially assigned and different orders of magnitude related to resolution and image field can be recorded (Caplan et al., 2011; Loussert Fonta and Humbel, 2015). Electron microscopy provides high resolution but has one central limitation

with a restricted field of view. Light microscopy has the advantage of having a large field of view which enables identification and localization of structures at low magnification (Loussert Fonta and Humbel, 2015). The combination of these two imaging techniques is a valuable approach to investigate structures at a submicrometer level in biological research (Bradley and Withers, 2016; Guérin et al., 2019; Verkade and Collinson, 2019).

In this study, correlative microscopy was used for morphological imaging of silver nanoparticles in *H. azteca*. After appropriate sample preparation, investigations at the macrometer to nanometer level were carried out by combining microscopic techniques and X-Ray diffraction analysis (micro computer tomography). This allowed evaluation of the state of tissue structures following preparation by different techniques as well as imaging the morphology of the nanoparticles and to detect their localization. Additionally, EDX was applied for the elemental analysis or chemical characterization of the samples. For a schematic overview of correlative micrcoscopy see SI Fig. S1.

In order to get an impression of the morphology of the nanoparticles used in this study, SEM investigations were carried out on the raw material (Fig. S2). For this purpose the Ag nanoparticles were diluted (100 µg/L), dispersed using ultrasound and applied to a glass slide for SEM investigations. The diameter of the Ag nanoparticles was determined to be between 18 and 20 nm.

An adapted sample preparation protocol was developed to enable investigations by correlative microscopy (Mulisch and Sauer, 2015). The experimental animals were embedded in polymethylmethacrylate (PMMA). During the embedding process, a staining technique was applied. Two staining protocols based on either osmium tetroxide or iodine were tested.

The embedded samples ($n = 10$ for each treatment, single animals) were subsequently examined by using micro computed tomography (equipment nanome|x 180NF) to assess the embedding quality and the determined macro- and microstructure. It was found that staining with osmium tetroxide can cause localized damage such as structural changes at the micrometer level (Fig. 2, left). However, the iodine stained samples showed no damage and iodine staining was thus the preferred procedure used in this study (Fig. 2, right).

For imaging by light and electron microscopy, a further sample preparation was carried out. Thin sections of the embedded samples with a thickness of about 80 µm were prepared according to the cutting-grinding technique of Karl Donath (Donath, 1995). The sections were analysed by light (Olympus BX 51) and scanning electron microscopy (SEM, equipment Quanta 3D FEG, FEI) to identify areas in the morphological structure of the animals where the nanoparticles could be located. Such areas were subsequently analysed by SEM combined with EDX to detect and localize silver. Images were produced using backscattered electron (BSE) material contrast in the low vacuum mode at an accelerating voltage of 10 kV after conductive coating with carbon. An additional sample preparation was conducted to allow further investigations by transmission electron microscopy (TEM, equipment FEI Tecnai G2 F20). The identified areas were dissected with an ultramicrotome [equipment RMC PowerTome PT-PC with CRX cryochamber (RMC, Tucson)] with a diamond knife (DIATOME angle 35°) to generate ultra-thin sections of 50 nm. Subsequently, TEM investigations were performed in combination with EDX analysis for the detection of silver nanoparticles (EDX Nanospot). For each sample at least 3 images were examined and each region of interest (see below) was examined at least 3 times.

2.9. Data analysis

The data analysis software Origin (OriginLab Corporation; OriginPro 2017G) was used for analysis of variance (ANOVA) for the data obtained in the different studies. All data were subjected to an outlier test (SQS 2013 Version 1.00 by J. Kleiner and G. Wachter). Replicates identified as an outlier were excluded from further data analysis. Time weighted average concentrations (TWA) of Ag in the aqueous phase of the different treatments were calculated for the experimental periods. Accumulation factors (AF_M) were obtained by dividing the total Ag concentrations in the amphipods by the TWA concentrations of the test medium AF_M. Due to the lack of evidence for obtaining steady-state conditions during the exposure studies, the calculated AF_M values do not necessarily reflect equilibrium conditions.

3. Results

3.1. First exposure tests

In the first exposure test (7d) with S_L sludge (7303 ± 873 mg Ag/kg) a TWA concentration of 5.11 µg Ag/L was measured in the water (Table 1, Table S1). In comparison, no measurable Ag concentration was present in the media of the S_0 treatment.

Animals collected at the end of the first exposure test (7d) from the S_0 treatment showed a negligible total Ag content (Fig. 8) independent of whether they were kept in the strainer (0.03 ± 0.03 mg Ag/kg) or at the bottom of the beaker (0.04 ± 0.03 mg Ag/kg). The animals which were kept in the strainer of the S_L treatment, without direct contact to the Ag/AgNP containing sludge, showed a low Ag body burden of 1.92 ± 0.82 mg Ag/kg. However, for the animals with direct contact to the S_L sludge, a high body burden of 201.36 ± 33.25 mg Ag/kg was measured (Fig. 8).

Accumulation factors were calculated as the ratio of tissue concentrations to medium (AF_M) concentrations for the first exposure test (Table 2). Due to non-measurable Ag concentrations in the media of the control group no AF_M could be calculated. In contrast, AF_M values of 376 and 39,404 were calculated for the strainer group fed control sludge and the bottom group of the S_L treatment, respectively (Table 2).

3.1.1. Histological investigations using correlative microscopy

Two staining protocols based on osmium teroxide or iodine were

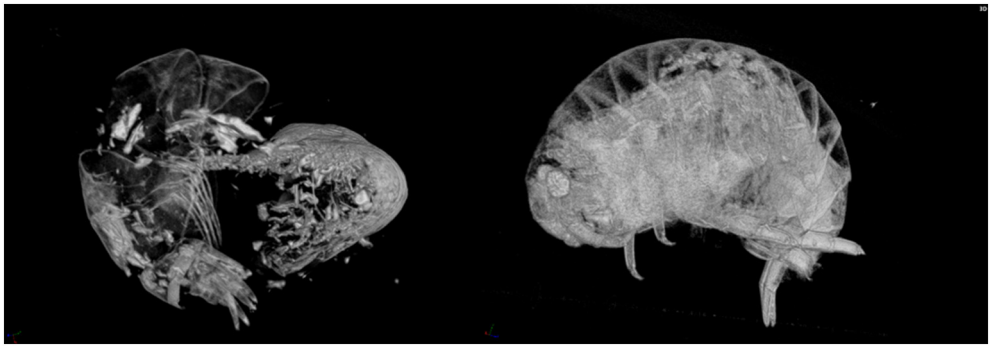

Fig. 2. Comparison of OsO_4 (left) and iodine (right) staining techniques via CT investigations.

Table 2
Calculated AF_M values for the amphipod groups in the two exposure studies.

Study	Treatment	Group	AF_M
Exposure Study I	S_0	Strainer	–
		Bottom	–
	S_L	Strainer	376
		Bottom	39,404
Exposure study II	S_0	Strainer	–
		Bottom	–
	S_L	Strainer	504
		Bottom	21,730
	$AgNO_3$	Strainer	363
		Bottom	396

tested to prepare the embedded experimental animals for correlative microscopy. Investigating the stained samples by CT showed that the iodine staining is obviously better suited for the maintenance of the tissue as described above (Fig. 2). Due to the size of the applied AgNPs the imaging of the Ag nanoparticles in the iodine stained *H. azteca* by CT would only have been possible after agglomerate formation. However, this could not be confirmed by the CT images which indicated a good dispersion of potentially ingested AgNPs even after direct exposure to NP enriched sewage sludge (Fig. 3, video material Video S1 and Video S2).

Using correlative microscopy, the morphology of *H. azteca* from the macro- to nanometer level of the thin and ultra-thin sections was investigated. Inspection of the SEM images showed that the nanoparticles were present in the intestine of the animals with direct contact to AgNP enriched sludge (S_L). Thus, this region (intestine and gut content) (ROI: region of interest) was noted for further investigations (Fig. 4; A).

The analysis with EDX in SEM showed a peak of Ag in the ROI (Fig. 4; C). This was confirmed by TEM investigations at a higher resolution after cutting the same region with an ultramicrotome. This nanospot analysis indicated an enormous Ag deflection during EDX analysis at the nanometer level, which confirmed the results obtained at the micrometer level in the SEM (Fig. 5).

SEM images of *H. azteca* (S_L treatment) with indirect contact to Ag during the first exposure test showed particles which are similar to the raw material of the applied nanoparticles in the intestine of the animals. However, this was not confirmed by the EDX analyses where no silver was detected but traces of P and Ca were found (Fig. 6). This was confirmed by the nanospot analyzes in the TEM (Fig. 7). Only a clear peak of Cu, potentially originating from the Cu-grid used in the ultramicrotome work, was observed.

3.2. Second exposure test

Considering the TWA Ag concentration measured in the media of the S_L treatment during the first exposure test (5.11 µg Ag/L; Table S1) a similar Ag media concentration was established for the $AgNO_3$ treatment with 5.41 µg Ag/L (Table S2). However, the TWA Ag

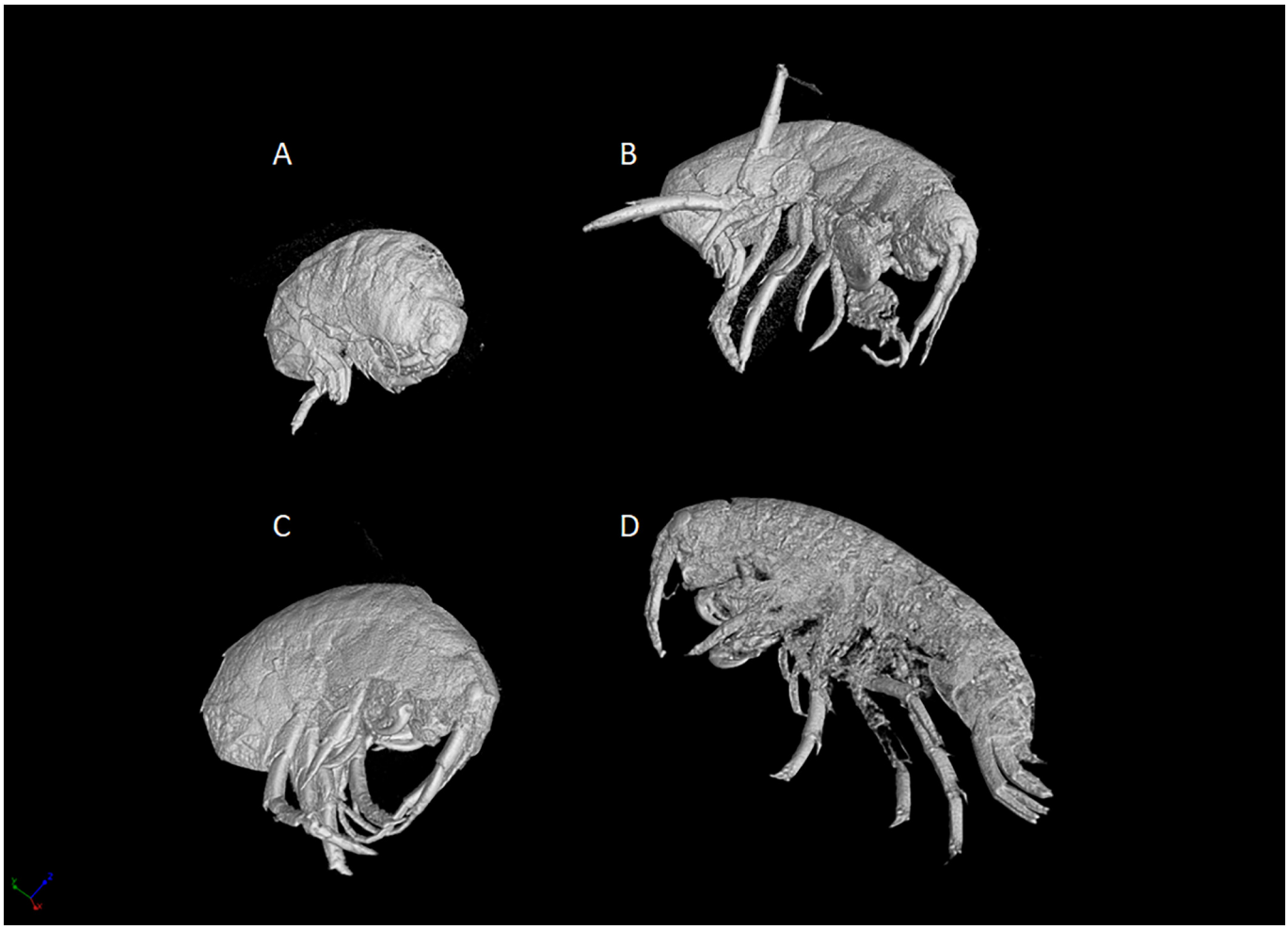

Fig. 3. 3D imaging after CT investigations of iodine stained *H. azteca*. Agglomerates or aggregates of NPs > 400 µm would have been visible as coloured dots. A: Hyalella from the strainer of S_L treatment; B: Hyalella from the bottom of the S_0 treatment; C: Hyalella from the bottom of the S_L treatment; D: Hyalella from the strainer of the S_0 treatment. Comparable to Video S1.

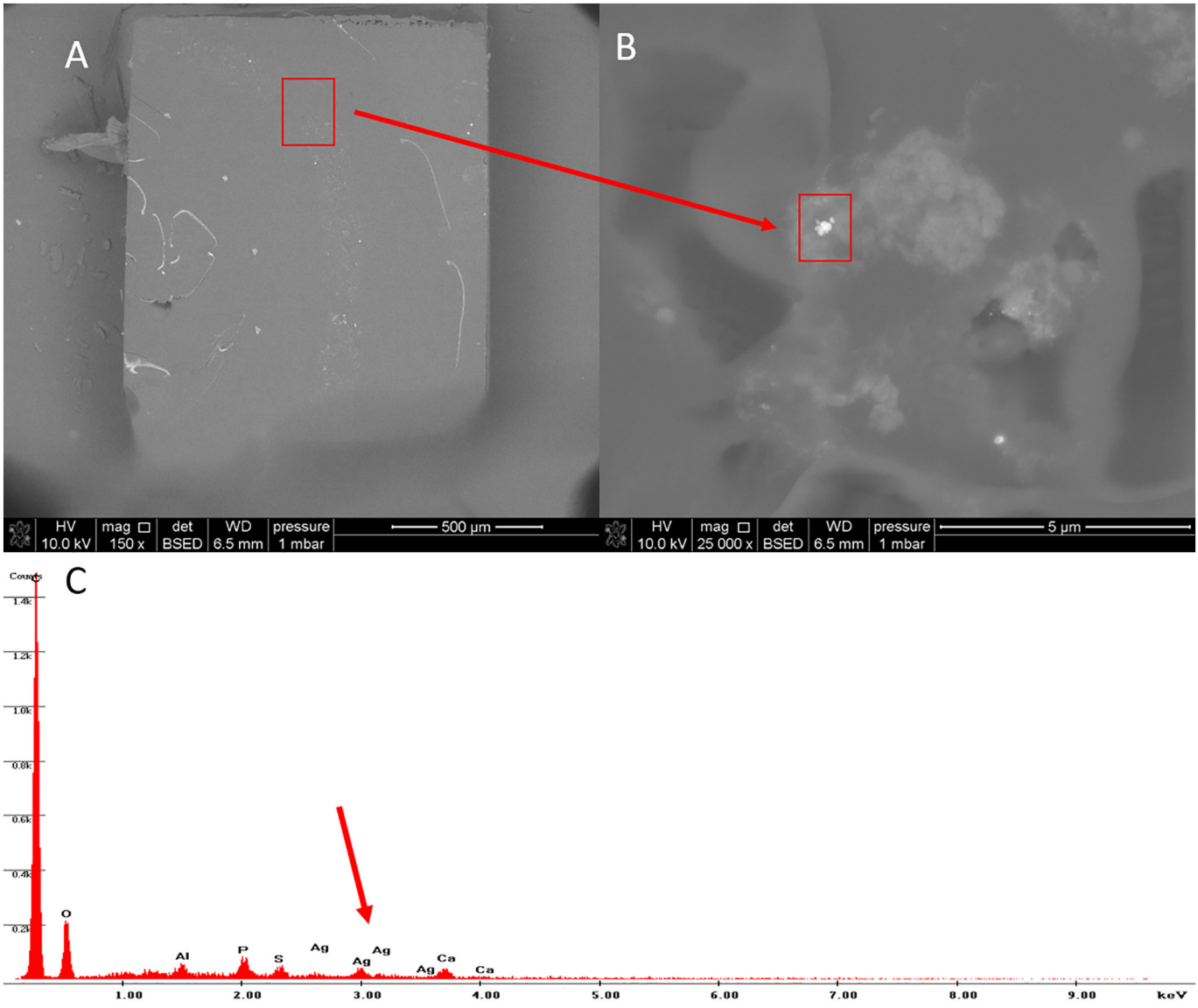

Fig. 4. SEM image of the intestine region of *H. azteca* from S_L treatment (first exposure test) with direct contact to the AgNPs enriched sludge. Marked ROI (red square) at A: gut content 150 x magnification and B: 25000 x magnification of the region from the red square of part A and EDX spectrum (C) with Ag peak (red arrow, bottom). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

concentration of the S_L treatment measured in the medium during the second exposure test was 16.74 µg Ag/L (Table S3) and thus around three times higher as compared to the first exposure test. Only the samples from day 7 of the S_L treatment showed a significant difference in media concentrations between bottom and strainer region (Table S4). There were no significant differences in $AgNO_3$ media concentrations measured at the bottom or in the strainer (Table S5). In both treatments, most of the particulate Ag or Ag^+ in the medium (around 99%) were supposed to be bound to organic colloids or precipitated as shown by the media concentrations measured after ultracentrifugation (Table S2 and S3). In agreement with the first exposure test, no measurable Ag concentration was present in the media of the S_0 control sludge treatment (Fig. 8).

The control animals of the second exposure test (7d) showed very low Ag tissue concentrations of 0.03 ± 0.02 mg Ag/kg and 0.18 ± 0.00 mg Ag/kg for the animals in the strainer and at the bottom of the beaker, respectively (Fig. 8). For the animals in the $AgNO_3$ treatment low Ag tissue concentrations were determined with 2.14 ± 0.27 mg Ag/kg and 1.96 ± 0.46 mg Ag/kg for the animals at the bottom of the beaker and in the strainer, respectively. The Ag concentrations in the animal tissue from the S_L treatment were higher than those from the first exposure study with 357.91 ± 77.09 mg Ag/kg and 8.31 ± 2.72 mg Ag/kg measured in animals with direct and indirect (strainer group) contact to the S_L-sludge, respectively (Fig. 8).

Also during the second exposure test no AF_M could be calculated for the control animals (fed with S_0 sludge only) due to the non-measurable Ag concentrations in the test media.

AF_M values of 504 and 21,730 were calculated for the animals collected from the S_L treatment (strainer and bottom, respectively). Lower AF_M values were determined for the two groups in the $AgNO_3$ treatment, with similar values of 363 and 396 for the strainer and bottom groups, respectively.

3.2.1. Measurement of total Ag in protein extracts of Hyalella after enzymatic protein digestion

At the end of the second exposure test, a small group of animals was

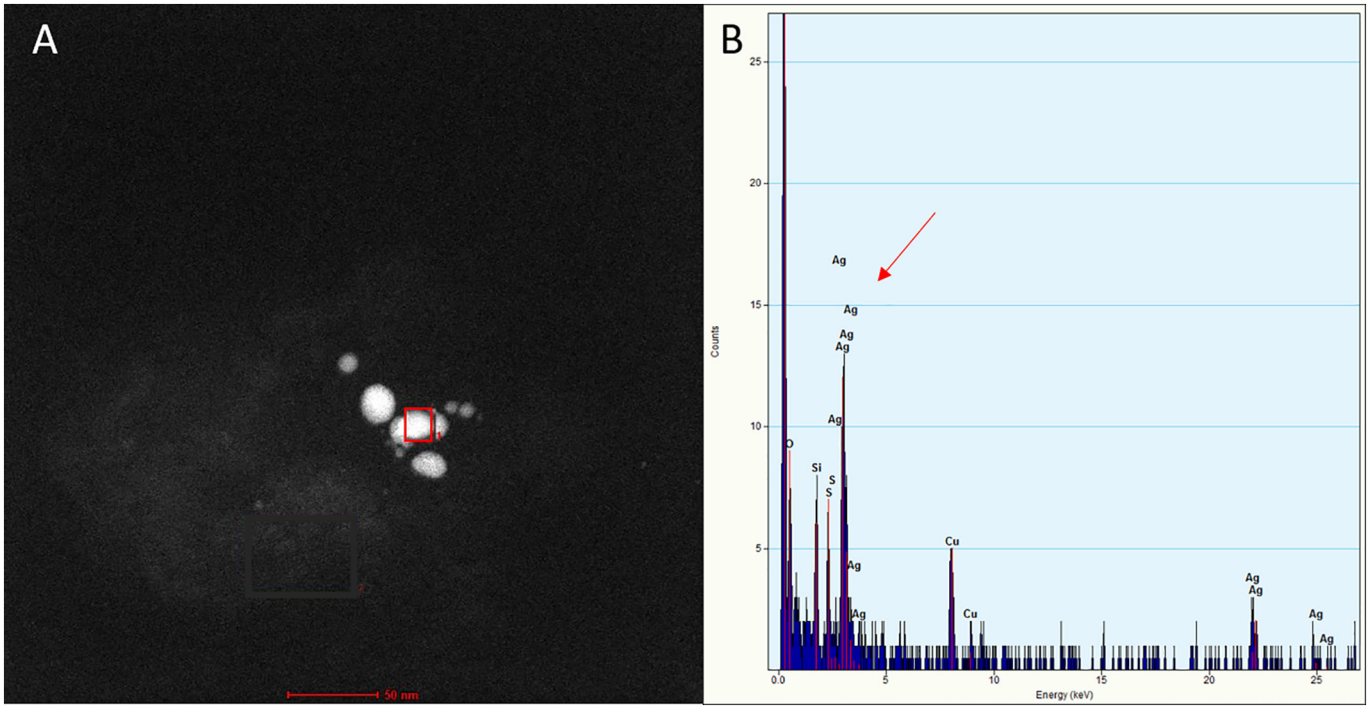

Fig. 5. A: TEM image of ROI after cutting with ultramicrotom and B: EDX spectrum of nanospot analysis with high Ag deflacltion.

collected from each treatment (same replicate beakers) to be enzymatically digested as described for the sp-ICP-MS samples. The total amount of Ag that was extracted with the proteins from the amphipods body was acid digested and determined using ICP-OES. For the $AgNO_3$ treatment, 57% of the total amount of Ag measured in the whole amphipods turned out to be extractable from the protein fraction. The total amount of silver (100%) was equivalent to the concentration measured after acid digestion of the whole animals. However, from the animals of the S_L treatment only 17% of the total amount of silver measured in the whole animals was extractable after enzymatic protein digestion.

3.2.2. Examination of aqueous test media and Hyalella tissue using single particle ICP-MS

By using sp-ICP-MS the number and mean size of Ag particles in the medium and animal samples collected during the second study were determined. Particle concentrations were determined in the media of S_L and $AgNO_3$ treatment. In both treatments the particle concentration increased during the exposure period. In the animal tissue from all treatments including the S_0 sludge particle concentrations were determined at the end of the test (day 7) with the highest value in the S_L treatment. The calculated mean particle sizes were determined for medium and tissue samples and are presented in Table 3.

4. Discussion

AgNPs reach the aquatic environment mostly as transformed hardly soluble silver sulphide (Ag_2S) after passing through the STP. It was recently reported that Ag from AgNPs, which are transformed and believed to be passivated, is still available for uptake by invertebrates exposed to STP effluents or sludge (Kampe et al., 2018; Kühr et al., 2018). This was confirmed in this study which was aimed at elucidating the pathways leading to the accumulation of silver from STP sludge containing AgNPs in *H. azteca*. The dietary uptake of AgNPs from the sludge as well as bioconcentration following dissolution of Ag^+ ions from the AgNPs was observed. However, no tissue penetration and accumulation of ingested particles was shown. This is in accordance with Zeumer et al. (2020) where the dietary uptake of wastewater borne AgNPs obviously did not lead to significant accumulation of Ag.

The AgNP containing sewage sludge used was obtained from a lab scale STP simulation unit running with NM 300 K spiked artificial influent according to OECD TG 303A (Organisation for Economic Co-operation and Develpement (OECD), 2001). The sludge was examined by TEM and EDX revealing that the AgNPs which were still present were sulfidized according to the authors (Kampe et al., 2018). The sludge used for the control groups (S_0) was taken from a control lab scale STP unit running on unspiked artificial influent. The measured total Ag content of around 1.2 mg/kg in the S_0 sludge should result from the initial active sludge used to inoculate the STP units obtained from a municipal STP (Kampe et al., 2018).

In all tests with STP sludge containing AgNPs measurable total Ag concentrations in the test media were found. With the $AgNO_3$ treatment an exposure scenario could be established which was comparable to the media concentrations measured in the STP-sludge treatment (S_L) of the first exposure test. The examination of the test media collected at the start of the second exposure tests using spICP-MS revealed that Ag particles were detected in the S_L and in the $AgNO_3$ treatments. The calculated median size of the particles detected in both media (aqueous phase) was nearly the same. However, a lower particle concentration was determined for the S_L treatment including STP sludge containing AgNPs. Kuehr et al. 2020 also observed Ag particles in an $AgNO_3$ exposure medium with comparable particle sizes. The observation was explained by the measurement of pseudo (presumable) particles (Kuehr et al., 2020) which is the result of two limitations of the spICP-MS method. First, the method cannot distinguish between the manufactured AgNPs and products of Ag^+ precipitations processes like e.g. AgCl or Ag_2S, or higher numbers of Ag^+ attached to colloids or proteins. Second, due to the small size of the NM 300 K AgNP that is near the instrumental size detection limit (around 10 nm for Ag, given by the Agilent MassHunter Software) or the particle detection limit of 16–20 nm as reported by (Lee et al., 2014). Also, the particles detected in the media collected at the start of the second exposure test were supposed to be pseudo particles. In the $AgNO_3$ treatment, which was carried out with control sludge containing no AgNPs, the detected particles could be Ag^+ that was complexed or associated with organic

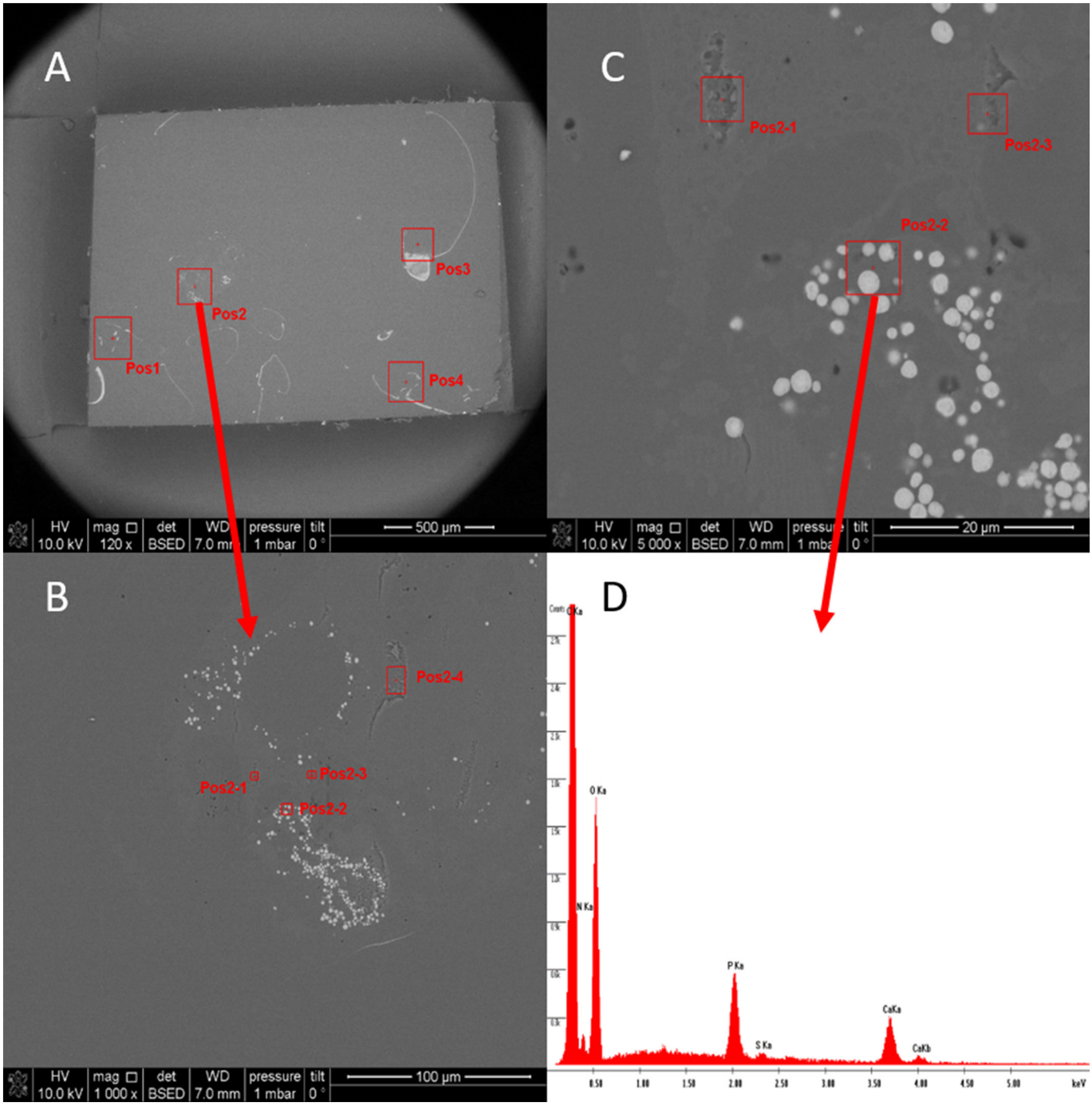

Fig. 6. SEM image of embedded *H. azteca* from S_L treatment (first exposure test) without direct contact to the AgNPs enriched sludge (strainer group) with marked ROIs (red squares, intestine and near region) at 120× (A), 1000× (B) and 5000× magnification (C) with EDX spectrum showing peaks for P and Ca but not for Ag (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

or inorganic colloids or precipitated Ag species like Ag_2S or AgCl (Degenkolb et al., 2018). Sulfidized AgNPs are still capable of releasing Ag^+ as described by Kampe et al. (2018) and Kühr et al. (2018). Particles detected in the S_L medium at the test start could be explained by the release of AgNPs from the STP sludge or by Ag^+ ions leaching from the AgNPs and following the same complexation pathway as described before. In the aged medium collected at the end of the second exposure test, nearly the same concentration of Ag particles was measured for the $AgNO_3$ medium. These pseudo particles showed a slightly higher calculated diameter, probably caused by a higher amount of Ag^+ associated with the colloids present compared to those measured at the test start. In contrast, the measured particle concentration in the aqueous media of the S_L treatment was three magnitudes higher in the aged media collected at the test end compared to the concentration in the fresh medium (after 24 h of aging) from the test start. Also the median particle size was higher compared to the test start and in comparison to the particles detected in the $AgNO_3$ medium, but nearly identical to the size of pristine particles (NM 300 K) and to the sulfidized particles measured in the S_L sludge by Kampe et al. (2018). Thus the particles which were measured at the test start were assumed to be an artifact similar to the particles found in the $AgNO_3$ medium.

Measurement of total silver concentrations in exposed animals

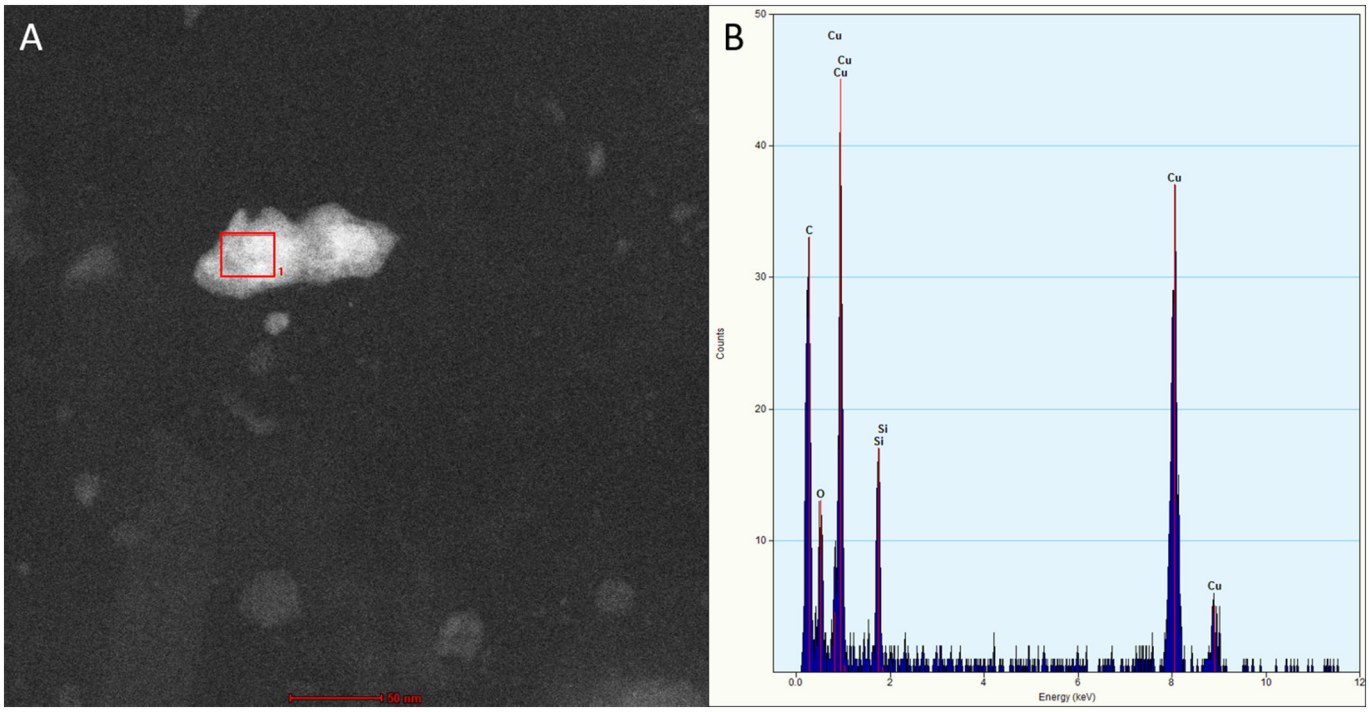

Fig. 7. TEM image of conspicuous (Figure 6) of an animal from the S_L treatment (first exposure test) without direct contact to the AgNPs enriched sludge (strainer group) showing high Cu but no Ag peak in the EDX spectrum (B).

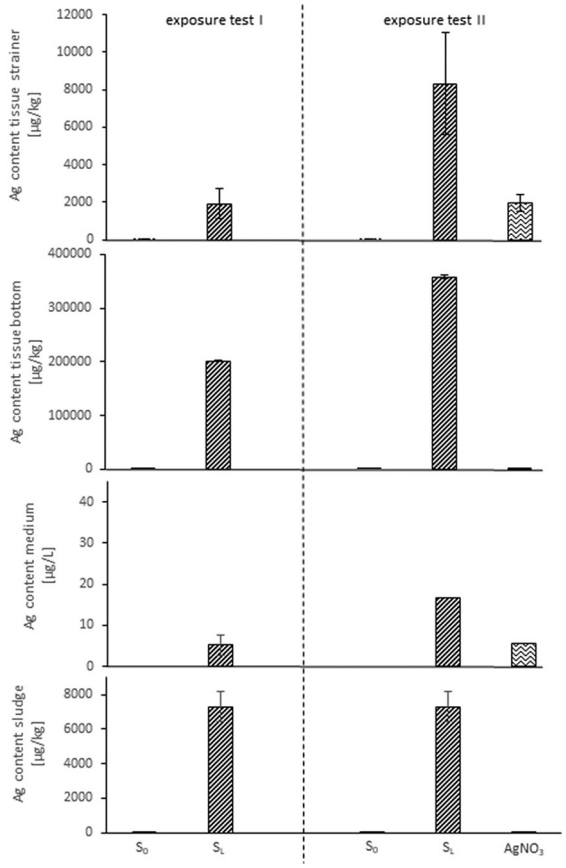

Fig. 8. Exposure test I-II. Measured total Ag concentration in sludge [mg/kg], media [µg/L] and tissues of experimental animals collected from groups exposed outside (bottoms) or inside the strainer (top) (Fig. 1).

showed that the highest tissue concentrations were observed in animals which were in direct contact with the contaminated sludge. Animals obviously ingested AgNPs as part of their diet. This was confirmed by single particle ICP-MS which was carried out to elucidate the presence of ingested particles in the previously exposed animals. Highest particle concentrations were measured in the animals which had access to contaminated sludge. However, particles could also be detected in animals of the $AgNO_3$ treatment and even in control animals. The spICP-MS method applied in this study is only suitable to prove the presence of AgNPs with a particle size > 20 nm. In addition, a differentiation between real AgNPs and measuring artifacts by the spICP-MS caused by high amounts of ions in the sample background is not possible. Thus, it cannot be excluded that the measured particles were only the result of an artifact due to the limitations of the measurement and calculation process of the spICP-MS. Due to the sample fitration using 0.45 µm syringe filters, we cannot exclude that particles or agglomerations of particles with a size higher than 450 nm had not been available for the spICP-MS measurements. However, such big particles would have been visible at least by the TEM observation during correlative microscopy.

Correlative microscopy allows the combination of different microscopic methods to bridge the macrostructural analysis like light microscopy (morphological orientation and information) with the nanostructural analysis like TEM for clear characterisations of special spots with ultra high resolution within ROIs. Furthermore, the correlative workflow allows examination of the same sample for all methods of each step of the workflow.

Correlative microscopy was applied in this study to confirm the uptake of AgNPs by *H. azteca* and to further elucidate the potential tissue penetration and accumulation of the ingested particles. TEM observations, which are carried out as part of the correlative microscopy workflow, allow detection of particulate Ag without false detection of Ag that is complexed or associated with organic matter (Kampe et al., 2018). Thus the risk of detecting artifacts, like the measurement of pseudoparticles, is not possible. Even though correlative microscopy provides results which are not quantitative, the methods applied allow a precise localization and characterization (indication of

Table 3
Particle concentrations (particles/L) in the media samples from day 4 and 7, and animals tissue from day 7. Diameter of particles in the samples were calculated median diameters in nm.

Treatment	Matrix	Concentration [particles/L]		Diameter [nm]	
		Day 0	Day 7	Day 0	Day 7
S_0	Media	–	–	–	–
	Animals	–	$2.2 \times 10^8 \pm 1.5 \times 10^7$	–	17.2 ± 0.2
S_L	Media	$3.7 \times 10^8 \pm 3.0 \times 10^7$	$1.5 \times 10^{11} \pm 2.2 \times 10^9$	11.5 ± 0.2	15.0 ± 0.0
	Animals	–	$2.5 \times 10^{12} \pm 3.4 \times 10^{11}$	–	18.5 ± 0.4
$AgNO_3$	Media	$5.0 \times 10^9 \pm 2.0 \times 10^9$	$6.0 \times 10^9 \pm 4.6 \times 10^9$	11.2 ± 0.5	13.5 ± 0.8
	Animals	–	$8.2 \times 10^9 \pm 4.6 \times 10^9$	–	17.6 ± 1.2

transformation) of the NPs. In this way it was shown that there were only very low amounts of AgNPs in the gut of the amphipods (S_L treatment) and no AgNPs within the animals tissue. Also, no AgNPs were found in the animals with no direct contact to AgNP enriched sludge. From this it can be concluded, that the AgNPs were only taken up from the sludge, but not incorporated into the organisms tissue or cells. Furthermore, the correlative microscopy revealed that the quantification of the Ag body burden by ICP-OES and spICP-MS was not influenced by NPs attached to the carapace. Thus the measured Ag body burden only represents the AgNPs in the gut content and potentially other Ag species like Ag^+ in non particulate form which were taken up via bioconcentration processes.

By using the method of spICP-MS high concentrations of measured particles (< 20 nm) were found in animals collected from control and all treatments at the end of the second study. However, no Ag particles could be identified by correlative microscopy in control animals even though a low level of Ag was detected in the tissue. This was potentially caused by the ingestion of the control sludge containing a low amount of silver as background contamination. Animals collected from the S_L strainer group, without direct contact to the STP sludge containing high Ag concentrations, showed accumulation of silver. However, no Ag particles could be observed in the animals using correlative microscopy providing clear evidence that Ag was supposed to be accumulated following uptake of Ag^+ ions released from the sludge.

As described for several invertebrates and aquatic species, the bioaccumulation of Ag, especially after the exposure of Ag ions, occurs rapidly and often leads to high body burdens, due to effective and active uptake mechanisms and binding of Ag to proteins as a detoxification strategy (Croteau et al., 2011; Hogstrand et al., 1996; Kuehr et al., 2020; Waalewijn-Kool et al., 2014). Also in this study, Ag^+ accumulated by bioconcentration processes or incorporated after release from the gut content seems to be associated to proteins like metallothioneins (Ahearn, 2010). This could explain, why around 57% of the total amount of the Ag body burden found in the animals collected from the $AgNO_3$ treatment was detected in the protein solution prepared from the same sample material. In comparison, only 17% of the total amount of Ag found in animals from the S_L treatment, with direct contact to the contaminated sludge, was detected in the animals protein fraction which may be explained by the lower concentration of Ag^+ due to the limited and delayed release of Ag^+ from the sulfidized AgNP surface.

Nevertheless, the measured accumulation of Ag in the groups separated in strainers was very limited. This can be explained by the observation that Ag^+ released from the sludge was apparently not freely dissolved as confirmed by the $AgNO_3$ treatment. The results of Ag measurements following ultrafiltration of the S_L media from the first and second exposure tests have both shown that around 99% of the total silver measured in the medium was present in a particulate or colloidal form that could not pass through 3 kD filter membrane. This could be explained by precipitates, suspended AgNPs, or Ag^+ bound to humic acids, proteins or other organic colloids (Degenkolb et al., 2018; Kaegi et al., 2011; Kampe et al., 2018). The assumption that Ag^+ is associated with organic or inorganic colloids is in agreement with the observation that around 99% of the measured total Ag in the medium of the $AgNO_3$ treatment were also present in a particulate form or associated with colloids bigger than 3 kD as shown by ultrafiltration.

The test design used for this study allowed investigation of the major pathways involved in the accumulation of Ag from AgNPs in STP sludge. The calculation of accumulation factors as the ratio of total Ag concentrations in the aqueous media helped to evaluate the impact of dietary uptake and bioconcentration processes leading to the Ag accumulation in *H. azteca* observed in this study.

No significant difference between the AF_M values of the top and bottom group of the $AgNO_3$ treatment was observed (363 vs. 396). Both groups were kept in contact with control sludge and exposed to the same test medium. In contrast, AF_M values calculated for the S_L treatment (second study) reveal that there was a significant difference regarding the accumulation of Ag in the top and bottom group (376 vs. 39,404). Groups of both treatments (S_L and $AgNO_3$) were exposed to the same medium concentration, but fed on different types of sludge in the bottom group underlining the major contribution of dietary uptake to the measured Ag body burden and the apparent accumulation of Ag in this exposure scenario.

AF_M values observed in the S_L treatment from the second exposure study confirm the significant difference in the calculated AF_M values regarding the accumulation of Ag in the top and bottom group (504 and 21,730). However, AF_M values calculated for the animals in the strainer (top) were higher compared to the first study. During the first and second study sludge with the same concentration was applied in the S_L treatment but leading to different media concentrations. This may be explained by the different amount of sludge that was provided to the vials to guarantee feeding ad libitum of the test animals. Bigger animals were exposed during the second study. Consequently, the application of the higher amount of sludge during the second study led to higher total Ag concentrations in the test media compared to the first test as reflected in the AF_M values.

Ag^+ is well known to induce lethal and sublethal toxicological effects, especially in aquatic species (Leblanc et al., 1984; Sakamoto et al., 2015; Wang et al., 2012; Zhao and Wang, 2011). However, no mortality or significant effects were observed in both exposure studies. The presumable complexion of Ag^+ from the $AgNO_3$ treatment and potentially released Ag^+ from AgNPs of the enriched sludges may explain the lack of significant effects or mortality in comparison to the S_0 control group in both studies, even if *H. azteca* is considered to be the most sensitive benthic species for Ag^+ exposure (Blaser et al., 2008). The lack of observed effects is in accordance with the results of Kühr et al. 2018, where the waste water borne AgNPs did not cause any negative effects in contrast to water borne pristine AgNPs. Comparable observations were made by Hartmann et al. (2019) when pristine AgNPs from NM 300 K caused a significant reduction of the reproduction in *Daphnia magna*, while wastewater borne NM 300 K AgNPs, which were proved to be transformed to Ag_2S, did not cause any significant differences in the number of offspring (Hartmann et al., 2019).

5. Conclusions

AgNPs such as NM 300K can be ingested by the benthic amphipod *Hyalella azteca* by dietary uptake from STP sludge as shown in this study. However, even though the NPs were shown to be ingested, we did not find any evidence for an incorporation of the NPs or transfer from the gut into the animals tissue or cells. However, the bioaccumulation of Ag^+ released from the sulfidized AgNPs, was confirmed. The accumulation of Ag from AgNPs may lead to a higher Ag body burden in the animals and the amphipods may thus accelerate the transfer of heavy metals from NPs accumulated in the sediment into the aquatic food chain (Vogt et al., 2019).

It was shown that investigations on the bioavailability and bioaccumulation of NPs in aquatic organisms should be complemented by the methods of correlative microscopy. The reason for this is that the established method of spICP-MS often detects artifacts, such as presumable and/or pseudo particles, but cannot confirm (or conclude) that they are in fact artifacts. Further investigations utilizing imaging methods with higher resolution, such like synchrotron radiation x-ray imaging, are required to gain more information on the distribution of the metal within the organism to allow a more precise conclusionon the bioavailability and bioaccumulation of AgNPs.

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Abbreviations

AF_M	Accumulation factor based on the Ag concentration in the medium
Ag^+	Silver (I) ion
AgNPs	Silver nanoparticles
ANOVA	Analysis of variance
BSE	Backscattered electron
EDX	Energy dispersive x-ray spectroscopy
SP-ICP-MS	Single particle inductively coupled plasma mass spectrometry
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma optical emission spectrometry
NP	Nanoparticle
OECD	Organization for Economic Co-Operation and Development
PMMA	Polymethylmethacrylate
ROI	Region of interest
SEM	Scanning electron microscopy
STP	Sewage treatment plant
TEM	Transmission electron microscopy
TWA	Time weighted average concentration
UHQ water	Ultra high quality water

CRediT authorship contribution statement

Sebastian Kuehr:Methodology, Investigation, Writing - review & editing.**Jessica Klehm**:Methodology, Investigation, Visualization, Writing - review & editing.**Claudia Stehr**:Methodology.**Matthias Menzel**:Methodology.**Christian Schlechtriem**: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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Supporting Information

Unravelling the uptake pathway and accumulation of silver from manufactured silver nanoparticles in the freshwater amphipod *Hyalella azteca* using correlative microscopy

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SA. Sewage treatment plant simulation and preparation of sewage sludge

For the production of the sewage sludge a lab scale STP simulation was carried out according to OECD TG 303A [1] providing sewage treatment plant sludge. Fresh active sludge was obtained from a municipal STP and used to inoculate the model STP units. STP simulation approaches were carried out with and without addition of AgNPs to generate Ag-STP and control-STP sludge. The addition of AgNPs to the test system was achieved by supplementing the influent (test sewage) with a concentration of 5 mg/L Ag from NM 300K for 18 days [2].

Sewage sludge collected from the model STPs was centrifuged for 15 mins at 4.700 rpm (4.149 g) (Avanti J-26SXP, Beckmann Coulter). The supernatant liquid was decanted and the sludge was centrifuged again under the same conditions. After removing the new supernatant liquid the sludge was dried for 72 h at 65 °C. The dried sludge was milled to a fine powder for 5 min using a ball mill (Retsch RM100).

SB. Preperation and analysis of media samples

The water samples (n=3 for each treatment) were analysed directly after ultracentrifugation for 60 min at 3500 rpm (2851 g) (Allegra® X-15R, Beckmann Coulter) using ultrafiltration units (Vivaspin 20, 3,000 MWCO PES, Sartorius). The filtrate was used to measure the concentration of Ag+, while the filter residue was analysed for its Ag content as an indication of AgNPs in the water phase.

Collected samples of aqueous test media were prepared and analyzed as described by Kühr et al. (2018) [3]. The samples werediluted with aqua regia at a ratio of 1:10 (1mL sample diluted with 9 mL aqua regia) and digested using a microwave (turboWave® Inert, MLS; max temperature 220 °C, max pressure 40 bar). Aqua regia was prepared by mixing nitric acid and hydrochloric acid at a ratio of 1:3. HNO_3 (69%, suprapure grade) was purchased from Roth (Karlsruhe, Germany) and HCl (30%, suprapure grade) from Baker (Netherlands). The digested solution was filled up to a final volume of 15 mL with UHQ water and analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES), as described by Kühr et al. (2018) [3]. For the analysis an Agilent 720 ICP-OES (Agilent Technologies) was used to measure the total silver concentrations in the aqueous media at a wavelength of 328.068 nm. Before each measurement the calibration of the instrument was carried out using blank and calibration solutions covering a range of 0.1 up to 100 µg/L. The calibration standards were prepared by dilution of a commercially available certified Ag ICP standard solution containing 1,000 mg/L Ag in 3% nitric acid (Merck, Darmstadt, Germany) with diluted aqua regia solution. The calibration function was calculated using a linear regression algorithm of the ICP-OES software. Aqueous certified reference material TM 25.4 (Environment Canada; certified concentration 22.0 µgAg/L) was analyzed along with all samples. The analytical method was also verified using a multi-element Merck IV Standard, diluted to 50 µg/L. For each standard and sample, at least three measurements were taken and the mean concentration determined by the ICP-OES software. The recovery for these quality assurance samples was $\pm$ 15%.

SC. Preparationand analysis of sewage sludge and *Hyalella acteca* samples

To meausure the total Ag content of sewage sludge loaded on glass fibre filters disposable scalpels (Braun, Cutfix®) were used to remove sludge samples from the filter surface (n = 5, each consisting of the loading of one single filter). The analysis of

total Ag concentrations in sewage sludge and *Hyalella azteca* collected during the studies were carried out according to Kühr et al. (2018) [3] where 8 ml of aqua regia (3:1; nitric acid: hydrochloric acid) were added to the samples prior to digestion in a microwave (turboWave® Inert, MLS; max temperature 220 °C, max pressure 40 bar). The heating time was set to 25 minutes. The samples were heated up to 220°C at a maximum pressure of 40 bar and the maximum temperature was kept constant for 30 minutes for the extraction in aqua regia. The digested samples were diluted to 15 mL using UHQ water. The silver analysis was performed by ICP-MS (Agilent 7700 ICP-MS, Agilent Technologies). Ag was quantified as described above, following the method described by Wasmuth et al. 2016 [4] using the isotope ^{109}Ag. A rhodium standard (Merck KGaA; CertiPUR) was applied as internal standard. All concentrations were evaluated using data obtained in the no-gas mode of the analytical device.

SD. Examination of Hyalella tissue and aqueous test media by single particle ICP-MS

All media and digested samples were diluted with UHQ water by a factor of 10^2 – 10^5 prior to the measurement to reach a particle concentration of 200 - 2000 particle events per minute and an Ag concentration in the range of ng/L [5, 6]. Peak detection and integration was conducted automatically by the Agilent MassHunter software and converted into particle sizes. Ag was measured as the isotope ^{107}Ag. The determination of the nebulization efficiency was carried out using freshly prepared dispersions of 60 nm gold nanoparticles (AuNPs 60 nm, BBI solutions, UK). The threshold between background and particle signals was defined and based on visual inspection of the measured signal distributions.

Sampling	Replicates [µg/L]	Mean of Replicates [µg/L]	TWA [µg/L]	percentage of total Ag found in the supernatant [%]	percentage of total Ag found in the filtrate [%]
day 0*	4.45 2.94 0.57	2.65		99.42	0.33
day 4	5.45 4.35 7.46	5.75	**5.11**	85.17	14.83
day 7	12.78 11.18 7.58	10.51		99.60	0.40

Tab. S1. Ag media concentrations of the samples used to determine the TWA for the calculation of AF_M of the S_L treatment in the first exposure test. * After 24 h of equilibration.

Sampling	Replicates [µg/L]	Mean of Replicates [µg/L]	TWA [µg/L]	Percentage of total Ag found in the supernatant [%]	Percentage of total Ag found in the filtrate [%]
day 0*	8.16 6.99 6.61	7.25	**5.41**	99.33	0.67
day 4	4.41 4.56 3.94	4.30		99.42	0.58
day 7	3.43 3.25 2.88	3.19		96.86	3.14

Tab. S2. Ag media concentrations of the samples used to determine the TWA for the calculation of AF_M of the $AgNO_3$ treatment in the second exposure test. Also, percentages of Ag found in the supernatant and filtrate with regard to the total measured Ag concentrations. * After 24 h of equilibration.

Sampling	Replicates [µg/L]	Mean of Replicates [µg/L]	TWA [µg/L]	percentage of total Ag found in the supernatant [%]	percentage of total Ag found in the filtrate [%]
day 0*	0.40 0.57 0.49	0.49		85.71	14.29
day 4	25.15 25.57 25.28	25.33	**16.74**	99.63	0.37
day 7	39.23 39.74 40.19	39.72		99.70	0.30

Tab. S3. Ag media concentrations of the samples used to determine the TWA for the calculation of AF_M of the S_L treatment in the second exposure test. Also, percentages of Ag found in the supernatant and filtrate with respect to the total measured Ag concentrations. * After 24 h of equilibration.

Sampling	Replicates [µg/L]	Mean of Replicates [µg/L]	± SD [µg/L]
Start/ day 0	strainer	0.46	0.11
	bottom	0.51	0.15
day 4	strainer	24.52	2.49
	bottom	26.15	2.84
day 7	strainer	37.84*	0.54
	bottom	41.59*	0.26

Tab. S4. Ag media concentrations of the samples taken from the top or bottom of the beakers of the S_L treatment in the second exposure test. * After 24 h of equilibration. Differences between the concentrations of the medium from inside the strainer and from the bottom of the strainer were only statistically significant ($p \leq 0.05$) between the replicates (n=3) from day 7.

Sampling	Replicates [µg/L]	Mean of Replicates [µg/L]	± SD [µg/L]
day 0*	strainer	7.72	0.78
	bottom	6.79	0.54
day 4	strainer	4.31	0.29
	bottom	4.29	0.24
day 7	strainer	3.07	0.17
	bottom	3.31	0.35

Tab. S5. Ag media concentrations of the samples taken from the top or bottom of the beakers of the $AgNO_3$ treatment in the second exposure test. * After 24 h of equilibration. Differences between the concentrations of the medium from inside the strainer and from the bottom of the strainer were not statistically significant ($p \leq 0.05$) between the replicates (n=3).

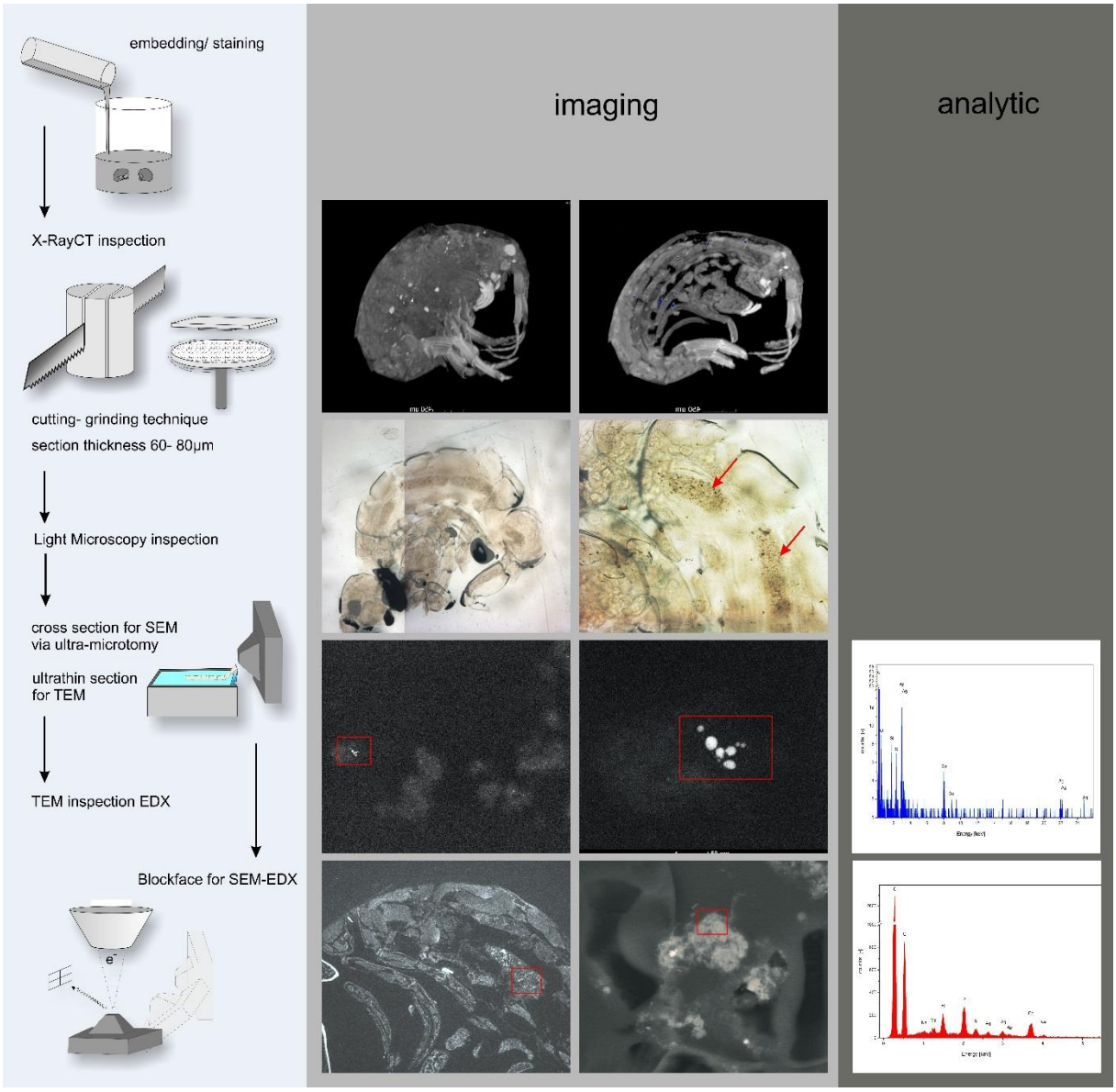

Figure S1. Overview of the work steps of the morphological imaging of AgNPs in H. azteca by correlative microscopy.

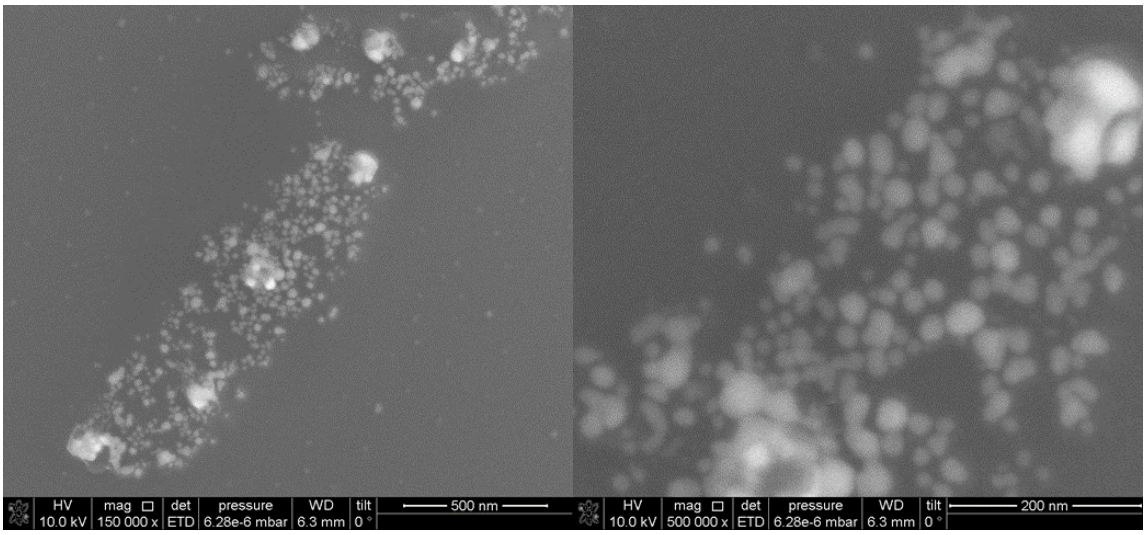

Figure S1. NM 300 AgNP raw material visualized using SEM with 150,000 x (left) and 500,000 x magnification (right).

Fig. S3. Single CT image of male *H. azteca*

Video S1. Sagital scan can trough the body of a male *H. azteca* for the identification of potentially bigger NP agglomerates.

Video S2. 3D imaging after CT investigations of iodine stained *H. azteca*. Can be best seen with 3D glasses. Made of a series of single CT images as presented in Fig. S1. Agglomerates, aggregates or bigger foreign particles would have been visible as colored dots.

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Chapter 6: Ingestion of bivalve droppings by benthic invertebrates may lead to the transfer of nanomaterials in the aquatic food chain

Chapter 6 consists of the following publication

Environmental Sciences Europe

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

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Funding acquisition; design of study, resources; writing – review & editing; supervision

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RESEARCH Open Access

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

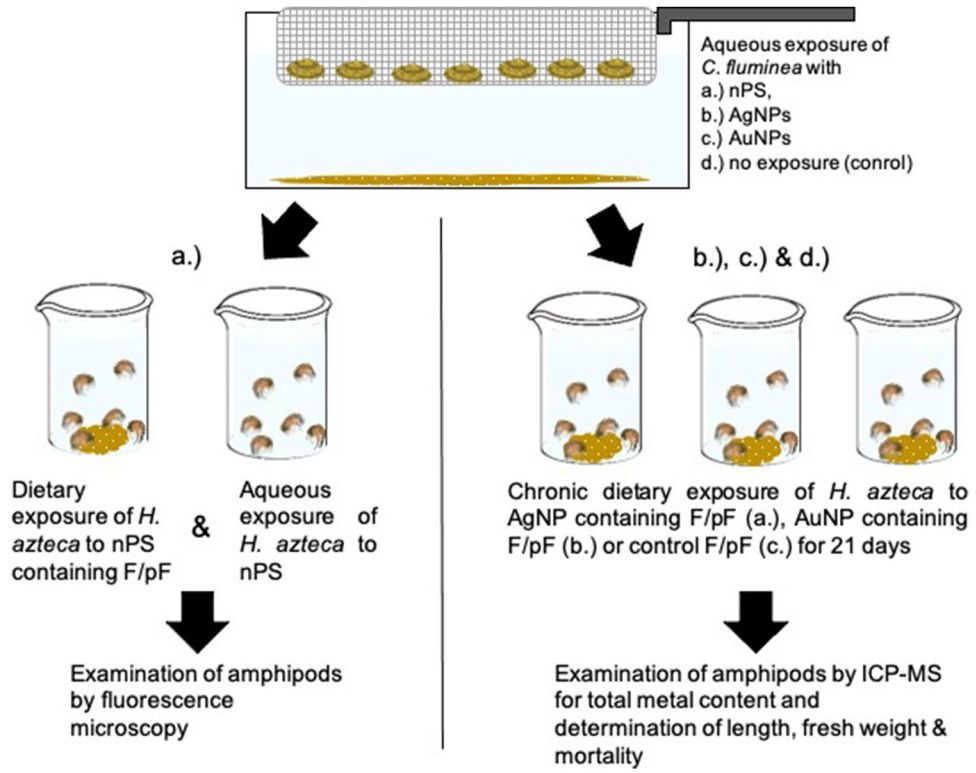

Fig. 1 Scheme of the general exposure and study design

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.

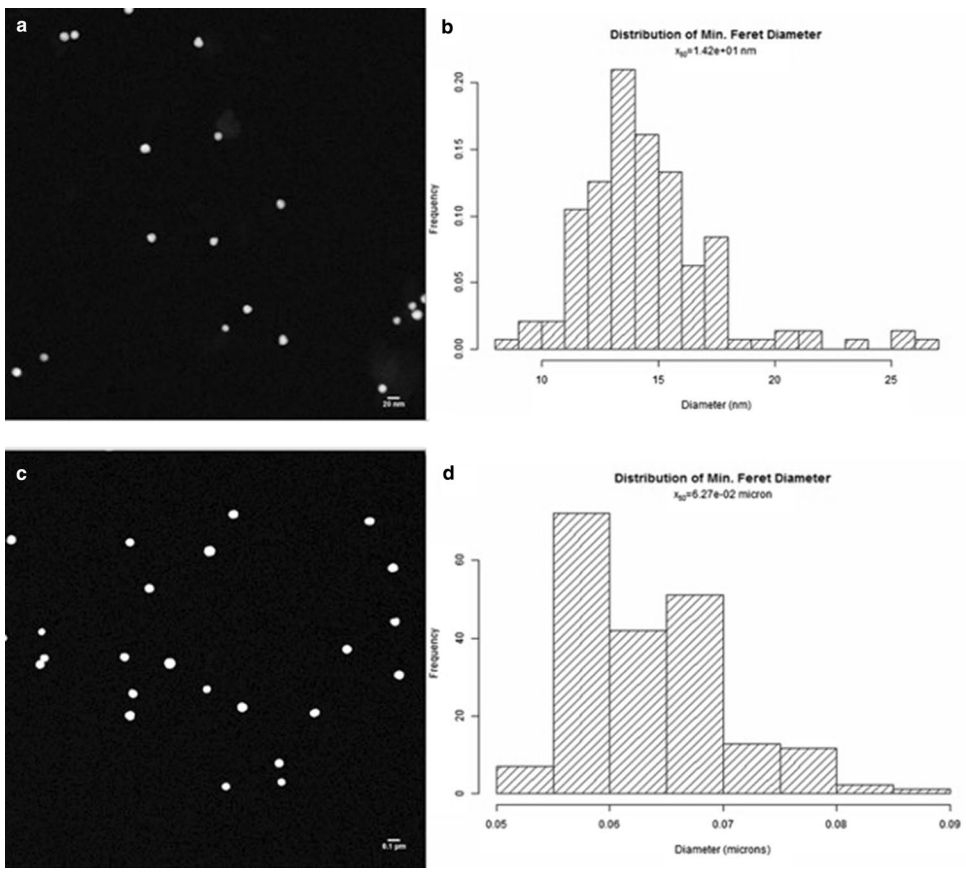

Fig. 2 TEM images (high angular annular dark field, HAADF) of NPs and histograms of the particle size distribution. **a** AgNPs, indicated magnification 35 kx, scale bar shows 20 nm; **b** AgNPs, histogram based on 143 particles; **c** AuNPs, indicated magnification 30 kx, scale bar shows 0.1 µm; **d**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

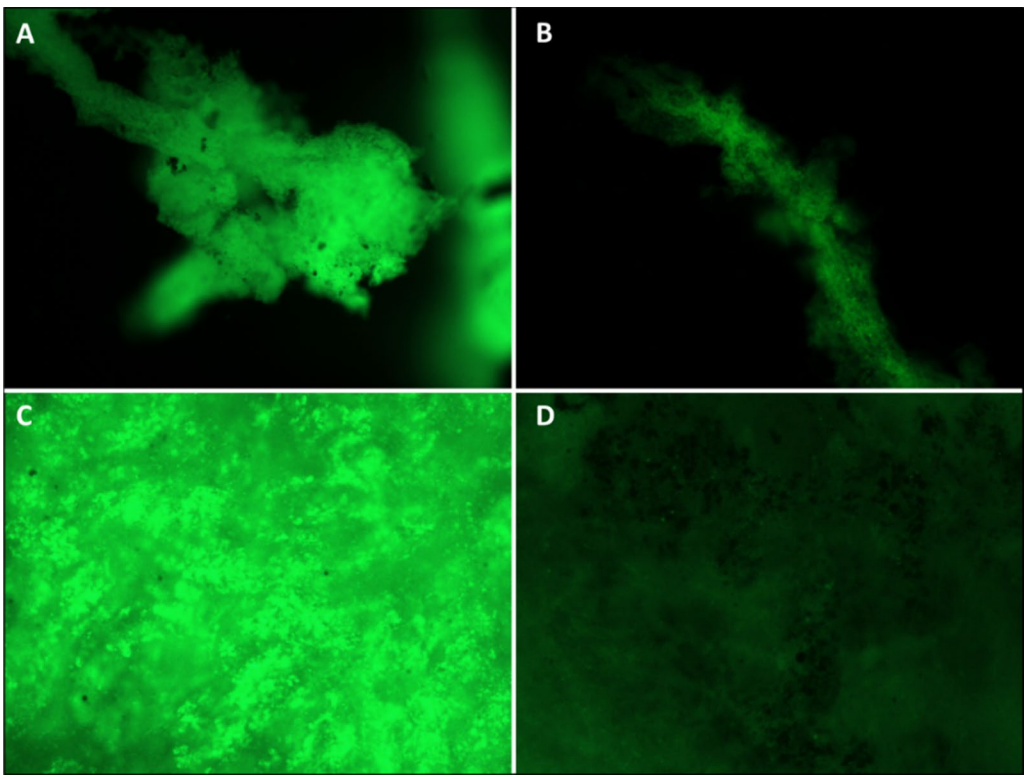

Fig. 3 Samples from the nPS bioavalibility test with *C. fluminea* observed under FM via fluorescence channel. **a** F/pF after 24 h of nPS exposure; **b** F/pF after 12 h of depuration; **c** viscera after 24 h of nPS exposure; **d** viscera after 12 h of depuration

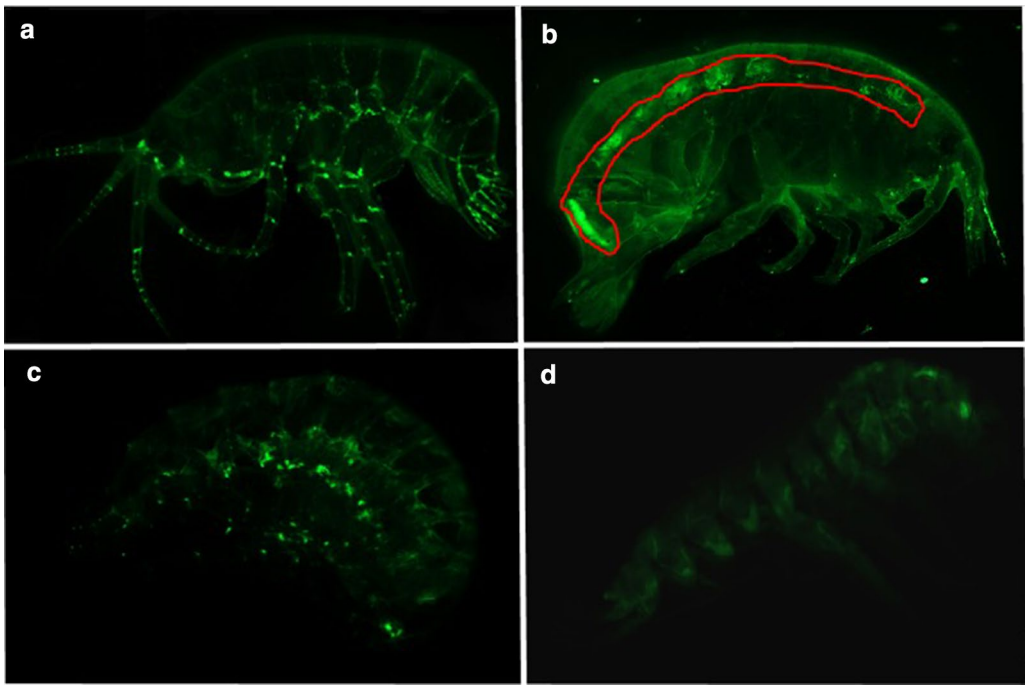

Fig. 4 FM pictures (flourescence channel) of *H. azteca* sampled from the nPS bioavailability tests. **a** After 24 h of aqueous exposure, **b** after 24 h of dietary exposure (the red mark shows the gut area), **c** after 24 h of depuration after aqueous exposure, **d** after 24 h of depuration after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

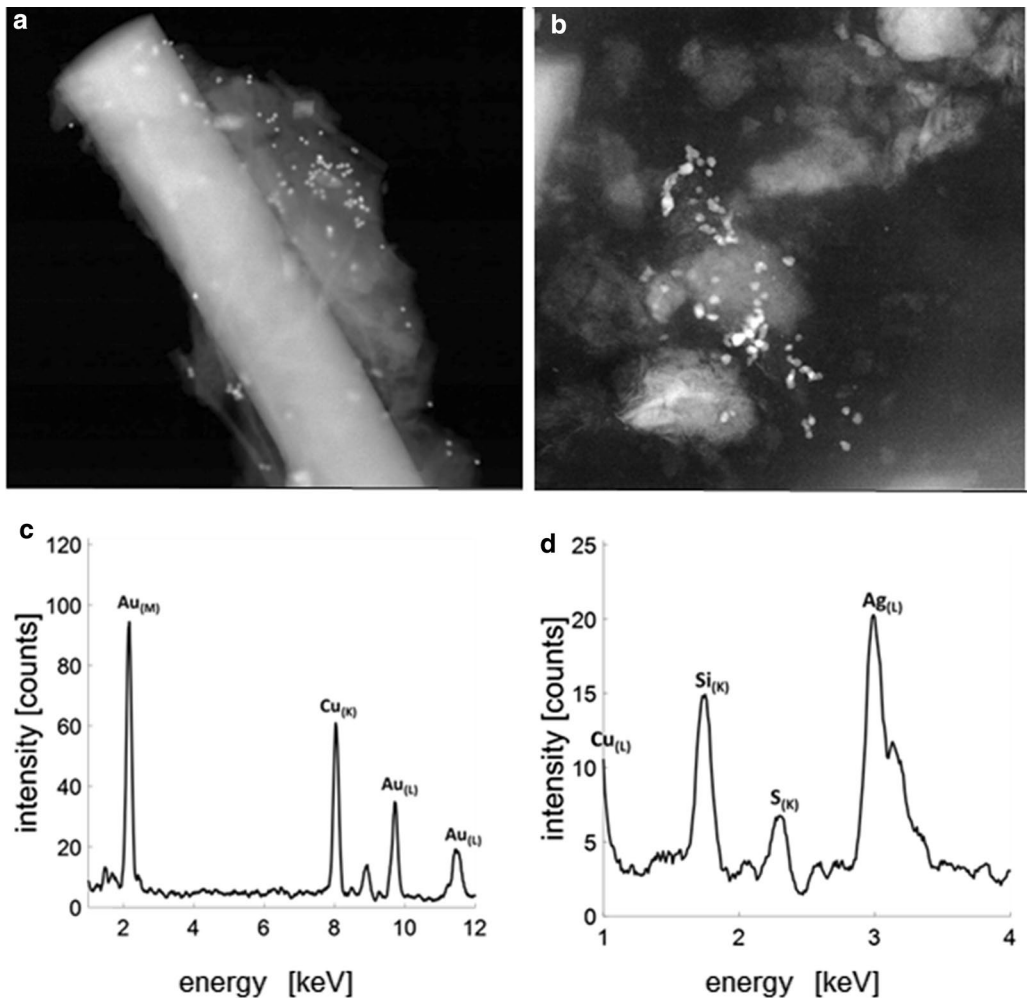

Fig. 5 TEM (high angular annular dark field, HAADF) images and the corresponding energy dispersive x-ray (EDX) spectrum of NPs enriched in the F/pF obtained from previously exposed *C. fluminea*. **a** AuNPs, magnification 15 kx; **b** AgNPs, magnification 110 kx; **c** AuNP, EDX signals; **d** AgNPs,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

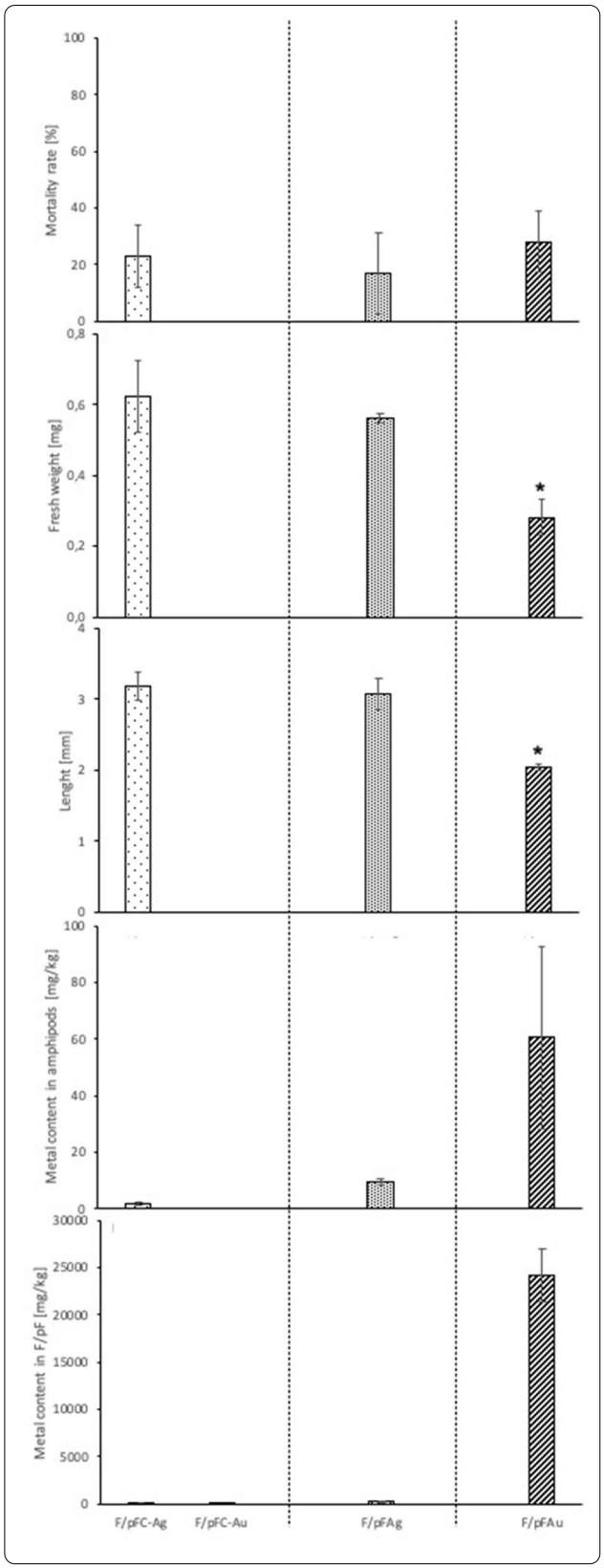

Fig. 6 Results of chronic exposure test with *H. azteca* fed F/pF enriched with AgNP or AuNPs. Measured Ag and Au mean content in generated F/pF (mg/L) ± SD from *C. fluminea* ($n = 4$) as well as the respective total metal body burden of amphipods ($n = 5$, replicates consisting of 11 to 19 amphipods) 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

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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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Declarations

Ethics approval and consent to participate
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Consent for publication
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Competing interests
The authors declare that they have no competing interests.

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Publisher's Note

Supporting Information

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

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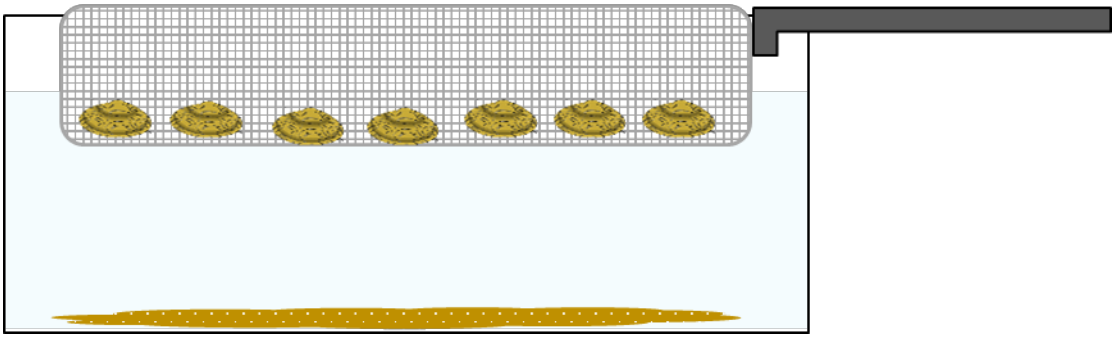

Figure SI 1: Exposure scenario for the production of NP enriched F/pF.

Figure SI 2: F/pF loaded feeding filters. Left: control F/pF; middle: AuNP containing F/pF$_{AunP}$; right: AgNP containing F/pF$_{AgNP}$.

Table SI 1: Chemical and physical parameters of the copper reduced tap water used for the acclimatization and test phases.

Parameter	Conductivity [µS/cm]	pH	Nitrate [mg/L]	Nitrite [mg/L]	Ammonium [mg/L]	Phosphate [mg/L]	NPOC [mg/L]	Cl [mg/L]	Ca [mmol/L]
Acclimatization	234	7.8	8	<0.005	<0.01	<0.10	0.6161	<0.02	0.82
Tests	245	7.9	10	<0.005	<0.01	<0.10	0.7435	<0.02	0.80

Parameter	Mg [mmol/L]	Cd [µg/L]	Cr [µg/L]	Cu [µg/L]	Fe [µg/L]	Mn [µg/L]	Ni [µg/L]	Pb [µg/L]	Zn [µg/L]
Acclimatization	0.4	0.00785	0.053	1.200	<0.443	0.275	0.380	<0.002	<0.576
Tests	0.5	0.00688	0.103	0.591	<0.443	0.270	0.251	<0.002	<0.576

Table SI 2: Values from temperature, pH and oxygen monitoring of the F/pF$_{control}$ treatment during the chronic exposure test with H. azteca.

Day	Temperature [°C]	pH	Oxygen	
			mg/L	%
0	22.0	7.81	8.81	111.5
1	23.1			
2	22.3	8.03	7.92	102.4
3	21.9			
4	21.3			
5				
6	22.7	7.85	8.28	104.5
7	22.5			
8	22.0			
9	21.9	7.72	7.95	99.7
10	22.5			
11				
12	22.4	7.74	7.81	97.5
13	23.3			
14	23.4	7.73	7.82	100.3
15	23.4			
16	22.6	7.69	7.66	97.2
17	22.2			
18	22.5			
19	23.8	7.74	7.53	96.8
20	23.6			
21	24.6	7.60	6.27	80.9
mean	**22.7**	**7.77**	**7.78**	**99.0**
± SD	**0.8**	**0.11**	**0.64**	**7.7**

Table SI 3: Values from temperature, pH and oxygen monitoring of the F/pF$_{AuNP}$ treatment during the chronic exposure test with H. azteca.

Day	Temperature [°C]	pH	Oxygen	
			mg/L	%
0	22.2	7.81	8.81	111.5
1	23.3			
2	25.0	7.85	8.37	105.2
3	21.9			
4	21.5			
5				
6	22.6	7.82	8.32	105
7	22.7			
8	22.0			
9	22.0	7.75	7.98	100.3
10	22.7			
11				
12	22.4	7.81	8.15	101.8
13	22.9			
14	23.4	7.91	8.25	107.4
15	23.4			
16	22.7	7.83	7.83	97.5
17	22.2			
18	22.5			
19	23.7	7.81	7.63	94.3
20	23.9			
21	24.6	7.68	6.90	87.7
mean	**19.8**	**7.81**	**8.03**	**101.2**
± SD	**7.2**	**0.06**	**0.51**	**6.8**

Table SI 4: Values from temperature, pH and oxygen monitoring of the F/pFAgNP treatment during the chronic exposure test with H. azteca.

Day	Temperature [°C]	pH	Oxygen	
			mg/L	%
0	22.1	7.81	8.81	111.5
1	23.2			
2	22.5	7.91	8.26	106.1
3	22.2			
4	21.7			
5				
6	22.7	7.83	8.31	106.5
7	22.7			
8	22.0			
9	22.2	7.70	7.77	97.3
10	22.8			
11				
12	22.1	7.80	8.14	101.8
13	23.1			
14	23.5	7.73	7.82	100.3
15	23.5			
16	22.7	7.81	7.72	95.6
17	22.4			
18	22.6			
19	23.5	7.75	7.54	93.8
20	23.8			
21	24.7	7.61	6.69	85.2
mean	**22.8**	**7.77**	**7.90**	**99.8**
± SD	**0.7**	**0.08**	**0.56**	**7.4**

Table SI 5: Examination of water burdens of nitrate, nitrite and ammonium in the F/pFcontrol treatment during the chronic exposure test with H. azteca.

Day	Nitrate [mg/L]	Nitrite [mg/L]	Ammonium [mg/L]
0	8	<0.005	<0.01
7 aged	9	<0.005	<0.01
7 fresh	8	<0.005	<0.01
14 aged	9	0.041	>0.5
14 fresh	8	<0.005	<0.01
21 aged	9	0.099	1.20
21 fresh	8	<0.005	<0.01

Table SI 6: Examination of water burdens of nitrate, nitrite and ammonium in the F/pFAuNPl treatment during the chronic exposure test with H. azteca.

Day	Nitrate [mg/L]	Nitrite [mg/L]	Ammonium [mg/L]
0	8	<0.005	<0.01
7 aged	9	<0.005	<0.01
7 fresh	8	<0.005	<0.01
14 aged	9	0.013	0.39
14 fresh	8	<0.005	<0.01
21 aged	9	0.024	0.80
21 fresh	8	<0.005	<0.01

Table SI 7: Examination of water burdens of nitrate, nitrite and ammonium in the F/pFAgNP treatment during the chronic exposure test with H. azteca.

Day	Nitrate [mg/L]	Nitrite [mg/L]	Ammonium [mg/L]
0	8	<0.005	<0.01
7 aged	9	<0.005	<0.01
7 fresh	8	<0.005	<0.01
14 aged	9	0.051	0.27
14 fresh	8	<0.005	<0.01
21 aged	8	0.048	0.70
21 fresh	8	<0.005	<0.01

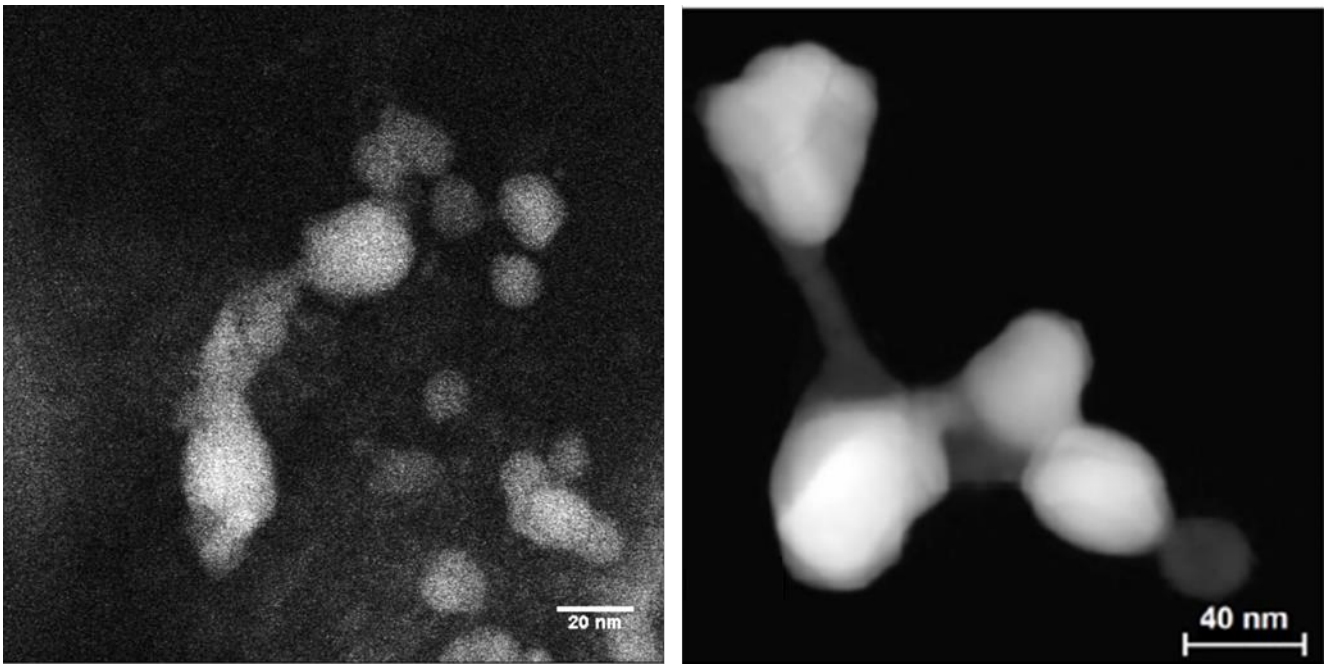

Figure SI 3: Left: TEM HAADF image of AgNPs from the F/pF$_{AgNP}$ showing aggregates of AgNPs fused by presumable Ag$_s$S – nanobridges;. Right: STEM-HAADF image of aggregates of sulfidized AgNPs connected with Ag$_s$S-nanobridges taken from Metreveli et al. (2020)[1]

1 G. Metreveli, J. David, R. Schneider, S. Kurtz and G. E. Schaumann, Morphology, structure, and composition of sulfidized silver nanoparticles and their aggregation dynamics in river water, *Sci. Total Environ.*, 2020, **739**, 139989.

Statistical analysis of biological endpoints

Length (mm)

One Way Analysis of Variance

Normality Test (Shapiro-Wilk): Passed (P = 0,520)

Equal Variance Test (Brown-Forsythe): Failed (P < 0,050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source: Data 1 in Notebook 1

Group	N	Missing	Median	25%	75%
Control (mm)	5	0	3,172	2,994	3,370
Ag (mm)	5	0	3,034	2,853	3,336
Au (mm)	5	0	2,389	2,370	2,456

H = 9,620 with 2 degrees of freedom. (P = 0,008)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0,008)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	P	P<0,050
Control (mm) vs Au (mm)	41,000	4,100	0,010	Yes
Control (mm) vs Ag (mm)	7,000	0,700	0,874	No
Ag (mm) vs Au (mm)	34,000	3,400	0,043	Yes

Note: The multiple comparisons on ranks do not include an adjustment for ties.

Fresh weight (mg)

One Way Analysis of Variance

Normality Test (Shapiro-Wilk): Passed (P = 0,659)

Equal Variance Test (Brown-Forsythe): Passed (P = 0,382)

Group Name	N	Missing	Mean	Std Dev	SEM
Control (mg)	5	0	0,623	0,113	0,0503
Ag (mg)	5	0	0,560	0,0927	0,0415
Au (mg)	5	0	0,281	0,0558	0,0250

Source of Variation	DF	SS	MS	F	P
Between Groups	2	0,332	0,166	20,419	<0,001
Residual	12	0,0975	0,00813		
Total	14	0,429			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0,001).

Power of performed test with alpha = 0,050: 0,999

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0,05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0,050
Control (mg) vs. Au (mg)	0,342	5,999	<0,001	Yes
Ag (mg) vs. Au (mg)	0,280	4,906	<0,001	Yes
Control (mg) vs. Ag (mg)	0,0624	1,094	0,296	No

Mortality (%)

Group Name	N	Missing	Mean	Std Dev	SEM
Control	5	0	23,000	12,550	5,612
Ag	5	0	17,000	16,047	7,176
Au	5	0	28,000	12,042	5,385

One Way Analysis of Variance (arcsin transformed data)

Normality Test (Shapiro-Wilk): Passed (P = 0,875)

Equal Variance Test (Brown-Forsythe): Passed (P = 0,771)

Group Name	N	Missing	Mean	Std Dev	SEM
Control	5	0	0,484	0,166	0,0744
Ag	5	0	0,400	0,197	0,0881
Au	5	0	0,551	0,135	0,0602

Source of Variation	DF	SS	MS	F	P
Between Groups	2	0,0567	0,0283	1,005	0,395
Residual	12	0,338	0,0282		
Total	14	0,395			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,395).

Power of performed test with alpha = 0,050: 0,051

The power of the performed test (0,051) is below the desired power of 0,800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Chapter 7: Bioaccumulation assessment of nanomaterials using freshwater invertebrate species

Chapter 7 consists of the publication

Bioaccumulation assessment of nanomaterials using freshwater invertebrate species

Sebastian Kuehr, Verena Kosfeld, Christian Schlechtriem

Environmental Sciences Europe 2021, 33, 9, DOI: 10.1186/s12302-020-00442-2

There are no supporting information for this article.

Authors' contribution Chapter 7:

Sebastian Kuehr

Conceptualization and coordination of the manuscript; literature research and analysis; development of the assessment scheme; visualization; writing – original draft;

Verena Kosfeld

Literature research (chapters "Amphipoda" and "Branchiopoda" of the manuscript)

Christian Schlechtriem

Conceptualization and coordination of the manuscript; development of the assessment scheme; writing – review & editing

Environmental Sciences Europe

REVIEW Open Access

Bioaccumulation assessment of nanomaterials using freshwater invertebrate species

Sebastian Kuehr[1,2], Verena Kosfeld[1,3] and Christian Schlechtriem[1,2,3*]

Abstract

Background: The high production volume of engineered nanomaterials (ENMs) may lead to high pressure on the environment, and a scientific assessment of ENMs that bioaccumulate in organisms and biomagnify in the food web is necessary. Within the regulation of chemicals in several jurisdictions, such as the European regulation REACH, the bioconcentration factor is the standard endpoint. The bioconcentration factor is mostly determined by flow-through fish tests. However, nanomaterials tend to agglomerate, which may lead to sedimentation in aquatic environments. The bioavailability of the tested nanomaterials may be thus impaired for pelagic species, including fish, in comparison to benthic or filtrating species. Several risk assessment regulations allow the usage of data gained during tests using invertebrates and such data may allow a waiver of further tests using vertebrates. The aim of this study was to elucidate the potential of different freshwater invertebrate species to be used in laboratory bioaccumulation studies on ENMs and to give some guidance for the use of bioaccumulation endpoints derived from studies using aquatic invertebrate species in the risk assessment process for ENMs.

Results: The existing literature related to the testing of nanomaterial bioaccumulation with freshwater invertebrates was screened and reviewed to find suitable test species with regard to their ecology and physiology, as well as laboratory test systems allowing to investigate the bioavailability/bioaccumulation of nanomaterials with the respective species. Bivalvia, gastropoda, isopoda, amphipoda, and branchiopoda were reviewed and their suitability for bioaccumulation testing was assessed. Amphipods and bivalves represent worst-case scenarios and show clear advantages to be used as test organisms. However, only amphipods allow the examination of two clearly independent exposure pathways (water and diet).

Conclusion: Amphipods are suitable test organisms for bioaccumulation testing of ENMs. The results from amphipod bioconcentration and biomagnification tests can be included in a tiered assessment suggested at the end of this study allowing a clear grading of the tested nanomaterials as "bioaccumulative" or "non bioaccumulative." Due to the worst-case scenario of the amphipod test, this approach may allow a waiver of further vertebrate tests.

Keywords: Risk assessment, Nanomaterials, Bioconcentration, Biomagnification, Invertebrates

Background

The high production volume of engineered nanomaterials (ENMs) may lead to high pressure on the environment and can only be long lasting and sustainable if environmental and human health protection is ensured. The identification and scientific assessment of compounds that bioaccumulate in organisms and biomagnify in the food web are important aspects in the regulation

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of chemicals in several jurisdictions, such as the European regulation concerning the registration, evaluation, authorisation and restriction of chemicals (REACH), the Turkish law KKDIK (Kaydi, Değerlendirilmesi, İzni, Kisitlanmasi), the High Production Volume Challenge Program of the USA, or the Toxic Chemicals Control Act of Korea [1–4]. The bioconcentration factor (BCF), as commonly determined by the flow-through fish test according to OECD TG 305 [5], is the standard endpoint in regulatory bioaccumulation assessment and describes the ratio between the body burden of a substance (mg/kg) taken up from the surrounding medium (water) and the exposure concentration (mg/L). The test system is well established and allows the comparison of results of different studies and is thus preferred for instance in the regulatory processes under REACH [1]. However, depending on the properties of the test items, the performance of fish bioconcentration tests can be challenging or may be even not suitable. This is a major concern with respect to the special characteristics of some ENMs [14]. ENMs tend to agglomerate, leading to sedimentation in aquatic environments [6]. Thus, the bioavailability of the tested ENMs may be impaired for pelagic species, including fish, in comparison to benthic or filtrating species. This aspect seems to be of relevance especially at the mostly very high exposure concentrations applied in laboratory studies [6–8]. Furthermore, several studies indicate that the major uptake pathway for NMs of fish is by oral uptake following dietary exposure. However, metal and metal oxide-based NMs ingested by fish in this way showed only limited transfer through the blood system to other organs [9–18]. The bioaccumulation potential of compounds that are ingested via the diet can be expressed as the biomagnification factor (BMF) describing the ratio between the body burden of a substance and the concentration of the substance in the diet. Alternatively, a BAF (bioaccumulation factor) can be calculated which is, however, less specific and corresponds to the body burden of an organism and the concentration in the animal's environment not distinguishing between the different uptake pathways. Hou et al. showed that the logarithmic BCF values of fish were 1–2 orders of magnitudes lower for the same ENM than in *Daphnia magna* and underlined the lack of suitability of fish as a test organism for regulatory bioaccumulation assessment of ENMs which obviously does not represent a worst-case scenario [19]. Kühnel and Nickel identified the need for the development of an amendment to OECD TG 305 and proposed the use of other species, like crustaceans and bivalves, for bioaccumulation testing [20]. REACH was developed without having regard to the mode of action of nanomaterials and their special behavior, and therefore, the development of other more suitable methods is required [21]. According to Annexes VIII-XIII of the REACH regulation, test methods could be adapted if the actual method induces no significant or an unexpected exposure [22]. Other taxonomic groups than fish are allowed to be used in bioconcentration studies providing endpoints which could be used for assessing a chemicals bioaccumulation potential [1]. The American Society for Testing and Materials (ASTM) mussel bioconcentration test is suggested as an alternative test concept [23]. The results of such studies should be used in combination with further information to investigate if B (bioaccumulative, under REACH: BCF value $\geq$ 2000) or vB (very bioaccumulative, under REACH: BCF value $\geq$ 5000) criteria are fulfilled (e.g., [1]).

In 2007, de Wolf et al. calculated the need of about 326,700 fish for the estimation of bioconcentration factors for nearly 5500 chemical compounds. This is only for REACH and represents the amount of test animals if just the minimum number of 108 fish per test is used [24]. The usage of invertebrates for bioaccumulation tests would fit to the principles of the 3Rs [25–27] and is in agreement with the European Council Directive 86/609/EEC [28] that ensures that the animal species with the lowest degree of neurophysiological sensitivity are used for scientific studies. According to the directive, invertebrates do not fall within the given definition of animals as all living vertebrates, without humans [28, 29]. The high number of fish required for testing does not only raise an ethical problem but there is also a need to rethink the test concept with regard to economic reasons. A single test may last up to several weeks leading to high costs for labor, water, feed, and further resources.

Handy et al. [30] proposed a tiered testing strategy for ENMs, including data from invertebrate studies at the second tier. According to the tiered approach, the tested material will only be tested on the next higher tier if the data from the lower tier show a credible risk for bioaccumulation. In this way only the conspicuous materials would go forward to in vivo testing (Tier 4) which is essentially the OECD TG 305 method for dietary bioaccumulation testing using fish. Thus, the data from invertebrate studies may allow a waiver of further tests using vertebrates. Even though the authors suggest the use of terrestrial invertebrate species, such as earthworms or nematodes, for testing the bioaccumulation of ENMs, we would rather suggest the use of aquatic invertebrate species to allow a more adequate prediction of the bioaccumulation potential of ENMs in fish.

The aim of this literature study is to elucidate the potential of different aquatic invertebrate species to be used in laboratory bioaccumulation studies on ENMs. The bioaccumulation of metal and metal oxide ENMs is the focus of this study, but the results may also be

applied to polymer- and carbon-only-based ENMs showing different characteristics and fate in the environment and aquatic organisms. First, general requirements are defined that must be fulfilled by the species and related test systems to be suitable for the proper bioaccumulation assessment of metal and metal oxide-based ENMs. Second, we give a summary of a broad literature review on selected invertebrate groups and species with regard to their use in bioaccumulation studies. Third, the pros and cons of the different organisms for bioaccumulation testing are discussed. Finally, some guidance for the use of bioaccumulation endpoints derived from studies using aquatic invertebrate species in the risk assessment process for ENMs is provided.

Suitability of aquatic invertebrate species for testing bioaccumulation of ENMs

Bioaccumulation of ENMs is the result of the dynamic interplay of different factors, including substance-specific physico-chemical properties, the biological and ecological properties of the receiving organism, as well as its habitat characteristics. Therefore, test systems need to fulfill a range of general requirements to be suitable for the assessment of the bioaccumulation of ENMs in aquatic organisms. For the required information a review was conducted using Scopus, Web of Science, and Google scholar. For the search we used the keywords "bivalve," "gastropod," "isopod," "amphipod," "branchiopod," and "daphnia" (and the plural forms) in combination with "nanomaterial," "nanoparticle," and "bioaccumulation," "bioconcentration," "biomagnification," "uptake," and "elimination." Field studies and studies using marine species were excluded (only with the exception for physiological or mechanistic information). In addition, studies using test items with diameters higher than 100 nm or test items not based on metals or metal oxides were also excluded.

Biology and ecology of test species

The ecology of an aquatic organism can have a significant impact on the bioavailability and thus the bioaccumulation of ENMs. In this context, the composition of the water is of crucial importance. In marine environments ENMs tend to aggregate and agglomerate on a larger scale than in freshwater, due to physico-chemical processes induced by the surface charges of the ENMs, as well as the decreased electrophoretic mobility and the higher ionic strength of marine water [31]. Thus, the bioavailability of ENMs and potentially released ions is decreased in marine waters with a high ionic load [32]. Furthermore, transformation of ENMs may be enhanced under marine conditions due to the high Cl^- content and thus may affect the bioavailability of the particles [33]. Due to the limited bioavailability of ENMs under marine conditions, only the bioaccumulation in fresh water species is considered in this study. Also, the natural habitat (pelagic vs. benthic) of an aquatic organism may affect the bioavailability of ENMs, with sediments being a likely sink for ENMs due to their potential for heteroagglomeration and sedimentation [34], [35]. Key factors affecting the ingestion of ENMs by aquatic organisms mainly relate to the specific way of breathing as well as feed uptake. If the processes of respiration and nutrition are coupled with ENM exposure pathways, an elevated probability of ENM ingestion is likely. Following ingestion of ENMs, it is of particular importance which physicochemical processes take place in the organism, and which may potentially lead to an accumulation of the ENMs or their metals [36, 37]. In this context, the binding of metals to proteins, like metallothioneins (MT), the binding into intracellular vesicles as granules, or the precipitation in mineral deposits or exoskeletons should be mentioned [38–40]. Furthermore, it should be considered whether there are any processes that may influence the ion regulation and uptake processes. For instance, the uptake of essential metal ions can be increased by specific transporters as is known for Cu or Zn. For non-essential metals, uptake due to ionic mimicry, as observed for Ag^+ or Cd^{2+} by Na^+ channels or Ca^{2+} uptake and transport mechanisms, is possible [41–45]. However, also the cellular uptake of ENMs is possible. For instance, processes of endocytosis/pinocytosis may allow ENMs to enter the cells via the animals surface, as described by Petros and De Simone [46], if this is not impeded by an impermeable cuticula. Testing bioaccumulation of metal and metal oxide-based ENMs under laboratory conditions requires a sound knowledge of the biological and ecological properties of the test organism as well as its natural habitat characteristics.

Culture and breeding of test species

The test species should be readily available and have an appropriate size to allow the performance of bioaccumulation studies. Animals can be collected in the field and maintained in the laboratory or reproduced and grown under laboratory conditions which are generally preferred to ensure consistency of the test animals. Field sampling requires an uncontaminated collection site which provides a sufficient number of animals of a suitable developmental stage. A method for age and size determination should be available to characterize the collected animals [5]. Culture of test animals in the laboratory requires a sound knowledge of the optimal species-specific breeding and husbandry conditions. Apart from the abiotic factors preferred by the respective species, such as light intensity, temperature, pH,

and chemical composition of the water, information on the feeding preferences are of elementary importance. Feed items which are commercially available or can be prepared in the lab using standardized preparation procedures as known for algal- or plant-based diets are recommended. Care needs to be taken that the diets ensure a sustainable and sufficient supply of nutrients promoting the growth and development of the animals.

Factors influencing the suitability of aquatic invertebrate species for bioaccumulation testing

For bioaccumulation testing a homogeneous batch of animals should be used to eliminate any differences of age or size of the animals on the results obtained. To avoid effects attributed to growth during a study performed over a time period of several weeks, adult or slow growing animals are preferred. Generally, reproduction during the test period should be excluded to avoid elimination of accumulated test item through the release of juveniles. This can be achieved by using only test animals of one sex or adjusting test conditions where no reproduction occurs. Only healthy animals showing normal behavior should be selected as test animals. Mortality of animals should be monitored before and during a test to demonstrate optimal test conditions. As for maintenance and breeding of test animals, optimal conditions for the test species should be applied during bioaccumulation testing.

It should be noted that changes in temperature in the experimental system may alter bioaccumulation kinetics of ENMs. Light conditions may allow phototransformation of tested nanomaterials [47–49]. Optimal oxygen concentration in the test medium has to be guaranteed because low oxygen concentrations in water may result in increasing water turnover rates in aquatic organisms. This is in order to satisfy their oxygen needs potentially leading to a faster uptake of contaminants from the water column. However, high oxygen and active aeration of the test system may increase the transformation of ENMs, e.g., by oxidation and potentially dissolution or the release of ions [50–52]. Flow-through conditions are preferred to avoid sedimentation of ENMs in the experimental system [6–8]. However, semi-static conditions are also acceptable as long as constant water concentrations of the test material can be guaranteed.

The feeding requirements of the test organisms during bioaccumulation testing are dependent on the test duration and the test species used. For short-term testing (only hours) it might not be necessary to provide food, but if tests last for several days or even weeks, constant feeding is required. Fasting of test animals may affect the uptake, metabolism, and elimination of a test substance and thus alter its bioaccumulation behavior. The feeding method should be selected carefully especially for bioconcentration testing (exposure via the water), since ENMs might sorb to food and thus lead to dietary intake. This can be avoided by the immediate uptake of food by the test organisms and the continuous removal of feed residues from the test vessels. A further key factor for bioaccumulation testing is the selection of appropriate test concentrations. Test concentrations should be low enough to avoid toxic effects in the test organisms. This should be investigated prior to bioaccumulation testing. Depending on the toxicity of an ENM, the application of low test concentrations may be required which can be challenging regarding the analysis of the test media and the resulting tissue concentrations.

All these aspects need to be taken into account when searching for suitable aquatic invertebrate species for bioaccumulation assessment. In the following, five groups of invertebrate organisms are presented that have been used for bioaccumulation studies, ideally on metals and ENMs. A summary of the related literature is presented in Table 1.

The biology and ecology of the different organisms is described as well as the species-specific key pathways of substance uptake and elimination. Information on the culture and breeding of the different species is provided. Concepts for bioaccumulation testing are summarized if available. Potential endpoints derived from bioaccumulation studies with the different organisms are described and discussed with respect to their potential contribution to the regulatory bioaccumulation assessment of ENMs.

Bivalvia

Biology and ecology

The class *Bivalvia* is part of the phylum *Mollusca* and includes more than 10,000 species, including animals, like scallops, clams, and mussels. Bivalves can be found in marine and fresh water systems and are benthic organisms that are usually burrowers and therefore have a sedentary existence on the sediment. Bivalves are literally compressed animals and possess a shell composed of two valves that completely enclose their body.

Bivalves have usually very large gills which are used for gas exchange as well as for food collection (Fig. 1). Most bivalves are mainly filter feeders with large water throughputs at high rates [53]. Filter feeding involves trapping particles, like sediment, plankton, and organic debris on the ctenidial filaments, subunits of the ctenidium, the feather-shaped gill-like respiratory organ of many mollusks. The labial palps sort too large particles for immediate removal as pseudofeces or as mucus entrapped particles by periodic contractions of the mantle cavity [54]. Therefore, respiration and nutrition are closely linked in most bivalves since the ctenidia have

Table 1 References including information on bioaccumulation studies using aquatic invertebrates

Category	Organism	ENM	(nominal) individual size [nm]	Hydrodynamic diameter [nm]	Exposure condition	Endpoint	References
Bivalvia	*A. cygnea*	AuNP	40	86 ± 14	Water	BCF; tissue distribution	Moëzzi et al. [421]
	C. fluminea	AuNP	17	n.d	Diet	BCF	Renault et al. [109]
	C. fluminea	Al_2O_3NP	11	4—12	Sediment/water	BCF	Stanley et al. [121]
	C. fluminea	AuNP	7.8, 15 & 46	n.d	Water	Clearance rate	Hull et al. [122]
	C. fluminea	AuNP	10	n.d	Water/ diet	Uptake & elimination rates	Arini et al. [89]
	C. fluminea	AgNP TiO_2NP	15 (AgNP) & 26 (TiO_2NP)	27 (AgNP) & 351 (TiO_2NP)	Water	BAF, tissue distribution	Kuehr et al. [98]
	C. fluminea	CuONP	12 ± 8 & 29.5	140.5 & 194	Water	Body burden; tissue distribution	Koehle-Divo et al. [422]
	C. fluminea	TiO_2NP	5—10	n.d	Sediment	Body burden; tissue distribution	Fan et al. [423]
	D. polymorpha	CeO_2NP	3	8	Water	BCF	Garaud et al. [303]
	D. polymorpha	CeO_2NP	3—4	8	Water	Body burden; tissue distribution	Garaud et al. [424]
	D. polymorpha	AgNP	50 ± 20	80	Water	BCF, subcellular distribution	Zimmermann et al. [425]
	D. polymorpha	TiO_2NP	10.4	n.d	Water/ diet	BCF/BAF	Bourgeault et al. [123]
Gastropoda	*L. stagnalis*	AuNP	15	28	Diet	Body burden	Hudson et al. [195]
	L. stagnalis	CuONP	7 ± 1	82 ± 1	Water	Body burden	Misra et al. [426]
	L. stagnalis	AgNP	13 ± 3 & 17 ± 5	n.d	Water/diet	Uptake & elimination rates	Croteau et al. [187]
	L. stagnalis	CuONP	7	77 ± 5	Water/diet	Uptake & elimination rates; body burden	Croteau et al. [427]
	L. stagnalis	AgNP	n.d	36 ± 1	Diet	Uptake rate	Oliver et al. [428]
	L. stagnalis	^{67}ZnONP	20 -70	245	Diet	Body burden	Dybowska et al. [206]
	L. stagnalis	AgNP	10.3 ± 3.4 & 12.8 ± 4.4	n.d	Water	Uptake rate	Luoma et al. [429]
	P. antipodarum	AgNP	10—15	12.1 ± 2.93—13.9 ± 3.17	Water/ sediment	Body burden	Ramskov et al. [430]
	P. antipodarum	CuONP	7	n.d	Sediment	Body burden	Ramskov et al. [176]
	P. antipodarum	CuONP	7 ± 1	n.d	Sediment	Body burden	Ramskov et al. [431]
	P. antipodarum	CuONP	6	n.d	Sediment	Body burden	Pang et al. [432]
	P. antipodarum	CuONP	6 & 100	19 & 204	Sediment	Body burden	Pang et al. [433]
	B. glabrata	AgNP	115 ± 55.6	n.d	Water	Body burden	Oliveira-Filho et al. [158]
	B. aeruginosa	AgNP	18 ± 7.7, 30.2 ± 9.4 & 57 ± 19.3	n.d	Sediment	BAF, tissue distribution	Bao et al. [177]
	B. aeruginosa	TiO_2	11.6 ± 2.4	n.d	Sediment	Body burden	Ma et al. [434]
	B. aeruginosa	CuONP	41.6 ± 4.6	n.d	Sediment	Body burden; tissue distribution	Ma et al. [188]
Isopoda	*A. aquaticus*	WCNPs	< 100		Water	Body burden	Ekvall et al. [212]

Table 1 (continued)

Category	Organism	ENM	(nominal) individual size [nm]	Hydrodynamic diameter [nm]	Exposure condition	Endpoint	References
Amphipoda	*H. azteca*	Al_2O_3NP	11	4—12	Sediment/water	Body burden	Stanley et al. [121]
	H. azteca	CuO	30—40	733.1 ± 143.4	Water	BCF	Burkal et al. [435]
	H. azteca	AgNP	15	27	Water	Body burden	Kuehr et al. [311]
	H. azteca	AuNP	15	28	Diet	Body burden	Hudson et al. [195]
	H. azteca	ZnONP	22.4 ± 2.8	n.d	Water	Body burden	Poynton et al. [312]
	H. azteca	AgNP	15	27	Water & diet	Body burden	Kuehr et al. [314]
	H. azteca	AgNP, AuNP, TiO_2NP	15 (AgNP), 26 (TiO_2NP) & 60 (AuNP)	51.2 (AgNP), 1,861 (TiO_2NP) & 61 (AuNP)	Water/ diet	BCF/ BMF	Kuehr et al. [315]
	G. fossarum	AgNP	20, 23 & 27	96 ± 8.4, 56 ± 3.2 & 63 ± 3.2	Water	BCF	Mehennaoui et al. [270]
	G. fossarum	AgNP, AuNP	20, 40 & 80 (AgNP); 20, 40 & 80 (AuNP)	22 ± 0.7—78 ± 2.9 (AgNP) & 39 ± 1.5—89 ± 0.9 (AuNP)	Water	Body burden	Mehennaoui et al. [304]
	G. roeseli	CeO_2NP	3	8	Water	BCF	Garaud et al. [303]
Branchiopoda	*D. magna*	AuNP	20	n.d	Water	Distribution	Lovern et al. [407]
	D. magna	CuONP	26 ± 8, 45 ± 11 & 75 ± 19	263 ± 38, 153 ± 45 & 241 ± 3	Water	Spatial distribution & burden	Santos-Rasera et al. [436]
	D. magna	AgNP	10—11	22 & 28.3	Diet	Body burden	Kalman et al. [437]
	D. magna	AuNP	10	33 ± 17.3	Water & diet	Body burden	Lee et al. [403]
	D. magna	CuONP	< 50	926 ± 35	Water & diet	Body burden	Wu et al. [376]
	D. magna	AgNP	20	40—50	Water	Body burden	Zhao and Wang [438]
	D. magna	AuNP	19 ± 2.5	24.2 ± 0.2	Water	Elimination rate	Khan et al. [414]
	D. magna	^{65}ZnONP	10—30	46—56	Water	Uptake & elimination rates	Li & Wang [391]
	D. magna	CdSe/ZnS QDs*	5.2 ± 0.4	n.d	Water	Body burden	Lewinski et al. [393]
	D. magna	TiO_2NP	21	580.5	Water	BCF	Zhu et al. [412]
	D. magna	ZnONP	30 ± 17	111.8 ± 24.9—670.2 ± 230	Water	Body burden	Skjolding et al. [386]
	D. magna	AuNP	14 ± 4	225 ± 61	Water	Uptake & tissue distribution	Jensen et al. [406]
	D. magna	AgNP	15.5 ± 3.6	50 ± 0.7	Water	Body burden	Kim et al. [409]
	D. magna	AuNP	10 & 30	14 ± 4—142 ± 53 & 23 ± 5—225 ± 61	Water	Uptake & elimination rates	Skjolding et al. [387]
	D. magna	TiO_2NP	< 25	20.9 ± 2.89—218 ± 47.3	Water	Body burden	Tan & Wang [392]
	D. magna	TiO_2NP	30—80	n.d	Water	BCF	Fan et al. [394]
	D. magna	AuNP	10	33 ± 17.3	Diet	Body burden	Lee et al. [403]
	D. magna	TiO_2NP	4.6 ± 2.6, 11.2 ± 2.9, 20.3 ± 4.3, 27.4 ± 5.2 & 103 ± 24.6	929 ± 62, 908 ± 3, 953 ± 50, 908 ± 591 & 763 ± 12	Water	BCF/ BMF	Chen et al. [389]
	D. magna	CdSe/ZnS QDs*	4.5 ± 0.5—4.9 ± 0.7	n.d	Water	Uptake & elimination rates	Lee et al. [405]

Table 1 (continued)

Category	Organism	ENM	(nominal) individual size [nm]	Hydrodynamic diameter [nm]	Exposure condition	Endpoint	References
	D. magna	AgNP, AuNP, PtNP	5 & 50 (Ag), 5 & 50 (Au) & 5 6 50 (Pt)	n.d	Water	Body burden	Krystek et al. [411]
	D. magna	CuNP, ZnONP	50 (CuNP) & 43 (ZnONP)	568 ± 72 (CuNP) & 1154 ± 252 (ZnONP)	Water	Body burden	Xiao et al. [408]
	C. dubia	TiO_2NP	< 25	n.d	Water & diet	BAF/ BMF	Dalai et al. [413]
	D. magna	AgNP	3—8	80	Water & diet	BCF / BMF	Ribeiro et al. [410]

become specialized for filter feeding. Some bivalve species are able to perform pedal feeding, a pinocytosis mechanism for the uptake of small particles or single-cell algae or microbes by the surface of their pedal/foot [55–57].

Filter-feeding mussels show a significant impact on the ecosystem they live in [58]. They incorporate huge amounts of suspended organic matter from the water phase into mucus that is released as undigested pseudofeces causing a strong shift of nutrients and changes in the structure of the sediment [56, 59–61]. Several benthic amphipods feed on the protein- and carbon-rich pseudofeces of the mussels which thus have an impact on the abundance of several benthic species, also by the transfer of toxins by pelagic–benthic coupling [61–73].

Bivalvia species have been shown to accumulate a wide range of environmental pollutants [74, 75]. Due to their sedentary lifestyle, high filtration rates [76–78], and their ability to accumulate high amounts of heavy metals, bivalves have been used as bioindicators for metal pollution in aquatic systems [79–81]. Accumulation of metals is mainly based on the production of high amounts of metal-binding proteins, like metallothioneins, produced by bivalves as a detoxification mechanism. The characteristics described make bivalves a preferred group of species to investigate accumulation of metal and metal oxide ENMs in aquatic systems [82–86].

Culture and breeding

Two strategies are available to obtain animals for bioaccumulation testing:

1. Field collection
   Bivalves used in former studies mostly originated from wild populations, as suggested in the ASTM guideline, [77, 87–89, 98]. It is known that organisms collected in the field might be contaminated with pollutants as it was shown for lindane [76]. Thus, although bivalves are preferably collected from non-polluted areas, animals should be acclimatized in the laboratory for a sufficient time to ensure the elimination of previously ingested contaminants present in the gut content or tissues. In addition, the animals need to acclimate to the new water conditions (ionic strength, pH, etc.) that may alter the filtration activity and thus the uptake kinetic during the exposure. Acclimation periods applied in former studies ranged from one to several weeks [88, 98] and up to 5 months [77]. Another problem of field-collected animals could be the infestation with parasites that may impact the performance of the test animals and thus the uptake of test items during bioaccumulation studies [90–94]. Therefore, bivalves should be checked carefully during acclimation to ensure that only healthy animals without any visible abnormalities are used as test animals. Husbandry of collected bivalves under laboratory conditions is possible over longer periods [95]. In general, sufficient food to support survival and growth should be provided. Living or freeze-dried algae, or plant powders, e.g., of stinging nettle leaves, are added to the holding water to provide sufficient nutrients for the filter-feeding animals allowing them to be kept alive for several months under laboratory conditions [96–98]. The laboratory conditions may vary depending on the species. Usage of filtered and or only aerated tap water is possible. Temperatures of 12 °C ± 4 °C up to 20 °C ± 2 °C and light for 16 h per day have been applied [77, 87, 96, 98].
2. Lab culture
   Even though the husbandry of the animals is possible, breeding of bivalves under laboratory conditions is highly challenging [99]. The mostly hermaphroditic individuals produce fertilized eggs [100] which evolve to glochidiums, parasitic larvae, that are released into the water. After that they must be ingested by fish (host) and implanted in their gills or need to be attached to the body surface of other vertebrates.

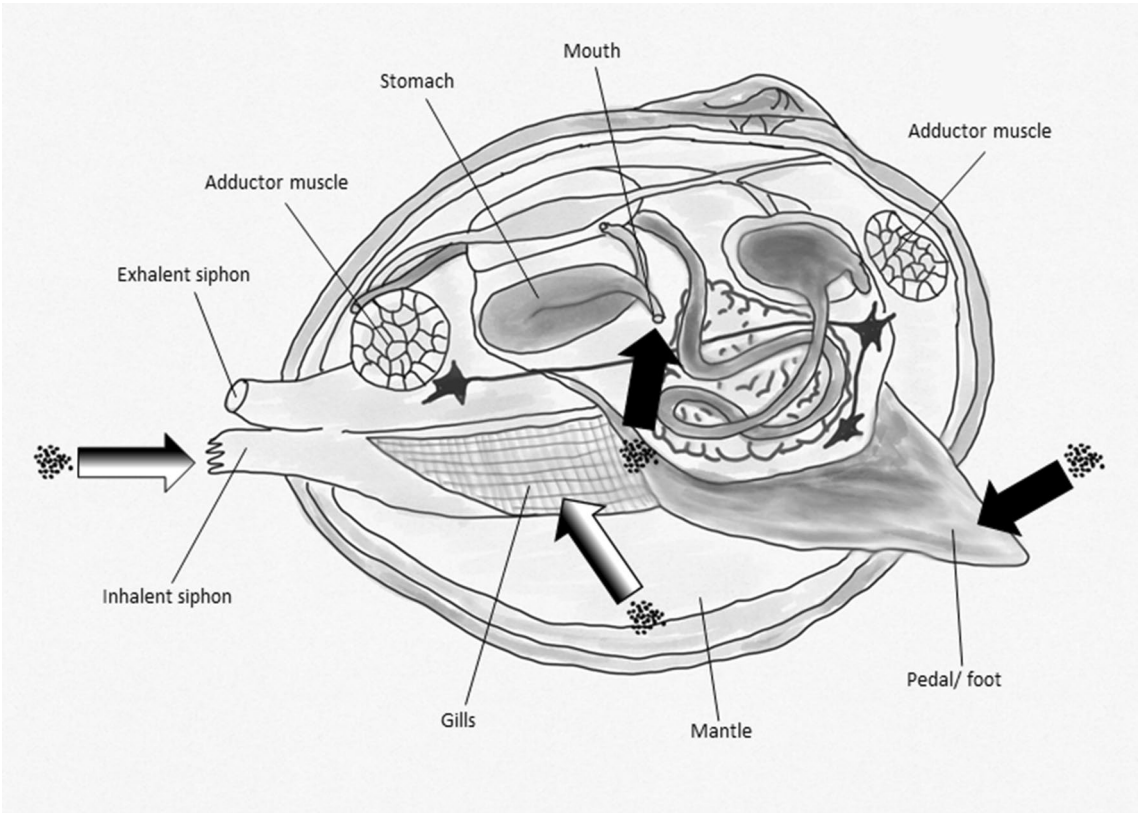

Fig. 1 Potential uptake routes of ENMs in *Bivalvia*. Black arrow: ingestion by the dietary route/ mouth and foot; white and black arrows uptake via inflow siphon and ingestion via gills for respiration and nutrition

After some time (depending on the species) the juveniles detach from their host and mostly burrow for several years into the substrate of the water system [101, 102]. Juvenile bivalves mostly show a very high mortality, and it may take several years to get mature bivalves that show a sufficient body size to be used in bioaccumulation studies. Thus, the breeding of test organisms is not considered feasible for bivalves.

Bioaccumulation testing

There are existing standardized test methods that are developed and optimized to investigate the bioaccumulation potential of soluble, non-particulate substances using marine bivalves under flow-through conditions [23]. The proposed species are the oyster (*Crassostrea gigas*) or the blue mussel (*Mytilus edulis*). Bivalves have already been used as test organisms to investigate the uptake, accumulation, and elimination of metals. Winter [103] carried out bioaccumulation studies with *Dreissena polymorpha*, *Corbicula fluviatilis,* and *Anadonta anatina* and showed that all of them accumulate dissolved Cd, whereby the estimated BCF was 19,000 for *Dreissena polymorpha* and decreased in the order as listed above. The accumulation of Cd in combination with Zn was also observed in *Mytilus edulis* [104]. *Mytilus* is able to accumulate a wide range of metals, [105, 106], proving the high bioavailability of metals for bivalves. *M. galloprovincialis* was also used by Montes et al. to investigate the bioaccumulation of nanoparticles (NPs). It was shown that insoluble CeO_2-NPs were primarily located in the pseudofeces and only a small part was accumulated in the tissue [107]. However, the soluble ZnO-NPs caused an accumulation of Zn (ions) in the tissue. Comparable results were published by Gomes et al. [108] comparing the accumulation of Cu from CuO-NPs and dissolved Cu^{2+}. In their studies they observed that the digestive gland is the main target of Cu accumulation and that the Cu accumulation from free Cu^{2+} is higher than that from CuO-NPs. For the metal uptake in bivalves, there are two pathways to be considered. Renault et al. [109] estimated tissue-specific BCFs (equivalent to BAF as described above) for Au of 4000 in gills and 26,000 in the visceral mass of *Corbicula fluminea* after dietary exposure with

algae loaded with Au-NPs. The accumulation of Au-NPs in *Scrobicularia plana* was investigated by Jin-Fen Pan [110]. Ferry et al. [11] mentioned that bivalves represent as filter feeders one of the most important pathways of nanomaterials into the human food chain. The bivalve species used for bioaccumulation testing were selected on the basis of availability, as well as their commercial, recreational and ecological importance, and the previous successful use and ease of handling in the laboratory. *Corbicula spec*. is often used as a brackish species that is also widely spread in fresh water systems in Africa, North and South America, Europe, and the Pacific Islands [111–116]. Apart from *Corbicula, Dreissena* is one of the most aggressive and successful freshwater invaders and thus also widely spread throughout Europe and North America [117, 118]. The invasive species with euryoecious characteristics show a very high filtration rate and a rapid growth that makes these species a potential test organism for bioaccumulation studies with ENMs [119, 120]. The freshwater species used for bioaccumulation tests with ENMs were *C. fluminea* [89, 98, 109, 121, 122] or *D. polymorpha* [123]. The size of the used animals was between 20 and 30 mm [98, 123]. A standardized test system for the assessment of the bioaccumulation potential of ENMs in bivalves was recently described by Kuehr et al. [98].

Mussels show a relationship between the size of the animals and the potential for bioaccumulation due to the higher filtration rate of juvenile mussels. Thus, smaller individuals accumulate significantly more organic test substance than larger ones, while older individuals display a longer terminal half-life than younger ones. Low temperatures slow down the filtration process, inhibit growth, and may expand the life expectancy of zebra mussel [124, 125]. Increasing temperature and the presence of higher particulate matter (food) also increase the uptake by increased filtration rates [126–128]. Thus, the feeding during the studies may alter the uptake rate of the animals leading to different BAF values. However, as observed by Kuehr et al. [98] the filtration behavior was minimized or nearly stopped when no feeding occurred during the test. The filtration rate can also be impacted by the exposure concentration. Bivalves show a well described protecting mechanism of valve closing and decreased filtration activity to avoid toxic effects caused by the surrounding medium [74, 98, 128–134]. Thus, preliminary tests should be carried out to determine exposure concentrations which do not affect the valve closing behavior or filtration rate. Other conditions, like light and oxygen levels and pH, were monitored during the given studies, but no effects on the animals were described.

The duration of uptake phases described in the literature ranges from one hour up to several days and depuration phases were applied lasting 3 up to 6 days under semi-static [123] or flow-through conditions [98]. In a recent study, *C. fluminea* was exposed in a zuger glass system which allowed constant exposure conditions for up to 170 animals at flow rated of 4 L/h. A further and more detailed description of the test system can be found in Kuehr et al. [98].

Bivalve samples collected during bioaccumulation studies mostly consist of the soft body dissected from 3 to 4 individuals depending on size and species. It is recommended that sampled animals are replaced by new ones, separated by a grid, to maintain the filtration capacity of the biomass in the exposure system [88].

Soft parts of bivalves have been mostly dissected for measurement of tissue concentrations [77, 78, 98, 135] allowing to draw conclusions on the bioavailability, incorporation, transport, and fate of ENMs or their released ions. The physiological and morphological structure of the mussel tissue also allows the enzymatic digestion as part of the tissue preparation process without dissolution of previously ingested or accumulated ENMs making the analysis of particles using methods, like single particle inductively coupled plasma mass spectrometry possible (spICP-MS) [98, 136, 137].

However, due to the filtration behavior for feeding and breathing and the resulting uptake mechanisms, only BAF values can be calculated based on the results of bioaccumulation studies using bivalves as test organism.

Gastropoda

Biology and ecology

With 30,000 described species the class *Gastropoda* is the largest class of molluscs. Gastropods are the only group of molluscs that have also successfully colonized terrestrial habitats. Gastropods play an important role in the decomposition of organic material in aquatic systems and serve as a food source for organisms of higher trophic levels, like leeches, crayfish, fish, and bugs [138–142]. By this, gastropods may contribute to trophic transfer and secondary poisoning by ingestion and bioaccumulation of ENMs.

Only few gastropods are filter feeders which are able to ingest suspended ENMs. Most gastropoda feed on a wide range of foods, containing algae, detritus, plankton, sponges, polychaets, a variety of cnidarians, crustaceans, other mollusks, fish, and body fluids. As grazing herbivores (or detrivores), gastropods have significant impact on the density of macrophytes in the ecosystem [143–148]. Feeding mostly involves rasping by the radular teeth on the food substratum followed by transfer of the dislodged particles to the mouth (Fig. 2). Salivary glands, the esophagus, and the intestine produce enzymes supporting the extracellular digestion of ingested feed particles

which takes place in the stomach. The digestive gland is the principal site for intracellular digestion. With regard to the ingestion of ENMs, dietary uptake or absorption through the permeable membrane (at least for very small ENMs or released metal ions) is of concern [149–151]. However, uptake by respiratory mechanisms may be less important or even non-existent.

Freshwater gastropods can be gill-breathing caenogastropods, like *Viviparus viviparus*, *Bithynia tentaculata,* or *Potamopyrgus antipodarum* with often only one ctenidium or gill. However, most freshwater gastropods are lung-breathing pulmonates, like *Lymnaea stagnalis or Biomphalaria glabrata* [151, 152]. The pulmonate freshwater snails are air-breathing animals without gills. For filling their mantle cavity or "lungs," they spend time at the water surface [153]. Differences in those residence times of snails on the surface may introduce variability of bioconcentration processes also under experimental conditions [154]. Different respiration modes were identified by Meredith-Williams et al. [155] as the main reason for species-specific differences in the uptake of dissolved contaminants, such as the pharmaceutical carvedilol with the pulmonate snail *Planorbarius corneus* showing a lower body burden compared to the gill-breathing amphipod *Gammarus pulex*.

Oliveira-Filho et al. [158] examined the soft tissue of *B. glabrata*, previously exposed to Ag-NP for four weeks, and measured high tissue concentrations of Ag but without observing any indications of toxicity [156–158]. This was explained with the presence of metal(ion)-binding proteins, like MT, causing detoxification of potentially incorporated metal ions [159]. The presence of these proteins, their gene expression, and activity has been described for a wide range of gastropods [160–169]. Goncalves et al. [170] described a further detoxification strategy for metals by *L. stagnalis* based on phytochelates. These are chelating peptides which detoxify several metal ions [170]. In addition to that, Desouky et al. [171] observed lysosomal granules formed by sequestration of metals with P and S, leading to an immobilization of free metal ions and thus allowing the detoxification and excretion of the respective metal by defecation or exocytosis. This mechanism allows exposure of gastropods to metal or metal oxide ENMs without increasing the risk of toxic effects. The resulting body burden may be transferred to higher trophic levels or cause secondary poisoning.

Culture and breeding

The combination of their lifestyle, feeding behavior, accumulation capacity for metals, and their ecological role make gastropods an interesting group of species to be considered in bioaccumulation testing of ENMs. One of the most commonly used gastropods for bioaccumulation studies is the pulmonate gastropod *L. stagnalis* which is abundant in European freshwaters in which they are widely distributed [172, 173].

Two strategies are available to obtain animals for bioaccumulation testing:

1. Field collection
   Freshwater snails, *such as Lymnaea spec.*, *Physa spec.*, *Potomopyrgus spec.,* or *Bellamya spec.,* have often been collected from ditches [174, 175], but animals can also be obtained from specialized suppliers [174–177]. Collected animals should be acclimatized to laboratory conditions before being used in experiments and it should be controlled that no parasites impair the animals. Several larvae and trematode worms use gastropods as host and may alter their growth and egg production. Parasites may also have an impact on the metabolism, respiration, and feeding behavior of the animals potentially influencing bioaccumulation processes [178–184]. In previous studies acclimation periods of 2 up to 6 weeks turned out to be sufficient to ensure adaptation of the animals to laboratory conditions [176, 177, 185–187]. Husbandry of field-collected animals has been mostly carried out under semi-static conditions with temperatures between 17 °C, 20 °C and 24 ± 1 °C for *P. antipodarum*, *L. stagnalis* or *B. glabrata* and *B. aeruginea,* respectively. Light:dark cycles of 12:12 or 16:8 h are suitable [158, 176, 188, 189]. For feeding (ground) commercial fish food, lettuce leaves and dried spinach can be used and should be added once per week to the culture vessel [154, 157, 158, 176, 177]. Goncalves et al. [157] mentioned that an oxygen content in the water for the culture of *Physa acuta* should be above 8 mg/L and a continuous aeration is thus recommended. The culture media should have a pH value above 7.9 [157]. A standard snail medium with reconstituted water for snail husbandry and laboratory testing was described by Thomas et al. [178], in other studies moderately hard water according to USEPA was used for the culture [178, 190].
2. Lab culture
   Van Duivenboden et al. [189], explained that under laboratory conditions animals show first sexual activity after seven or 8 weeks. Egg production usually follows two or three weeks after first observed sexual activity. Per day, up to 68 eggs per snail can be produced [189].
   Most pulmonates, like the species of the genera *Lymnaea* and *Physa,* are monoecious and show high reproduction rates. However, the mortality rate is

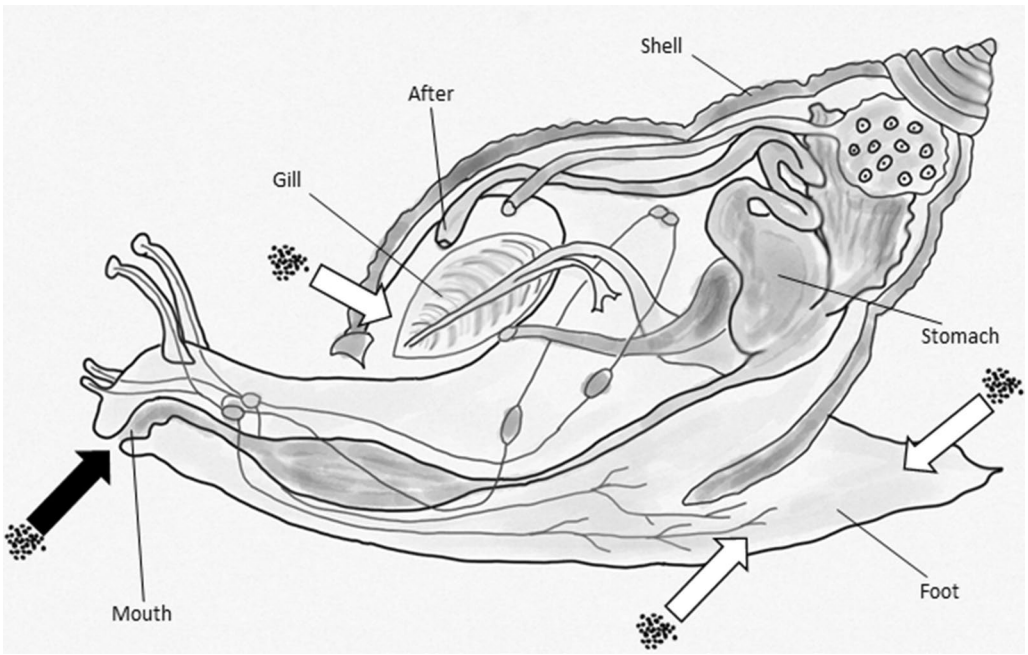

Fig. 2 Potential uptake routes of ENMs in *Gastropoda*. Black arrow: ingestion by the dietary route/ mouth; white arrows: uptake by the respiration/ gill (pulmonates) and passive uptake by the soft body tissues surface, e.g., foot

often higher than 99% until juveniles reach maturity [151, 184, 191]. Culture conditions are the same as described above for animal husbandry.

Bioaccumulation testing

Ideally, bioaccumulation experiments with snails are carried out under flow-through conditions. For flow-through studies with organic soluble compounds, flow rates ranging from 7.5 L per day up to 22 L/h were recommended [174, 192, 193]. Bioaccumulation tests with ENMs may need to be carried out with high flow rates to ensure a stable and homogeneous distribution of the test compound in the water [154]. The use of sediment in the test system is not recommended for bioconcentration studies which may be distorted by the dietary uptake of contaminated sediment. In contrast, several studies have used sediment as a source of ENM exposure [176, 177, 188]. In this case, a BAF/BMF rather than a BCF will be derived as the result of the bioaccumulation tests.

However, most bioaccumulation studies using ENMs or metals were carried out under semi-static conditions [185, 194] in different volumes of test media from 500 mL [195] to 10 L [196]. The age of the used animals in bioaccumulation tests with metals or ENMs ranged from 14 to 21 days [195] and up to 5 months [188]. Replicates were taken during the bioaccumulation studies and mostly consisted of several pooled animals, ranging from 10 animals (*L. stagnalis*) up to 30 animals (*P. antipodarum*) per replicate [176, 195] to reach sufficient biomass for sample analysis.

For tissue analysis usually only the soft tissue has been considered. In some studies the soft tissue was dissected into several compartments, like hepatopancreas, gonads, muscle tissue/foot, kidney, and digestive tract, giving the opportunity to investigate the distribution of the tested metals or ENMs [171, 177, 197]. By this, the hepatopancreas has been identified as the tissue with the highest accumulation potential for metals, presumably caused by the high content of metal-binding proteins [177, 188]. However, Legierse et al. [154] mentioned that for measuring internal concentrations of the test item, the gut should be removed from the soft tissue and excluded from weight determination and tissue analysis.

Uptake phases of bioaccumulation experiments ranged from 6 h [198] up to four weeks [158, 188] for water exposure, and from 3 h [198] up to one week [176] for dietary exposure studies. The duration of the depuration phases were mostly in the same range, and in the study of Croteau et al. [199] the duration of the depuration phase was to two times the uptake phase.

Continuous feeding of snails during the test period is recommended to prevent starvation or alteration of metabolism due to fasting conditions. Daily feeding of lettuce, dried spinach, or fish food and removal of feces and feed residues has been the preferred method [158, 176, 177, 188]. Another feeding procedure is adding

Spirulina algae powder or ground fish food to the vessel which sediments and can be ingested by grazing the bottom of the experimental tank [157, 200]. However, as a consequence, a rise of total organic carbon content in the test system is observed, which enhances adsorption of the test substances to organic matter [5, 197]. This potentially influences its bioavailability and thus the result of the bioconcentration experiment. To avoid this, further studies were carried out without feeding of the snails but only for short exposure periods to avoid the risk of fasting as mentioned above [175, 193].

For metal bioaccumulation the digestive gland plays an important role acting as a metal sink as shown for aluminum [186]. The gut of gastropods secretes mucus with a strong affinity for Al [185, 201]. Thus, the mucus could be a factor altering the uptake of metals because mucus glycoproteins are able to bind trivalent metals, like Fe, Zn, Pb, and Al [202–204]. For the mostly bivalent metal Cd, Coeourdassier estimated BCFs of 90–262 in the foot and 662–2602 in the viscera of *L. stagnalis* that are dose dependent with the highest values obtained for the highest concentrations [196]. However, for *L. palustris*, the estimated BCF values for Cd were up to 6000 and thus 9.4 times higher than those calculated for *L. stagnalis*. Accumulation of Cd (in combination with Zn) was also observed in the species *Melanoides tuberculata* and *Helisoma duryi* after a short aqueous exposure phase of about 6 h [198]. For the bioaccumulation of NPs, *Gastropoda* are often exposed via the dietary pathway. However, few studies have investigated the bioaccumulation of NPs in *Gastropoda* and these are mostly in combination with the dissolved form (e.g., Ag-NPs and $AgNO_3$) of the same element to compare the bioaccumulation, uptake, and elimination of both forms [205].

It was shown that the accumulation of water-borne Ag and Cu from the dissolved form (Ag^+ and Cu^{2+}) is much higher than that from the nanoparticulate form [32, 177, 188].

Bao et al. [177] exposed the freshwater gastropod *B. aeruginosa* to Ag-NPs and dissolved Ag and observed a higher uptake of Ag following exposure to the dissolved form leading to the highest burden in the hepatopancreas, followed by the gonads and digestive tract. Only low amounts of Ag were found in the foot tissue [177]. The distribution of the metals could be explained by the amount of metal-binding proteins and chelates in the respective tissues. Also, for dissolved Cu (Cu^{2+}) a higher uptake compared to Cu-NP exposure was observed in the same species, resulting in comparable tissue distributions [188].

Croteau et al. [199] described a protocol that allows the feeding of snails with algae which were previously exposed to NPs. That method was used for a dietary exposure study with CuO-NPs. Feeding of the benthic diatom *Nitzschia palea* which was enriched with Ag-NPs and Ag^+ to *L. stagnalis* showed that the uptake rates were faster for Ag^+ than for Ag-NPs when both exposure scenarios are compared. During the depuration phase following dietary exposure, a very slow elimination of Ag with nearly no differences between both forms of Ag was observed. However, for aqueous exposure a faster elimination of Ag was observed if the Ag was presented as Ag-NPs. That means that the aqueous exposure may mainly lead to a simple ingestion of Ag-NPs that can be eliminated easily. A similar approach was successfully applied for ^{67}ZnO-NPs. Pre-exposed diatoms were used to expose *L. stagnalis*, resulting in comparable observations [206]. In a further study on *L. stagnalis,* only low bioaccumulation was observed for Au-NPs [11, 195] following dietary exposure.

Isopoda

Biology and ecology

The order *Isopoda* represents the first group of crustaceans considered in this work and contains more than 4000 described species. Besides, a few terrestrial species most isopods live in aquatic habitats. Aquatic isopods are benthic animals which are also able to swim. Gills for respiration are located at the base of the thoracic appendage and the integumentary surface is also used for gas exchange (Fig. 3).

Although the main part of the aquatic species lives in the sea, some species are native to freshwater systems. As benthic organisms, they are often used for ecotoxicological studies with sediment [207].

Asellus aquaticus has been described to be a useful biomonitor for organic and metal pollution in aquatic systems [208–214]. Due to their low sensitivity for brackish water, this species can be used in tests under freshwater and estuarine conditions [215–217].

Asellus aquaticus can be found in most European freshwater systems, streams, lakes, and nearly every surface water in Europe and North America [218–222]. It is often present in water systems close to sewage treatment plants or in lakes and other water systems with organic pollutants in higher concentration [223]. In the water system they live in littoral and sublittoral zones and reside on macrophytes, filamentous algae, litter, and stones [224, 225]. As most isopods, *A. aquaticus* is an omnivore and deposit feeding organism. As a non-selective feeder it feeds on detritus, periphyton, microscopic algae, small invertebrates, decaying vegetation, fungi, and bacteria [218, 219, 221, 225]. *A. aquaticus* is a very robust species and survives extreme environmental conditions, including hypoxic phases [207], and tolerates

water temperatures of up to 28 °C, and pH values down to 4.3 [207, 224, 226–229]. In contrast, *A. aquaticus* is very sensitive to heavy metal exposure being as sensitive as *Gammarus* or *Daphnia* [230]. The accumulation of metals from food and water in *A. aquaticus* has been described to strongly differ across several metals with some metals even showing no bioaccumulation potential [231]. For instance, aluminum is not accumulated from water at neutral pH conditions [232]. The differences in metal bioaccumulation were explained by the amount, binding affinity, and capacity of metal-binding proteins present in the isopods tissue used as part of the detoxification system [207, 214, 233, 234]. Sensitivity to metal exposure has been shown to be different for male and female animals as well as for different life stages (mature or juvenile) [230].

Another detoxifying strategy involves the sequestration of metals in Type B granules based on S-Type cells of invertebrates, leading to metal accumulation especially in the soft tissue close to the carapace and in the exoskeleton [235]. Rauch and Morris observed more than 20 times higher metal concentrations in the molted carapace than in the soft tissue of *A. aquaticus* [233]. This may also be explained by physical adsorption of metal ions to the exterior surface of isopods [232]. This must be considered in bioaccumulation studies, especially with respect to isopods which shed their exoskeleton causing an uncontrolled elimination [232].

The accumulation of metals and organic compounds in *A. aquaticus* plays an important role in the transfer of pollutants in the aquatic food chain [236]. As macro-invertebrate species, *A. aquaticus* represents an important food source for predatory invertebrates, several fish, amphibians, and waterfowl in the northern hemisphere [225, 237–242].

Culture and breeding

Most laboratory studies have been carried out with animals collected in the field which were then acclimated for a period of a few weeks up to several months under laboratory conditions [230–232, 243, 244]. The preferred culture conditions in a semi-static system include temperatures between 10 and 19 °C with around 240 mg/L $CaCO_3$, a pH range of 7.2 to 7.4, and a light:dark cycle of 14:10 or 16:8 h [230–232]. However, due to the risk of parasite infection, e.g., by acanthocephalans, and the higher abundance of animals in contaminated water systems with high organic load and potentially leading to a high metal burden, laboratory culture of test animals is recommended [245–247].

Protocols for the laboratory culture of *Asellus* were described by Plahuta et al. [247] and McCahon and Pascoe [248]. 500–1500 juveniles per breeding container (1 L plastic containers) [248] were obtained from 100 females maintained in dechlorinated, aerated tap water. The isopods can be fed with *Elodea* or ash leaves [232]. However, the leaves should age in water or the applied culture media to enable colonization of a microbial community that can provide nutrients, such as vitamins, which are essential for the animals [221].

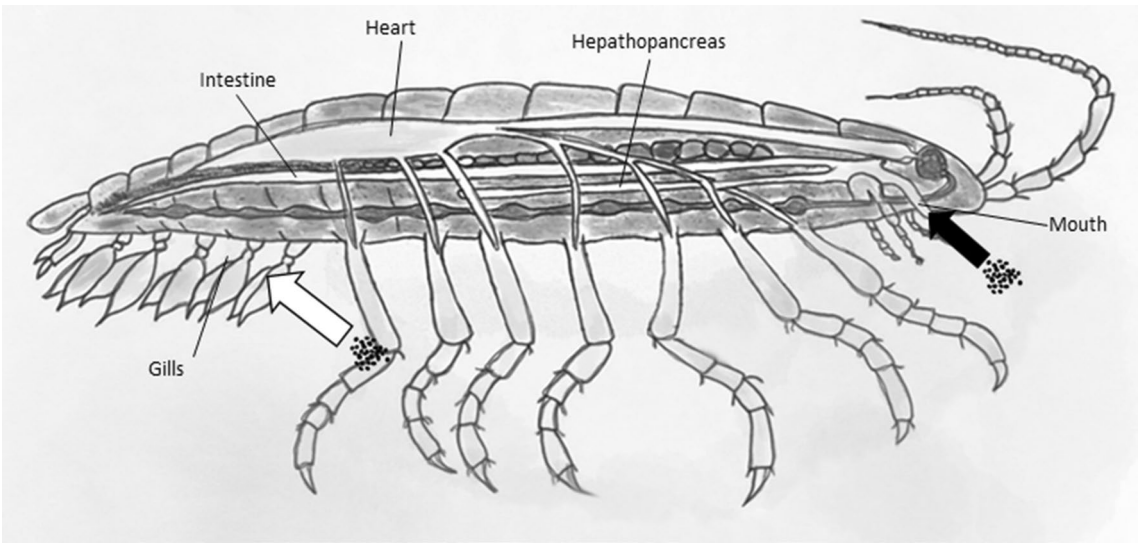

Fig. 3 Potential uptake routes of ENMs in *Isopoda*. Black arrow: ingestion by the dietary route/ mouth; white arrow: uptake by the respiration/gills

Bioaccumulation testing

Asellus has been used for bioconcentration and biomagnification studies, in particular for investigations on metal accumulation [207, 241]. Water is the primary route of exposure for *A. aquaticus* [249] regarding environmental pollutants. Only for highly lipophilic chemicals uptake via food is important due to adsorption to organic particles [251, 252], which may also be the case for ENMs [249, 250].

Both male and female animals of *A. aquaticus* have been used for bioaccumulation testing. However, only animals showing no apparent sexual or reproductive activity were used [249]. The size of animals used in the experiments ranged from 4 to 20 mm [230, 232, 244], which had fresh weights ranging from 7 to 28 mg [220].

During tests the temperature ranged from 15 to 21 °C and a photoperiod of 16:8 h of light: dark was maintained [244]. In the case of short uptake periods, such as 2 to 5 days, animals were not fed [207, 243, 251]. During longer test periods isopods were fed with ash or *Elodea* leaves [232, 244].

Static test systems have often been used for metal bioaccumulation tests with spiked sediment as the source of exposure [207, 220, 231, 252]. Van Hattum et al. [231] used deionized and previously aerated (24 h) tap water as test medium, whereas Elangovan et al. [232] used standard snail water as described by Thomas et al. [178]. For the investigation of bioaccumulation of PAHs, van Hattum and Montañés [244] carried out a semi-static test with an uptake and elimination phase lasting 7 and 12 days, respectively [244]. Organic compounds were mostly tested in flow-through systems which should also be considered for testing ENMs to ensure homogeneous exposure conditions. Richter and Nagel [249] applied a medium exchange rate of around 1.5 exchanges per day (1 mL/min) and observed no mortality during the test period, whereas van Hattum et al. [231] replaced the medium once per day in test vessels containing 20 animals. In this study cannibalism of the test animals was observed, which indicates an insufficient supply of nutrients or unsuitable test conditions causing stress [231]. Samples collected for chemical analysis included 4 to 25 pooled animals to ensure sufficient biomass for sample extraction [207, 220]. Due to the potential of metal ions to sequester in Type B granules in S-Type cells located close to the carapace or exoskeleton, the soft tissues and exoskeleton should be analyzed as single compartments, e.g., as described for the terrestrial isopod *Porcellio scaber* by Kampe et al. [253].

Only one bioaccumulation study with ENMs using aquatic isopods has been described in the literature. Ekvall et al. [212] exposed *A. aquaticus* for 2 months to WC-NPs under static conditions. The body burden of pooled samples consisting of ten individuals was measured [212]. However, no BCF or BAF was calculated.

However, several studies have been carried out on the bioaccumulation of metals. van Hattum et al. [241] exposed *A. aquaticus to* Cd and calculated a BCF of 18,000 and a BMF of 0.08. The uptake was shown to be independent of the pH of the exposure medium. The uptake by the dietary path represented only 50% of the complete Cd uptake [241] even at very high Cd concentrations in the food (220 times higher than in the water). For *A. meridianus,* both the uptake of Cu and the uptake of Pb, following dietary and aqueous exposure, are described by Brown [254], whereby Eimers et al. [255] showed that for Cd the dissolved form is more bioavailable for *A. racovitzai* than Cd associated with particles [255]. In their work from 1993 van Hattum et al. described that cadmium and copper seem to be accumulated and stored in the organisms, whereas zinc and lead are rapidly eliminated [231]. Further, it has been reported that the accumulation of the metals palladium (Pd), platinum (Pt), and rhodium (Rh) seem to depend on the exposure time (BCFs: 150, 85, 7) rather than the exposure concentration [207]. The uptake rates of Cd measured in *A. aquaticus* are comparable with those reported for the bivalve *Ruditapes decussatus* and the snail *L. stagnalis* [165, 256, 257]. However, if exposed to mixtures of Pb, Cd, Cu, Ni, and Zn, the uptake rates of Cd in *A. aquaticus* were shown to be higher than those reported for *Daphnia magna* when exposed to the same mixture of metals [258].

Amphipods

Biology and ecology

Amphipods often represent the dominant part of the benthic macro-invertebrate species in fresh water ecosystems [259]. They represent an important food source for several groups, like fish, predatory insect larvae, amphibian species, and water birds and are present during the whole year in contrast to insects which show a largely seasonal distribution [260–264]. The group of amphipods contains several species that are key species in aquatic ecosystems, playing an important role in the energy and nutrient cycling within the ecosystem, e.g., by breaking down leaf litter and their important role as a prey organism [263, 265–267]. By shredding decomposing leaves they mobilize small particles that can be ingested by filter-feeding organisms. The released feces can be readily decomposed by other organisms. Important food sources of amphipods can be water plants, such as *Elodea spp*., but also other macroinvertebrates and even small/wounded fish species [259]. Also cannibalism has often been observed in amphipod populations. Gammarids are the most widespread group of amphipods in Europe

(e.g., *Gammarus fossarum* or *G. pulex*) but are also present in North America (e.g., *G. fasciatus* or *G. limnaeus*) [268–270]. *Hyalella azteca* is an epibenthic amphipod which is widespread in North and Middle America [262, 271–274]. In the following sections we mainly focus on *Gammarus sp*. and *Hyalella*.

Gammarus pulex can reach an age of 1–2 years, depending on the environmental conditions, and mainly feeds on decaying leaves of deciduous trees, algae, insects, and other invertebrates [218, 261, 275]. Their growth is strongly temperature dependent and generally slower under low temperature conditions [276]. The approximate length of *G. pulex* is about 2 cm, with males being larger and usually growing faster than females [275, 277]. *G. pulex* can represent up to 17% of the dietary-based energy ingested by young salmonids [278], and in some macroinvertebrate communities different *Gammarus* species can represent 28–38% of the biomass [259, 278].

H. azteca, in contrast to *Gammarus*, feeds on detritus by grazing on the sediment [279]. At the age of 20–30 days the first pairings appear. The number of offspring varies between 3 and 17 juveniles, depending on the age of the female [279]. *H. azteca* is described to be very tolerant against varying temperatures and low oxygen levels [262, 280].

Respiration of *H. azteca* mainly occurs via gills that are located at the thoracic segments (2–7), whereas *G. pulex* sometimes have an additional pair of gills on the first pair of gnathopds or pereopods [281, 282] (Fig. 4). The thin epithelium of the gills is primarily responsible for the exchange of ions, like Na^+, Ca^+ , and NH_4^+, but also other areas of the animals surface seem to be permeable for ions and are thus further locations of metal ion uptake [283–286]. While Vellinger [287] mentioned three theoretical ways of metal uptake for amphipods: (i) directly from food or water, (ii) through the cuticle, and (iii) diffusion across the exchange surface, Rainbow [288] explains that the crustacean exoskeleton is supposed to be almost impenetrable for dissolved metals [287, 288]. This is in line with the results of Vellinger [287], who showed for *G. pulex* that the strongest uptake of metals, as observed for Cd, is through the permeable surfaces, e.g., of the gills. In this study the accumulation of Cd and As was observed, whereby the uptake of Cd was shown to happen via the gill membranes, whereas As (as As^{5+}) was ingested through phosphate channels [287].

Following aqueous exposure of Cd, *G. pulex* has been observed to reduce the ventilator activity by reduction of the pleopods beats [289]. Comparable observations have been described for Cu and other heavy metals of sublethal concentrations [290, 291]. Another strategy to avoid toxic effects of metal exposure is the sequestration of free metal ions as described before for other species. Water born Cd is known to accumulate primarily in the gills and hepatopancreas of amphipods [292–294]. However, accumulated Cd and Pb were mainly found in insoluble fractions and particularly in metal-rich granules [295]. The role of metal-binding proteins and precipitation granules are further described by Koropatnick [296] or Ahearn [297]. Accumulated Ni was primarily localized in the cytosolic fractions of *Gammarus sp.* [298].

Another amphipod which has often been used for bioaccumulation tests is *Diporeia spp* a benthic organism which is known to be the most abundant macroinvertebrate in the Great Lakes. But its occurrence has sharply declined since the 1990s as a result of a change in the food web composition, probably caused by invading dreissenid mussels [299, 300]. Their formerly high abundance made them an interesting organism to test the toxicity and accumulation behavior of different compounds in the Great Lakes' region. For sediment toxicity tests it has been included as an alternative test organism in the ASTM guideline 1706–05 [301].

Culture and breeding

In previous laboratory studies, *Gammarus sp.* were mostly collected in the field and acclimatized for 1 up to more than 20 days to temperatures between 12 and 20 °C and kept under a light:dark regime of 16: 8 h or even under complete darkness [155, 250, 270, 302–305]. During the acclimation phase the animals are usually kept in dechlorinated tap water, artificial pond water, or commercial mineral water [306–310]. In contrast to *Gammarus*, which are difficult to breed in the lab, *Hyalella* can easily be cultured under laboratory conditions [311–315].

Using *Gammarus sp.* from the field brings the risk of using animals that are infected by parasites, like the acanthocephalan parasite *Pomphorhynchus laevis*. Infected animals show an altered feeding behavior that may be caused by damaged internal organs, including the hepatopancreatic ceca which is involved in the detoxification of stored metals. Thus, checking samples of the populations from which test animals are collected is essential. Parasites can be identified by dissection or by looking for orange spots that are visible through the carapace providing a clear indication for the parasite infection [316–324]. Effects of the infection on the concentration on metal-binding metallothioneins have been described, e.g., by Frank et al. [325] and Marijic et al. [326]. Further, a recent study showed that the cannibalistic behavior of *G. duebeni celticus* was greatly enhanced when infected with the microsporidian *Pleistophora mulleri* [327].

For *G. pulex* there is no broadly applied protocol that enables easy culture in the laboratory. First trials to

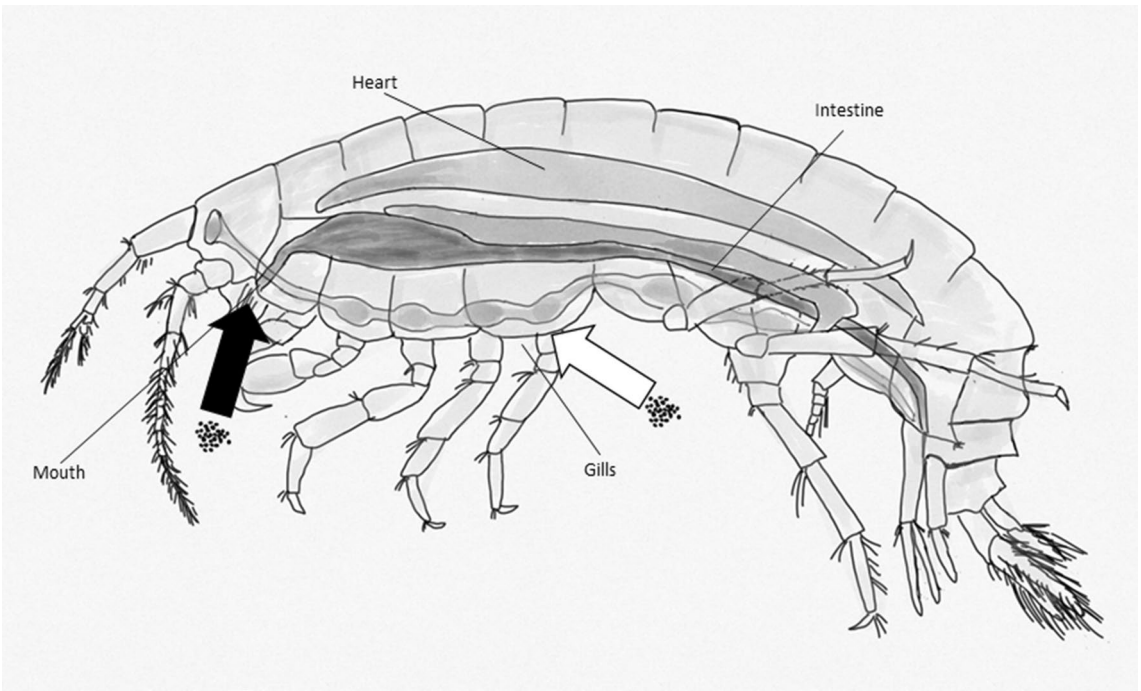

Fig. 4 Potential uptake routes of ENMs in *Amphipoda*. Black arrow: ingestion by the dietary route/ mouth; white arrow: uptake by the respiration/ gills and treacheae

culture it for accumulation studies date back to the 1980s [275]. Probably the best known culturing procedure was described by McCahon and Pascoe who reported that approximately 70% of the juveniles survived and reached maturity obtained in a flow-through system with dechlorinated tap water which gets exchanged approximately every four hours [248, 306]. Furthermore, illumination was for 12 h with a light intensity of 750 lx; food consists of conditioned leaves of preferably horse chestnut leaves. Other feeding options are conditioned leaves of elm, oak, or sycamore. Algae are another food option, early juveniles feed on the adults' feces before they can feed on leaves [248, 270, 305, 306].

Brood sizes in *Gammarus* vary from approximately 6 to 29 eggs per female. 2 to 3 broods can be delivered per breeding season that in general lasts from spring to late autumn, followed by a breeding pause during winter [328]. McCahon and Pascoe [248] were able to determine a correlation between the age of *G. pulex* and the mean body length and mean antennal segment counts which simplifies age determination in test situations [248, 306]. An increased temperature and excess food reduces the time *Gammarus* needs to reach sexual maturity and to enable breeding in the laboratory throughout the year. Bloor et al. [329] adapted a culture method requiring approximately 200 gravid females for an output of 500–1000 juveniles with no or only negligible mortality. Although these protocols apparently seem to provide an option to cultivate *G. pulex* in the laboratory, up to now these procedures are not broadly applied and a test procedure to establish *G. pulex* as a standard test organism is still absent [330]. A big problem in the culture of *G. pulex* appears to be cannibalism, with bigger animals often preying on smaller animals [331].

For *H. azteca* mass culture conditions have been reported by de March [332] and optimized by Borgmann et al. [334] and were used in different laboratories [121, 311–313, 332–335]. Animals have been mostly cultured in 1–2 L of standard artificial media containing bromide in polyethylene containers with a small piece of cotton gaze (3 × 3 cm) providing a place of refuge. A 16:8 h light: dark photoperiod, water temperatures of 23–25 °C, and feeding three times a week with periphyton or ground fish feed (TetraMin®) are optimal conditions for the culture of *H. azteca* [279, 334, 336, 337]. Wilder showed that the population density strongly influences growth, reproduction rate, and mortality of *H. azteca* in lab culture [338]. However, the easy culture providing a stable and homogeneous population makes *H. azteca* a clearly more suitable species for laboratory testing compared to *Gammarus sp.*

Diporeia spp. cannot be cultured in the lab and have to be collected from the field. In the lab, collected animals should not be held for longer than 2 months under static

conditions because deterioration can be observed after this culturing period [301]. However, in some cases the lab culture could be maintained for up to several months [339].

Bioaccumulation testing

Gammarus pulex tests have been commonly conducted with static renewal of the test media, sometimes also as flow-through studies [155, 307, 340, 341]. Reported mortalities of control animals in *G. pulex* test systems are low and ranged from 0% in short-term experiments to below 2% mortality for populations held for two weeks under experimental conditions [155, 298, 340]. The low mortality confirms the suitability of the test systems for laboratory experiments. In several studies daily renewal of the test media was carried out [155, 298, 308, 342]. Xu and Pascoe [343] used a flow-through system providing a full exchange of the test medium in approximately 3 h. Exposure periods ranged from 24 h to 4 days in tests with ENMs, the duration of the following depuration phases was in a comparable range [303, 312]. Feeding either consists of alder leaves or no feeding during the tests [270, 303, 305]. Mostly only males with a length of at least 8 mm were used for the tests [270, 303–305]. At each sampling point 3 or 4 replicates were collected, each consisting of 4 to 5 adult [303–305] or 10 juvenile animals which were further processed and analyzed as pooled samples [312].

Known to be good metal accumulators the species from the genus *Gammarus* may constitute a primary source of metals in the food chain [344]. The ability of Cu accumulation of *Gammarus* is comparable to that from *Daphnia*, but 9 times higher than reported for *H. azteca* [258, 345, 346]. Clason et al. [347] obtained BCFs for Cd (80–202), Cr (80–122), Cu (306), Pb (251–520), and Ni (23) in Gammarus by using a two-compartment model. In contrast Xu and Pascoe [343] used a modified first-order kinetic model, taking the basal level of essential trace metals into account, to calculate the uptake and elimination rate to predict the BCF of Zn in *G. pulex*. They proved the suitability of the model by agreement of the predicted and measured BCFs. Mehennaoui et al. [270] determined BCF values of 147 to 4238 for different water-borne Ag-NPs in *G. fossarum.* The BCF for $AgNO_3$ was determined to be 2277. A comparable low BCF value of 176 was determined for CeO_2NPs in *G. roeseli* by Garaud et al. [303].

H. azteca has been suggested by USEPA and Environment Canada, as a model organism to investigate sediment toxicity covered by an ASTM test method [190, 273, 301]. The potential of *H. azteca* as a test organism for bioconcentration studies with organic compounds was demonstrated by Schlechtriem et al. [313]. *H. azteca* has also been tested in bioaccumulation studies using ionic organic compounds [335, 348, 349].

Experimental setups with nanomaterials were conducted under (semi-)static and flow-through conditions with flow-rates of around 3 to 4 L/h [311, 312, 315]. Feeding was carried out during the studies by using ground fish feed (TeraMin®) or DECOTABs as described by Kuehr et al. [315] that minimize the risk of artifacts due to uncontrolled exposure to ENMs that adsorb to the feed [315, 350].

Duration of uptake phases ranged from 24 h to 14 days, with similar durations for the following depuration phases [195, 311, 312]. For analysis, mostly 3 to 5 replicates were used each consisting of 10 to 20 animals [195, 311, 312, 314, 315]. Survival rates were not presented for all studies but ranged under flow-through conditions between 80 and 100% [313].

Studies carried out according to the official guidelines usually assess sediment toxicity. Also some of the accumulation studies were conducted investigating the accumulation of contaminants from the sediment rather than from the water column [121, 312, 345].

The age of test animals varied in the bioaccumulation studies which have been conducted so far, but no detailed comparison regarding bioaccumulation potential depending on the life stage of *H.* azteca has been found in the literature. However, Alves et al. [345] stated that uranium uptake from sediments does not depend on the age of *H. azteca*.

H. azteca has been used in some laboratories as an organism to assess accumulation of metals, like Cu, Cd, Zn, Pb, U, Tl, or Hg. Alves et al. [345] showed by the example of U that bioaccumulation of metals in *H. azteca* sediment tests is primarily based on the dissolved part and less from the sediment. Similar observations were described for other metals by other authors [346, 351–353]. Alves et al. [345] also showed that the bioaccumulation of U is dose dependent and could be described and explained by the Biotic Ligand Modell of Paquin et al. [354]. Shuhaimi-Othmann and Pascoe [355, 356] showed that there is a rapid uptake and elimination of metals, with the exception of Cd that showed virtually no elimination [355, 356]. This explains the high BCF of 31,803 for Cd that is significantly higher than those of Cu or Zn (BCFs of 1535 and 1060). It was also proven that a co-exposure of these metals influences their accumulation negatively.

The observation of the primary role of ions in the uptake of the metal is in agreement with the results of Kuehr et al. [314] who used methods of correlative microscopy to identify the main uptake pathway of silver from wastewater and/or sewage sludge-borne Ag-NPs. They also made similar observations for silver as made

for Cd by Shuhaimi-Othmann and Pascoe [356] who observed virtually no elimination, potentially caused by the binding of Cd to metal-binding proteins [314, 356]. Similar observations regarding the incomplete elimination of Ag were made by Kuehr et al. [98] after water and dietary exposure to Ag-NP or $AgNO_3$. A special flow-through system was applied described by Kuehr et al. [315] allowing the constant and homogeneous exposure of the amphipods by water or by using a diet that minimizes the dissolution of ions. During their studies they were able to determine BCF, BMF, and BAF values for Ag from $AgNO_3$, Ag-NPs, TiO_2 from TiO_2NPs, and Au from Au-NPs [315]. However, Hudson et al. used juvenile *H. azteca* in the age range of 7 to 14 days and observed no measurable uptake of Au-NPs by the young amphipods after 24 h of dietary exposure [195].

Branchiopoda

Biology and ecology

Cladocerans belong to the class of *Branchiopoda* (fairy shrimps) and are more commonly known as water fleas and encompass a group of so far 620 described species. In general, Cladocerans live in fresh water and usually represent a very important part of the food web. However, a small fraction has been identified living in brackish to saline waters, or are even considered as truly marine species [357]. The genus *Daphnia* has been studied for approximately 250 years already, resulting in a vast pool of information about this genus that so far has more than 100 known different species [358]. Depending on the species their body size varies from 0.5 to 6 mm [359]. Commonly used for ecotoxicological testing are the two *Daphnia* species *Daphnia magna* and *Daphnia pulex*.

The information about the biology of *Daphnia* are collected from reviews conducted by Herbert [358], Ebert [360], and Stollewerk [361] if not cited differently.

Daphnia are filter feeders, living pelagically in open waters. Animals consume small particles, such as algae, bacteria, fungi, protozoa, and organic debris, and represent an important part of the diet of a wide range of vertebrate (planktivorous fish) and invertebrate species, such as animals of the genus *Chaoborus* or *Notonecta,* and thus play a significant role in energy and nutrition flow of their ecosystems [362, 363].

For feeding they use their appendages to generate a water flow that funnels suspended material toward their mouth [364]. It is reported that *Daphnia* are only able to ingest particles bigger than 200 nm after filtering the water, due to the size of the space between the setulae on the thoracic limbs that are used to generate and sieve the water flow [365]. Also, the extraction efficiency of suspended particles depends on their size [366, 367]. Nevertheless, ENMs with smaller particle sizes suspended in the surrounding media may be ingested by direct intake due to passive drinking or incidental ingestion of the medium [368–370] (Fig. 5).

A *Daphnia* colony consists mainly of female organisms that reproduce via parthenogenesis into genetic clones. The diploid eggs reside within a dorsally placed brood chamber. The reproductive process is influenced by a variety of environmental factors with temperature being the most important one. A water temperature of around 20 °C represents optimal conditions for reproduction. At this temperature young hatch from their eggs in the brood chamber after only one day, then stay there for about 3 days before being released. A mature Daphnid is capable of releasing a brood every three to four days, lower temperatures increase this time. Furthermore, the onset of sexual maturation of Daphnids is delayed at low temperatures. The amount of offspring depends on the mother's size; therefore, a nutrient-rich environment promotes growth, resulting in bigger animals that in return can give birth to larger broods. When food becomes scarce or environmental conditions deteriorate, a trigger for sexual reproduction can be activated inducing sexual reproduction instead of parthenogenesis.

Waters inhabited by *Daphnia* show pH values of 6.5–9.5, the optimum appears to be in the range of 7.2–8.5. Their osmoregulation works via an uptake of chloride ions via absorbing glands. Oxygen distribution is facilitated by their hemoglobin-containing hemolymph, which Daphnids can regulate according to the oxygen availability in the water.

Blood cell-derived phagocytosis has been observed in *Daphnia* already in 1884 that may also allow the incorporation of previously ingested ENMs [371]. Bianchi and Wood [372] described for Ag^+ that the branchial epithelial cells of *D. magna* are capable of ingesting metal ions through Na^+ channels. It has been further described that the exposure to heavy metals and their released ions induce the expression of MTs and MT-like proteins in *Daphnia* [373, 374]. Fraysee et al. [375] observed that 75% of the complete body burden of *D. magna* after exposure to Cd was bound to MT. Wu et al. [376] described that the Cu content of molted carapaces of *D. magna* that have previously been exposed to CuO-NPs was as high as the total body burden of the Daphnids. Thus, the storage of metals in the carapace and the molting process that occurs approximately every two days may also represent a detoxifying and elimination strategy for metals in *Daphnia*.

Their worldwide abundance, sensitivity toward contaminants, important role in the food chain, and ability to

be cultured in the lab have made *Daphnia* popular organisms for ecotoxicological testing. As a result, two OECD test guidelines have been developed over the years to assess the toxicity of chemicals with respect to two different factors. These are acute toxicity (OECD TG 202) and chronic toxicity (OECD TG 211) [377, 378] with immobilization and reproduction as the respective endpoints. Although both *Daphnia pulex* and *Daphnia magna* are accepted species for studies following both test guidelines, *Daphnia magna* is the commonly preferred species.

Culture and breeding

One of the reasons why *Daphnia* has gained popularity as a test organism is the option to easily culture it in the laboratory. As test medium well-filtered or spring water can be used [379, 380], but due to the demand for a standardized medium and general culturing conditions, the M4 and M7 media by Elendt [381] are recommended by the OECD guidelines [378]. However, *Daphnia* cultures do not appear to readily survive in Elendt media from the beginning, which is why a gradual acclimation to the medium over a period of up to one month is recommended.

A *Daphnia* culture should be monitored frequently. If *Daphnia* start to develop ephippia and male animals can be spotted, the culture is under stress and females produce eggs that can withstand unfavorable conditions. This has to be avoided and animals from such a culture should not be used for tests.

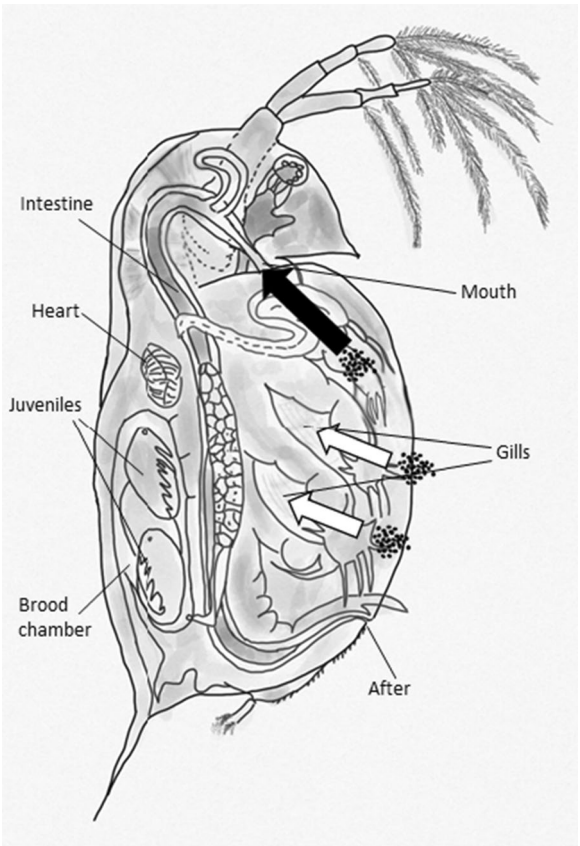

Fig. 5 Potential uptake routes of ENMs in *Daphnia*. Black arrow: ingestion by the dietary route/ mouth; white arrows: uptake by the respiration/gills

Bioaccumulation testing

Accumulation studies with *Daphnia* have been conducted already in the 1970s, investigating accumulation potentials for substances, such as DDT [382], anthracene [380], and α-HCH [383]. For long-term experiments the usage of a medium with stable characteristics is sensible. Therefore, the media suggested for culturing should be also used for longer-term studies. In the case that metals or substances containing metals are tested, the use of test media, such as M4 and M7, containing EDTA, a chelating agent that binds metals, should be avoided. The OECD recommendation for an alternative test medium is reconstituted hard fresh water described in the ASTM guideline E 729–96 [384].

Dissolved oxygen of more than 3 mg/L can be achieved by constant aeration of the test medium prior to use. However, aeration during testing should be avoided (OECD TG202, TG211) since *D. magna* may die from small air bubbles in the medium [385].

Temperatures in *Daphnia* tests are usually in a range of 20–25 °C [386–389], but should not vary by more than 2 °C within a test [378]. Light intensities should be in a range of 1000–1500 lx of cool white light [378]. The light–dark cycle in bioaccumulation studies with ENMs varied from complete darkness [386, 387], 12:12 [390] and 14:10 [391, 392] to 16:8 h [393, 394].

The OECD guideline 211 allows to either run a static-renewal or a flow-through study which may help ensure constant exposure conditions. The ASTM guideline E1193-97 [395] describes different delivery systems suitable for *Daphnia* tests [396–398]. Accumulation studies using a flow-through system were described by Biesinger et al. [399] and Meinertz et al. [400].

The OECD TG 211 guideline suggests daily feeding in static renewal systems, but feeding in accordance to media exchange is also accepted. The food addition should not be too concentrated or too diluted. Food for *Daphnia* consists of green algae cells. Several algal species are suggested in the official guidelines. Other supplements proposed by the ASTM guideline E 1193–97 [395] are trout chow and yeast, but is only rarely applied [400–402]. However, in bioaccumulation studies on ENMs feeding generally poses the risk of ENMs attaching to the

food potentially leading to altered bioavailability and the uptake via different pathways (water and food) and thus only allowing the determination of BAF values.

So far accumulation studies have been performed with different compounds, including several metal and metal oxide ENMs. The age of Daphnids used for (bio)accumulation studies varied significantly and there is no recommendation for a preferred age. In the literature studies are found using neonates [386–388, 391, 403–406] as well as using adults [393, 394, 407–410]. In most cases, 3 replicates per sampling point were used, each consisting of 10 adult individuals. In the case of collecting neonates, replicates could consist of up to 100 animals [388, 403]. The volume of the test/exposure medium was mostly in a ratio of 5 to 10 mL per animal and all tests were conducted under static or semi-static conditions. Information on the feeding regime during bioconcentration studies is not always available. However, in some studies no feeding was applied [408, 411]. Feeding of the animals during bioconcentration studies was leading to a faster and more effective elimination of body burden, but no alteration in the uptake of the test items was observed [387, 388, 412]. Some studies investigated the uptake of ENMs by the dietary path and described methods to generate algae-based, pre-exposed diets [389, 410, 413].

The length of the uptake phase of *Daphnia* bioaccumulation studies ranged from 30 min [391] and 5 or 6 h [388, 414] up to 2 days [392, 404, 408, 410]. Depuration phases were sometimes longer than the corresponding uptake phase ranging from 24 h [387, 414] up to 3 days [388].

Several studies observed the uptake of ENMs by *D. magna* and their accumulation in the gut region [19, 387, 389, 390, 403, 404, 406, 412]. The availability of the particles is explained by the mostly higher hydrodynamic diameter that allows the extraction and uptake of these particles from the surrounding medium, even with core sizes smaller than the mesh size of the daphnids setula [414]. Regarding the uptake of ENMs into epithelial cell membranes, the transfer by clathrin-mediated endocytosis is discussed [414–416]. However, there was no clear evidence for the real bioaccumulation/incorporation of ingested ENMs into the tissue or single cells or their membranes [404, 406, 407, 417, 418] as confirmed by histological investigations using electron microscopy. However, the simple ingestion of ENMs may still cause increased body burdens as described for Cu-NPs and ZnO-NPs [387, 408]. High BCF values were determined for ionic Ag which can be explained by the fast uptake mechanism due to the high affinity of Ag^+ to membrane transporters, like Na^+ channels [372, 410]. Also the release of ions from ENMs may thus contribute to the body burden of exposed animals. In most studies on metal bioaccumulation in Daphnids, uptake and elimination kinetics were derived but no steady-state BCF/ BAF or BMF values were reported. Only in a few studies bioaccumulation endpoints were determined. For TiO_2-NPs BCF values ranging from 1230 (after 24 h of exposure at 1 mg/L, with food) to 118,000 (after 24 h of exposure at 1 mg/L without food) for adults [412] and from 21,200 to 145,350 for juveniles (after 24 h of exposure to 1 and 10 mg/L, respectively) were reported [389]. BMF values for TiO_2-NPs were concentration dependent with higher values for higher exposure concentrations and ranging from 5.7 to 122 in juveniles after 24 h of exposure [389]. Higher BAF values of 132–214 were observed after 48 h of exposure in a bioaccumulation study with *Ceriodaphnia dubia* [413]. In contrast, lower BCF and BMF values were reported for silver after exposure of Ag-NPs for 48 h to adult *D. magna* [410] with values of 3–5 and 0.5, respectively.

Evaluation of aquatic invertebrate species for testing bioaccumulation of ENMs

Data that are suitable to be used in the regulatory risk assessment of ENMs and that potentially allow a waiver of further tests using vertebrates, like fish, need to be generated in bioaccumulation studies carried out using experimental procedures with test species that fulfill special requirements. These requirements include constant exposure conditions and ideally a worst-case scenario regarding the availability and accumulation of ENMs by the test organisms. Several groups of invertebrates were described in this literature study that may be suitable test organisms for bioaccumulation studies with ENMs. A summary of the characteristics of the different species as test organisms is provided in Table 2.

Due to the high filtration rate and thus potentially increased ingestion of ENMs from the exposure media, filter feeding seems to represent a worst-case scenario regarding the bioaccumulation of ENMs. Bivalves and *Daphnia sp.* (*Branchiopoda*) are filter-feeding species with different morphological and physiological characteristics.

For *Daphnia sp.* breeding protocols are available and well established, whereas bivalves must be collected from the field due to a lack of suitable lab breeding protocols. Nevertheless, the high reproduction rate of daphnids can be disadvantageous. The release of the parthenogenetic juveniles as well as the molting rhythm of around 48 h constitutes an irregular, inhomogeneous elimination mechanism for previously accumulated contaminants. This only allows generation of suitable data in studies with short durations and this may explain the lack of evidence for incorporated and thus bioaccumulated ENMs in *Daphnia sp.* Due to the lack of a flow-through system which has been proven to allow the continuous and

homogeneous exposure of ENMs, *Daphnia sp.* seem to be a less suitable group for bioaccumulation testing. This is the case even if there is a broad experience in testing metals and their bioaccumulation in these species. Another limitation of bioaccumulation testing with *Daphnia* is the endpoint obtained which rather represents a BAF than a clear BCF or BMF. This is due to the filtration activity for respiration and feeding and the passive drinking for osmoregulation. This also limits the use of bivalves which show, however, further benefits for bioaccumulation testing. For instance, a flow-through system is available allowing the constant exposure of the test animals with ENMs. The duration of bivalve bioaccumulation studies is in a time frame that may allow the incorporation of ENMs by mechanic and physiological processes but is still shorter than the time required to perform a classic fish study according to OECD TG 305. During bivalve bioaccumulation studies no reproduction and thus uncontrolled elimination by the release of larvae/ juveniles occur and there is no risk of significant growth effects. The size of the individuals allows the determination of tissue-specific accumulation and distribution factors. The characteristics of the soft tissue also enables the preparation of samples which can be further investigated for particle concentrations replacing total mass concentration. In addition, the size allows characterization of the test animals and the usage of homogeneous test batches. This is due to the fact that the shell size correlates with the filtration activity and thus the uptake and accumulation potential for ENMs. A broad literature base is given for the accumulation processes of metals in bivalves, including the pronounced detoxifying strategies including MT.

The biological-related group of gastropods is comparable to bivalves but shows several limitations for bioaccumulation testing. Although there are protocols for dietary exposure of ENMs, the highly permeable body surface (soft tissue) of the animals may allow the uptake and thus co-exposure of ENMs or ions, via dietary and aqueous exposure. The most used pulmonate *L. stagnalis* should be used with caution because air-breathing pulmonates move to the water surface and may remain some time without being exposed to the contaminated water/test medium. Regarding biological and individual variations, the time spent without full exposure may cause artifacts in BAF calculation due to inhomogeneous exposure conditions within one treatment or study.

Even if isopod bioaccumulation studies seem to be a promising approach, there are only a few data available on the bioaccumulation of ENMs and metals in this group. The high sensitivity to metals is critical, and therefore, appropriate exposure concentrations are required, resulting in concentrations that can be challenging for the subsequent sample analysis. Further studies are required to allow a thorough assessment of isopods as suitable species for bioaccumulation testing of ENMs. Using isopods for bioaccumulation testing would allow derivation of distinct BCF or BMF values.

A suitable flow-through system for ENM exposure and protocols for dietary exposure without the risk of uncontrolled co-exposure via the water are available for amphipods, in particular for *H. azteca* which allows derivation of distinct BCF or BMF values. The easy culture and breeding methods described allow the production of high amounts of test animals of the same age under laboratory conditions. With respect to the duration of the exposure and elimination phases of the given bioaccumulation studies and short times required to reach steady-state conditions, the usage of adults with an age above 8 weeks (showing slow growth) will reduce the risk of growth effects to a negligible level. Even if the organism is too small to allow a tissue or compartment-based analysis after dissection, there are protocols and methods available that may allow the localization of ENMs in the different tissues by imaging methods or measurements, like spICP-MS. Considering the broad knowledge on the

Table 2 Characteristics of the reviewed invertebrates; * metal or metal oxide-based ENMs; ** dissection of different compartments for investigations on biodistribution possible; n.d. = not described;

Category	Bivalvia	Gastropoda	Isopoda	Amphipoda	Branchiopoda
Endpoints	BAF	BAF (BMF)	BCF, BMF	BCF, BMF	BAF
Literature—biology & physiology	++	++	+	++	+++
Literature & data—metal uptake/ accumulation	+	++	++	++	++
Literature & data—ENM* uptake/ accumulation	+	+	-	+	++
Reproduction in lab	-	+	++	+++	+++
Lab culture	+	++	++	+++	+++
Size of organisms	+++, **	+++, **	+	++	++
ENM* exposure test system	++, for BAF	n.d	n.d	+++, for BCF & BMF	+

ecology, physiology, metal uptake process, and detoxifying mechanisms of amphipods as well as the long-term experience obtained from metal bioaccumulation studies with amphipods and the growing experience with ENMs, the benthic species *H. azteca* could play a key role in the bioaccumulation assessment of ENMs. Even though *H. azteca* represents the most promising species for bioaccumulation studies with ENMs as summarized in Table 2, further ENMs with different characteristics need to be tested to extend the knowledge on ENM bioaccumulation in freshwater amphipods and to allow a final assessment of their general suitability to be used as test organisms within a tiered testing strategy for ENMs.

Note of guidance for the use of bioaccumulation endpoints derived from studies using aquatic invertebrate species in the risk assessment process for ENMs

We propose a testing strategy representing a modified version of the tiered approach proposed by Handy et al. [30] (Fig. 6, left), including an assessment scheme with the aim of defining a "bioaccumulative" or "non bioaccumulative" grading for ENMs without using fish (Fig. 7). In addition, we propose decision criteria/endpoints that are more robust regarding the analytical challenges involved in ENM studies as described by Petersen et al. [33]. Due to the special properties of ENMs, the classical endpoints gained in bioaccumulation studies with ENMs, like BCF, BMF, or BAF, cannot or should not be derived as is normally the case for dissolved/ water-soluble test items.

Petersen et al. [33] explained that the usage of kinetic endpoints, like BCF_{kin} or BAF_{kin}, that are not based on steady-state conditions would be of more value. They mentioned that steady-state conditions may be observed in laboratory studies, but may not occur in aquatic systems under environmental conditions [33]. However, steady-state conditions that are observed during the laboratory studies still represent a worst-case scenario and are thus suitable for regulatory assessment. In addition, as mentioned by Handy et al. [30] the calculations of uptake and elimination kinetics (k_1 and k_2) used for calculation of kinetic BCF_k are based on the usage of the Michaelis–Menten kinetics and the Fick equation. Thus, information on the thermodynamical stability and free ion activity are required [30]. But ENMs diffusion in liquids is based on Brownian motion and the particle concentration and the given energy of the system (suspension/dispersion). Further the collision frequency determines the fate of the particles, regarding the behavior to form agglomerates or not and thus this is much more complicated to be modeled and kinetic approaches will be more inaccurate [419, 420]. Thus, a non-kinetic test design is suggested as outcome of this literature review. A tiered approach for testing ENMs in invertebrates is described allowing to derive endpoints (BCF and BMF) which can be assessed using the established threshold values for bioaccumulation assessment.

In Tier 1 the chemical and physical properties of the ENM are reviewed.

At the first step of Tier 1, (Fig. 6) the potential ability of dissolution or ion release in water or lipids is tested. If the material produces ions or dissolves in water or lipids, the ionic/dissolved form of the ENM should be tested as a second treatment (aqueous exposure) in the following in vivo bioaccumulation tests with invertebrates of Tier 2.

As a second step of Tier 1 the ENM's potential to generate a homogeneous and stable exposure scenario under aquatic conditions is elucidated (Fig. 6). If there is only a limited or even no chance to generate a continuous and homogeneous exposure scenario by the surrounding media /water, a dietary-only exposure scenario is recommended as described by Kuehr et al. [98] for the amphipod *H. azteca* using ENM-enriched DECOTABs.

In contrast to the assessment scheme proposed by Handy et al. [30] exposure pre-tests with the amphipod *H. azteca* are carried out in the first step of Tier 2 to determine suitable test concentrations for the bioaccumulation tests. In addition to that, the availability of the test item for the test organisms and the resulting potential loading capacity of the ENM and its potential ions/ dissolved form is determined. *H. azteca* is suggested as test organism to determine clear BCF and BMF values as endpoints that can be used within regulatory assessment.

As mentioned by Kühnel and Nickel [20] an endpoint based on the measured body burden needs to be assessed with caution due to the challenging differentiation between the burden resulting from ENMs attached to the animals surface or simply ingested and those really incorporated and thus bioaccumulated. Information about the half-lives of the body burden offers important information about this and thus plays an important role in the presented assessment scheme (Fig. 6; Part 2 of Tier 2).

As a first step, the bioavailability of the test compound (ENM) needs to be confirmed. The exposure should last 7 days and the exposure concentration should be at a low level, if possible, mirroring the predicted environmental concentration to avoid artificial results regarding the bioavailability and bioaccumulation of ENMs [6]. If the measured body burden of the animals is higher than the measured initial natural background plus the limit of quantification (LOQ) defined as 9 times the limit of detection of the respective test item, a significant loading capacity for the tested ENMs is given. The measurements should be carried out on the level of total mass concentrations. If

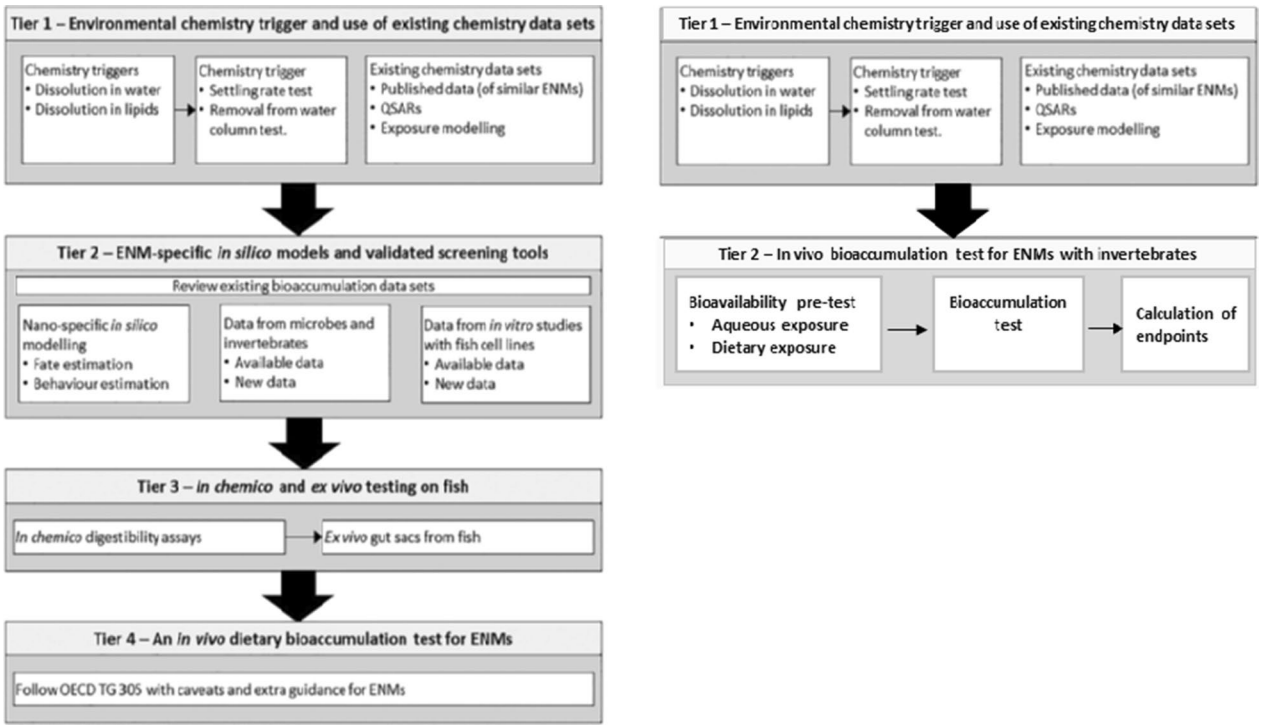

Fig. 6 Left: tiered approach for testing nanomaterials by Handy et al. [30], right: modified test approach according to Handy et al. [30]

no significant loading capacity can be achieved during 7 days of exposure, the tested ENM could be graded as "non bioaccumulative." If a significant loading capacity is reached within 7 days or less, bioconcentration and biomagnification tests for the ENM and, if necessary, a bioconcentration test for the ionic/dissolved form should be carried out. These tests must include a depuration phase to allow estimation of the half-life of the previously ingested/accumulated (?) ENMs.

The uptake phase of the bioaccumulation tests (bioconcentration or biomagnification approach) should last at least as long as required that three subsequent body burden measurements ($\Delta_t \geq 12$ h) show no variation higher than 20% allowing the calculation of a time-weighted loading capacity (TWLC) for the end of the uptake phase based on the three last measurements.

As a result of the second study bioconcentration and biomagnification factors are calculated by dividing TWLC by the exposure concentration in water or food, respectively. If the values are below the threshold value of 2000 (BCF_{TWLC}) or 1 (BMF_{TWLC}), the tested ENM can be graded as "non bioaccumulative," provided that the common threshold values are accepted for ENM bioaccumulation assessment. Generally, if one of the calculated endpoints shows a value above the set thresholds, the results need to be further validated based on the depuration behavior of the previously accumulated test item.

Thus, the body burden of the animals needs to be further elucidated during a depuration phase following exposure of the second study. For the depuration phase two outcomes for the elimination of the ENMs are possible, leading to two grading scenarios: First, the elimination of the previously measured ENMs occurs fast and completely; the level of the body burden has a half-life of ≤ 1 day. This scenario has been observed for AuNPs in *H. azteca* by Kuehr et al., where the determined BCF value of >400 indicated a certain loading capacity [315] and a level below the measured initial natural background plus the LOQ was reached within ≤ 1 day of depuration. This scenario indicates that the body burden and the loading capacity may primarily result from simply ingested, but not incorporated, and thus not bioaccumulated material, i.e., located in the gut (content) or attached to the animals surface. This outcome would lead to a decision for a "non bioaccumulative" grading if fast elimination is observed following aqueous or dietary exposure.

In the second scenario a slow or incomplete elimination is observed during the depuration phase following one or both exposure scenarios: If the elimination half-life is >1 day, the depuration phase is extended until

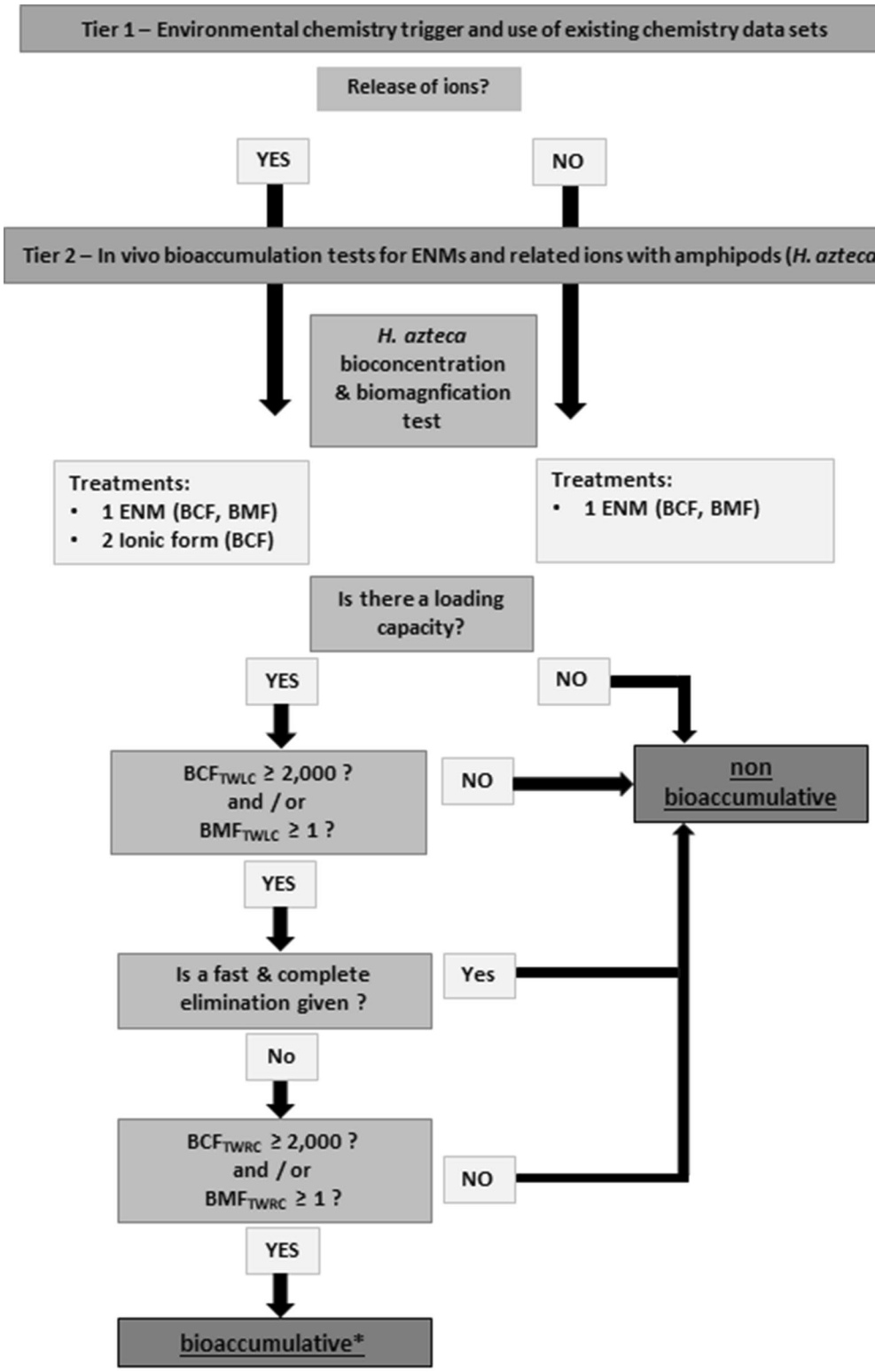

Fig. 7 Assessment scheme for bioaccumulation assessment of ENMs; * if BCF_{TWRC} is ≥ 2000 or ≥ 5000, and $BMF_{TWRC} \geq 1$, the test item is classified as "bioaccumulative" or "very bioaccumulative," respectively, and a higher tier test could follow to refine the calculated endpoint

either the burden reaches the initial natural background concentration or three subsequent body burden measurements ($\Delta_t \geq 12$ h) show that the residual body burden values do not vary by more than 20% (sink). Under these conditions bioconcentration and biomagnification factors are calculated based on the time-weighted residual capacity (TWRC) and the exposure concentrations. The ENM is graded as "bioaccumulative" if these values are above 2000 for BCF_{TWRC} and 1 for BMF_{TWRC}. Such a scenario was observed in an amphipod bioaccumulation study with Ag-NPs as well as in a bivalve bioaccumulation study with the same test item by Kuehr et al. [98, 315].

If only the ionic/dissolved form shows $BCF_{TWLC/TWRC}$ values above the threshold of 2000, but not the respective ENM tested in the bioconcentration approach, the ENMs have to be considered as "non bioaccumulative." With the *H. azteca* bioaccumulation tests leading to higher BCF/BMF values compared to fish as shown for Ag-NPs and TiO_2-NPs [17, 315], the invertebrate test can be seen as a worst-case approach and further tests using fish can be waived if a clear grading was made following the assessment scheme presented in Fig. 7. If the calculated value for BCF_{TWRC} is ≥ 2000 or ≥ 5000, the test item should be graded as "bioaccumulative" or "very bioaccumulative."

Although the focus of the review part of this work is on metal and metal oxide-based ENMs, the proposed testing approach (Fig. 6), including the assessment scheme (Fig. 7), can be used for all types of ENMs, e.g., polymer- and carbon-only-based ENMs. This approach may also be useful for further testing and the regulation of microplastics. However, more information and data are required to confirm the suitability of this approach for the testing and assessment of microplastics.

Conclusion

The literature study has elucidated the potential of different aquatic invertebrate species to be used in laboratory bioaccumulation studies on ENMs. Amphipods were identified as the most promising species for ENM testing. Individual BCF and BMF values can be calculated that fulfil the requirements of endpoints needed for the bioaccumulation assessment of ENMs under regulations, like REACH. The results from amphipod bioconcentration and biomagnification tests can be included in a tiered assessment allowing a clear grading of the tested nanomaterials as "bioaccumulative" or "non bioaccumulative." Due to the worst-case scenario of the amphipod test, this approach may allow to waive further vertebrate tests if there are no indications for bioaccumulation of ENMs. In case, that bioaccumulation of ENMs is observed in *Hyalella azteca* a fish test as the golden standard of bioaccumulation testing should be considered.

Abbreviations

BAF: Bioaccumulation factor; BCF: Bioconcentration factor; BMF: Biomagnification factor; ENM: Engineered nanomaterial; LOQ: Limit of quantification; MT: Metallothioneins; NPs: Nanoparticles; TWLC: Time-weighted loading capacity; TWRC: Time-weighted residual capacity.

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Authors' contributions

SK and CS conceptualized and coordinated the preparation of the manuscript and developed the assessment scheme. SK carried out the literature research and analysis of the literature. VK contributed to the literature research of the chapters "Amphipods" and "Branchiopoda." SK mainly wrote the manuscript. CS supported to further elaborate the manuscript and contributed specific aspects. All authors read and approved the final manuscript.

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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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Chapter 8: Conclusions and Further Perspectives

8.1 Conclusion

In this thesis, investigations on the bioaccumulation of NMNs in aquatic systems were carried out and test systems and methods were developed to enhance the understanding of the MNMs fate within aquatic organisms. Different test species were checked regarding their suitability for bioaccumulation testing with MNMs. The results obtained from the studies and the available literature were used to develop a proposal for a new assessment scheme for the bioaccumulation assessment of MNMs under regulations like REACH [39].

… biomagnification studies using fish for bioaccumulation assessment on (metal/ metal oxide based) MNMs are less suitable.

Chapter 2 of this thesis, consists of a study using rainbow trouts to investigate the uptake and accumulation of Ag from either waste water borne and transformed (sulfidized) AgNPs or from AgNPs which were applied as pristine particles. The exposure tests with rainbow trout were conducted according to the OECD TG 305 using both, the bioconcentration and the biomagnification approach [92].

The evaluation of the studies showed that no real bioaccumulation in fish tissues occurred during the BMF approach. Ag burden were only detected in the gut and probably resulted only from either AgNPs remaining in the mucus present in the intestine or Ag^+ ions (Ag^+) bound to proteins present in the intestinal mucus. This observation is consistent with the observations described by Khan et al. (2017) in studies on the intestinal uptake of AgNPs in rainbow trout [113]. The fast elimination of the Ag burden measured in the gut samples taken during the depuration phase supports this assumption and could be explained by the regular excretion of the intestinal mucus in combination with defecation and thus elimination of AgNPs or Ag^+ adsorbed or complexed within the mucus matrix.

These proteins are called metallothioneins (MTs). They have a high binding affinity and capacity for (heavy) metals and also Ag^+ and can be found in nearly all higher species and play a key role in the detoxification and regulation of the homeostasis of metals [114], [115]. These proteins may bind the Ag^+, presumably released from the exposed AgNPs, before they can enter the organism, e.g. by ionic mimicry via Na^+ channels of the intestinal epithelial cells [116]–[120]. This mechanism of trapping the Ag^+ ions by MTs may also be the reason for the delayed transfer of Ag from the gills to the carcass as observed during the bioconcentration approach of the same study. Thus, the fish BMF test seems to be of limited suitability for testing the bioaccumulation potential of MNMs in aquatic species. The bioconentration approach resulted in higher accumulation and metal transfer tissue transfer from the gills to the carcass, but only for AgNPs applied as pristine particles. These findings, as well as the observation that

only the aqueous exposure of AgNPs applied as pristine particles caused a significant Ag uptake and accumulation from AgNPs in the carcass of the used fish underlines the key role of ions in the bioaccumulation of metals after MNM exposure.

… metal bioaccumulation from MNMs is mainly based on the soluble species of the metals/ their ions.

Vogt et al. (2019) examined fish from a prealpine lake. Although sulfidized AgNPs were found in the lake sediment, no Ag contamination was detected in the collected fish samples [121]. This is in accordance with the results from the exposure studies of **Chapter 2** with sulfidized waste water borne AgNPs and indicates that the concentration of available Ag^+ ions was too low to cause a significant accumulation of Ag.

AgNPs spiked as pristine particles to dilution water were used in the bioconcentration study described in **Chapter 2.** Particles may be subjected to the chemical process of oxidation, which leads to an increase of Ag^+ release [122], [123]. The resulting high Ag^+ concentration observed in this study was presumably high enough to cause a significant uptake of Ag by the fish. The high Ag^+ concentration may have reached a level that cannot be buffered by the MTs present in the gills tissue, due to their limited loading capacity of around 12 mol Ag per mol MTs [124]–[126]. Thus, the further uptake may have led to the measured delayed Ag burden in the carcass samples. The fast increase of the Ag concentration in the carcass potentially started after a few days of exposure, due to Ag^+ that were not trapped by the MTs of gills. Further, the incomplete elimination of Ag during the depuration phase may be a result of Ag residues complexed with the MTs present in different tissue of the fish.

These finding of limited elimination of previously accumulated Ag, is in accordance with the results from the bivalve exposure studies of **Chapter 3**. Mussels exposed to $AgNO_3$ also showed a lack of complete elimination of the previously accumulated amount of Ag. The toxicokinetics and distribution factors gained for the different tissues also indicate a central role of MTs and thus of Ag^+ for the observed bioaccumulation of Ag. The high distribution factors of Ag estimated for the viscera in all treatments ($AgNO_3$, AgNPs, TiO_2NPs) may be explained by the flow of the ingested exposure medium towards the viscera. The viscera contains the filtering cilia and gills that extract the NPs from the water as well as the dissolved Ag^+ ions, e.g. by Na^+ and Cu^+ uptake and transport mechanisms [118], [127], [128]. Nevertheless, this does not explain the high distribution factors of Ag in the tissue of the mantle that were caused by the exposure of AgNPs and $AgNO_3$, but not by the TiO_2NPs exposure. An explanation of the higher distribution factor for the viscera and the mantle for Ag in contrast to TiO_2 may again be the binding of Ag to induced MTs, that are mainly present and released in the gills (belonging to the viscera) and mantle tissue of bivalves [129],[130]. MTs expression is triggered by metal ions, which were lacking in the TiO_2 treatment.

A mesocosm study carried out to investigate the fate of sulfidized AgNPs/ Ag_2SNPs in the different compartments of a floodplain revealed a comparable sink for Ag in the bivalve *C. fluminea* as described in **Chapter 3** (Metreveli et al.; results presented at the SETAC SciCon SETAC Europe 30th Annual Meeting; 03.-07.05.2020; publication in preparation). A stable level of Ag burden for more than 4 weeks (end of samplings) was observed after a previous initial single Ag_2SNP spiking event.

Similar observations regarding the formation of a sink of Ag were made during the bioaccumulation studies with *H. azteca* which were exposed to $AgNO_3$ and AgNPs **(Chapter 4)**. Investigations on the protein fraction of *Hyalella,* which have previously been exposed, revealed that nearly the complete part of Ag residue in the amphipods during the depuration was located in the protein fraction. Moreover, around 54% of the amount of Ag measured during the steady state condition was connected to the protein fraction. Thus, presumable only the half or less of the steady state Ag burden seems to result from AgNPs taken up or attached to the animals. The same investigation on the protein fraction of *H. azteca* from the study described in **Chapter 5** showed that 57% of the total Ag burden in *H. azteca* after exposure to $AgNO_3$ was located in the protein fraction.

A negative correlation between the exposure concentration of (soluble) metal species and the resulting BCF has been described for several aquatic invertebrates [131]–[133]. A possible explanation could be the limited expression of MTs and their limited binding or loading capacity [131], [134]. This may be supported by the observation that the residual amount of Ag during the depuration (representing the observed Ag sink) of the bioconcentration studies **(Chapter 4),** carried out with $AgNO_3$ or AgNPs, were in a range of approximately 1.5 mg Ag/kg. The amount of Ag present in the protein fraction of the amphipods of the further study **(Chapter 5)**, where animals were exposed without direct contact to the AgNP was determined to be around 17%. The whole Ag burden of these animals was determined to be 8.3 mg Ag/kg. Thus, the amount of Ag present in the protein fraction was around 1.4 mg Ag/kg. It can only be speculated whether this value of approximately 1.5 mg Ag/kg might be the maximum loading capacity for Ag in *H. azteca*.

In conclusion, all these results seem to confirm that at least in the case of Ag, the bioaccumulation of metals in aquatic species during exposure to MNMs is significantly affected by the released ions.

… aquatic invertebrates play an important role for the transport of MNMs along the aquatic food chain.

Filter-feeding mussels play an important role regarding the impact and fate of MNMs in aquatic environments. As expected regarding the extremely fast elimination of the TiO_2NP burden during depuration **(Chapter 3)**, the mussel accumulate the MNMs absorbed from the filtered water

in their (pseudo)feces and are thus able to eliminate them effectively by defecation. Organisms that feed on the released feces enriched with MNMs, might be exposed to high concentrations of particles, which they might not be exposed to without this exposure pathway. This was shown by the bioavailability test with fluorescence labeled polystyrene NPs with *H. azteca,* described in **Chapter 6**. While there was no uptake of nano polystyrene particles in *H. azteca* during aquatic exposure, an uptake of the particles was observed after dietary exposure to feces collected from previously exposed bivalves. Thus, we can assume that benthic invertebrates play a key role in the transport of MNMs into/ along the aquatic food chain [135]–[138].

… aquatic invertebrates represent a worst case scenario for testing MNMs in aquatic systems regarding their interaction with MNMs.

Even if no biomagnification (BMF value > 1) of the tested MNMs in amphipods was observed during the *Hyalella* BMF tests **(Chapter 4)**, the uptake and resulting body burdens allow to compare this test setup with the established BMF approach according to OECD 305 [92] as conducted with rainbow trout **(Chapter 2)**. The experimental diet applied in the fish feeding study **(Chapter 2)** was comparable with the diet used in the *Hyalella* tests with regard to the use of an agar-agar matrix embedding the MNM contaminated food. The experimental diet of the fish study was measured to hold a burden of 121.5 µg Ag/kg. The dietary exposure caused a maximum body burden of 34.3 µg Ag/kg in the fish, followed by a complete elimination (initial natural background was reached) during the depuration phase of the study. In contrast, concentrations of 70 and 0.75 µg Ag/kg were measured in the experimental diets applied in the *Hylaella* tests **(Chapter 4)** and resulted in a body burden of 180 and 70 µg Ag/kg for the respective treatments. Even under consideration of the higher initial (natural) background concentration of Ag in the amphipods, an increase of the Ag body burden of at least 30 µg Ag/kg was observed at the end of the dietary exposure phase lasting 7 days. The body burden was thus in the same range as measured for the two times higher contaminated diet applied for 14 days during the fish test. Further, the measured body burden seems to be the result of bioaccumulated Ag leading to an incomplete elimination during the depuration phase, presumably caused by Ag^+ bound to MTs as explained above.

Therefore, bioaccumulation in *H. azteca* seems to be a worst case scenario compared to fish, regarding the calculated BCF as well as BMF values. Further, the bivalve and amphipod approaches represent a worst case scenario due to the fast uptake rate of MNMs and their potential contribution to the transfer of the particles along the food chain.

…aquatic invertebrates represents suitable substitute for fish (vertebrates) in alternative test methods that could be integrated into regulatory risk assessment.

Investigations on exposed amphipods using methods of correlative microscopy proved that the animals are able to ingest AgNPs even if no real incorporation of the NPs occurs **(Chapter 5)**.

AgNPs were only detected in the gut lumen. A relocalization of these NPs into the tissue or other regions was not observed.

The suitability of the benthic amphipod *H. azteca* as test organism for bioaccumulation studies with MNMs was evaluated by the use of representative MNMs selected from different groups of MNMs having different characteristics **(Chapter 4)**. The reproducibility of the results was shown by a 2-lab comparison. Similar uptake and elimination kinetics and comparable BCF values were obtained for the different AgNP treatments. In addition, data on the uptake and elimination kinetics for all used MNMs were gained, at least in the biomagnification approaches. The test setup enabled us to estimate distinct BCF and BMF values which are established endpoints for regulatory bioaccumulation assessment. The adapted test system that was originally developed for flow-through studies with the freshwater bivalve *C. fluminea* **(Chapter 3)** allowed to carry out aqueous and dietary exposure studies with *H. azteca* under constant exposure conditions without causing artifacts due to inhomogeneous or discontinuous exposure concentrations. A comparison of the accumulation factors gained for *H. azteca* after exposure to either AgNP enriched fecal matter of *C. fluminea* or to an artificial diet (DECOTABs) enriched with AgNPs **(Chapter 4)** confirmed the suitability of this test method.

Regarding the requirements of the regulatory risk assessment, the amphipod *H. azteca* seems to be more suitable as test organism for bioaccumulation testing of MNMs when compared to filter feeding species like the freshwater bivalve *C. fluminea*. Although *C. fluminea* proved to be the worst case for the uptake of MNMs under aquatic exposure **(Chapter 3**), resulting in BAF values of >9,000 for TiO_2NPs. Nevertheless, there are some drawbacks for the use of the bivalve test. Due to the process of filtration, it is not possible to distinguish between the uptake of dissolved particles through the respiratory process and the intake through food uptake mechanism. Thus, only the less precise BAF value can be determined as bioaccumulation endpoint. Thus, the amphipod tests allowing the calculation of BCF and BMF values is recommended for regulatory bioaccumulation assessment.

In addition, the filtration process of bivalves is subjected to a protective mechanism which reduces the filtration rate of the animals under unfavorable conditions and therefore the uptake can vary, independent of the bioavailability of the test item, and may thus lead to artificial results. Nevertheless, the mussel test can still provide valuable data due to the opportunity to gain toxicokinetic data and to estimate distribution factors for several compartments that allow an understanding of real bioavailability vs. simple ingestion. Such information could be of certain value for bioaccumulation assessment allowing to refine the calculated endpoints especially if values slightly above or below the respective threshold value are obtained.

Based on the results gained from the different studies, it can be concluded that invertebrate species could be a potential alternative for fish as test species which may help to improve the

risk assessment regarding the bioaccumulation potential of MNMs. Due to their small size, the vertebrates can be examined as whole organism for instance by the methods of correlative microscopy **(Chapter 5)** allowing to investigate the bioavailability and fate of MNMs in the respective test species.

A detailed overview of the biology and pros and cons of some potential invertebrate groups for bioaccumulation testing are presented in **Chapter 7**. The test concept of using *H. azteca*, **(Chapter 4)** was rated to be the most feasible and suitable test method for bioaccumulation studies with MNMs. The data collected from such tests may under certain conditions allow to waive further vertebrate tests for the bioaccumulation assessment of MNMs.

The flow-through test system allows to apply constant exposure conditions, and enables dietary exposure excluding the risk of co-exposure by MNMs or ions that leached into the medium from the diet or fecal matter. The design of the flow-through system also prevents unintentional accumulation of MNMs at the bottom of the exposure tank as it may occur in a simple aquarium.

Based on the knowledge obtained in the PhD project, a new assessment scheme for bioaccumulation of MNMs was described **(Chapter 7).** Alternative endpoints are recommended also considering the often ignored factor of real incorporation vs. simple ingestion of MNMs. The proposed endpoints also take into account the risk of secondary poisoning caused by the animals' high loading capacities for MNMs which are for instance simply ingested or attached to the body surface.

The described and proposed test method allows to derive the established endpoints, BCF and BMF, even if the usage of alternative (adapted) endpoints is recommended. Being a central part of the proposed assessment scheme, the amphipod test with *H. azteca* may represent a suitable substitute to fish in the regulatory bioaccumulation assessment under regulations like REACH. The amphipod tests deliver data within a significantly shorter test duration and underlies no animal well fare restrictions.

In case that the calculated endpoint estimated by using the proposed assessment scheme are slightly below or above the regulatory threshold, the assessment may be complemented by further studies (higher tier test) involving more realistically or environmentally relevant conditions. This may include studies using WWTP effluent for the exposure of the amphipods, as described by Kühr et al. 2018 [67]. The bivalve test **(Chapter 3)** may help to further elucidate the toxikokinetics and the tissue distribution under worst case conditions regarding the uptake of MNMs from the exposure media.

8.2 Further Perspectives

The studies carried out in this work provided important insights into the uptake pathways and bioaccumulation processes of MNMs in aquatic organisms and thus delivered the basis for the bioaccumulation assessment scheme that may help to reduce the need for fish for regulatory testing.

However, there are still some aspects, which need to be further clarified:

The hypothesis of the key role of metal ions, (e.g. in combination with MTs) for the metal bioaccumulation during MNM exposure, as observed and discussed for Ag, need to be further tested for a larger spectrum of metal NPs to allow a more general statement.

With regard to the analytical methods of correlative microscopy, it would be useful to further expand the spectrum of high-resolution methods and to develop imaging techniques that can also detect non-metallic MNMs. This would provide a great opportunity to elucidate the bioaccumulation of further compounds such as micro/ nanoplastics in aquatic organisms.. For this purpose, it would be required to investigate up to which size such particles are available for *Hyalella* or other amphipods/ invertebrates. The mussel test system would certainly provide several advantages for this purpose due to their specific morphology and uptake/ filtration mechanism.

In general, this work mainly refers to metallic or metal oxide based MNMs. Further investigations on polymer based MNMs or carbon nanotubes or fullerenes should be considered. Such compounds may provide the opportunity to use ^{14}C-labelled material that would allow a highly sensitive analytics but also a localization in the tissue by autoradiography as recently demonstrated for *H. azteca* by Raths et al. (2019) using a ^{14}C-radiolabeled organic compound [139].

To establish the assessment scheme developed as part of this project, the suitability of the *Hyalella* test for testing MNMs would have to be proven in further studies. An international ring test for inter-laboratory comparisons as currently performed for the *Hyalella* bioaccumulation test (HYBIT) for organic compounds [112] should be considered.

As a final summary, the methods described in this paper and the resulting assessment scheme may help to further improve the regulatory bioaccumulation assessment of MNMs and to reduce the need for fish for such studies. Moreover, the suggested approach for bioaccumulation testing with *H. azteca* would allow to reduce the time needed to carry out the studies and thus make the regulatory process faster and more economical.

Abbreviations

Ag^+	Ag^+ ions
BAF	Bioaccumulation factor
BCF	Bioconcentration factor
BMF	Biomagnification factor
MTs	Metallothioneins
NMs	Nanomaterials
nm	Nanometer
MNMs	Manufactured nanomaterials
NPs	Nanoparticles
REACH	European Chemical Regulation
WWTP	Waste water treatment plant

References Chapters 1 and 8

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