


REVIEW

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Bioaccumulation assessment of nanomaterials using freshwater invertebrate species

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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 filtering 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 KKDİK (Kaydi, Değerlendirilmesi, İzni, Kısıtlanması), 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 filtering 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 ≥ 2000) or vB (very bioaccumulative, under REACH: BCF value ≥ 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 physico-chemical 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	<i>A. cygnea</i>	AuNP	40	86 ± 14	Water	BCF; tissue distribution	Moëzzi et al. [421]	
	<i>C. fluminea</i>	AuNP	17	n.d.	Diet	BCF	Renault et al. [109]	
	<i>C. fluminea</i>	Al ₂ O ₃ NP	11	4—12	Sediment/water	BCF	Stanley et al. [121]	
	<i>C. fluminea</i>	AuNP	7.8, 15 & 46	n.d.	Water	Clearance rate	Hull et al. [122]	
	<i>C. fluminea</i>	AuNP	10	n.d.	Water/ diet	Uptake & elimination rates	Arini et al. [89]	
	<i>C. fluminea</i>	AgNP TiO ₂ NP	15 (AgNP) & 26 (TiO ₂ NP)	27 (AgNP) & 351 (TiO ₂ NP)	Water	BAF, tissue distribution	Kuehr et al. [98]	
	<i>C. fluminea</i>	CuONP	12 ± 8 & 29.5	140.5 & 194	Water	Body burden; tissue distribution	Koehle-Divo et al. [422]	
	<i>C. fluminea</i>	TiO ₂ NP	5—10	n.d.	Sediment	Body burden; tissue distribution	Fan et al. [423]	
	<i>D. polymorpha</i>	CeO ₂ NP	3	8	Water	BCF	Garaud et al. [303]	
	<i>D. polymorpha</i>	CeO ₂ NP	3—4	8	Water	Body burden; tissue distribution	Garaud et al. [424]	
	<i>D. polymorpha</i>	AgNP	50 ± 20	80	Water	BCF, subcellular distribution	Zimmermann et al. [425]	
	<i>D. polymorpha</i>	TiO ₂ NP	10.4	n.d.	Water/ diet	BCF/BAF	Bourgeault et al. [123]	
	Gastropoda	<i>L. stagnalis</i>	AuNP	15	28	Diet	Body burden	Hudson et al. [195]
		<i>L. stagnalis</i>	CuONP	7 ± 1	82 ± 1	Water	Body burden	Misra et al. [426]
<i>L. stagnalis</i>		AgNP	13 ± 3 & 17 ± 5	n.d.	Water/diet	Uptake & elimination rates	Croteau et al. [187]	
<i>L. stagnalis</i>		CuONP	7	77 ± 5	Water/diet	Uptake & elimination rates; body burden	Croteau et al. [427]	
<i>L. stagnalis</i>		AgNP	n.d.	36 ± 1	Diet	Uptake rate	Oliver et al. [428]	
<i>L. stagnalis</i>		⁶⁷ ZnONP	20 -70	245	Diet	Body burden	Dybowska et al. [206]	
<i>L. stagnalis</i>		AgNP	10.3 ± 3.4 & 12.8 ± 4.4	n.d.	Water	Uptake rate	Luoma et al. [429]	
<i>P. antipodarum</i>		AgNP	10—15	12.1 ± 2.93—13.9 ± 3.17	Water/ sediment	Body burden	Ramskov et al. [430]	
<i>P. antipodarum</i>		CuONP	7	n.d.	Sediment	Body burden	Ramskov et al. [176]	
<i>P. antipodarum</i>		CuONP	7 ± 1	n.d.	Sediment	Body burden	Ramskov et al. [431]	
<i>P. antipodarum</i>		CuONP	6	n.d.	Sediment	Body burden	Pang et al. [432]	
<i>P. antipodarum</i>		CuONP	6 & 100	19 & 204	Sediment	Body burden	Pang et al. [433]	
<i>B. glabrata</i>		AgNP	115 ± 55.6	n.d.	Water	Body burden	Oliveira-Filho et al. [158]	
<i>B. aeruginosa</i>		AgNP	18 ± 7.7, 30.2 ± 9.4 & 57 ± 19.3	n.d.	Sediment	BAF, tissue distribution	Bao et al. [177]	
<i>B. aeruginosa</i>		TiO ₂	11.6 ± 2.4	n.d.	Sediment	Body burden	Ma et al. [434]	
<i>B. aeruginosa</i>		CuONP	41.6 ± 4.6	n.d.	Sediment	Body burden; tissue distribution	Ma et al. [188]	
Isopoda	<i>A. aquaticus</i>	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	<i>H. azteca</i>	Al ₂ O ₃ NP	11	4–12	Sediment/water	Body burden	Stanley et al. [121]
	<i>H. azteca</i>	CuO	30–40	733.1 ± 143.4	Water	BCF	Burkal et al. [435]
	<i>H. azteca</i>	AgNP	15	27	Water	Body burden	Kuehr et al. [311]
	<i>H. azteca</i>	AuNP	15	28	Diet	Body burden	Hudson et al. [195]
	<i>H. azteca</i>	ZnONP	22.4 ± 2.8	n.d.	Water	Body burden	Poynton et al. [312]
	<i>H. azteca</i>	AgNP	15	27	Water & diet	Body burden	Kuehr et al. [314]
	<i>H. azteca</i>	AgNP, AuNP, TiO ₂ NP	15 (AgNP), 26 (TiO ₂ NP) & 60 (AuNP)	51.2 (AgNP), 1,861 (TiO ₂ NP) & 61 (AuNP)	Water/ diet	BCF/ BMF	Kuehr et al. [315]
	<i>G. fossarum</i>	AgNP	20, 23 & 27	96 ± 8.4, 56 ± 3.2 & 63 ± 3.2	Water	BCF	Mehennaoui et al. [270]
	<i>G. fossarum</i>	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]
	<i>G. roeseli</i>	CeO ₂ NP	3	8	Water	BCF	Garaud et al. [303]
Branchiopoda	<i>D. magna</i>	AuNP	20	n.d.	Water	Distribution	Lovern et al. [407]
	<i>D. magna</i>	CuONP	26 ± 8, 45 ± 11 & 75 ± 19	263 ± 38, 153 ± 45 & 241 ± 3	Water	Spatial distribution & burden	Santos-Rasera et al. [436]
	<i>D. magna</i>	AgNP	10–11	22 & 28.3	Diet	Body burden	Kalman et al. [437]
	<i>D. magna</i>	AuNP	10	33 ± 17.3	Water & diet	Body burden	Lee et al. [403]
	<i>D. magna</i>	CuONP	< 50	926 ± 35	Water & diet	Body burden	Wu et al. [376]
	<i>D. magna</i>	AgNP	20	40–50	Water	Body burden	Zhao and Wang [438]
	<i>D. magna</i>	AuNP	19 ± 2.5	24.2 ± 0.2	Water	Elimination rate	Khan et al. [414]
	<i>D. magna</i>	⁶⁵ ZnONP	10–30	46–56	Water	Uptake & elimination rates	Li & Wang [391]
	<i>D. magna</i>	CdSe/ZnS QDs*	5.2 ± 0.4	n.d.	Water	Body burden	Lewinski et al. [393]
	<i>D. magna</i>	TiO ₂ NP	21	580.5	Water	BCF	Zhu et al. [412]
	<i>D. magna</i>	ZnONP	30 ± 17	111.8 ± 24.9–670.2 ± 230	Water	Body burden	Skjolding et al. [386]
	<i>D. magna</i>	AuNP	14 ± 4	225 ± 61	Water	Uptake & tissue distribution	Jensen et al. [406]
	<i>D. magna</i>	AgNP	15.5 ± 3.6	50 ± 0.7	Water	Body burden	Kim et al. [409]
	<i>D. magna</i>	AuNP	10 & 30	14 ± 4–142 ± 53 & 23 ± 5–225 ± 61	Water	Uptake & elimination rates	Skjolding et al. [387]
	<i>D. magna</i>	TiO ₂ NP	< 25	20.9 ± 2.89–218 ± 47.3	Water	Body burden	Tan & Wang [392]
	<i>D. magna</i>	TiO ₂ NP	30–80	n.d.	Water	BCF	Fan et al. [394]
	<i>D. magna</i>	AuNP	10	33 ± 17.3	Diet	Body burden	Lee et al. [403]
<i>D. magna</i>	TiO ₂ NP	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]	
<i>D. magna</i>	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
	<i>D. magna</i>	AgNP, AuNP, PtNP	5 & 50 (Ag), 5 & 50 (Au) & 5 & 50 (Pt)	n.d	Water	Body burden	Krystek et al. [411]
	<i>D. magna</i>	CuNP, ZnONP	50 (CuNP) & 43 (ZnONP)	568 ± 72 (CuNP) & 1154 ± 252 (ZnONP)	Water	Body burden	Xiao et al. [408]
	<i>C. dubia</i>	TiO ₂ NP	< 25	n.d	Water & diet	BAF/ BMF	Dalai et al. [413]
	<i>D. magna</i>	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.

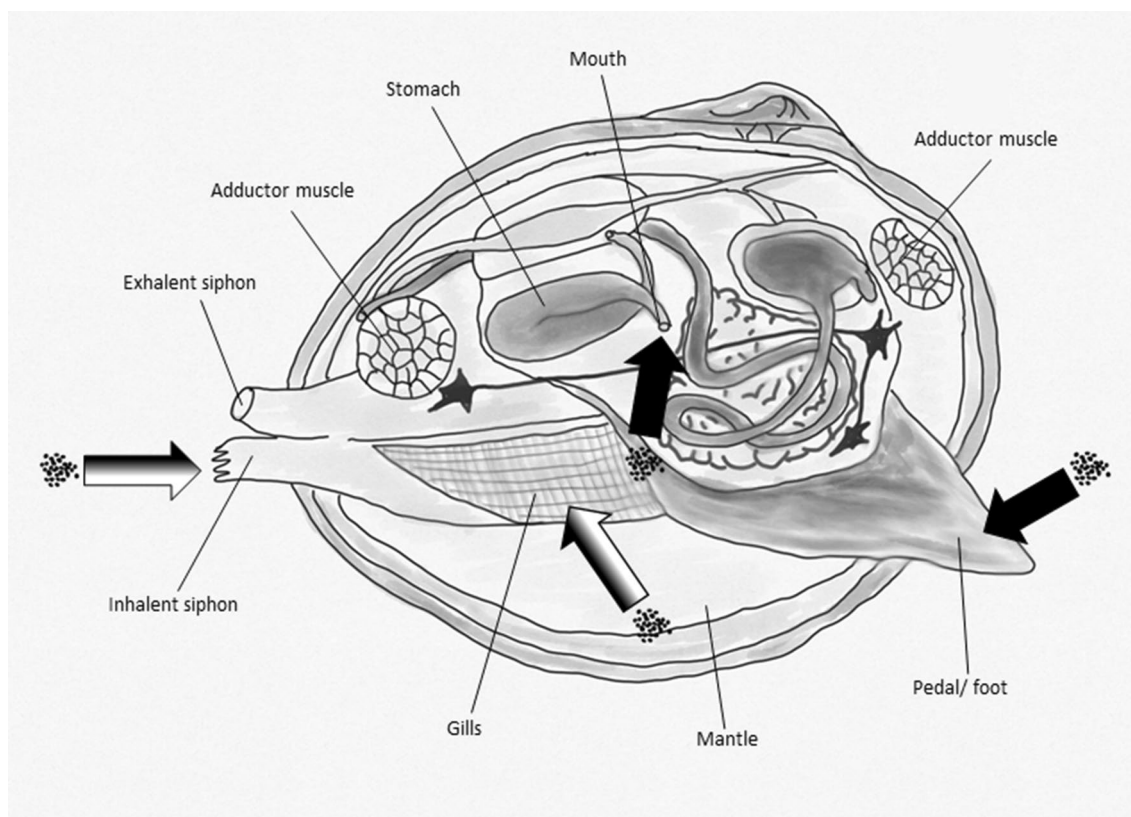


Fig. 1 Potential uptake routes of ENMs in *Bivalvia*. Black arrow: ingestion by the dietary route/ mouth and foot; white and black arrows uptake via inflow siphon and ingestion via gills for respiration and nutrition

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 fluminea*, 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 rate 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 detritivores), 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 phytochelatins. 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.*, *Potamopyrgus spec.*, or *Bellamyia 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. aeruginosa*, 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

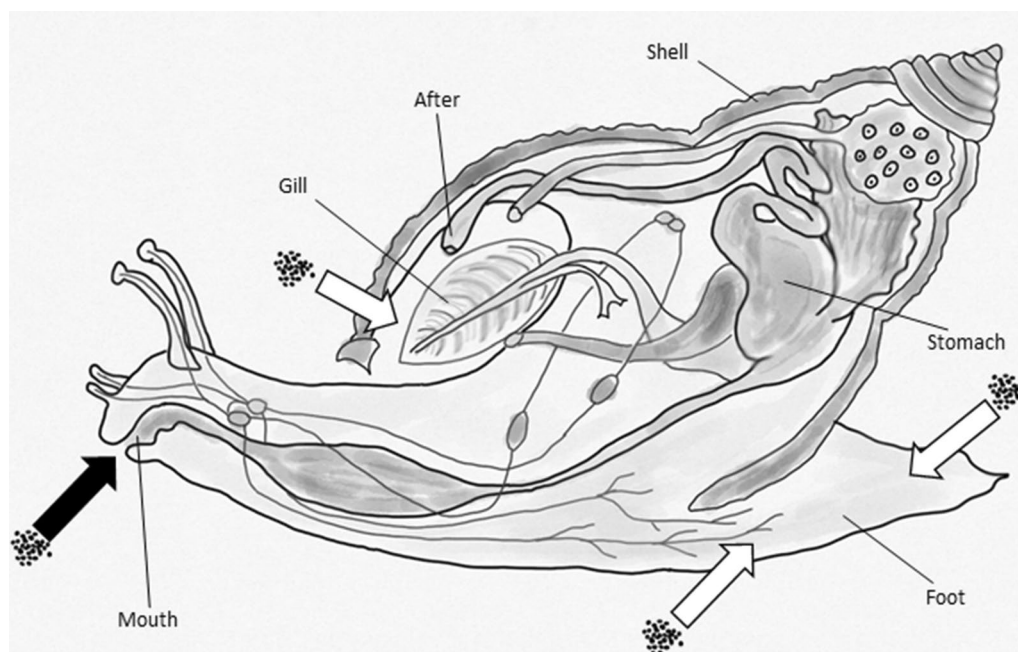


Fig. 2 Potential uptake routes of ENMs in *Gastropoda*. Black arrow: ingestion by the dietary route/ mouth; white arrows: uptake by the respiration/ gill (pulmonates) and passive uptake by the soft body tissues surface, e.g., foot

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 AgNO₃) 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 Cu²⁺) 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 (Cu²⁺) 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 ⁶⁷ZnO-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 bio-monitor 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 CaCO₃, 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].

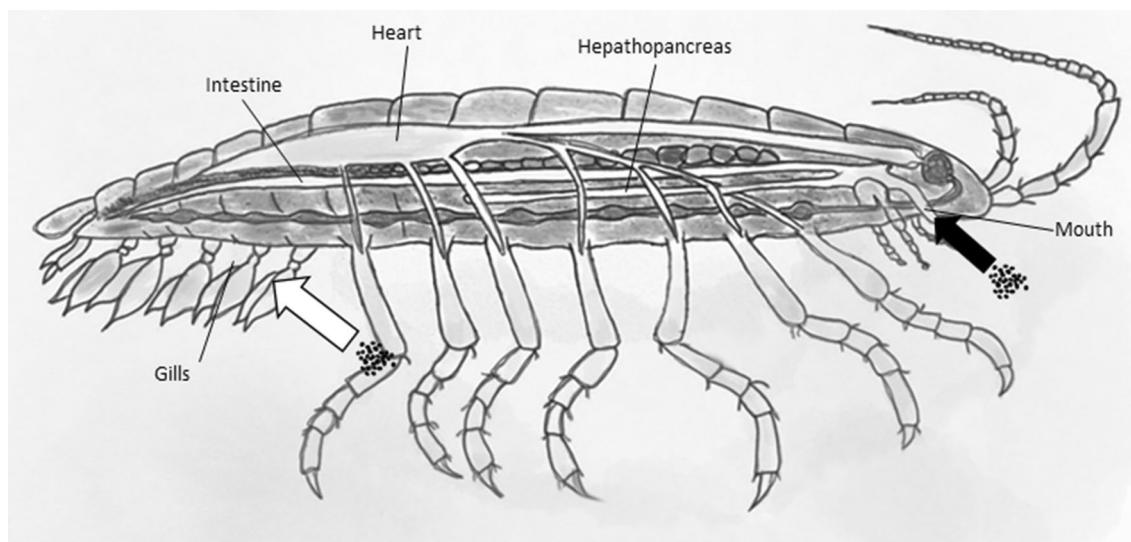


Fig. 3 Potential uptake routes of ENMs in *Isopoda*. Black arrow: ingestion by the dietary route/ mouth; white arrow: uptake by the respiration/gills

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 gnathopods 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

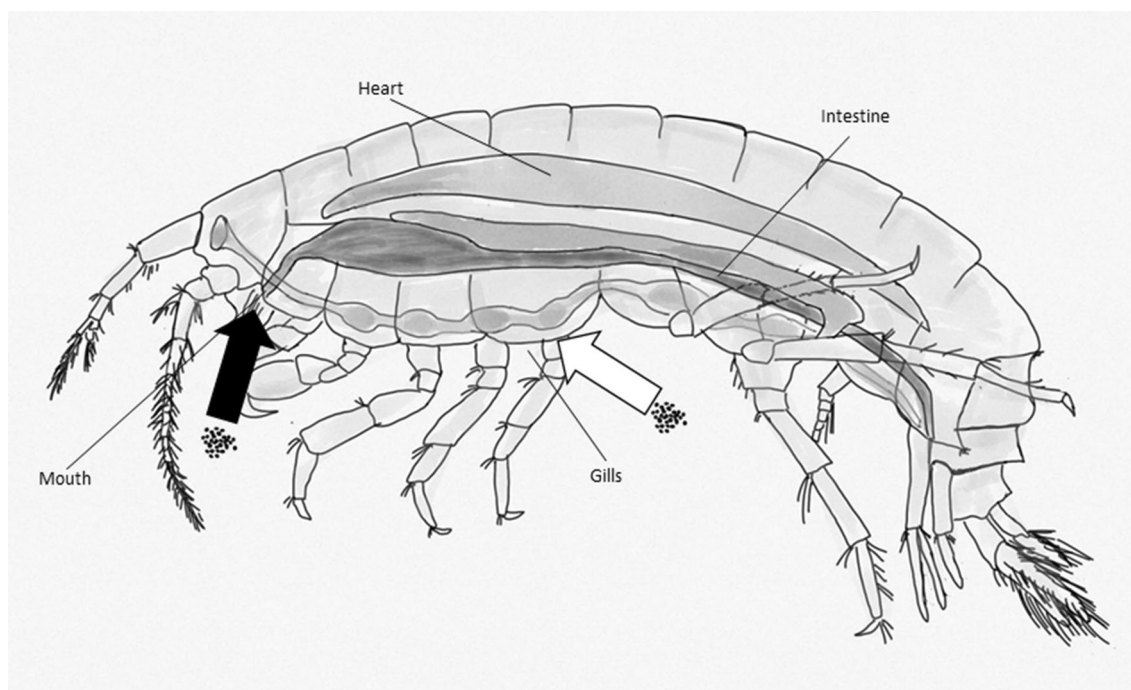


Fig. 4 Potential uptake routes of ENMs in *Amphipoda*. Black arrow: ingestion by the dietary route/ mouth; white arrow: uptake by the respiration/ gills and treacheae

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 AgNO₃ was determined to be 2277. A comparable low BCF value of 176 was determined for CeO₂NPs 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 Schleichtrien 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 Model 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 AgNO₃. 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 AgNO₃, Ag-NPs, TiO₂ from TiO₂NPs, 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* (fairly 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.

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

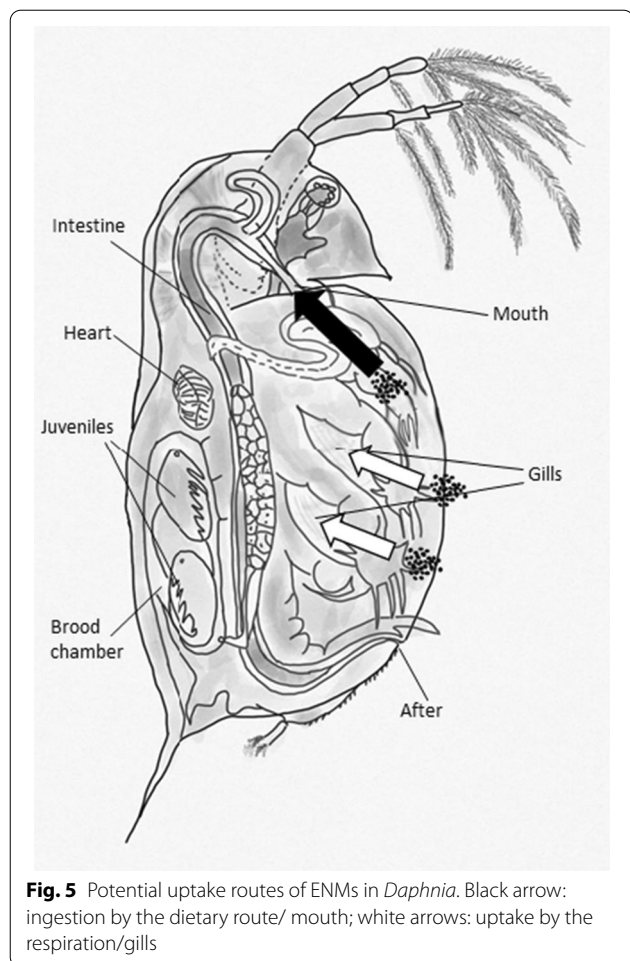


Fig. 5 Potential uptake routes of ENMs in *Daphnia*. Black arrow: ingestion by the dietary route/ mouth; white arrows: uptake by the respiration/gills

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 TiO₂-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 TiO₂-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

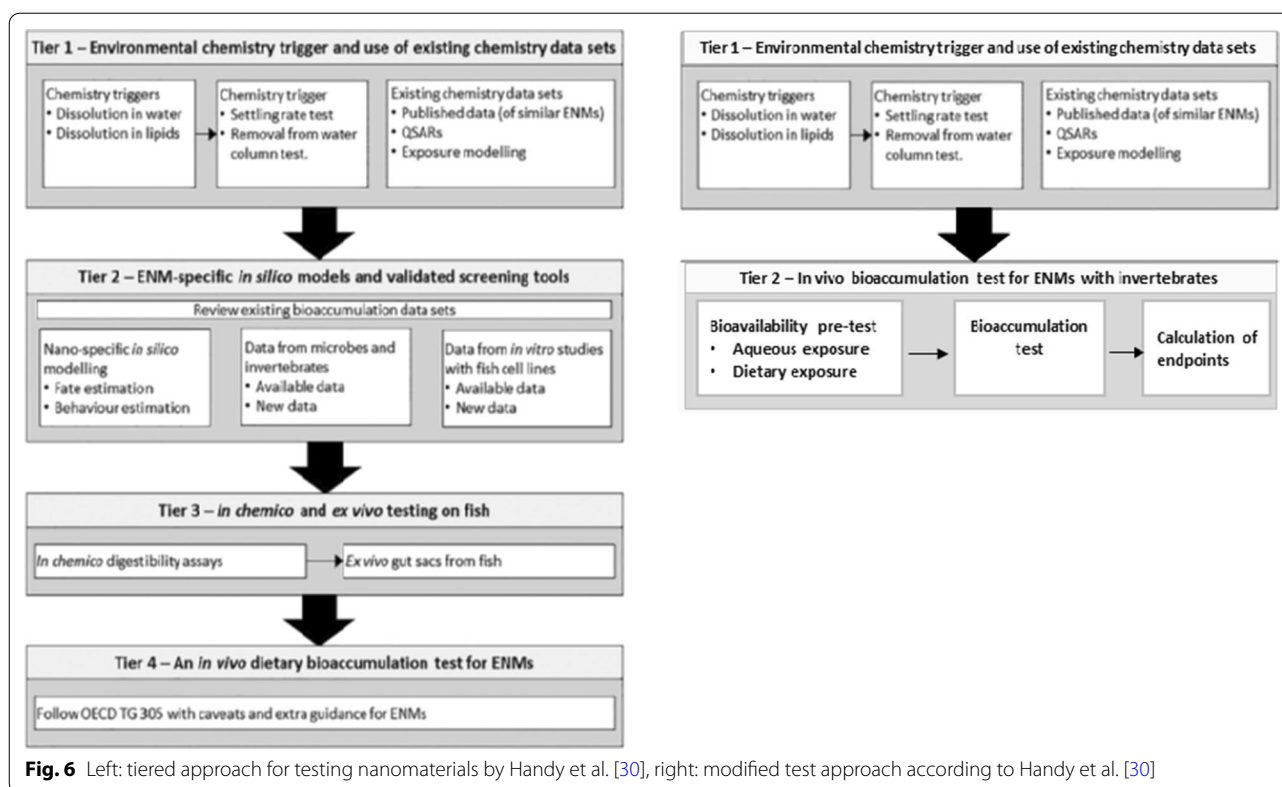


Fig. 6 Left: tiered approach for testing nanomaterials by Handy et al. [30], right: modified test approach according to Handy et al. [30]

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.

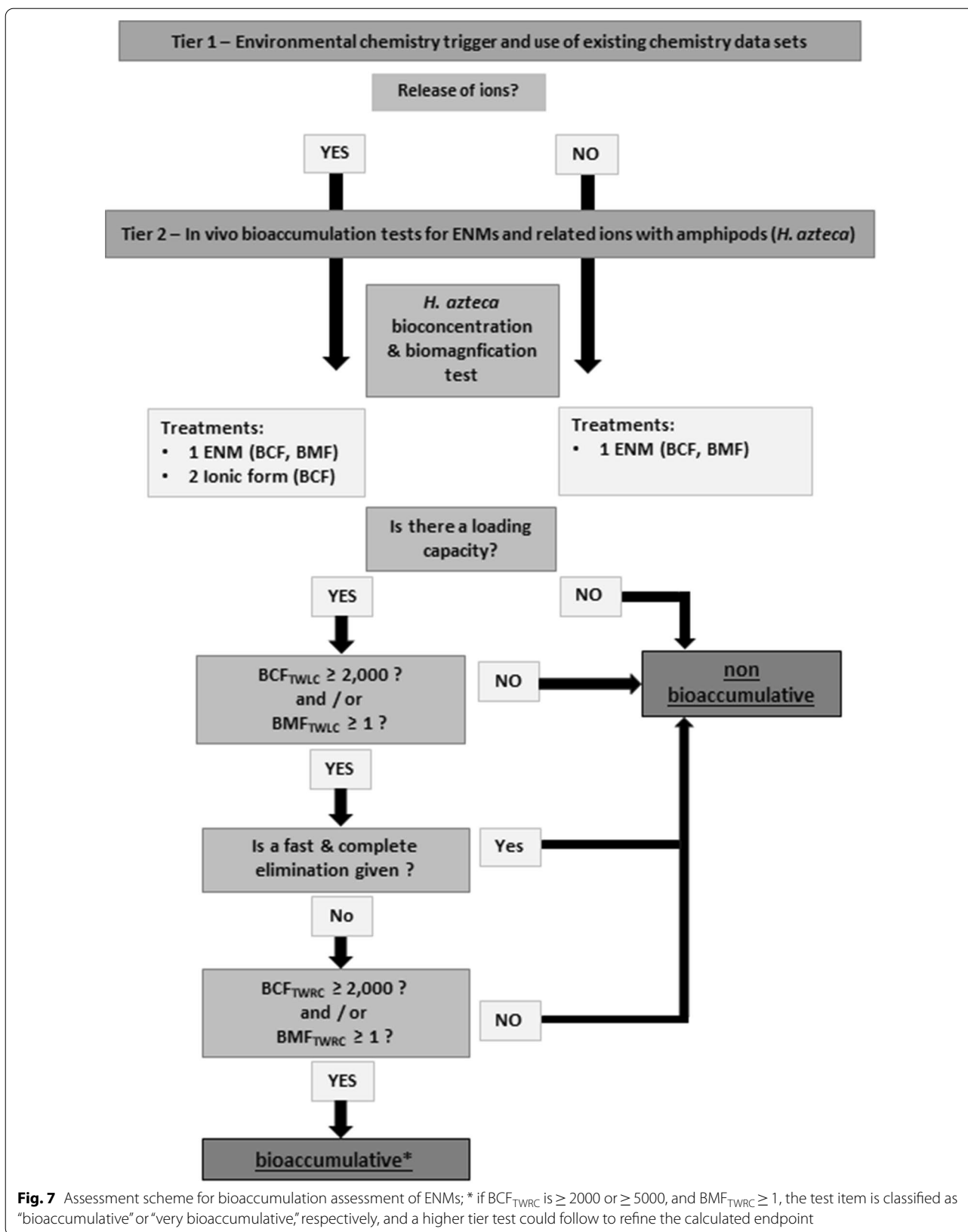
The uptake phase of the bioaccumulation tests (bioconcentration or biomagnification approach) should last at least as long as required that three subsequent body burden measurements ($\Delta t \geq 12$ h) show no variation higher than 20% allowing the calculation of a time-weighted loading capacity (TWLC)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



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 $BCF_{TWRC}/TWRC$ 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 *Hyaletella 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

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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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