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Upon Exposure to Cu Nanoparticles, Accumulation of Copper in the Isopod *Porcellio scaber* Is Due to the Dissolved Cu Ions Inside the Digestive Tract

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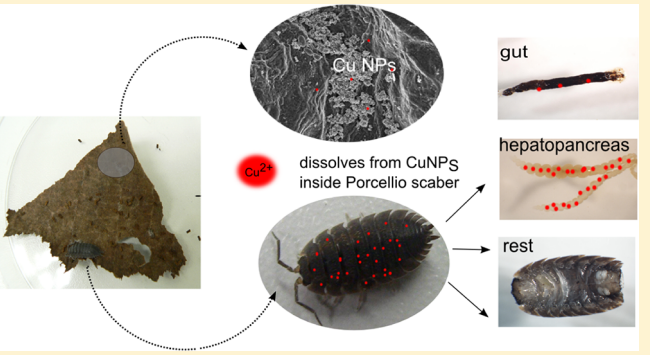
Supporting Information

ABSTRACT: The fate of nanoparticles in organisms is of significant interest. In the current work, we used a test system with terrestrial isopods (*Porcellio scaber*) fed with food spiked with Cu NPs or soluble Cu salt for 14 days. Two different doses were used for spiking to yield final concentrations of 2000 and 5000 μg Cu/g dry food. After the exposure period, part of the exposed group of animals was transferred to clean food to depurate. Cu content was analyzed in the digestive glands, gut, and the 'rest' of the body. Similar patterns of (i) assimilated and depurated amounts of Cu, (ii) Cu body distribution, and (iii) effect on isopods feeding behavior were observed regardless of whether the animals were fed with Cu NPs or soluble Cu salt spiked food. Thus, Cu ions and not CuO NPs were assimilated by the digestive gland cells. Solubilization of the Cu NPs applied to the leaves was also analyzed with chemical methods and recombinant Cu-sensing bacteria. The comparison of the in vitro data on solubilization of Cu NPs and in vivo data on Cu accumulation in the animal tissues showed that about 99% of accumulated copper ions was dissolved from ingested Cu NPs in the digestive system of isopods.



INTRODUCTION

In complex environments, engineered metallic nanoparticles (NPs) undergo various changes, including the dissolution process, which results in release of metal ions. Knowing the changes of nanoparticles in such environments is important for many reasons, including environmental chemistry (monitoring nanoparticles in air and water), materials processing (monitoring nanoparticle growth during synthesis), and in vivo modifications (monitoring nanoparticles inside organisms).[1] Very little information is currently available on how metallic nanoparticles are modified inside the organism. One reason for that is a shortage of suitable biological model systems which would not be oversensitive to ingested particles or metal ions and would allow assessment of accumulated metal ions in concentrations high enough to be distinguished from control.

In many papers, terrestrial isopods have been reported to be used in metal bioaccumulation studies.[2] Isopods have been shown to accumulate the highest concentrations of metals such as zinc, cadmium, lead, and copper so far recorded in any soft tissue.[3,4] Therefore, data on stored amounts of metals gives insight into bioavailable amounts of metals ingested with food. Metal accumulation in digestive glands is explained also as a detoxifying mechanism, which diminishes the potential adverse effect of ingested metal ions.[3]

In isopods, the hepatopancreas is the major digestive organ with intestinal, hepatic, and pancreatic functions.[3] It is the main site of synthesis and secretion of digestive enzymes, absorption of nutrients, storage of metabolic reserves (lipids, glycogen), and excretion of wastes. The hepatopancreas consists of two specialized epithelial cell types. The big B cells contain type C granules with oxygen-donating, phosphate-bearing ligands that normally have high iron contents. The small S cells contain type B granules with a more homogeneous matrix with sulfur-donating ligands and normally have high Cu content.[3] In the isopods from metal-contaminated sites, type B granules also

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contain zinc, cadmium, and lead while type C granules may also accumulate zinc and lead. In isopods, apart from type B and type C granules, hybrid A/B granules have also been reported. In hybrid A/B granules, Zn was shown to be associated with phosphates (type A material in A granules) and form a rim around Cu/S-rich B granules.

This work was inspired by our recent study on ZnO NPs,[5] where we showed that the assimilation of Zn by *P. scaber* when fed on food spiked with ZnO particles depending on Zn dissolution from the particles. Assimilation of Zn had a similar pattern when originating from the soluble zinc salt ($ZnCl_2$) or from ZnO NPs. We were interested in whether the same rule would apply also for Cu NPs. Thus, the main aim of the current research was to study the behavior of ingested Cu NPs with a presumption that assimilated copper originates from the dissolved Cu from Cu NPs.

Literature data clearly show that Cu has the potential to dissolve from CuO NPs. Knowledge on the solubilization of Cu from CuO capsules has been historically used in different fields. As copper is essential for growth and prevention of a range of clinical and pathological disorders in all types of farm animals,[6] CuO supplementation in the form of 'boluses'/'capsules' (also referred as 'needles', 'bullets', or 'wires') has been extensively used as a means of supplementing Cu to ruminants (sheep, deer, cattle). CuO 'capsules' are believed to enter the animal's rumen transiently, before passing to the abomasum, where particles are retained in the folds of the lining and release Cu under acidic conditions favorable for absorption (reviewed in Castillo-Alcala et al.[7]). Handeland et al.[8] have shown that in a deer herd (*Cervus elaphus*) with a diet low in Cu, supplementation with CuO capsules at intervals of a few months maintained adequate serum Cu levels in the animals. A more recent study on *C. elaphus* demonstrated also the efficiency of CuO 'wires' as food supplements: mean liver concentrations increased significantly in the deer treated with 10 g of bolus of CuO wires either 30 or 60 days earlier from 255 to 597 and 244 to 447 μmol/kg, respectively. In comparison, mean liver Cu concentrations declined from 229 to 80 μmol/kg over the 60-day study period in untreated control deer.[7] Also, a study by Langlands et al.[9] on feeding grazing sheep and cattle comparatively with CuO powder and CuO particles (wires) showed that CuO powder was not efficient but CuO wires remarkably increased the Cu concentration in animal livers compared to the control group. Apparently, the powder passes through the acid environment in the abdomasum before much Cu can be solubilized.

Solubilization of CuO NPs is also the main reason for the toxicity of CuO NPs to aquatic test organisms.[10−16] Physico-chemical data indicate that Cu can dissolve from Cu NPs. In general, Cu NPs are known to have an oxide coating, and the variability in the thickness of the coating can give rise to differences in NP dissolution. In addition, dissolution kinetics depends on crystallinity and structural disorder on the surface as well as the presence of different crystallographic planes. The Cu NPs size distribution changes in a complex manner in acid environments and becomes multimodal as dissolution occurs.[1]

In the work presented here, we hypothesized that analogously to ZnO nanoparticles studied in our previous paper[5] Cu ions and not Cu nanoparticles would be assimilated when Cu NPs are added to the food. We also expected similar assimilation and depuration patterns of Cu when fed on the Cu NPs or the soluble Cu salt ($Cu(NO_3)_2{\cdot}3H_2O$)-spiked food. In addition, we assumed that assimilated Cu would not be entirely depurated after transfer of animals to clean food/leaves as Cu tends to be stored in S cells of hepatopancreas, which are not subjected to elimination of their content in these exposure periods. If depuration is similar in Cu NPs fed animals and soluble Cu salt fed animals, this will be additional proof that ions and not particles are assimilated in the case of exposure of *P. scaber* to Cu NPs. We also discuss the dissolution of ions from particles inside the digestive system, which could not be predicted by ex vivo analysis of the solubilization of Cu NPs.

MATERIALS AND METHODS

Characterization of Cu Nanoparticles. Commercially available Cu NPs (Sigma-Aldrich, copper nanopowder, <50 nm, 99.9% purity, CAS 7440-50-8) were investigated. Additional characterization of the NPs was performed. After the samples were dried and degassed with nitrogen prior to analysis, BET analysis (Brunauer−Emmett−Teller surface area analysis; Tristar 3000, Micrometrics) was performed to obtain information on the relative surface area of the Cu nanopowder. TEM and XRD were used to investigate the presence of oxide coating on the NPs (JEOL 2100, Tokyo, Japan coupled with an EDS microanalysis system that was operated at 200 kV). SEM was used to inspect the size of Cu NPs as a powder (Jeol JSM-6500F).

After exposure, remnants of selected leaves were dried and attached to mounts with silver paint (SPI), gold−palladium sputtered (Sputter coater SCD 050, BAL-TEC), and investigated by field emission scanning electron microscopy (SEM) (Jeol JSM-6500F). Energy-dispersive X-ray analysis (EDX) was used to prove their chemical composition (Figure 1) (EDS/WDS Oxford Instruments INCA, Jeol JSM-6500F, at the Institute of Metals and Technology, Ljubljana).

Dissolution of Cu from Cu NPs. *Quantification of Dissolved Fraction of Cu by the Recombinant Copper-Sensing Bacteria E. coli.* For analysis, dispersions of Cu NPs (1330 and 3300 mg/L, respectively) were prepared in Ultrapure water (Elga Purelab Option-Q; 18.2 MΩ-cm) in the same way as for in vivo tests and thereafter filtered through a sterile Minisart 0.1 μm filter (Sartorius). Ssuspensions were mixed (400 rpm) on a magnetic stirrer at ambient temperature for 1 h and sonicated in the ultrasonic bath (Sonis 2 GT ultrasound cleaner, Iskra PIO, Šentjernej na Dolenjskem, Slovenia) for 1 h. Recovery of the ionic solution after filtering was proven by filtering soluble Cu salts followed by analysis. Under the conditions applied, Cu ions were not trapped in the filter (data not shown).

The filtrate was analyzed for dissolved copper ions using recombinant bioluminescent Cu-sensor bacteria *Escherichia coli* MC1061 (pSLcueR/pDNPcopAlux)[17] and also constitutively luminescent control strain *E. coli* MC1061 (pDNlux) to take account of the possible toxic effect of the tested compound.[18] The tests with the sensor and control bacteria were performed essentially as described by Heinlaan et al.[11] Briefly, 100 μL of filtrate of Cu NPs suspension and 100 μL of sensor/control bacteria in the analysis medium (0.9% NaCl, 0.1% casamino acids (acid hydrolyzed casein) and 0.1% glucose, pH 6.1) were mixed on a white 96-well microplate (ThermoLabsystem, Finland) and incubated for 2 h in the dark at 30 °C. Luminescence was recorded with a Floroskan Ascent Luminometer (ThermoLabsytem, Finland). The amount of solubilized Cu ions was quantified using the $CuSO_4$ calibration curve in Ultrapure water, assuming its 100% bioavailability to the sensor bacteria.

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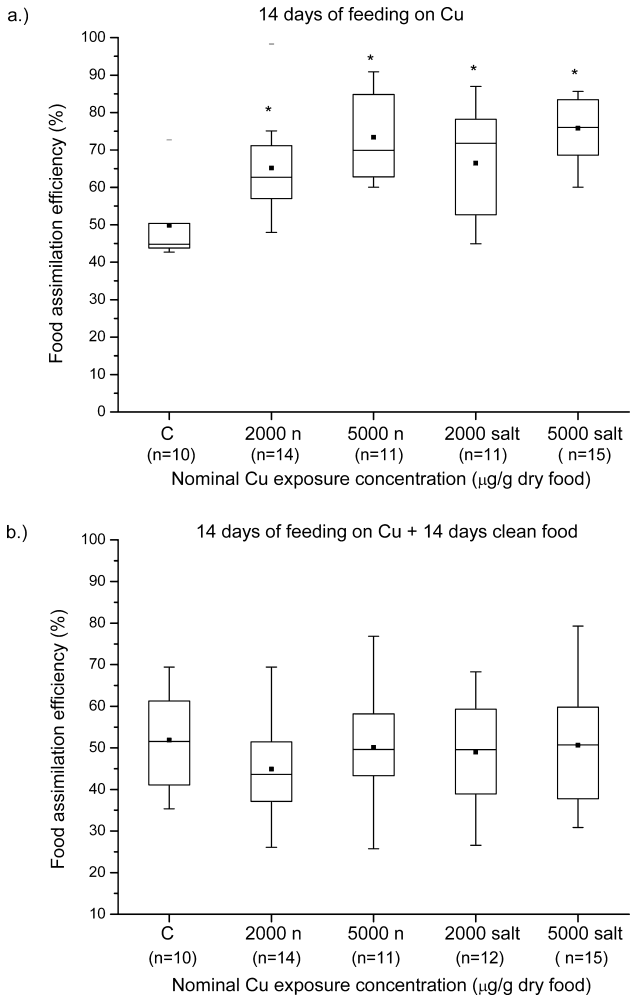

Figure 1. Food assimilation efficiency of isopods *P. scaber* after feeding for 14 days Cu-spiked food (a, experiment 1) and 14 days on Cu-spiked food followed by 14 days on clean (not Cu-spiked) food (b, experiment 2). In experiment 2 food assimilation was calculated only for the elimination period. Isopods were fed with $Cu(NO_3)_2{\cdot}3H_2O$ (designated as 2000 salt and 5000 salt) and Cu nanoparticles (2000 n and 5000 n) spiked food. Nominal exposure concentrations of Cu are provided on the *x* axis. Symbols on the box plot represent maximum and minimum value (whiskers: ⊥), mean value (■), outliers (—), and $p < 0.05$ (*).

Analysis of Dissolved Fraction of Cu by Chemical Analysis. For analysis, dispersions of Cu NPs (1330 and 3300 mg/L of Cu^{2+}) were prepared in Ultrapure water (Elga Purelab Option-Q; 18.2 MΩ-cm) in the same way as for in vivo tests. The obtained dispersions were ultracentrifuged twice at 100 000*g* twice for 30 min at 20 °C. Samples were analyzed for the concentration of Cu by flame atomic absorption spectrometry (Perkin-Elmer AAnalyst 100, Department of Biology, Biotechnical Faculty, University of Ljubljana).

It was expected that ultracentrifugation would cause sedimentation of nanoparticles; however, there is still the possibility that some of the particles remain in the supernatant. Therefore, the supernatant containing dissolved Cu ions and particles themselves was treated with 37% hydrochloric acid (Merck) (0.5 M) according to Elzey and Grassian.[1] It is expected that acidification will increase the amount of Cu ions if nanoparticles are present in the supernatant. However, the concentrations of Cu in the acidified and nonacidified part of the same supernatant did not differ (nonacidified, 0.09 ± 0.003 and 0.46 ± 0.019 mg/L in the case of 1330 and 3300 mg/L of Cu NPs, respectively; acidified, 0.10 ± 0.004 and 0.36 ± 0.017 mg/L in the case of 1330 and 3300 mg/L of Cu NPs) (mean ± SD; $n = 6$). We conclude that the ultracentrifugation procedure was efficient to remove the nanoparticles. Additionally, we checked the supernatant under SEM, and no particles were observed. The efficiency of removal of CuO NPs by ultracentrifugation prior to AAS quantification was also shown in a recent study by Bondarenko et al.[19] where DLS analysis of supernatants confirmed the absence of NPs.

Exposure of Isopods to Cu Nanoparticles and $Cu(NO_3)_2{\cdot}3H_2O$. *Test Organisms.* Adult specimens of the terrestrial isopods (*P. scaber*, Latreille 1804) were collected in September 2010 from the compost heap in a nonpolluted garden in Dobrova pri Ljubljani, Slovenia. Animals were kept in a controlled chamber at a constant temperature (20 ± 2 °C) and light regime (16 h light, 8 h darkness) and fed with dry common hazel (*Corylus avellana*) or black alder (*Alnus glutinosa*) leaves and freshly collected dandelion (*Taraxacum officinale*) rosettes during 2 weeks before exposure. Cultivation of animals in the laboratory was performed according to Drobne et al.[20] The adults of *P. scaber* of both sexes, intermoult and early premoult stages (PE1, according to Zidar et al.[21]). The average fresh body weight of animals was 46.6 ± 6.3 mg (mean ± SD; $n = 150$). There was no significant difference between masses of control animals and animals from any of the treatment regimes neither before nor after the experiment ($p < 0.05$; Mann−Whitney U test, Table 1, Supporting Information).

Preparation of the Food. During the experiments the animals were fed with dry common hazel (*C. avellana*) leaves spiked with Cu NPs and $Cu(NO_3)_2{\cdot}3H_2O$, respectively. Methods for the dispersion of NPs in Ultrapure water (Elga Purelab Option-Q; 18.2 MΩ-cm) (pH = 5.7) were the same as those described in Pipan-Tkalec et al.[5] for ZnO NPs. Two stock suspensions in concentrations 1330 and 3300 mg/L of Cu were prepared for both Cu NPs and $Cu(NO_3)_2{\cdot}3H_2O$. Suspensions were mixed (400 rpm) on a magnetic stirrer at ambient temperature for 1 h and sonicated in an ultrasonic bath (Sonis 2 GT ultrasound cleaner, Iskra PIO, Šentjernej na Dolenjskem, Slovenia) for 1 h. A 150 μL amount of this dispersion per 100 mg of leaf was applied onto the abaxial leaf surfaces. Each solution was prepared freshly prior to experiment. This resulted in two final concentrations of Cu on leaves: 2000 and 5000 μg/g dry leaf, respectively (nominal values). Cu concentrations tested were selected based on previous studies.[22,23] Also, Pipan-Tkalec et al.[5] analyzed the effect of the same exposure concentrations of ZnO nanoparticles allowing comparison of the results.

Experimental Design. Two separate experiments were performed. Experiment 1 included feeding of animals on Cu-spiked food for 14 days followed by 1 day depuration for removal of Cu-spiked food from the digestive system. Experiment 2 consisted of two stages where in the first part the animals were fed on Cu-spiked food for 14 days followed by feeding on clean food for another 14 days.

Conditions during the experiment were the same as in previous experiments.[20] Briefly, each animal was placed individually in a plastic Petri dish to which individual pieces of Cu NPs and Cu salt-spiked dry leaves were added, respectively. The food was not replaced during the exposure period, and fecal pellets were collected weekly to allow

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calculation of the feeding rate during the exposure period. At the end of the experiment, remnants of the leaves were collected, air dried, and weighed. Fecal pellets were also weighed after drying in a desiccator for 48 h. Fifteen animals per each concentration were exposed, but the number of analyzed animals after the experiments was lower due to mortality caused by molting and due to development of marsupia in females. The latter animals were excluded from further data processing. The number of analyzed animals is presented in the figures.

After the feeding experiments, the animals were dissected and Cu content was analyzed in three body parts: digestive glands (hepatopancreas), gut, and the 'rest' of the body (body remnants). Each body part (hepatopancreas, gut, and 'rest' of the body) was placed on a separate piece of filter paper (approximately 4 mm × 7 mm size) and stored in a plastic tube until analysis.

AAS Measurements. Copper was measured on Cu-spiked leaves of experiment 1 and in different body fractions (digestive gland, gut, and 'rest' of the body) of isopods. Prior to analysis, samples were digested in a concentrated nitric/perchloric acid mixture (HNO_3:$HClO_4$ = 7:1). After evaporation of the acid, the residue was dissolved in 0.2% HNO_3. Total Cu concentrations in digestive glands, gut, and the 'rest' of the body were analyzed by flame atomic absorption spectrometry (Perkin-Elmer AAnalyst 100, Department of Biology, Biotechnical Faculty, University of Ljubljana). Within each measurement certified reference material (TORT-2, National Research Council of Canada) was used to check the accuracy and precision of the analytical procedure. Along with the samples also 10 replicates of a known amount of certified reference material were acid digested and each sample was measured in triplicate. The concentration of the reference material was 106 ± 10 mg/kg; our measurement was 100.03 ± 3.69 mg/kg (mean ± SD, n = 30). Precision was always within 5% (relative standard deviation).

Data Analysis. Food assimilation efficiency was calculated as a percentage of assimilated food (difference between consumed food and defecated material) in comparison to consumed food. The amount of the total consumed Cu was calculated from the mass of consumed Cu-spiked leaves and the corresponding actual measured concentration of Cu on remnants of leaves at the end of the experiment. Cu assimilation efficiency was the ratio between the amount of total Cu in the whole body of each isopod exposed to the Cu-spiked food and the amount of consumed Cu by the same animal. Food assimilation was calculated separately for experiment 1 (where the animals were fed only with contaminated food) and experiment 2 (2 weeks on contaminated food, 2 weeks of an elimination period on clean food). In experiment 2, food assimilation was calculated only for the elimination period where animals were fed with clean food. The amount of Cu in the whole body was calculated as the sum of Cu in all body fractions (digestive gland, gut, and 'rest'). In these calculations, the average amounts of Cu measured in control animals were subtracted from the amounts in the exposed animals. Data are presented as mean values, and uncertainties are expressed as standard deviations (SD). All data presented in the figures refer to nominal concentrations of Cu (2000 and 5000 μg/g dry leaf). Statistical significance of differences between the control and the exposed groups of animals was assessed by the Mann−Whitney U test ($p < 0.05$) using Statgraphics software (Statgraphics Plus for Windows 4.0, Statistical Graphics, Herndon, VA, USA).

RESULTS

Characterization of Cu Nanoparticles. The BET-estimated specific surface area for Cu nanopowder was 7.80 ± 0.04 m^2/g. Scanning electron micrograph of Cu nanoparticles' powder is shown in the Supporting Information (Figure S1). The primary particles were found to be in the size range 50−150 nm and form aggregates. This was also seen from SEM images of an aqueous dispersion of Cu NPs applied on leaves, which showed that different sizes of aggregates up to a few micrometers were present and particles were spread over the entire leaf surface (Figure S2, Supporting Information). TEM and XRD revealed that a thin coat of oxygen was present on the surface of the NPs.

Actual Cu Exposure Concentrations on Leaves. The background Cu content of chemical-free (not spiked) leaves which were fed to control animals was 11.2 ± 0.6 μg Cu/g leave dry weight (mean ± SD, n = 30). Measured concentrations of Cu on Cu-spiked leaves were close to nominal values: 2590 ± 100 μg/g dry leaf at 2000 μg/g dry leaf Cu (Cu NPs); 5040 ± 240 μg/g dry leaf at 5000 μg Cu/g dry leaf (Cu NPs); 2070 ± 30 μg/g dry leaf at 2000 μg/g dry leaf ($Cu(NO_3)_2{\cdot}3H_2O$); and 5360 ± 280 μg/g dry leaf at 5000 μg Cu/g dry leaf $Cu(NO_3)_2{\cdot}3H_2O$ (mean ± SD, n = 30 for each concentration).

Quantification of Dissolved Fraction of Cu by the Recombinant Copper-Sensing Bacteria *E. coli*. Dissolution as assessed by the recombinant copper-sensing bacteria *E. coli* MC1061 (pSLcueR/pDNPcopAlux) showed that in the case of 1330 and 3300 mg/L of Cu (from Cu NPS), 0.41 ± 0.11 mg/L of Cu ions (0.031%) and 0.59 ± 0.09 mg/L of Cu ions (0.018%) were measured, respectively (mean ± SD, n = 3 for each concentration).

Concentration of Dissolved Ions in Cu NPs Suspensions Measured after Ultracentrifugation by Chemical Analysis. After separation of the dissolved fraction of copper ions from Cu NP suspensions by ultracentrifugation, the concentration of measured copper was 0.09 ± 0.003 and 0.46 ± 0.019 mg/L (mean ± SD; n = 6) in the case of 1330 and 3300 mg/L of Cu NPs, respectively. This represents a very small share of dissolved Cu (0.007% and 0.014% in the case of 1330 and 3300 mg/L of Cu NPs, respectively). It is important to note that the actual concentration of copper ions quantified in the supernatants of ultracentrifuged Cu NPs suspensions may also be smaller than the total concentration of copper quantified by AAS (comprising of copper ions and small fraction of nonremoved NPs). The concentrations of Cu ions measured by Cu-sensing bacteria and AAS were quite similar (Table 1).

Survival and Feeding Pattern of Isopods. Prior to the experiments, the isopods were inspected for moult stage (according to Zidar et al.[21]) and only animals in the intermoult and early premoult (PE1) stage were chosen for the experiments. However, some animals still started moulting during the 4 week duration of the experiment and died as this physiological state makes them very vulnerable. Some females which developed a marsupium were also excluded from further analyses. Up to 20% of animals (both dead individuals and ovigerous females) at each concentration were removed and not included in further data processing.

After 14 days of exposure to copper compounds, food assimilation efficiency was increased in all treated groups in comparison to the controls (Figure 1a). This parameter indicates a significant effect of Cu on the feeding physiology

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Table 1. Bioavailability of Copper and Zinc Assessed by Bacterial Sensors (in vitro), Chemical Analysis, and Isopods *P. scaber* (in vivo)[a]

			calculated from the NP solubility data analyzed by biosensors			calculated from the NP solubility data measured by chemical analysis			
actual (measured) exposure concentration (μg metal/g dry leaf weight)[b,c]	consumed metals during the whole test period (2 weeks for Cu, 4 weeks for Zn)[b,d] (μg/g animal dry weight)	actual (measured) assimilated metal in whole body[b,e] (μg/g animal dry weight)	predicted assimilated metal in whole body[b,f] (μg/g animal dry weight)	estimated metal dissolved inside *P. scaber* (μg/g animal dry weight)	estimated share of metal dissolved inside *P. scaber* (%)	predicted assimilated metal in whole body[b,g] (μg/g animal dry weight)	estimated metal dissolved inside *P. scaber* (μg/g animal dry weight)	estimated share of metal dissolved inside *P. scaber* (%)	metal assimilation efficiency (%)
	A	B	C (A* dissolution rate)[f]	D (B−C)	E (D/B)	F (A* dissolution rate)[g]	G (B−F)	H (G/B)	I
Cu NPs	Cu	Cu	Cu	Cu	Cu	Cu	Cu	Cu	Cu
2586 ± 95.9	5060 ± 420	449 ± 71	1.56 ± 0.1	447	99.6	0.35 ± 0.03	448.6	99.9	6.61 ± 0.1.1
5039 ± 243	9869 ± 740	449 ± 59	1.8 ± 0.5	447	99.6	1.38 ± 0.1	447.6	99.6	2.88 ± 0.74
ZnO NPs[h]	Zn	Zn	Zn	Zn	Zn	Zn	Zn	Zn	Zn
1500 ± 82	5293 ± 555	77 ± 41	29 ± 3	48	62				4.92 ± 2.71
3800 ± 227	12365 ± 1767	243 ± 43	32 ± 4.6	211	87				10.1 ± 2.33

[a]Data for ZnO NPs are taken from Pipan-Tkalec et al.[5] [b]Mean ± SD. [c]Nominal exposure concentrations were the same (2000 and 5000 μg metal/g dry weight of a leaf), but actually measured levels in the case of Zn were significantly lower; therefore, actual levels are provided. [d]Calculated based on the amount of consumed leave per animal dry weight and amount of measured metal on leaves. [e]Measured body burden with subtracted values of control. [f]Assuming that only bioavailable metals determined with recombinant bacterial sensors would be assimilated. These were calculated from dissolution rates: 0.031% of Cu dissolved at 2000 μg Cu/g dry weight of a leaf and 0.018% dissolved at 5000 μg Cu/g dry weight of a leaf; 0.55% of dissolved Zn at 1500 Zn/g dry weight of a leaf and 0.26% of dissolved Zn at 3790 Zn/g dry weight of a leaf. [g]Assuming that only bioavailable metals determined with chemical analysis would be assimilated. These were calculated from dissolution rates: 0.007% of Cu dissolved at 2000 μg Cu/g dry weight of a leaf and 0.014% dissolved at 5000 μg Cu/g dry weight of a leaf. [h]Pipan-Tkalec et al.[5] Four weeks feeding experiment.

of isopods: copper-exposed isopods retained more food than unexposed animals. No differences between animals fed on leaves with the same concentration but different origins of Cu (NPs, salt) were observed. The increase was dose dependent ($p < 0.05$). After exposure to untreated food, food assimilation efficiency in all treated groups returned to the control level (Figure 1b).

Distribution of Copper in Different Body Parts of Isopods. After 14 days of exposure to leaves spiked with Cu NPs or $Cu(NO_3)_2{\cdot}3H_2O$, the amount of Cu increased statistically significantly in all body fractions (hepatopancreas, gut, and 'rest') in comparison to the nontreated control organisms (Figure 2a and 2c, experiment 1 and first stage of experiment 2; data for 'rest' not shown). No differences in Cu concentrations were observed among animals fed on food with equal Cu concentrations of different origin. Cu assimilation, however, was not dose dependent (Figure 2a and 2c, data for 'rest' not shown). The highest concentration of Cu was found in hepatopancreas (11 836 ± 1002 μg Cu/g dry weight), followed by gut (3706 ± 678 μg Cu/g dry weight) and 'rest' (118 ± 7.89 μg Cu/g dry weight) (mean ± SD, n depicted on Figure 2).

After the feeding stage on nonspiked leaves for 14 days (experiment 2, second stage), Cu was efficiently depurated from the gut and the 'rest' of the body but still remained accumulated in the hepatopancreas (Figures 2b and 2d, data for 'rest' not shown). No differences in Cu concentrations were observed among animals fed on equally spiked food but with two different origins of Cu. Retention of Cu in hepatopancreas was again not dose dependent, namely, even if animals were fed on food containing 5000 μg Cu/g dry weight, the accumulated amount of Cu was similar as in the case of 2000 μg Cu/g dry weight in the food. We explain this by the copper accumulation capacity of animals which was already reached at 2000 μg Cu/g dry food level feeding.

Copper Assimilation Efficiency. The estimated amount of assimilated Cu in the body after 14 days of feeding was calculated as a sum of Cu in all body fractions minus the average Cu concentration in the control animals. No differences were observed among animals fed on leaves with the same concentration but with two different origins of Cu ($p > 0.05$; Mann−Whitney test) (Table 1; in the case of Cu salt $Cu(NO_3)_2{\cdot}3H_2O$: 372 ± 7.6 μg/g animal dry weight at 2000 μg Cu/g dry food; 527 ± 4.2 μg/g animal dry weight at 5000 μg Cu/g dry food; mean ± SD; n = 11 and 14 for 2000 and 5000 μg Cu/g dry food, respectively). On the basis of the amount of assimilated Cu, Cu assimilation efficiency (%) was calculated. No differences were observed between animals fed with leaves dosed with two different origins of Cu (NPs, salt) but of the same concentration. Both parameters were significantly lower at 5000 μg/g dry food in comparison to 2000 μg/g dry food (Figure S3, Supporting Information).

The bioavailability of copper assessed by bacterial sensors, chemical analysis, and isopods is compared in Table 1. On the basis of the amount of consumed leaf per animal dry weight and amount of measured copper on leaves we calculated the amount of ingested copper during the test period (column A). These data served to calculate the predicted assimilated copper in the whole body (columns C and F) where we assumed that the animals assimilated only the dissolved fraction assessed by recombinant Cu−biosensors assay or chemical analysis. By comparing the actual measured assimilated copper in the whole body and the values on dissolved copper obtained with bacterial

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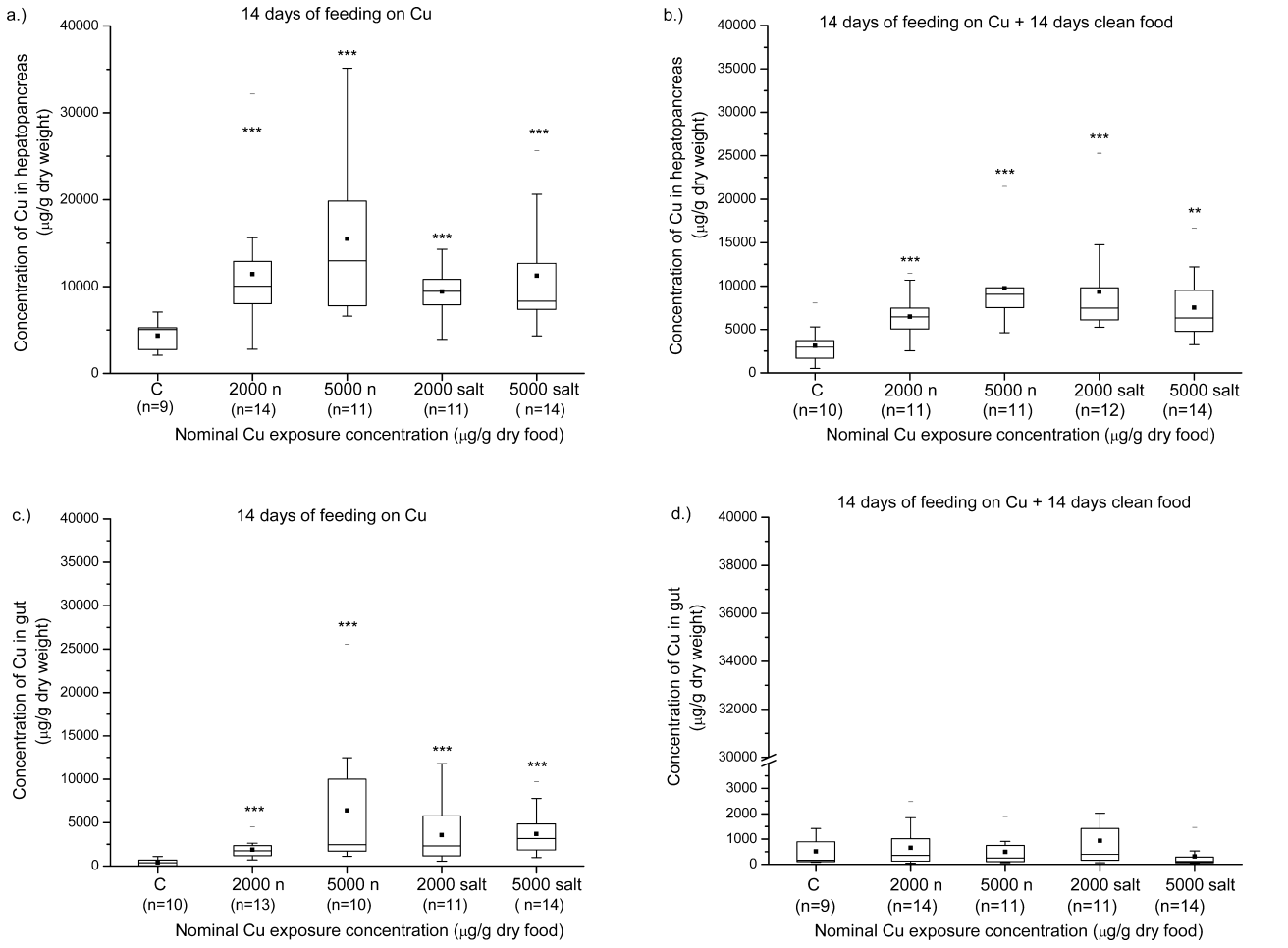

Figure 2. (a–d) Measured concentration of Cu in hepatopancreas (a and b) and gut (c and d) of isopods *P. scaber* after feeding for 14 days on Cu-spiked food (a and c experiment 1) and 14 days on Cu-spiked food followed by 14 days on not Cu-spiked (clean) food (b and d experiment 2). Isopods were fed with $Cu(NO_3)_2{\cdot}3H_2O$ (2000 salt and 5000 salt) and Cu nanoparticles (2000 n and 5000 n) spiked food. Nominal exposure concentrations of Cu are provided on the *x* axis. Symbols on the box plot represent maximum and minimum value (whiskers: ⊥), mean value (■), outliers (—), $p < 0.01$ (**), and $p < 0.001$ (***).

assay, we found that they do not match. Animals assimilated a significantly higher amount of copper than estimated from the bacterial dissolution quantification assay performed in vitro (or ex vivo). Similarly, when the AAS-analyzed dissolved amount of Cu was compared to the actual assimilation rate, significant discrepancies were evident. Actual assimilated amounts of Cu were much higher than estimated from in vitro dissolution experiments of Cu NPs followed by AAS analysis. Therefore, it is reasonable to assume that most of the dissolved Cu ions further assimilated and accumulated by the isopods were dissolved from Cu NPs in the digestive system of the animal, i.e., in vivo.

■ DISCUSSION

Copper is an essential metal for isopods, and its accumulation and depuration dynamics have been extensively studied. It was shown to accumulate in large amounts in the type B granules of small S cells in the digestive gland.[3] We hypothesized that if the assimilation pattern of Cu in the case of Cu NPs would be the same as is known for Cu salt ($Cu(NO_3)_2{\cdot}3H_2O$), this would prove that dissolved Cu ions from Cu NPs are assimilated and not the NPs themselves. The results of this work confirmed the hypothesis, since the same distribution pattern of Cu in three-body compartments, level of clearance from the body, and effect on the feeding behavior were observed regardless of the origin of Cu (salt or NPs). Similar results were also confirmed by a previously published study with ZnO NPs in the same experimental setup with the same species of isopods, where dissolved Zn ions from ZnO NPs were assimilated[5] (Table 1). Furthermore, another isopod study with the same experimental set up provided a detailed micro-PIXE analysis and TEM investigation and showed that in the case of Ag NPs, Ag is accumulated in S cells of isopods, but no NPs were observed inside these cells.[24]

The initial fraction of dissolved Cu in Cu NPs dispersion in Ultrapure water, which was applied onto leaves, was very low (less than 0.1%) as assessed by recombinant Cu-sensor bacteria as well as chemical analysis. If this is the bioavailable fraction, it could be expected that very low levels of Cu would be accumulated (0.35–1.38 μg/g animal dry weight in the case of chemical analysis, 1.56–1.8 μg/g animal dry weight in the case of biosensor assay). However, the amount of assimilated Cu in isopods was significantly higher. We therefore explain that animals consumed Cu NPs and a very low amount of dissolved Cu, whereas Cu ions dissolved from Cu NPs in the digestive system were accumulated in the body. According to the calculations about 99.6% of accumulated Cu is dissolved from Cu NPs within the digestive system of isopods, which was also

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the case in our previous experiments with Zn NPs, where a very high share of assimilated Zn dissolved inside the isopods (up to 97%)[5] (Table 1). These findings opened a new view on dissolution of ions from metallic NPs which may occur in biological fluids and could not be detected when only particle suspensions in the supplemented food are analyzed for dissolution. The possibility of modification of NPs inside organisms should therefore be taken into account, and further studies are suggested.

The dissolution behavior of metallic oxide nanoparticles is not well understood, but obviously it depends on the physico-chemical conditions of the exposure environment (reviewed in Quik et al.[25]). Previous studies using recombinant microbial sensors have shown that the dissolution of Cu from CuO NPs largely depends on the properties of the test medium.[26] For example, approximately 3% of copper was dissolved from CuO NPs in Milli Q medium,[27] 25% in algal medium,[12] and 2% in medium used for *Tetrahymena thermophila* assays.[28] The concentration of dissolved Cu also depends on the time of incubation in the test media,[13,19] particularly on the pH (dissolution is expected to be high in acid environment).[29]

The pH in the digestive system of isopods differs in different gut sections to provide an appropriate environment for digestive enzymes.[3] In the hepatopancreas, the pH was found to be 6.2 ± 0.2, in the typhlosole region of anterior hindgut pH = 6.5 ± 0.2, and in the posterior part pH = 6.5 ± 0.2. Specimens of *P. scaber* are able to buffer a wide range of pH values if food with different pH (from 4.0 to 7.5) is consumed. We therefore do not expect that the increase in dissolution of Cu from Cu NPs is a straightforward consequence of pH inside the gut, since Cu NPs were prepared in Ultrapure water of pH = 5.7, and a slightly more alkaline environment in the gut predominantly should not affect the dissolution.

It is therefore most probable that other dissolution mechanisms are involved in the digestive tract, among them the ligand-promoted dissolution and organic dissolution.[30] This knowledge arises from studies on the bioaccessibility and mobilization of copper from natural environments, such as soil and sediment. It has been shown that the solubility of Cu is significantly increased in the presence of amino acid histidine (commonly present in the gut of deposit feeders),[30] bovine serum albumin (a surrogate for proteinaceous material in the digestive tract),[30] citric acid,[31] and sodium taurocholate (anionic surfactant present in the digestive tract of deposit feeders).[32] It is known that very high concentrations of surfactant lipids are present in the gut fluid of isopods to reduce the potential impact of ingested tannins via food.[33] However, the exact composition of the gut fluid is unknown.

Copper affected the feeding behavior of isopods. After 14 days of feeding on Cu NPs or ($Cu(NO_3)_2{\cdot}3H_2O$), the feeding rate significantly decreased and food assimilation efficiency increased. The effect on the feeding behavior was the same for Cu salt and Cu NPs. A decrease in consumption and decrease of faecal production was accompanied by increased food assimilation activity due to the longer retention time of food in the digestive system and is a well-known phenomenon in isopods.[34] The change in comparison to control was dose dependent, and after exposure to nonspiked food, feeding was restored to the level of control animals.

Mean Cu assimilation efficiencies (in %) estimated in this study were 6.61 ± 0.1.1 at 2000 Cu−Cu NPs, 2.88 ± 0.74 at 5000 Cu−Cu NPs, 7.62 ± 1.51 at 2000 Cu−$Cu(NO_3)_2{\cdot}3H_2O$, and 3.64 ± 0.5 at 5000 Cu−$Cu(NO_3)_2{\cdot}3H_2O$. These are lower than previously reported for Zn[5] (Table 1). Cu assimilation efficiency decreased with increasing concentration, which was also observed by Zidar et al.[23] This is a common observation and can be explained by the fact that isopods have a limited capacity to accumulate metal ions in a given period of time. Since the metal assimilation efficiency is calculated as a ratio between accumulated metal and consumed metal, a higher amount of consumed metal at a higher concentration will result in a lower metal assimilation efficiency when the threshold amount for assimilation of Cu is reached. Since a similar amount of Cu was found to be assimilated in both exposure concentrations, we assume that the assimilation capacities of isopods were reached already at 2000 μg/g dry food exposure concentration.

We conclude that Cu ions were assimilated when isopods were fed with Cu NPs. Exposure to Cu NPs causes adverse effects on the feeding behavior of isopods, and this effect is mediated by solubilized Cu ions. Actual assimilated amounts of Cu were much higher than estimated from in vitro Cu dissolution studies from Cu NPs by chemical analyses or biosensor assay. Study of the modulation of NPs properties inside the organism (in vivo) needs appropriate robust, reproducible, and repeatable biological tests. The *P. scaber* in vivo method accompanied by chemicals or biosensor assays demonstrated in the current paper could be suggested as one of these methods.

■ ASSOCIATED CONTENT

Supporting Information

Scanning electron micrograph of Cu nanoparticles powder, scanning electron micrograph of Cu nanoparticles applied onto leaves and EDX analyses of particles, copper assimilation efficiency (%) of isopods *P. scaber* after feeding for 14 days on Cu-spiked food, and masses of test animals in experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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