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Trophic transfer of CuO NPs from sediment to worms (*Tubifex tubifex*) to fish (*Gasterosteus aculeatus*): a comparative study of dissolved Cu and NPs enriched with a stable isotope tracer (^{65}Cu)†

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Metal nanoparticles (NPs) released into the aquatic environment will likely accumulate in sediment and be available for sediment-dwelling invertebrates that serve as food for other organisms, such as fish. The aim of the present study was to investigate trophic transfer of copper oxide (CuO) NPs and dissolved Cu ($CuCl_2$) from natural sediment to the sediment-dwelling worm *Tubifex tubifex*, and then to the predatory fish *Gasterosteus aculeatus* (three-spined stickleback). Cu enriched in the stable isotope ^{65}Cu was used to increase detection and discriminate newly added/assimilated Cu from background Cu levels. Worms were exposed to sediment spiked with $^{65}CuCl_2$ or ^{65}CuO NPs (~20 nm) at environmentally relevant concentrations for 7 days and subsequently fed to fish for 7 days. Worms accumulated ^{65}Cu during sediment exposure to both $^{65}CuCl_2$ and ^{65}CuO NPs (0.7 and 1.1 μg ^{65}Cu per g dw tissue, respectively), resulting in ^{65}Cu body burdens significantly different from control. Furthermore, significantly more ^{65}Cu was released from the sediment into the overlying water in the ^{65}CuO NP exposures compared to the $^{65}CuCl_2$ exposures. ^{65}Cu accumulation in fish feeding on $^{65}CuCl_2$ and ^{65}CuO NP-exposed worms was limited (intestinal tissue: 80 and 65 ng g^{-1} dw; fish carcass: 7 and 10 ng g^{-1} dw, and liver: 50 and 10 ng g^{-1} dw, respectively). Glutathione peroxidase (*gpx*) mRNA expression was significantly higher in fish feeding on $^{65}CuCl_2$-exposed worms compared to ^{65}CuO NP-exposed worms (though ^{65}Cu tissue concentrations were similar). No significant effects were detected for the other investigated genes (*ctr1*, *mta*, *gcl*, *gr*, *sod*, *cat*, *zo-1*). Our results show that NP-derived Cu can enter freshwater food webs, but bioaccumulation and trophic transfer under environmentally realistic exposures is low (detectable with a tracer) and not different from that of dissolved Cu.

Environmental significance

Engineered metal nanoparticles (NPs) released into the aquatic environment will likely accumulate in sediment, where they may be available for uptake by sediment-dwelling invertebrates such as *T. tubifex* that serve as food for higher trophic level organisms, such as fish. Through this dietary uptake NPs may be transferred in aquatic food webs with risk of biomagnification. In the present study, we compared transfer of ^{65}CuO NPs with dissolved copper (added as $^{65}CuCl_2$) in an experimental food chain involving sediment, sediment-dwelling worms, and predatory fish. Isotopically enriched ^{65}Cu was used to enhance detection and discriminate between newly accumulated and background Cu, making it possible to use environmentally realistic Cu concentrations in sediment (100 μg Cu per g dw sediment). Our findings suggest that dietary transfer of metal NPs may occur to a limited extent in freshwater environments.

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1. Introduction

Over the past few decades, there has been a dramatic increase in the industrial production and use of copper (Cu)-bearing nanoparticles (NPs), such as Cu, CuO, Cu_2O and $Cu(OH)_2$ NPs.[1] For CuO NPs, the total production amount was estimated to be 20 tons in Europe in 2016, of which less than 1% was predicted to end up in the aquatic environment.[2] Possible entry routes of these NPs into the aquatic environment include urban stormwater, untreated

wastewater, wastewater treatment plants effluents,[2–4] runoff from agricultural land treated with NP-containing biosolids and pesticides,[3,5,6] and release from weathered surfaces treated with NP-containing wood coatings or antifouling paints.[2,7] After release into natural water bodies, Cu-bearing NPs are likely to undergo a series of transformations that may affect their fate, bioavailability, and toxicity. For instance, they are likely to aggregate, settle out and accumulate in sediments.[8–12] Furthermore, dissolution may occur in both water and sediment. CuO NPs tend to dissolve less than other Cu-bearing NPs and may thus prevail in the environment.[8,12–17] Using a multimedia fate and transport model simulating ten years of release of manufactured nanomaterials including CuO NPs in the San Francisco Bay area, Garner *et al.* (2017) predicted that CuO NPs will accumulate in freshwater and marine sediment beds potentially reaching concentrations in the ng kg^{-1} to μg kg^{-1} range.[4] Similarly, Caballero-Guzman and Nowack, estimated that alone the use of CuO NP-containing wood coatings in Europe could result in an increase of their concentration in sediments by about 400 ng kg^{-1} anually.[2] The increasing occurrence of CuO NPs in sediment beds augments the likelihood for exposure of benthic deposit-feeders. Previous research has established that sediment-associated CuO NPs are available for endobenthic worms such as *Nereis* (*Hediste*) *diversicolor* and *Lumbriculus variegatus* living in estuarine and freshwater systems.[9,18,19] Since benthic invertebrates represent a major food source for fish, it is hypothesized that CuO NPs may be further transferred along the food chain.

Today a range of studies have focused on the fate of manufactured nanomaterials in aquatic food webs (for reviews see ref. 20 and 21) but only a few on their transfer along food chains including predatory fish as a secondary consumer.[22–32,33] Furthermore, most studies focused on pelagic food chains using, for instance, artemia,[22,25,27,29,32] or daphnia[23,24,28,31] as the model prey, and zebrafish (*Danio rerio*) as the model predator.[23–28,31,32] An exception is published by Wang *et al.* (2016), who studied the trophic transfer of TiO_2 NPs in a marine benthic food chain from the clamworm (*Perinereis aibuhitensis*) to juvenile turbot (*Scophthalmus maximus*).[30] The authors reported trophic transfer, but no biomagnification of TiO_2 NPs between trophic levels. Even though metal(oxide) NPs constitute the biggest group of manufactured nanomaterials that has been tested in food chains including fish,[22,23,25,28–32] CuO NPs appear to have been used in only one study so far.[22] The authors found that dietary exposure was a likely uptake route of CuO NPs and that accumulation primarily occurred in the intestine. There are only a few studies assessing dietary uptake of nanoparticulate Cu in fish, and findings concerning the intestinal absorption efficiency and accumulation in inner tissues compared to dissolved Cu species vary.[34–36] Some information on NP transfer from invertebrate prey organisms to fish can be inferred from studies that examined intestinal uptake and accumulation of metal(oxide) NPs from spiked artificial diets.[37–45] Altogether, the data from the few studies published on dietary exposure of fish to Cu(O) NPs suggest that Cu from NP-spiked diet is available, but uptake is limited compared to dissolved Cu.[34,38,40,41] The present study is a continuation of such a study where we examined Cu uptake from Cu salt and CuO NPs in spiked artificial diets (worm homogenates).[42] Such test design provides considerable logistical advantages, as well as the possibility of strict control of the exposure dose *etc.* However, it is important to bear in mind that these studies ignore physical, chemical, and biological processes occurring in the environment and the influence that the prey organisms may play in altering the availability of NPs to predators.

In a review, Tangaa *et al.* defined four processes that influence trophic transfer of metal NPs: environmental transformations of metal NPs, uptake and accumulation in the prey organism, internal fate and localization in the prey, and the digestive physiology of the predator.[20] Therefore, in the present study, fish were exposed *via* live worms to increase environmental realism of the exposure scenario regarding both food type (live worms), complexation with biological tissue and realistic exposure dose (dependent on actual bioaccumulation of Cu in worms during sediment exposure).

The objective of this work was to study whether transfer of CuO NPs from sediment to worms (prey) to fish (predator) occurs at environmentally relevant exposure concentrations and conditions. In addition, the aim was to evaluate if potential changes in gene expression resulting from NP exposure could be detected. Dissolved Cu applied as $CuCl_2$ was used as a reference (soluble Cu counterpart). CuO NPs and $CuCl_2$ enriched in the stable isotope ^{65}Cu (^{65}CuO NP and $^{65}CuCl_2$)[34] were used to trace the transfer of Cu along the different compartments of the simulated food chain (sediment, worms, and fish). The model prey and predator organisms chosen for this study were the sediment-dwelling freshwater oligochaete *Tubifex tubifex* and the fish three-spined stickleback (*Gasterosteus aculeatus*). *T. tubifex* is a widely used standard test organism for bioaccumulation testing,[46] and is, as a deposit-feeder, at particular risk of exposure to sediment-associated metal NPs and metals. In addition, previous research has established that *T. tubifex* is relatively insensitive to Cu-induced toxicity and can accumulate high amounts of the metal before lethality occurs.[47–51] This makes it a good model prey organism for studying trophic transfer of Cu including Cu-bearing NPs. Three-spined stickleback (*Gasterosteus aculeatus*) is a widely used fish model in ecotoxicology and as a bottom-feeder a natural predator of tubificid worms.[52–54]

2. Methods

2.1 Synthesis and characterization of ^{65}CuO NPs

The synthesis of ^{65}CuO NPs were described previously.[34] In brief, the metallic precursor ^{65}Cu (isotope purity >99%, Trace Sciences International, USA) was dissolved in a mixture of HCl and H_2O_2. Solvent removal on rotatory evaporator under vacuum at 90 °C yielded a powder of $^{65}CuCl_2$. ^{65}Cu NPs were

synthesized by thermolysis of ^{65}Cu-oleate obtained by reflux of a mixture of $^{65}CuCl_2$ and sodium-oleate in water, ethanol and hexane. ^{65}CuO NPs were produced by oxidation of the ^{65}Cu NPs, and were finally dispersed in 1 mmol L^{-1} sodium citrate solution. All data on the characteristics of the ^{65}CuO NPs including size (20 nm; TEM; JEOL2100F transmission electron microscope), shape (spherical) and surface chemistry (mixture phase of CuO, Cu_2O, and Cu; X-ray diffraction) are shown in Lammel *et al.* (2020).[34]

2.2 General experimental design of trophic transfer study

An overview of the overall experimental design is given in Fig. 1. Trophic transfer of ^{65}CuO NPs and dissolved ^{65}Cu ($^{65}CuCl_2$) from sediment to worms to fish was examined by conducting two successive dietary exposures, each lasting 7 days. In the first exposure, *T. tubifex* were exposed to clean sediment and sediment spiked with ^{65}CuO NPs or $^{65}CuCl_2$. At the end of this exposure, the worms were recovered from the exposure vessels and used as live food for three-spined sticklebacks (second exposure). In total, seven separate repetitions of the worm exposure were carried out. The repetitions were staggered by one day to ensure that, each day, fish received worms that had been exposed for the same duration (Fig. 1B).

2.3 Worm exposure

2.3.1 Experimental organism, *T. tubifex*. *T. tubifex* (EAN 4038358100154, Batch-No.: 1518413 produced by Live is Life for Zooschatz, Berlin, Germany) were purchased through ZooCenter Bäckebol (Hisings Backa, Göteborg, Sweden). The worms were reared in the laboratory in glass beakers (250 mL Schott bottles) containing 150 mL *T. tubifex* medium (described below) continuously aerated by a glass Pasteur pipette connected by silicon tubing to the in-house pressurized air system. The culture was kept at 17 °C at a 12 h/12 h darkness/light cycle (conditions in the aquarium facility).

2.3.2 Tubifex medium preparation. Tubifex medium (artificial freshwater) was prepared as specified in OECD Guideline 203. In brief, 25 mL of a calcium chloride stock solution (11.76 g L^{-1} $CaCl_2$–$2H_2O$, 10043-52-4, Merck), a magnesium sulfate stock solution (4.93 g L^{-1} $MgSO_4$–$7H_2O$, 10034-99-8, Sigma-Aldrich), a sodium bicarbonate stock solution (2.59 g, $NaHCO_3$, 144-55-8, Merck) and a potassium chloride stock solution (0.23 g L^{-1} KCl, 7447-40-7, Merck) were mixed and the total volume made up to 1 L with deionized water. The final solution was kept at 17 °C and aerated for at least 24 h before being used.

2.3.3 Sediment preparation. Sediment was collected from Roskilde Fjord, Munkholmbroen (55°40′25″N 11°48′44″E) in October 2016 by scraping of the top few centimeters of the sediment surface and subsequently sieved at the site (<0.5 mm). After settling, the overlying water was removed and the sediment was frozen at −20 °C to kill macro- and meiofauna. Thereafter, the sediment was thawed, sieved to <125 μm, rinsed with fresh *T. tubifex* medium and stored at 4 °C until experimental use. Dry weight (dw) to wet weight (ww) ratio determined using a Metler Toledo moisture analyzer was 0.374 ± 0.001 (mean ± sd, n = 2). The background Cu concentration of the sediment (<125 μm) was 29.4 ± 3.7 μg Cu per g dw sediment (n = 7; Cu concentrations inferred by either of the two naturally occurring stable Cu isotopes, ^{63}Cu or ^{65}Cu, as described below).

For exposures, sieved sediment (about 240 g ww sediment per treatment) was spiked with ^{65}CuO NPs or $^{65}CuCl_2$ at a nominal concentration of 100 μg ^{65}Cu per g dw sediment (the measured concentrations were 88 and 95 μg ^{65}Cu per g dw sediment, respectively; see Table 2). In addition, control sediment was prepared. This sediment was spiked only with the solvent in which ^{65}CuO NPs and $^{65}CuCl_2$ spiking solutions were prepared (2 mM citrate solution, see above). To obtain a homogenous Cu distribution, the sediment was first stirred by hand and then by automated shaking at 300 rpm and room temperature overnight (Heidolph Unimax 1010 Orbital Shaker). Thereafter, the sediment was kept at 4 °C until use for exposures (one week).

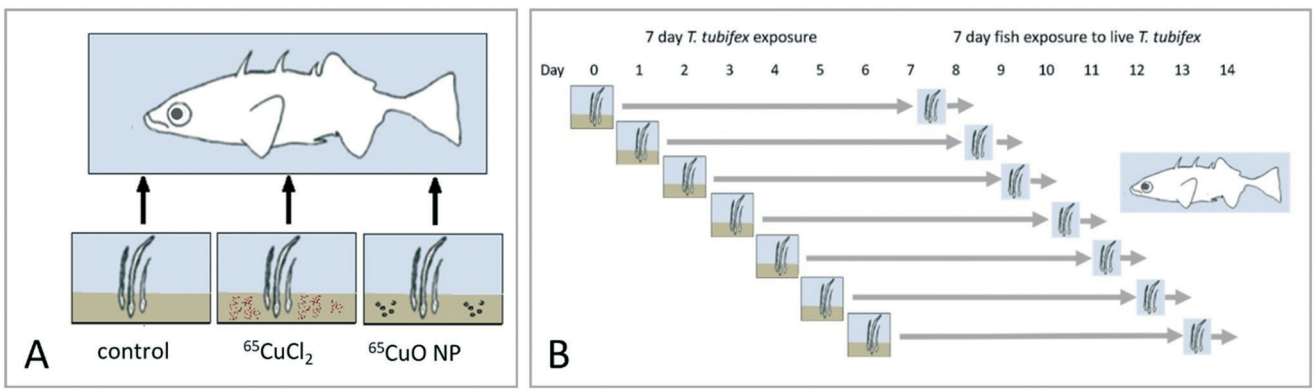

Fig. 1 Overall experimental design. A. Illustration of the overall approach where worms were exposed to sediment and subsequently fed to fish. B. New *T. tubifex* exposures were initiated daily for seven days. Each worm exposure was terminated after seven days and worms were fed to fish. Thus, dietary fish exposures were initiated seven days after the first worm exposure was initiated.

2.3.4 Exposures of *T. tubifex* to ^{65}CuO NP and $^{65}CuCl_2$-spiked sediment

Initiation of T. tubifex exposures. Beakers (100 mL Schott bottles, 56 mm in diameter) were loaded with 3 g of natural non-contaminated (control), $^{65}CuCl_2$- or ^{65}CuO NP-spiked sediment (100 μg ^{65}Cu per g dw sediment; see above). Subsequently, 100 mL of aerated tubifex medium was added to the beakers and the sediment left to settle for 24 h. Before experimental start, the overlying water was carefully removed and replaced with fresh aerated tubifex medium. Then, ~1.2 g ww healthy and intact *T. tubifex* of similar size were selected, sub-divided into three portions of ~0.4 g ww each, and added to the exposure beakers. A random sub-sample of ten worms was taken and their ww determined to ensure that worm sizes were comparable between treatment groups and independent repetitions (Table S1†). *T. tubifex* exposures lasted seven days and were carried out in a room with controlled air temperature (17 °C). The overlying water was gently aerated (~1–2 bubbles per second) throughout the experiment *via* a glass Pasteur pipette positioned 2 cm above the sediment surface. The temperature, dissolved oxygen content and conductivity of the water overlying the sediment was 17.0 ± 0.1 °C, 94.3 ± 1.0% and 553.9 ± 6.2 μS cm^{-1}, respectively.

Sampling of overlying water and sediment. For three of the seven independent repetitions, sub-samples (1 mL) of overlying water of each exposure vessel were taken at experimental day 0, 1, 4, and 7 to determine ^{65}Cu remobilization from sediment into water (determined by inductively coupled plasma-mass spectrometry, ICP-MS; see below). The samples were taken from four different locations (4 × 250 μl) around the beaker's circumference (staggered by 90°) and ~1.5 cm above the sediment surface. In addition, representative samples of sediment (0.2–0.3 g ww) were taken of each exposure vessel at the end of the exposure. All samples were stored at −20 °C until analysis.

Sampling of worms. At the end of experimental day 7, worms were carefully sampled from the exposure vessels using a 1 mm sieve. The worms were collected in a beaker with fresh aerated tubifex medium and all sediment associated with the outer surface of the worms was removed by repeatedly rinsing and transferring the worms into new beakers with clean aerated tubifex medium. Following determination of the total number and weight of worms recovered from each exposure (data shown in Table S2†), the cleaned worms were incubated for additional 20 h to purge the gut from sediment particles and unassimilated Cu, and then fed to fish (see below). A sub-sample of ten purged worms of each group was used to determine the average body ww of individual worms (data shown in Table S3†) needed to calculate the number of worms corresponding to the fish' food ration (representative images of proportionated purged *T. tubifex* fish were fed during exposures are shown in Fig. S2†). Moreover, two purged worms of each treatment group and independent repetition were set aside for quantification of ^{65}Cu body burden using ICP-MS (n = 14).

2.4 Fish exposure

2.4.1 Experimental organism: three-spined stickleback (*Gasterosteus aculeatus*).

Three-spined sticklebacks (*G. aculeatus*) were caught from a small channel (58° 13′ 57.4″ N, 11° 28′ 22.9″E) close to the marine research station Kristineberg, Sven Lovén Centre for Marine Infrastructure, on the island of Skaftö on the Swedish west coast in September 2018. The fish were transported to the animal facility at the Department of Biological and Environmental Sciences, University of Gothenburg, where they were transferred to an acclimatization tank with environmental enrichment, aeration, and ~14 °C-cold, brackish water (~18 ppt). After one week, the fish were slowly acclimatized to freshwater by gradually replacing the brackish water with filtered, UV-treated tab water (~155 μS cm^{-1}, ~10 °C) originating from the in-house semi-recirculating system. The fish were maintained at these conditions under continuous flow-through and were fed *ad libitum* with frozen blood worms (*Röda Mygglarver*, AkvarieTeknik) until used for experimentation in November 2018. All animal procedures were performed in accordance with national and international guidelines for care and use of laboratory animals following the policy for animal experimentation of the University of Gothenburg and were approved by the Animal Ethics Committee of the Swedish Board of Agriculture (Ethical permit 15986-2018).

2.4.2 Dietary exposure of three-spined sticklebacks to live ^{65}CuO NP and $^{65}CuCl_2$-exposed *T. tubifex*.

Two weeks before experimental start, 45 fish with similar weight were selected and transferred to an experimental flow-through aquaria system (fish were of male and female sex; see Table S5† for sex distribution of analyzed fish). The experimental tanks contained 1.5 L of filtered, UV-treated tap water (10 °C) supplied by the in-house semi-recirculating system. The water turnover in the experimental tanks was ~0.1 L min^{-1}. Each tank contained one fish and was assigned to one of the three treatment groups (control, $^{65}CuCl_2$, ^{65}CuO NP). During the first seven days of the study (experimental day 1–7) all fish were fed live, uncontaminated *T. tubifex* collected from the *T. tubifex* culture (see above). On experimental day 8, feeding was suspended so that fish could empty their guts and the tanks be cleaned from feces before starting with the dietary exposure. From experimental days 9 to 15, the fish were fed live *T. tubifex*, which were previously exposed to non-contaminated (control), $^{65}CuCl_2$-spiked or ^{65}CuO NP-spiked sediment for seven days (see above). Each fish was fed six to seven worms each day corresponding to an average daily food ration of approximately 26 ± 0.4 mg and 4% of the fish body ww (0.627 ± 0.114 g, n = 45; the average body ww between treatment groups was similar). More detailed information on the number, weight and ^{65}Cu body burden of worms fed to each fish on each day is shown in Table S5.† In addition, feces egested by each fish were collected at the end of each experimental day (*i.e.*, prior to feeding) and stored at −20 °C until ICP-MS analysis. Furthermore, water samples (50 mL) were taken from three experimental tanks of each treatment

group on a daily basis. Water samples originating from tanks belonging to the same treatment group were pooled and analyzed by ICP-MS to determine Cu background levels and control for potential ^{65}Cu release from feces. The temperature, dissolved oxygen content and conductivity of the water in the flow-through system were monitored daily and were 10.0 ± 0.2 °C, 79.3 ± 3.3% and 159.3 ± 4.3 μS cm^{-1}, respectively. Furthermore, acidity (pH), general hardness (GH), carbonate hardness (KH), nitrite (NO_2^-), nitrate (NO_3^-) and chlorine (Cl) levels of the water in the system were controlled at the beginning of the experiment and the end of the exposure. All parameters were within the quality criteria (pH: 6.8 to 7.2, GH: >4°, KH: 3 to 6°, NO_2^-: 0 mg L^{-1}, NO_3^-: 0 to 10 mg L^{-1}, Cl: 0 to 0.8 mg L^{-1}). There were no significant differences among treatment groups.

2.4.3 Sampling and tissue dissection. After the 7 day exposure period (end of experimental day 15), all fish were stunned by a sharp blow to the head and killed by transection of the spinal cord. Following determination of the body ww and standard length, liver and intestine were excised. The intestine was gently flushed with a physiological buffer solution to remove any remaining content (chyme). Liver and intestinal tissue samples of each fish were split into a small tissue sample for gene expression analysis (abscised from the proximal end of the intestine and the distinct, pointy end of the liver) and a large tissue sample for ICP-MS analysis. Tissue samples were placed in cryotubes, snap-frozen and stored in liquid nitrogen. The remainders of the fish (in the following referred to as carcass) were frozen on dry ice and stored at −80 °C. Tissue samples of 8 of the 15 fish in each treatment group were analyzed (see below).

2.5 Gene expression analysis

Liver and intestine samples were homogenized in RLT plus buffer using a TissueLyser II apparatus and stainless-steel beads (5 mm) according to the manufacturer's instructions (all stated materials and instruments were from Qiagen). Total RNA was isolated using RNeasy Mini Kit (Qiagen). RNA concentration and purity were measured using a NanoDrop 2000c spectrophotometer (ThermoFisher). cDNA was synthesized using the iScript™ cDNA synthesis kit from BioRad. Quantification of relative mRNA expression levels (cDNA copy numbers) was carried out using SsoAdvanced™ Universal SYBR Green Supermix (BioRad) and appropriate primer pairs (Eurofins Genomics, Ebersberg, DE).[42] The sequence and concentrations of the oligonucleotide primers, and the efficiencies of the respective qPCR (quantitative polymerase chain reaction) assays are presented in Table 1. All qPCR reactions were run in duplicate on a Bio-Rad CFX Connect™ Real-Time System (one cycle of 3 min-cycle at 95 °C followed by 40 cycles of 10 s at 95 °C (denaturation) and 30 s at 60 °C or 62 °C (annealing and extension)). ΔCq values were calculated subtracting the mean Cq value of each target gene by the average of the mean Cq values of the two reference genes.

2.6 Concentrations of background Cu and newly accumulated ^{65}Cu

2.6.1 Sample analysis with ICP-MS. Cu concentrations in fish and worm tissues, sediment, water, and fish feces were measured by ICP-MS (7900, Agilent). Samples were dried in an oven at 40 °C for at least 24 h. Larger samples (fish carcass and sediment samples) were weighed into Weflon™ vials

Table 1 List of analyzed reference and target genes and information on primers used for qPCR including sequence, concentration, annealing temperature and efficiencies of optimized assays

Gene name and abbreviation	Sequence (5′ ≥ 3′) of forward (fw) and reverse (rv) primers		Conc. [nM]	Temp. [°C]	Eff. [%]	Ref.
β-Actin (*β-act*)	fw	CTGTCTTTCCCTCCATCGTC	500	60	103.4	55
	rv	CTCTTGCTCTGGGCTTCATC	500			
Ubiquitin (*ubq*)	fw	AGACGGGCATAGCACTTGC	500	60	93.4	56
	rv	CAGGACAAGGAAGGCATCC	500			
Tight junction protein 1a (*zo-1*)	fw	CTCTCTTAGGAGGCCCACCA	250	62	98.6	42
	rv	TCTCCCCGTGTTTTCTACGC	250			
High affinity copper transporter (*ctr1*)	fw	TCAACGTCCGCTACAACTCC	500	60	103.0	42
	rv	ACCTGGACGATGTGCAACAG	500			
Metallothionein-A (*mta*)	fw	CCCCTGCTGCCCGACTG	500	60	102.4	57
	rv	TGTTCAAACTGCCGCCATCTC	500			
Glutamate-cysteine-ligase, catalytic subunit (*gcl*)	fw	CGTGTTGAAATGGGGCGATG	250	60	104.8	42
	rv	TCCAAAGGGTGGGGTGATTG	250			
Glutathione reductase (*gr*)	fw	GCTGCAAAACTCTGGTGTGG	500	60	98.0	42
	rv	CATTTCCAAACCCATGGCGG	500			
Glutathione peroxidase (*gpx*)	fw	ATCAGGAGAACTGCAAGAATGAAG	100	60	110.3	58[a]
	rv	GTTCACCTTCTCAAGGAGCTG	100			
Superoxide dismutase-1 (*sod-1*)	fw	AGCAGGAGAGCGATAAAGCG	250	60	102.5	42
	rv	TCATCATTAGGGCCTGCGTG	250			
Catalase (*cat*)	fw	ACCAAGGTTTGGTCCCACAAAG	500	60	102.4	58[a]
	rv	TGCTCCACCTCTGCAAAGTAG	500			

[a] Oligonucleotide sequence was shortened.

(Milestone, Germany) and dissolved with 65% ultrapure nitric acid (HNO_3) and Milli-Q water (1 : 1) in a volume of 6.25 mL. All smaller sized samples were weighted into Teflon™ inserts, which made it possible to dissolve samples in smaller volumes of HNO_3 and Milli-Q water (1 : 1) (0.625 mL). Three Teflon™ inserts were placed in each Weflon™ vial containing 10 mL Milli-Q water and 2 mL 30% H_2O_2. All samples were subsequently digested according to ISO15587-2. Briefly, samples were heated in a microwave oven (Start D microwave digestion system, Milestone, Germany). All samples were then transferred into volumetric flasks, resulting in a known volume resulting in 8% HNO_3. Finally, the Cu concentration in each sample was determined directly after digestion or after a short storage period (<48 h). A series of standard Cu solutions (8% HNO_3) was used to calibrate Cu concentrations (six standards were selected from 0, 0.1, 2, 5, 10, 50, 100, and 1000 μg Cu per L to cover the range of expected Cu concentrations in the sample batch). During each analytical run, at least one of these standards were re-analyzed (approximately for every ten samples analyzed) to check for analytical drift and were all in agreement with expected Cu concentrations. Samples were analyzed in duplicate (each analysis averaged 32 measurements) for the naturally occurring stable isotopes ^{63}Cu and ^{65}Cu by ICP-MS (Agilent) as described in Lammel and Thit *et al.* (2019).[42] Samples were set to zero if their Cu concentrations inferred from ^{63}Cu were higher than those of ^{65}Cu.

Cu recovery was examined using certified mussel tissue (European reference materials, ERM® – CE278k; 5.98 μg Cu per g dw tissue), and certified freshwater sediment (RIZA, Trace metals WD CRM-CNS301-050; 44.2 μg Cu per g dw sediment), which were digested and analyzed together with different sample batches (at least one reference sample per batch). Results of certified tissue and sediment were in good agreement with the certificate of analysis (5.54 ± 0.27 μg Cu per g dw tissue, n = 4 and 41.45 ± 2.30 μg Cu per g dw sediment, n = 4, respectively). All equipment used for sample digestions was thoroughly acid-washed before use.

2.6.2 Calculation of concentrations of newly accumulated ^{65}Cu. Concentrations of newly accumulated or added ^{65}Cu (referred to as ^{65}Cu in the following) were calculated based on ICP-MS measurements of the two naturally abundant stable isotopes, ^{65}Cu, and ^{63}Cu, as described in Croteau *et al.* (2004).[59] Briefly, the relative abundance of the two isotopes in natural Cu samples in the absence of a spike (p^{65}) was set to 0.309.[60] Concentrations of newly accumulated ^{65}Cu in the experimental organisms ($[^{65}Cu]_{org}$) were calculated as the product of p^{65} and the total Cu concentrations inferred by the ICP-MS software from ^{65}Cu intensity ($[T^{65}Cu]$).

$$[^{65}Cu]_{org} = p^{65} \times [T^{65}Cu] \quad (1)$$

The original load of tracer ($[^{65}Cu]_{org}{}^{0}$) that occurred in each sample in the absence of a ^{65}Cu spike was calculated as the product of p^{65} and the total Cu concentrations inferred from the intensity of the most abundant Cu isotope, (*i.e.*, $[T^{63}Cu]$):

$$[^{65}Cu]_{org}{}^{0} = p^{65} \times [T^{63}Cu] \quad (2)$$

The net tracer uptake ($[^{65}Cu]_{org}$) was derived from the total Cu concentration inferred from ^{65}Cu signal ($[^{65}Cu]_{org}$ minus the pre-existing concentration of tracer ($[_{65}Cu]_{org}{}^{0}$)):

$$\Delta[^{65}Cu]_{org} = [^{65}Cu]_{org} - [^{65}Cu]_{org}{}^{0} = p^{65} \times ([T^{65}Cu] - [T^{63}Cu]) \quad (3)$$

2.7 Statistical analysis

In general, data are presented as mean ± sd (standard deviation) unless otherwise stated. One-way Analysis of Variance (one-way ANOVA) was conducted when data met the test's requirements for normality and equal variances. When requirements for parametric analysis were not met, non-parametric Kruskal–Wallis Analysis on ranks was conducted. The strength of the association between pairs of variables (for instance, ^{65}Cu concentration and target gene expression level) was measured by Pearson product–moment correlation analysis. Student's *t*-test were conducted to test the null hypothesis that the mean ^{65}Cu concentration in the overlying water of $^{65}CuCl_2$ and ^{65}CuO NP sediment exposures are equal. Data for ^{65}Cu concentrations in control treatment were zero/close to zero and hence failed the normality test. Thus, comparison by one-way ANOVA could not be performed. Kruskal–Wallis Analysis on ranks had too little statistical power to detect significant differences between treatments because of the low sample size and a Student's *t*-test was conducted. Statistical analysis was conducted using SigmaPlot for Windows Version 14.0 (Systat Software, Inc., San Jose, CA, USA).

3. Results and discussion

3.1 Bioaccumulation study of sediment-associated ^{65}Cu and ^{65}CuO NP in worms

^{65}Cu concentrations in sediment and water. The nominal sediment exposure concentrations (~100 μg ^{65}Cu per g dw sed) were selected on the basis of a worst-case scenario, where CuO NP concentrations in the sediment were assumed to be as high as the concentrations of non-particulate Cu in some sediments in Europe today (often 5–50, but reaching up to 4000 μg Cu per g dw sed at contaminated sites).[48,61] There was no significant difference between the measured sediment ^{65}Cu concentrations of the two ^{65}Cu treatments, though they were slightly higher in the sediment spiked with $^{65}CuCl_2$ compared to ^{65}CuO NP (by approximately 7%; Table 2). The bioturbation activity of *T. tubifex* resulted in resuspension of sediment particles (Fig. S1†), but only little ^{65}Cu was remobilized into the overlying water. Hence, it can be assumed that exposure to waterborne ^{65}Cu was negligible during the experiment (Table 2). Interestingly, the mean ^{65}Cu concentration measured in the overlying water at day 7 was significantly higher in the ^{65}CuO NP exposure than in the $^{65}CuCl_2$ exposure (p = 0.018, Student's *t*-test). Several processes leading to remobilization of Cu (as nanoparticulate

Table 2 Exposure concentrations of ^{65}Cu (*i.e.*, all forms) in sediment and overlying water in *T. tubifex* exposures, presented as mean ± sd. Significant differences between the means of the $^{65}CuCl_2$- and ^{65}CuO NP-exposure are indicated by an asterisk (*) (*t*-test, $p < 0.05$)

Sample	Sampling time	*n*	Control	$^{65}CuCl_2$	^{65}CuO NP	Unit
Newly accumulated ^{65}Cu						
Sediment	7 d	7	<0.01 ± 0.01	94.68 ± 27.26	88.24 ± 14.68	μg g^{-1} dw
Water	7 d	3	0.00 ± 0.00	5.44 ± 1.28	*10.56 ± 2.48	μg L^{-1}
Background Cu						
Sediment	7 d	7	29.35 ± 3.66			μg g^{-1} dw
Water	7 d	7	2.18 ± 2.50			μg L^{-1}

or dissolved form) may have been affected differently by the two ^{65}Cu treatments. 1) The conveyer-belt feeding behavior of *T. tubifex*, where sediment is ingested at depth and egested as fecal matter above the sediment surface,[62] can increase the concentration of metals in top sediment layers facilitating their remobilization into the overlying water.[63,64] Thus, differences in remobilization between treatments may be owed to differences in feeding activity (resulting in a lower amount of fecal pellets deposited on the surface in $^{65}CuCl_2$-treatment) or differences in gastrointestinal ^{65}Cu absorption[18,42] (resulting in lower ^{65}Cu concentration in fecal pellets egested in $^{65}CuCl_2$-treatment). 2) Cu stress can induce caudal autotomy (tail loss),[49] which precludes worms from defecating until the posterior end has been regenerated and is operational again (often after more than five days).[65] We did not specifically investigate this endpoint, but if exposure to dissolved Cu had caused caudal autotomy, egestion would have been decreased, resulting in decrease Cu remobilization. 3) Cu exposure has been reported to reduce locomotion and bioturbation activity of tubificid worms at sublethal concentrations (down to 10 μg Cu per g dw sediment),[66,67] and may affect remobilization of Cu. Bioturbation by *T. tubifex* likely increases the transport of metals from sediment to overlying water; thus, a reduced bioturbation activity will depress the concentration in the overlying water.

Body burden of ^{65}Cu in *T. tubifex*. The mean weight-specific body burden (WSBB) of ^{65}Cu in *T. tubifex* was higher after exposure to ^{65}CuO NP than after exposure to $^{65}CuCl_2$ (1.1 ± 1.0 *vs.* 0.6 ± 0.4 μg g^{-1} dw tissue, respectively; $n = 14$), though not statistically significant (Fig. 2). The amount of newly accumulated Cu (measured as ^{65}Cu) was relatively low compared to Cu background concentrations (22.3 ± 9.6 μg Cu per g dw tissue) and would likely have been indistinguishable from background without the use of the tracer, as previously reported by Misra *et al.* (2012)[68] and Lammel and Thit *et al.* (2019).[42] Our findings suggest that ^{65}Cu from both the dissolved and nanoparticulate Cu forms in the sediment was bioavailable and accumulated to a similar extent in the worms. This observation is consistent with several other studies reporting that the difference in WSBB of worms (*N. diversicolor*, *L. variegatus*, *T. tubifex*) exposed to different Cu forms is marginal.[9,19,42] It should be noted that worms in the NP treatment were not only exposed to ^{65}CuO NPs but also dissolved ^{65}Cu (*i.e.*, non-particulate ^{65}Cu). The proportion of dissolved (*i.e.*, non-particulate) ^{65}Cu in the ^{65}CuO NP dispersion used to prepare the dispersion spiked into sediment was ~34%.[34] The relative amount of dissolved (*i.e.*, non-particulate) ^{65}Cu in the exposure sediment may even have been higher, since the presence of chelating organic ligands (*e.g.*, humic acids) can increase CuO NP dissolution.[42,69,70] Hence, it is likely that accumulation of dissolved ^{65}Cu by worms in ^{65}CuO NP-spiked sediment occurred, partly concealing potential differences between treatments.

3.2 Transfer of ^{65}Cu and ^{65}CuO NPs from worms to fish

Tissue concentrations of ^{65}Cu in fish. Due to limited ^{65}Cu concentrations in worms, the dietary exposures doses of ^{65}Cu that fish received were very low (~4 ± 1 and 7 ± 1 ng g^{-1} body ww per day in the $^{65}CuCl_2$- and ^{65}CuO NP-treatment, respectively; for more details see Tables S5 and S6†). Furthermore, in both treatments, the dose of ^{65}Cu represented only a small portion (~5%) of the total dietary Cu dose fish received (the Cu background concentration was ~22 μg g^{-1} dw, which is in the range of nutritional dietary Cu requirements of fish[71]). At such low exposure doses, low dietary uptake is expected,[71,72] and overall ^{65}Cu

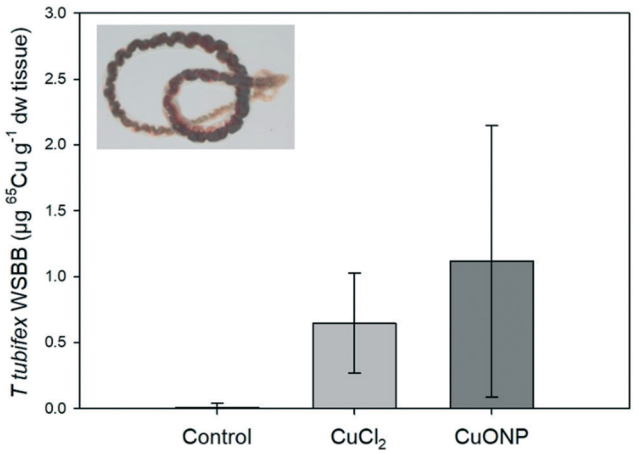

Fig. 2 ^{65}Cu WSBB in *T. tubifex*. Worms were exposed for 7 days in clean sediment or sediment with $^{65}CuCl_2$ or ^{65}CuO NPs at about 100 μg ^{65}Cu per g dw sediment. Mean ± sd ($n = 14$).

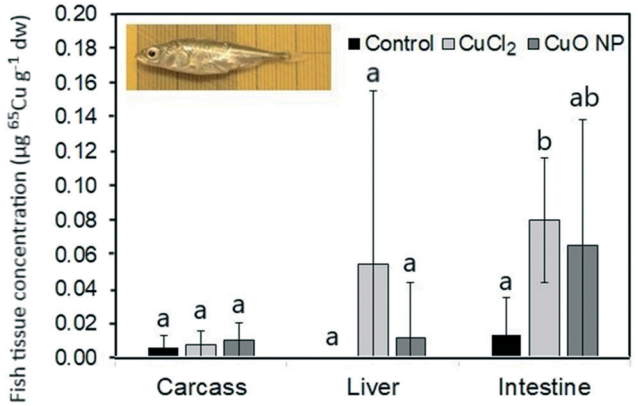

Fig. 3 ^{65}Cu accumulation in three-spined stickleback after seven days of dietary exposure to *T. tubifex* exposed in non-contaminated sediment (control) or sediment spiked with $^{65}CuCl_2$ or ^{65}CuO NPs for seven days. Bars represent the mean tissue concentrations of ^{65}Cu ± sd (n = 8). Different letter combinations indicate statistically significant differences ($p < 0.05$).

concentrations in fish tissues were low (Table S6†). Generally, highest concentrations were found in intestine, followed by liver and carcass (Fig. 3). No statistically significant differences in mean ^{65}Cu tissue concentrations were observed between treatments, though they were generally higher in fish exposed to $^{65}CuCl_2$ than in fish exposed to ^{65}CuO NPs (Table S6† and Fig. 3). However, it must be noted that *de facto* hepatic ^{65}Cu accumulation was detected in only two of the fish feeding on $^{65}CuCl_2$-exposed worms, and in only one of the fish feeding on ^{65}CuO NP-exposed worms. Similar to our previous findings,[42] Cu background concentrations were highest in liver tissue and Cu accumulation highest in the intestine of fish fed with $CuCl_2$-exposed worms or $CuCl_2$-spiked food packages.

Egestion of ^{65}Cu in feces. The mean ^{65}Cu concentration in the feces of fish fed $^{65}CuCl_2$-exposed worms was about half of that measured in feces of fish fed ^{65}CuO NP-exposed worms $^{65}CuCl_2$ and ^{65}CuO NP exposure, respectively (see Table S6† and Fig. 4). This result is largely consistent with the differences in the received dietary exposure dose (*i.e.*, ^{65}Cu WSBB of $^{65}CuCl_2$- and ^{65}CuO NP-exposed worms) and the differences in gastrointestinal ^{65}Cu assimilation between the two treatments (see Table S6† and Fig. 3). Altogether, ^{65}Cu concentrations in fish tissues and feces suggest that ^{65}Cu assimilation from ^{65}CuO NP-exposed worms might be lower compared to that from $^{65}CuCl_2$-exposed worms, albeit not significantly.

3.3 Effects of dietary ^{65}Cu and ^{65}CuO NP exposure in fish

In accordance with the low ^{65}Cu tissue concentrations, limited alterations in the expression of investigated target genes were observed (Fig. 5). Only in intestinal tissue of fish feeding on $^{65}CuCl_2$-exposed worms, a statistically significant upregulation of one of the assessed target genes, namely *gpx*, was evident (compared to control fish and fish feeding on ^{65}CuO NP exposed worms) (Fig. 5A). Furthermore, *gpx* expression levels were found to be positively correlated with intestinal ^{65}Cu concentrations (r = 0.9, p = 0.002), showing that the effect was related to Cu exposure (Table S7†). These results suggest that even minimally elevated dietary Cu levels can induce a disturbance in the cellular redox balance in intestinal tissue. This is in general agreement with previous research reporting Cu-induced oxidative stress and the induction of antioxidant defense mechanisms in intestinal cells and tissues.[73,74] Similarly, *gpx* appeared to be upregulated (though not significantly) in our previous study where three-spined sticklebacks were exposed to a practical diet prepared from $CuCl_2$-spiked worm homogenate (though at higher exposure dose).[42]

It must be noted that in intestine of fish fed with ^{65}CuO NP-exposed worms, *gpx* mRNA levels were not upregulated (*i.e.*, not different from the control), although mean ^{65}Cu tissue concentrations were similar to those in fish fed with $^{65}CuCl_2$-exposed worms. This may point to differences in the subcellular distribution[19] and or form of ^{65}Cu inside

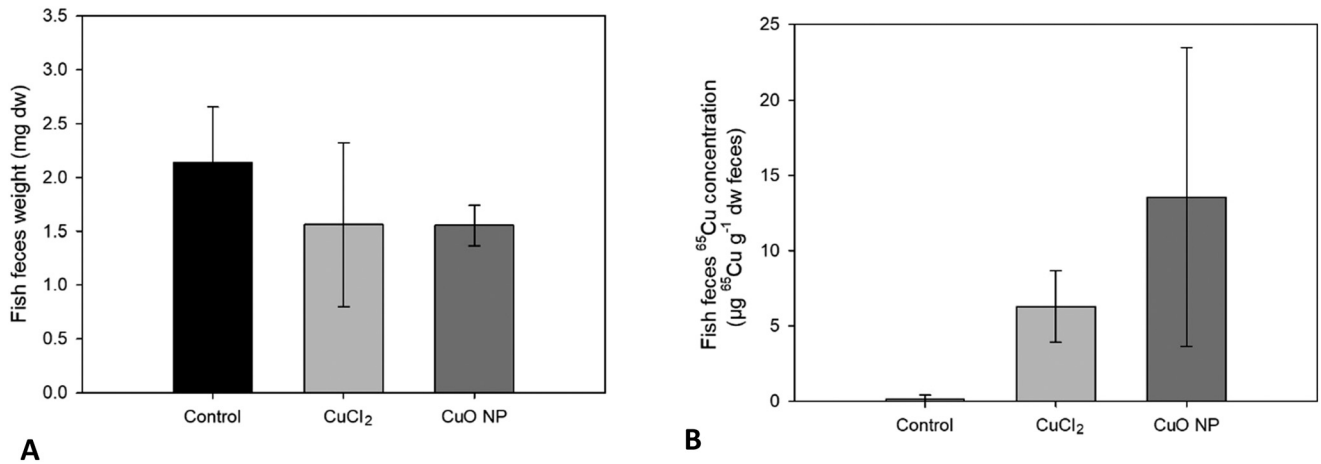

Fig. 4 Fish feces collected during seven days of dietary exposure to sediment exposed *T. tubifex*. Weight (A) and ^{65}Cu concentration (B) in fish feces produced by individual fish during seven days of exposure and collected daily. Data are presented as mean ± sd, n = 8.

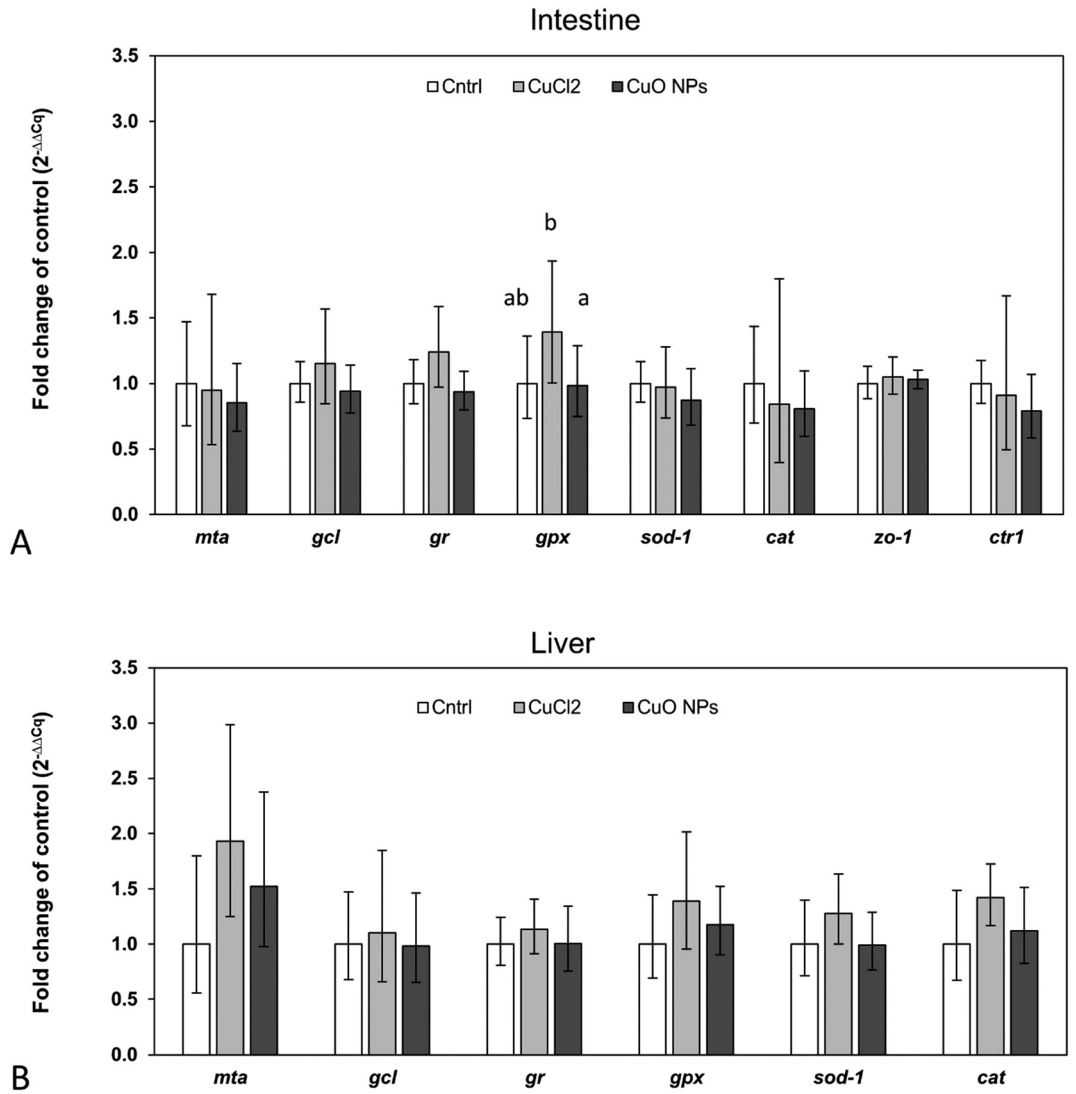

Fig. 5 Relative expression levels of selected target genes in intestinal (A) and hepatic tissue (B) of three-spined stickleback fed *T. tubifex* exposed to control, $^{65}CuCl_2$- or ^{65}CuO NP-spiked sediment (white, grey and dark grey bars, respectively). Bars and error bars represent the mean and sd, respectively. Letters above bars indicate statistically significant differences between the means. Bars that do not share the same letter are different at a significance level of 0.05 (One Way ANOVA followed by all pairwise multiple comparison using Holm–Sidak method).

intestinal cells between the exposure groups: However, this hypothesis needs further investigation.

Trophic transfer studies using live worms as feed for fish

Often, dietary bioaccumulation and toxicity of Cu in fish are studied using practical diets, which allows incorporation of Cu in excess (dietary exposure doses in previous studies range from ~0.4 μg to ~185 μg Cu per g fish body ww per day[71]). This may lead to unrealistic exposure scenarios in regards to both food type, complexation with biological tissue, and exposure dose. In contrast, the use of live feed offers the possibility of environmentally realistic exposure in laboratory tests. However, there are two major challenges associated with the use of live feed in trophic transfer studies. The first challenge is to achieve similar WSBB of test compounds and reference compounds in prey organisms (and reproducible WSBBs between individuals and replicates). Here, we found high variation in ^{65}Cu WSBB of sediment-exposed *T. tubifex* within treatments. However, the mean ^{65}Cu fish exposure doses were not significantly different between the two Cu treatments.

The second challenge is to obtain a high enough WSBB of the test compound in the prey organism for it to be detectable in the predator organism. Here, we found limited ^{65}Cu accumulation from sediment by *T. tubifex* worms, which translated into a very low dietary exposure dose for fish. Thus, demonstrating trophic transfer was only possible with the use of a stable isotope tracer. If such tracers are unavailable in future studies, higher dietary exposure doses may potentially (depending on when steady-state body burdens are reached) be achieved by extending the time of

worm exposure and subsequent fish exposure, rather than increasing exposure concentrations in sediment to unrealistic levels. Yet, it needs to be taken into consideration that this strategy will increase the experimental complexity giving rise to logistical challenges such as the requirement for more spiked sediment and more time-consuming, labor-intensive exposures of worms and fish that need to be run in parallel.

Conclusion

Isotopically labeled NPs offer unique possibilities for studying NP bioaccumulation and trophic transfer under environmentally realistic conditions. The findings from this study show that exposure to Cu at environmentally relevant concentrations in sediment, either in dissolved or nanoparticulate form, leads to increased concentrations in *T. tubifex* and can be transferred to fish (three-spined stickleback). Increases in body burdens *via* dietary exposure and trophic transfer of dissolved Cu and CuO NPs appears to be largely similar, which may be in part explained by partial dissolution of the NPs. In quantitative terms, trophic transfer of dissolved/nanoparticulate CuO across two trophic levels seems to be negligible. Based on our data, an increased risk for biomagnification of CuO NPs within the aquatic food web compared to dissolved Cu seems unlikely, which is well in accordance with the published literature. However, considering the complexity and diversity of abiotic and biotic interactions in the environment, more research is required before general conclusions can be drawn with regard to their bioaccumulation and trophic transfer potential, as well as overall environmental risk.

Conflicts of interest

There are no conflicts to declare.

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