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Effects, Uptake, and Depuration Kinetics of Silver Oxide and Copper Oxide Nanoparticles in a Marine Deposit Feeder, *Macoma balthica*

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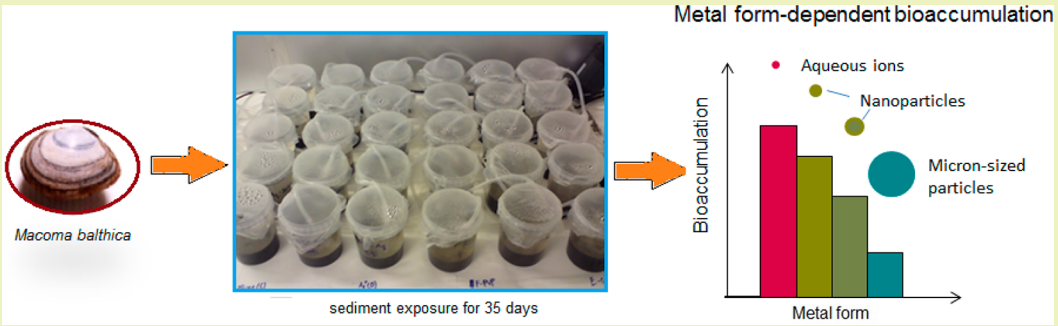
Supporting Information



ABSTRACT: Ag and CuO engineered nanoparticles (ENPs) have wide applications in industry and commercial products and may be released from wastewater into the aquatic environment. Limited information is currently available on metal ENP effects, uptake, and depuration kinetics in aquatic organisms. In the present study, a deposit-feeding clam, *Macoma balthica*, was exposed to sediment spiked with Ag and Cu in different forms (aqueous ions, nanoparticles, and micrometer-sized particles) in three experiments. In all experiments, no effects on mortality, condition index, or burrowing behavior were observed for any of the metal forms at measured sediment concentrations (150−200 μg/g) during 35 d of exposure. No genotoxicity was observed following exposure, measured as DNA damage with the single-cell gel electrophoresis assay (comet assay). Bioaccumulation of both Ag and Cu in the clams was form dependent such that bioaccumulation from sediment spiked with aqueous ions > nanoparticles > micrometer-sized particles. Cu uptake and depuration kinetics were studied in more detail yielding net uptake rates (μg Cu/g dw soft tissue/d) in soft tissue of 0.640, 0.464, and 0.091 for sediment spiked with aqueous Cu ions, CuO nanoparticle,s and micrometer-sized CuO particles, respectively, supporting that net uptake was dependent on form. Depuration rate constants (d^{-1}) from soft tissue were −0.074, −0.030, and 0.019 for Cu added to sediment as aqueous Cu ions, CuO nanoparticles, and micrometer-sized CuO particles, respectively. Ensuring sustainable use of nanotechnology requires the development of better methods for detecting and quantifying ENPs, particularly in sediment.

KEYWORDS: *Mollusk, Ecotoxicology, Uptake and depuration kinetics, Nanoparticles, Metals, Comet assay*

INTRODUCTION

Engineered nanoparticles (ENPs) containing metals or metal oxides (Me-ENPs) have been produced on a large scale and have been applied widely in commercial products such as clothing, toiletries, and food storage containers for several years.[1,2]

ENPs refer to particles designed to have at least one dimension between 1 and 100 nm. Associated with the reduction of particle size down to the nanoscale, Me-ENPs have specific properties due to increased surface to volume ratio and particle−quantum effects.[3] It has been demonstrated that Me-ENPs and corresponding metal ions released from Me-ENPs enter aquatic environments via sewage treatment plants.[4] Sediments are likely the ultimate sink for most metals including Me-ENPs, and deposit feeders in particular may thus be at high risk of exposure to Me-ENPs.

The extent to which Me-ENPs are more or less bioavailable and/or toxic than other forms of metals to aquatic organisms remains unclear. For instance, Pang et al.[5] found that CuO ENPs mixed into sediment caused greater adverse effects than Cu added to sediment in other forms (i.e., ionic Cu or micrometer-sized CuO particles) on growth, feeding rate, and reproduction in the freshwater snail, *Potamopyrgus antipodarum*. Burrowing activity was affected in the deposit feeder, *Scrobicularia plana*, exposed to sediment spiked with both Cu ions and CuO ENPs,[6] whereas no effects were observed on the burrowing activity of *Nereis (Hediste) diversicolor* exposed to sediment spiked with Ag ENPs and CuO ENPs.[6,7] In addition,

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exposure to Ag ENPs added to sediment caused higher DNA damage in *N. diversicolor* than Ag added as ions, and this difference in effect could not be explained by a difference in body burden of Ag between forms.[8] These results suggest that bioavailability and toxicity of Me-ENPs to aquatic organisms are not easily predictable. Furthermore, the bioaccumulation of Me-ENPs has been reported in several species (*N. diversicolor*, *S. plana*, *Perna viridis*, *Lymnaea stagnalis*, and *Peringia ulvae*);[6,8−11] however, uptake and depuration kinetics of Me-ENPs are still not fully understood.

Macoma balthica is one of the most common and numerous macrobenthic species in estuarine and marine habitats such as the Baltic Sea[12] and is an important food source for flatfish and wading birds. *M. balthica* is a filter feeder in sandy sediment and a deposit feeder in muddy sediment.[13] Thus, *M. balthica* is potentially exposed to Me-ENPs in sediments and plays an important role in marine food webs.

The goal of the present study was to investigate the bioaccumulation and effects of sediment-associated Ag and CuO ENPs in *M. balthica* in three interrelated experiments. The two metals were added to sediment as Ag^+ and Cu^{2+} (i.e., aqueous ions), as Ag^0 and CuO ENPs, and as micrometer-sized particles. A nominal concentration of 200 μg metal/g dry weight sediment (dw sed) was chosen with reference to environmental concentrations of both Ag (more than 100 μg/g dw sed) and Cu (more than 200 μg/g dw sed) in highly polluted sediment.[14,15] The effects of metal form were evaluated both at the organism level (mortality, condition index, and behavior) and at the suborganismal level (DNA damage by the comet assay). Furthermore, the biokinetics parameters (k_u and k_d) were quantified to examine bioaccumulation patterns of the tested Cu forms.

MATERIALS AND METHODS

Three experiments were performed in the present study. Experiment 1 tested effects and bioavailability of Ag added to sediment in different forms after 35 days of exposure. Aqueous Ag ($AgNO_3$, Sigma-Aldrich, U.S.A.), Ag ENPs-20 and Ag ENPs-80 (20 and 80 nm particles in powder, with w/~0.3% PVP coating, Nanostructured & Amorphous Materials, Inc., U.S.A.), and micrometer Ag (2−3.5 μm Ag particles in powder, Sigma-Aldrich, U.S.A.) were used. Experiment 2 tested effects and uptake of Cu added to sediment in different forms (aqueous Cu, CuO ENPs, and micrometer-CuO) during 35 days of exposure. Aqueous Cu ($CuCl_2{\cdot}2H_2O$, Sigma-Aldrich, Denmark), CuO ENPs (<100 nm, National History Museum, U.K.), and micrometer-CuO (<5 μm, cat. 20, 884-1, assay 98%, Sigma-Aldrich, Denmark) were used. Experiment 3 assessed the depuration of accumulated Cu in the same forms as in experiment 2, i.e., 35 days of uptake followed by 15 days of depuration. Sediment and *M. balthica* were collected from Isefjorden, Denmark (55°40′ N, 11°47′ E) in 2010 and 2011. The sediment was sieved to 500 μm and spiked with a nominal concentration of 200 μg metal/g dw sed (both Ag and Cu). After acclimation for 14 days in the laboratory, the clams were exposed to spiked or control sediment.

Burrowing behavior and condition index were evaluated in all experiments. The body burden of accumulated metal in clams was quantified by AAS. Net uptake rates (k_u) of Cu (experiment 2) were calculated following Spacie and Hamelink[16] by eq 1.

$$k_u = (\Delta C/\Delta t + k_d \times C)/C_{sed} \quad (1)$$

where C (μg Cu/g dw) was weight-specific Cu concentration in soft tissue or shell, and C_{sed} was Cu concentration in spiked sediment (μg Cu/g dw sed) at the beginning of experiment 2. ΔC was the difference in C for each time interval (Δt, d). Depuration rate constants (k_d) of Cu (experiment 3) were calculated by a simple one-compartment depuration model for both tissue and shell by eq 2.

$$\text{Ln } C = \text{Ln } C_0 - k_d \times t \quad (2)$$

where C_0 was Cu concentration in soft tissue or shell after 35 days of accumulation, and C was Cu concentration in soft tissue or shell during 15 days of depuration. Half-life ($t^{1/2}$, time to 50% reduction in body burden) was calculated by eq 3.

$$t^{1/2} = \ln 2/k_d \quad (3)$$

In addition, DNA damage was evaluated by the comet assay after 35 d of uptake in experiment 3. Metal concentrations in all samples (i.e., sediment, soft tissue, and shell of clams) were measured on either graphite-AAS or flame-AAS. See Supporting Information for further details on materials and methods used.

Statistics. Parametric or nonparametric analysis of variance (ANOVA or Kruskall−Wallis), depending on data homogeneity as tested by Levine's test, was used to test effects of metal form and/or nominal concentration on metal concentrations in sediment, mortality, burrowing, condition index (CI), clam size, weight-specific body burden (WSBB), and average k_u values. Tukey's test was used to compare WSBB among metal treatments. Linear regression was used to quantify burrowing activity (as the slope of Ln (% unburied clams + 1) during the first 30 min with a fixed intercept of 4.62 corresponding to Ln (100% unburied clams + 1) at time 0) in spiked sediment and k_d of accumulated Cu over time in experiment 3. The slope comparison of simple linear regression was made on k_d values from different Cu treatments.[17] All data in the present study were expressed as the mean ±1 standard deviation unless otherwise noted. Differences were considered statistically significant if $p \leq 0.05$ and marginally significant if $0.05 < p < 0.10$.

RESULTS

Metal Concentrations in Spiked Sediment. In experiment 1, no significant differences were found in Ag concentrations among treatments (one-way ANOVA, p = 0.477). The measured concentrations were 178.0 ± 13.7 μg/g dw sed for aqueous Ag, 134.4 ± 22.4 μg/g dw sed for Ag ENPs-20, 174.6 ± 49.1 μg/g dw sed for Ag ENPs-80, and 172.1 ± 48.6 μg/g dw sed for micrometer-Ag, respectively. The Ag concentrations were below the detection limit (0.02 μg Ag/g dw sed) in control and PVP control sediments.

In experiment 2 and during the uptake period of experiment 3, no significant differences were found in Cu concentrations among treatments (one-way ANOVA, p = 0.828). The Cu concentrations were 153.9 ± 9.0 μg/g dw sed for aqueous Cu, 147.7 ± 5.9 μg/g dw sed for CuO ENPs, and 152.0 ± 18.9 μg/g dw sed for micrometer-CuO, respectively. The Cu concentrations in control and clean sediment for depuration were low but measurable at 4.2 ± 1.0 μg/g dw sed.

Mortality. In experiment 1, no mortality was observed after 35 d of exposure to Ag-spiked sediment (data not shown). In experiment 2, mortality for the different treatments was 4.8 ± 8.2% in control, 2.4 ± 6.3% in aqueous Cu, 2.4 ± 6.3% in CuO ENPs, and 14.3 ± 15.0% in micrometer-CuO, respectively. No significant differences in mortality for these Cu forms were detected (Kruskal−Wallis, p = 0.173). In experiment 3, mortality during the exposure period was 4.5% in the control, 7.5% in aqueous Cu, 15.5% in CuO ENPs, and 4.5% in micrometer-CuO. During the depuration period of experiment 3, mortality was 6.7% in control, 10.0% in aqueous Cu, 11.1% in CuO ENPs, and 2.9% in micrometer-CuO. The mortality of clams in CuO ENPs was highest; however, the experimental design did not allow statistical comparisons of mortality among treatments to be made.

Burrowing Activity. Burrowing activity in all treatments after the first 24 h of exposure is shown in Figure S1 of the Supporting Information. In experiment 1, clams buried rapidly

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in the first 30 min and the percentage of unburied clams reached a steady state from 30 to 60 min with 25.8% (±19.1%) of the clams left on the sediment surface after 60 min (pooled data for all treatments). No significant differences were observed among any treatments during the first 30 min of burrowing activity (i.e., no significant differences in slopes; Table S1, Supporting Information), despite that it appeared that clams in all Ag treatments except Ag ENPs-80 burrowed faster than in controls. Thus, no avoidance behavior of clams in Ag-spiked sediment was observed. Furthermore, there were no significant effects on the percentage of unburied clams among Ag forms at 60 min (one-way ANOVA, p = 0.429). In experiment 2 and during the uptake period of experiment 3, there were no significant differences in burrowing activity among treatments in the first 30 min (Table S1, Supporting Information). Clams burrowed more slowly and irregularly in these two experiments, however. In addition, the percentages of unburied clams were 27.4% (±5.9) and 36.2% (±9.5) after 7 h in experiment 2 and the uptake period of experiment 3, respectively. There were no significant differences in the percentage of unburied clams among Cu forms after 7 h (one-way ANOVA, p = 0.624 and p = 0.388, respectively). Clams in experiment 2 burrowed faster than those in experiment 3 during the first 7 h in Cu-spiked sediment (Kruskal–Wallis, p < 0.001). It should be noted that clams in experiment 2 were significantly smaller than those in experiment 3 (Kruskal–Wallis, p < 0.001).

Condition Index. In experiment 1, CI was not affected significantly by exposure to any form of Ag (Figure 2(a); one-way ANOVA, p = 0.591), but CI decreased significantly over time (one-way ANOVA, p = 0.006). In experiment 2, a significant interaction of Cu form and time was detected (two-way ANOVA, p = 0.006) on CI of exposed clams. The CI of clams in aqueous Cu (1.3 ± 0.4) was greater than the CI of clams in CuO ENPs (0.6 ± 0.3) or micrometer-CuO (0.7 ± 0.1) on day 3 (Figure 1). However, the condition index of clams in all treatments was similar on day 35 with 0.6 ± 0.3 in control, 0.6 ± 0.1 in aqueous Cu, 0.5 ± 0.2 in CuO ENPs, and 0.6 ± 0.2 in miron-CuO, respectively (n = 7). Hence, a decrease in CI was due to time (one-way ANOVA, p < 0.001) but not related to Cu exposure (one-way ANOVA, p = 0.674).

Weight-Specific Body Burden of Metals in Soft Tissue after Exposure. All forms of Ag were bioavailable to clams (Figure 2). In experiment 1, significant differences in Ag weight-specific body burden (WSBB) in soft tissue for different Ag forms were detected on day 35 (one-way ANOVA, p = 0.005). The specific body burden of Ag was significantly lower in the soft tissue of clams in micrometer-Ag relative to aqueous Ag and Ag ENPs-20 (Tukey's test, p = 0.004 and p = 0.027). For the control and PVP control, Ag concentrations in soft tissue were below the detection limit of graphite-AAS (0.26 μg Ag/g dw soft tissue).

In contrast, in experiment 2 there were no significant differences among Cu forms in the WSBB of Cu in soft tissue of clams (one-way ANOVA, p = 0.152), even though there appeared to be a trend that WSBB of Cu in soft tissue decreased with increasing metal particle size (Figure 2). Compared to the control group (111.5 ± 50.7 μg/g dw data pooled over time, n = 18), WSBB increased approximately 12 times, 10 times, and 5 times in clams exposed to sediment spiked with aqueous Cu, CuO ENPs, and micrometer-CuO, respectively.

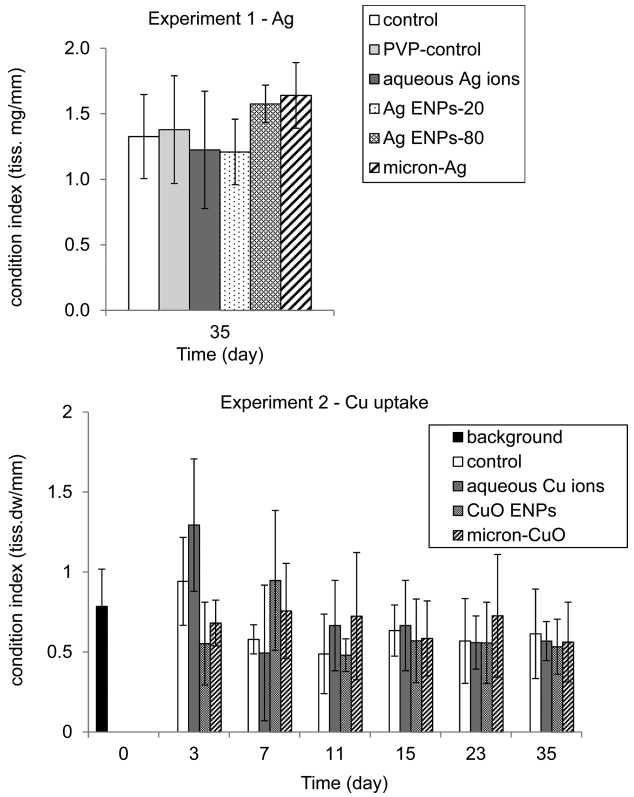

Figure 1. Condition index of exposed clams in experiment 1 on day 35 (n = 20) and in experiment 2 (n = 42). Error bars stand for 1 standard deviation.

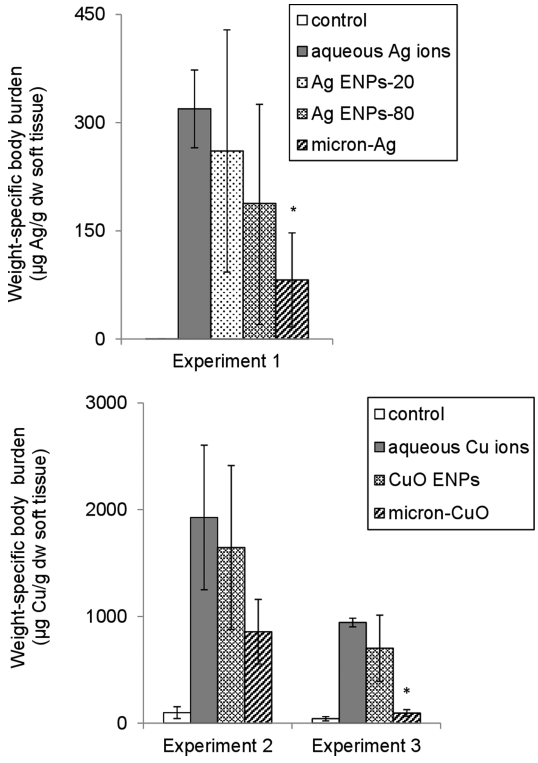

Figure 2. Weight-specific body burden of metals in clams after the 35 d exposure period for (a) experiment 1 and (b) experiments 2 and 3 (n = 3). Error bars = 1 standard deviation. ∗ refers to a significant difference among treatments (explained in the text).

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In the uptake period of experiment 3, there were significant effects of Cu form on WSBB of Cu in soft tissue of clams (one-way ANOVA, $p = 0.011$). WSBB increased more than 22 times in aqueous Cu, more than 17 times in CuO ENPs, and twice in micrometer-CuO compared to the control (41.9 ± 20.2 μg/g dw, $n = 18$).

Net Uptake (Experiment 2). In soft tissue, WSBB increased during the 35 d of exposure (Figure 3), which was

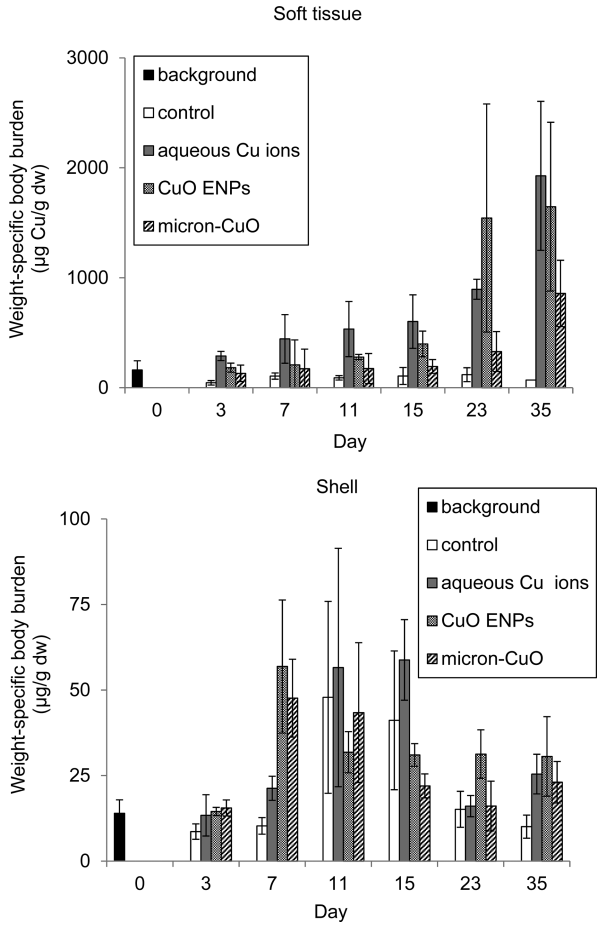

Figure 3. Weight-specific body burden of Cu in soft tissue and in shell of clams over time in experiment 2 ($n = 3$). Error bars stand for 1 standard deviation.

reflected by the positive k_u in all Cu treatments (Table 1). The average k_u of Cu was significantly dependent on Cu form (one-way ANOVA, $p = 0.026$) and was highest in clams exposed to sediment spiked with aqueous Cu followed by CuO ENPs and lowest in micrometer-CuO. Given the increased WSBB (one-way ANOVA, $p = 0.010$) and the fluctuating k_u values during exposure, a trend toward steady-state body burden levels was observed for these exposure scenarios.

The measured Cu in shells reached a peak at different times in the different treatments (Figure 3). From day 7 to day 15, it increased approximately 2-fold for control clams, 4-fold for clams exposed to aqueous Cu, and 3-fold for clams exposed to CuO ENPs and micrometer-CuO compared to the background concentration of Cu in shell on day 0. However, the k_u value of Cu was much lower in shells than in soft tissues.

Depuration (Experiment 3). The depuration, k_d, of Cu in soft tissue was only significant for clams in the aqueous Cu treatment (Table 2, Figure 4), where WSBB decreased by 65.5% after 15 d in clean sediment. The WSBB of Cu did not decrease significantly in CuO ENPs or micrometer-CuO treatments (one-way ANOVA, $p = 0.305$ and $p = 0.437$, respectively), and most Cu was retained in soft tissue after 15 d. A significantly higher k_d was detected for aqueous Cu than for CuO ENPs and micrometer-CuO ($0.025 \leq p < 0.050$).

No measurable reduction of WSBB of Cu in shell was detected (one-way ANOVA, $p > 0.05$), and most Cu remained in shell during depuration (i.e., 15 d). The exception was the micrometer-CuO treatment where WSBB of Cu in shell decreased significantly (one-way ANOVA, $p = 0.002$), with 43% of the initial Cu remaining after 15 d. In addition, a significant difference among Cu forms was detected for k_d values in shell ($0.003 \leq p < 0.005$), which was due to a significant difference between micrometer-CuO and both aqueous Cu and CuO ENPs (both $p < 0.050$, and $p > 0.100$ for aqueous Cu vs CuO ENPs).

Comet Assay. No significant genotoxic effects were observed for any of the Cu exposed specimens (one-way ANOVA, $p = 0.160$) after the Cu exposure in Experiment 3. The average tail moment was approximately 10.0% in all Cu treatments (Figure 5). However, exposing *M. balthica* cells to H_2O_2 (positive control) did lead to significant DNA damage (one-way ANOVA, $p = 0.020$ with the positive control) indicating that the lack of effect was not due to an experimental artifact.

DISCUSSION

Effects of Metals. Both Ag and Cu are two of the most toxic metals to invertebrates that have been documented in marine and estuarine environments, but the toxicity ranking of these two metals is species specific.[14] For *M. balthica*, the concentration of Cu ions (added as $CuSO_4$) resulting in 50% mortality has been reported in the range of 210−1290 mg/L in seawater (25‰, pH 7.9) for an exposure period of 11 d.[18] To our knowledge, no toxicity values for Ag ions have been reported for this species. Toxicity studies with Ag ions and Cu ions in sediment are complicated by many factors, such as organic matter, acid volatile sulfide, and species tolerance,[19] which means that it is difficult to compare toxicity values among studies. In the present study, exposure to sediment-associated aqueous Cu and Ag caused less than 10% mortality of *M. balthica* indicating a relatively low toxicity of both metals to this species.

It has been reported that CI is a sensitive sub-lethal endpoint in evaluating the effects of metal exposures on *M. balthica*, such as Cu and Cd[20,21] because stress caused by metal exposure can consume energy, affect net glycogen accumulation, and reduce the CI of clams.[22] However, the overall, general decrease in CI in the present study may be due to lack of nutrition, which may conceal the effect of metal exposure. On the other hand, different responses of CI to metal exposures have been found in clam populations from different localities,[18,20,23,24] which may be due to both genetic differences among populations and physiological conditions. CI of some specimens (Baalhoek and Westerschelde, The Netherlands) decreased during water exposure to Cu up to a concentration of 36 μg/L, while it increased in Gironde specimens (France) over the same concentration range.[20] In addition, CI of Gdánsk specimens (Poland) did not reflect Cu concentrations in the field, which may have been due to interference by decreasing body weight during reproduction in May−June.[23] In the present study, CI

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Table 1. Net Uptake Rates (k_u, μg Cu/g dw worm/d) in *M. balthica* for Different Time Intervals during Exposure to Cu in Various Forms in Experiment 2 (n = 3)[a]

clams	day	control	aqueous Cu ions	CuO ENPs	micrometer-CuO
soft tissue	3	−0.308	0.415	0.103	−0.084
	7	0.154	0.466	0.102	0.088
	11	−0.008	0.403	0.215	0.002
	15	0.062	0.400	0.341	0.040
	23	0.044	0.669	1.552	0.139
	35	−0.015	1.485	0.470	0.362
average k_u (SD)		−0.025 (0.200)	0.421 (0.031)	0.190 (0.113)	0.011 (0.073)
shell	3	−0.015	0.000	0.001	0.011
	7	0.003	0.016	0.087	0.087
	11	0.077	0.065	−0.052	0.010
	15	−0.014	0.011	−0.002	−0.034
	23	−0.027	−0.033	0.000	0.001
	35	−0.003	0.008	0.000	0.015
average k_u (SD)		0.013 (0.044)	0.023 (0.029)	0.009 (0.058)	0.018 (0.050)

[a] The average k_u was based on k_u values during the first 15 days. SD = standard deviation.

Table 2. Depuration Rate Constant (k_d, d^{-1}) of Accumulated Cu in Various Forms in *M. balthica* during the Depuration Period in Experiment 3 (n = 1 or 2, ±1 standard error) Estimated from the Slope of Linear Regressions (Figure 4)[a]

	soft tissue				shell			
forms	k_d (±SE)	p	r^2	$t^{1/2}$	k_d (±SE)	p	r^2	$t^{1/2}$
control	−0.032 (0.022)	0.171	0.114	–[b]	0.009 (0.014)	0.514	0.027	–
aqueous Cu	−0.074 (0.024)	0.006	0.423	9	−0.020 (0.014)	0.178	0.126	–
CuO ENPs	−0.030 (0.029)	0.305	0.066	–	0.014 (0.015)	0.343	0.060	–
micrometer-CuO	0.019 (0.028)	0.437	0.044	–	−0.053 (0.015)	0.002	0.509	13

[a] SE = standard error. [b] "–" means that $t^{1/2}$ cannot be determined because of insignificant linear regression of k_d.

appears not to have been a sensitive endpoint to Ag and Cu exposures in *M. balthica* collected in Isfjorden, Denmark.

The active avoidance of metal-spiked sediment has been observed in benthic organisms. For instance, McGeer[25] reported that *M. balthica* avoided burrowing in sewage-contaminated sediment containing a range of metals, including Cu (<150 μg/g dw sed), Pb (<74 μg/g dw sed), Zn (<172 μg/g dw sed), Cr (<90 μg/g dw sed), and Ag (<3.2 μg/g dw sed). In addition, Wentsel et al.[26] suggested that adsorbed or exchangeable forms of Cd and Zn (less than 1%) were responsible for avoidance in chironomids. Thus, the avoidance behavior of benthic organisms is related not only to metal concentration but also to metal speciation. In the present study, no avoidance behavior of clams was observed in sediment spiked with Ag or Cu in different forms. One explanation may be that both Me-ENPs and any released metals from them have bound to ligands such as sulfur to a high extent in the sediment used in the present study.

We also observed size-dependent burrowing activity in our study. Tallqvist[27] reported a positive correlation of shell length with burrowing time and time to start burrowing in *M. balthica*, such that small clams were more active than large ones. In addition, it was observed that large clams burrowed in shallow sediment because of relatively good oxygen conditions.[28,29] De Wilde[30] found higher oxygen consumption in small-sized clams than in large-sized clams. It is possible that the slower burrowing activity of large-sized clams in our study may have been due to their lower metabolic rate or preference for more oxygen-rich surface sediments.

There were no observed genotoxic effects in *M. balthica* after Cu exposure in experiment 3. This was somewhat surprising because other studies have demonstrated genotoxic effects of Cu at lower exposure concentrations for a related organism, *S. plana*.[31] The lack of effect was not due to a lack of sensitivity of the assay because positive controls showed significant effects when compared to negative controls. Furthermore, the negative controls had values that were in accordance with those found in comparable studies.[32] One possible reason for the lack of effect could be the activation of repair enzymes in organisms during the exposure period. Bihari et al.[33] showed that marine bivalves do have such repair mechanisms. This could explain the difference between the Cu-exposed clams and the positive controls because the latter were made by exposing cells in vitro. Tumor suppressing genes involved in DNA repair have been identified in the marine clam *Mya arenaria*[34] illustrating that this group of animals does have the capacity to cope with and repair DNA damage. Further studies are needed to verify whether repair mechanisms do play an important role in *M. balthica*'s ability to cope with genotoxic effects of Cu. This is relevant for the future usefulness of DNA damage as an ecotoxicological endpoint because it has otherwise been shown to be sensitive to effects of Me-ENPs.[8]

Compared to reported environmental concentrations of Ag (11−24 μg Ag/g dw sed) and a loading rate of Ag ENPs (195−1203 ng/kg sed/year),[4,35] sediment-associated Ag in all forms is unlikely to cause toxicity to *M. balthica* in the aquatic environment. However, the exposure level of Cu used in the present study is lower than concentrations reported in highly polluted sediments (320−1093 μg/g),[36,37] and the proportion of total Cu that is composed of ionic versus nano-Cu cannot be accurately assessed due to difficulties in detection and quantification of Me-ENPs in complex media such as sediment.

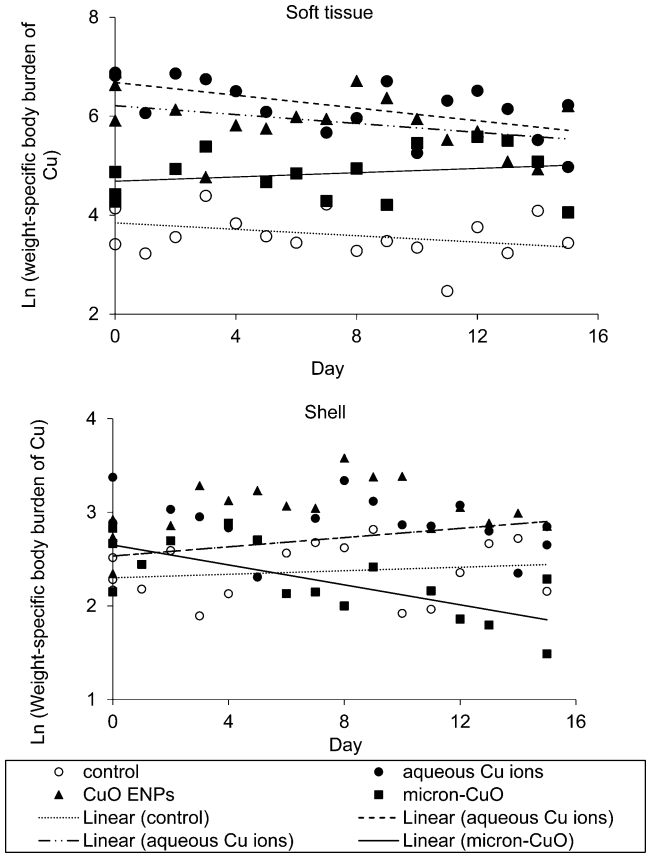

Figure 4. Linear regression of Ln (weight-specific body burden of Cu) in various forms in soft tissue and in shell over time during depuration period in experiment 3.

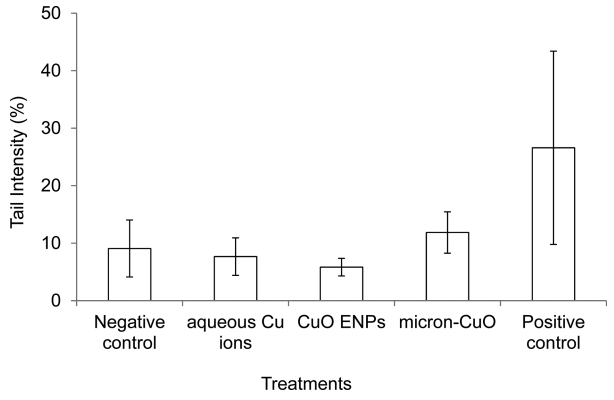

Figure 5. DNA damage expressed as tail intensity from the comet assay for experiment 3 (n = 4). Error bars stand for 1 standard deviation.

Bioaccumulation. In the present study, increased body burdens of Ag and Cu indicated that metals in all forms were bioaccumulated by *M. balthica* after 35 d of exposure. Ratios of body burdens to sediment concentrations of metals were approximately 2 for aqueous Ag (clam size of 11.0 ± 2.0 mm), approximately 12 for aqueous Cu in small clams (clam size 9.6 ± 1.4 mm), and 6 for aqueous Cu in large clams (clam size 12.0 ± 1.0 mm). In the field, the bioaccumulation factor of *M. balthica* can vary from 0.04 to 60 for Ag and from 0.05 to 8 for Cu based on metal concentrations in soft tissue.[18,22] We did not design the present study to ensure that body burdens reached steady state. However, the body burdens of both metals were extremely high compared to body burdens of clams from unpolluted sites (Cu: up to 300 μg/g dw tissue. Ag: up to 100 μg/g dw tissue[22]), which may reflect the very high exposure concentrations in the experiment (up to 200 μg/g dw sed) compared to unpolluted sites (Cu: up to 100 μg/g dw sed. Ag: up to 2.5 μg/g dw sed[22]). Such high body burdens of Ag and Cu without resulting toxicity, especially in small clams (experiment 2), may be due to effective metal detoxification. Adult bivalves seem to be able to detoxify accumulated Ag and Cu ions by binding them to metallothionein (MT)/MT-like proteins and by enclosing them in sulfide-rich granules and basal membranes of cells.[38,39] Body burdens of Cu from Cu ENPs and micrometer CuO particles were higher in small clams than in large clams. Strong and Luoma[40] found size-dependent uptake in some populations of *M. balthica* from San Francisco Bay. This is consistent with Lee et al.,[41] who found that the weight-specific influx rate of cadmium was negatively related to the size of *M. balthica*. However, effects of organism size on bioaccumulation of CuO in different forms need to be further investigated.

A form-dependent net uptake of Cu and Ag was observed in the present study, such that there was a trend that k_u decreased with increasing particle size. This may be related to effective mechanisms for particle sorting in bivalves. During exposure to glass wool (SiO_2), particle sorting has been observed in the bivalve, *Mytilus edulis*, where larger glass fibrils were found in gill epithelial cells in high amounts, and only small and fine glass particles (up to 200 nm) entered the primary tubules after 12 h and appeared finally in the secondary tubules after 24 h.[42] In addition, a longer gut retention time of polystyrene nanoparticles than 10 μm beads indicated that most nanoparticles were taken up in the digestive gland via endocytosis.[43] Uptake of Cu ions involves transport across the cell membrane through available protein carriers,[44] while uptake of Me-ENPs occurs via endocytosis.[45] Different endocytotic pathways have been shown to be involved in direct internalization of nanoparticles in different cells. For example, positively charged nanoparticles are preferentially internalized into HeLa (a cervical cancer cell line) cells by the clathrin-mediated endocytotic pathway at a higher uptake rate than negatively charged nanoparticles.[46] Uptake rates of negatively charged cerium oxide nanoparticles were found to be higher than positively charged nanoparticles in adenocarcinoma lung cells.[47] In a study of cellular internalization pathways of nanoparticles, Gratton et al.[48] found that larger particles of 50−100 nm were internalized through the caveolae-mediated endocytotic pathway. Thus, different uptake pathways may be involved in the internalization of CuO ENPs and micrometer CuO particles.

In the present study, the k_d for aqueous Cu (7.4% d^{-1}) was close to a typical depuration rate constant of 5% d^{-1} and was described best by a one-compartment model (cited in Amiard et al.[49]). As mentioned above, accumulated Cu ions are sequestered in cytosolic proteins (i.e., MT/MT-like proteins) followed by transport to lysosomes for degradation into insoluble residual bodies, which are excreted.[50,51] However, there was no significant decrease of accumulated Cu from CuO ENPs in the present study. This difference in loss dynamics suggests that the form of Cu accumulated in *M. balthica* from sediment-associated CuO ENPs was probably not Cu ions. The depuration mechanism of accumulated CuO ENPs is not very clear. Compartmentalization of accumulated CuO ENPs should be studied further in *M. balthica* to understand the mechanisms of Me-ENP detoxification in this species.

Incorporation of metals into bivalve shells commonly involves two sources: (1) metals accumulated in the soft tissue of bivalves transferred to the shell during shell deposition from mantle tissue[52] and (2) adsorption onto the shell surface from the surrounding external environment. Cu accumulated in shells of *S. plana* was shown to be a result of passive sorption of Cu in seawater.[53] Because no growth of shells occurred during the present study, the bioaccumulation of Cu in shells (including control clams) is thus most likely due to the sorption of different Cu forms from the sediments onto shell surfaces. Although shell sorption of Ag ENPs and CuO ENPs likely have little impact on the clam itself, shell-sorbed metal may still be of importance for bioavailability to predators of clams and thus to trophic transfer of ENPs and Me-ENPs.

In conclusion, our results show some statistically significant differences between Me-ENPs and the same metals in other forms. In most cases, the differences are not large enough to suggest that novel methods for risk assessment of sediment-associated Me-ENPs are needed. However, there remain major challenges in distinguishing Me-ENPs from other forms of metals in complex media, such as sediment, so that the sustainable development of nanotechnologies can be ensured.

■ ASSOCIATED CONTENT

Supporting Information

Detailed information on sediment handling, spiking with metals in different forms, experiment setup of *M. balthica* in three experiments, and burrowing activity. 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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