Understanding the Transformation, Speciation, and Hazard Potential of Copper Particles in a Model Septic Tank System Using Zebrafish to Monitor the Effluent

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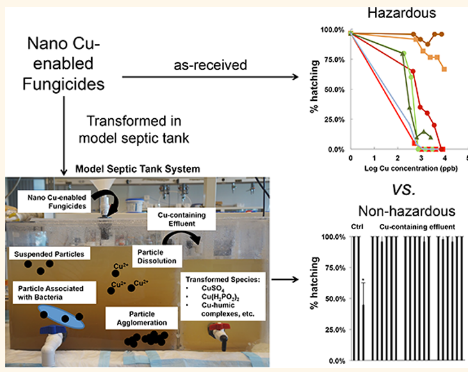
ABSTRACT Although copper-containing nanoparticles are used in commercial products such as fungicides and bactericides, we presently do not understand the environmental impact on other organisms that may be inadvertently exposed. In this study, we used the zebrafish embryo as a screening tool to study the potential impact of two nano Cu-based materials, CuPRO and Kocide, in comparison to nanosized and micron-sized Cu and CuO particles in their pristine form (0−10 ppm) as well as following their transformation in an experimental wastewater treatment system. This was accomplished by construction of a modeled domestic septic tank system from which effluents could be retrieved at different stages following particle introduction (10 ppm). The Cu speciation in the effluent was identified as nondissolvable inorganic $Cu(H_2PO_2)_2$ and nondiffusible organic Cu by X-ray diffraction, inductively coupled plasma mass spectrometry (ICP-MS), diffusive gradients in thin-films (DGT), and Visual MINTEQ software. While the nanoscale materials, including the commercial particles, were clearly more potent (showing 50% hatching interference above 0.5 ppm) than the micron-scale particulates with no effect on hatching up to 10 ppm, the Cu released from the particles in the septic tank underwent transformation into nonbioavailable species that failed to interfere with the function of the zebrafish embryo hatching enzyme. Moreover, we demonstrate that the addition of humic acid, as an organic carbon component, could lead to a dose-dependent decrease in Cu toxicity in our high content zebrafish embryo screening assay. Thus, the use of zebrafish embryo screening, in combination with the effluents obtained from a modeled exposure environment, enables a bioassay approach to follow the change in the speciation and hazard potential of Cu particles instead of difficult-to-perform direct particle tracking.



KEYWORDS: copper particles · transformation · speciation · wastewater treatment · zebrafish · high content screening

Nanoenabled Cu products are increasingly being used for commercial applications, including as antibacterial and antifungal agents that can be applied for spraying of vegetation or as a marine antifouling paint on the hulls of boats and ships.[1−7] For example, CuPRO and Kocide are $Cu(OH)_2$-based nanoproducts used as antifungal agents to spray agricultural crops and lawns. While clearly beneficial for eradicating bacterial and fungal growth, inadvertent exposure of other environmental species, such as fish or fish embryos, has not received sufficient attention because it is difficult to model complicated exposure environments. In addition to identifying relevant environmental species to serve as organisms for predictive toxicological assessment,[8,9] it is important to consider the fate, transport, and transformation of Cu particles in the exposure environment.[10] Not only is it challenging to track the presence and behavior of commercially applied nanoparticles in complex

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exposure environments but we also need to consider the impact of pH, ionic strength, light exposure, and the presence of natural organic matters on the fate, transformation, and possible hazardous impact of these materials.[10,11]

Because of the complexity of tracking the environmental fate and transformation of commercial nanomaterials, it is helpful to use simulated exposure scenarios to obtain information that can be used to support environmental risk assessment.[12,13] One approach is the use of life cycle analysis (LCA) to delineate potential hotspots of exposure that can be used to obtain predictive environmental concentrations (PEC) for risk assessment.[14] In a recent study of Cu-based nanomaterials, Keller *et al*. have provided assessments of environmental exposure routes, based on which the proportional distribution of commercial products to air, landfill, soil, and the aquatic disposal sites could be estimated.[15] This work has identified Cu entry into wastewater treatment systems (industrial, community, or private houses) as an important life cycle stage during which aquatic exposure can occur. Since 20−30% of American households use a septic tank system for sewage treatment,[16,17] we have established a laboratory-scale septic tank system to model the fate, transport, and speciation of nanoparticles. In contrast to intensive monitoring of industrial wastewater treatment plants (WWTPs),[18−28] household septic tanks are not scrutinized or regulated to the same degree.[29−37] Moreover, up to 40% of domestic septic tank systems do not function properly,[37] and the impact of commercial nanomaterials has not been considered for the function and efficiency of these wastewater treatment (WWT) systems.

Given this background, we designed a study wherein we combined the use of a model septic system with our zebrafish high content screening (HCS) platform for assessing the toxicological potential of nano- and micron-sized Cu and CuO, including the commercial nano-$Cu(OH)_2$-based particulates, CuPRO and Kocide. The septic tank system allows modeling of the fate, transport, and transformation of these materials in a decentralized WWT utility. The zebrafish embryo is a sensitive screening platform to access nanoparticle release and speciation of the Cu at a molecular level, namely, the active center of the zebrafish hatching enzyme 1 (ZHE1).[38] Moreover, this metalloprotease enzyme serves as a delicate abiotic marker that can predict the ionic metal and metal oxide species that can disrupt embryo hatching.[39] We demonstrate that even though nanosized Cu particles are more toxic than micron-scale particulates, particle transformation and Cu speciation at different stages of the WWT process led to a significant change in hazard potential, regardless of the particle composition or size. The hazard reduction was accompanied by the formation of insoluble inorganic as well as nondiffusible organic Cu species, which are not bioavailable to the hatching apparatus. Our results demonstrate an alternative approach to assessing the environmental transformation of nano-Cu without the necessity of direct particle tracking.

RESULTS

Acquisition and Physicochemical Characterization of a Cu Particle Library. To conduct our study, we assembled a library of particles that included nanosized Cu and CuO, micron-sized Cu and CuO, and two $Cu(OH)_2$-based commercial fungicides, Kocide and CuPRO. Comprehensive physicochemical characterization of the particles was undertaken, and the results are shown in Table 1. Transmission electron microscopy (TEM) showed a nano-CuO size range of 20−100 nm, with nano-Cu exhibiting a broader size distribution of 200−1000 nm. Representative images are shown in Figure S1. By comparison, micron-sized Cu and CuO particles were ≥ 2 μm in size. Although the commercial fungicides, Kocide and CuPRO, claim to include nano-$Cu(OH)_2$ as an active ingredient, only CuPRO showed discernible particles of ∼20 nm, while the Kocide TEM images showed amorphous materials with no definable particles. Most particles observed in our library had irregular shapes, except micro-Cu, which had a dendritic appearance (Figure S1). X-ray diffraction (XRD) analysis confirmed the presence of orthorhombic $Cu(OH)_2$ as the main chemical ingredient of CuPRO and Kocide. Highly crystalline, monoclinic CuO was the only phase identified in nano- and micron-sized CuO samples. Although no oxides were detected in the micron-sized Cu sample, a significant amount of Cu_2O was present in nano-Cu, likely as a result of surface oxidation.

When introduced into deionized water and Holtfreter's medium, the hydrodynamic diameters of the Cu particles ranged from 400 nm to 2 μm. All particles had a narrow range of zeta-potentials (−16 to −22 mV) in Holtfreter's medium, likely as a result of surface coating by alginate, which was included as a dispersal agent that is present in natural aquatic environment (Table 1). Particle purity was assessed by inductively coupled plasma optical emission spectroscopy (ICP-OES) and the presence of each ingredient was expressed as weight percentage (wt %) relative to the weight of the powdered formulation. The $Cu(OH)_2$ content of Kocide and CuPRO were 40 and 47 wt %, respectively, which is similar to the manufacturers' data. The Cu purity content was used to convert the nominal particle concentrations into elemental Cu concentrations for the planning of zebrafish exposure experiments.

Particle dissolution plays a critical role in hazard generation by metal and metal oxide nanoparticles.[38−41] We have previously demonstrated that dissolution of CuO nanoparticles in Holtfreter's medium can affect hatching interference in zebrafish embryos as a result of

TABLE 1. Physicochemical Characterization of Cu Particles

physicochemical characterizations	technique	unit	particles					
			Kocide	CuPRO	micro-Cu	micro-CuO	nano-Cu	nano-CuO
primary size	TEM	nm	N/A[a]	∼10	>10000	200−2000	200−1000	20−100
phase and structure	XRD		orthorhombic $Cu(OH)_2$, impurities	orthorhombic $Cu(OH)_2$, impurities	cubic Cu	monoclinic CuO	cubic Cu, cubic Cu_2O	monoclinic CuO
shape/morphology	TEM		irregular	irregular	dendritic	irregular	irregular	irregular
size in DI H_2O	HT-DLS	nm	1397 ± 143	889 ± 156	n/a[c]	1316 ± 176	1164 ± 202	420 ± 15
zeta potential in DI H_2O	ZetaPALS	mV	−53.8 ± 0.7	−45.1 ± 0.8	−32.5 ± 2.9	−28.5 ± 0.9	−46.3 ± 1.6	−16.5 ± 0.8
size in H buffer (w/alginate)	HT-DLS	nm	1172 ± 104	953 ± 88	n/a[c]	1349 ± 62	2714 ± 719	459 ± 4
zeta potential in H buffer (w/alginate)	ZetaPALS	mV	−19.9 ± 0.8	−22.9 ± 0.6	−19.9 ± 0.8	−16.2 ± 1.5	−15.9 ± 1.4	−18.8 ± 0.9
purity[b]	ICP-OES	wt %	39.9 ± 1.4	47.1 ± 2.6	94.9 ± 1.4	92.8 ± 1.1	84.8 ± 2.7	88.3 ± 1.3

[a] Primary size cannot be obtained because particles are of undefined morphology. [b] Purity refers to weight percentage of each main component (*i.e.*, $Cu(OH)_2$ in Kocide and CuPRO; Cu in micro-Cu and nano-Cu; CuO in micro-CuO and nano-CuO, respectively). [c] Hydrodynamic size cannot be obtained because of fast particle sedimentation.

the inhibitory effect of Cu^{2+} on the active center of the metalloprotease hatching enzyme, ZHE1.[39] Use of ICP-OES to determine Cu particle dissolution in Holtfreter's medium revealed three dissolution categories based on the wt % dissolution (Figure 1A). That is, while the two fungicides as well as nano-Cu were highly dissolvable (>8 wt % dissolution), nano-CuO occupied an intermediate range (2−8 wt % dissolution), with micro-Cu and -CuO showing <2 wt % solubility.

Use of As-Received Cu Particles to Study Their Impact on Zebrafish Embryo Hatching. We used our robotic arm for picking and plating fertilized zebrafish embryos into 96-well plates, followed by automated imaging to identify embryo hatching in the presence of the particles in our library. Healthy embryos were exposed to particles at concentrations of 0−10 ppm at 4 h postfertilization (hpf) before determining hatching outcome at 72 hpf, using our automated imaging equipment and phenotyping software.[38,39,42] Please note that we converted the nominal particle concentrations into elemental Cu concentrations to allow comparison of materials with different levels of impurity. Expression of the % embryo hatching vs. log[Cu] concentration (ppb) demonstrated dose-dependent hatching interference by the fungicides as well as nano-Cu and -CuO, with the micron-sized materials showing comparatively little effect (Figure 1B). The same data was expressed as a linear concentration (0−2.5 ppm) range vs % hatching in Figure S2; error bars indicate standard deviation. Using $CuCl_2$ as positive control allowed us to express the hierarchical hatching interference as $CuCl_2$ > nano-Cu > CuPRO = Kocide > nano-CuO > micro-Cu = micro-CuO. Statistically significant hatching interference was observed at 0.1 ppm of $CuCl_2$, 0.25 ppm of nano-Cu, 0.3 ppm of CuPRO and Kocide, and 0.5 ppm of nano-CuO, respectively. The particle ranking is in good agreement with the dissolution profiles, showing a Pearson's correlation coefficient of 0.873 for the IC_{50} values (concentration yielding 50% hatching interference) vs wt % particle dissolution. Overall, higher dissolution rates were strongly correlated to lower IC_{50} values. We also monitored other toxicological outcomes, including morphological abnormalities and mortality, throughout embryo development. No significant effects were observed at the concentration ranges used for all the particles.

Use of Septic Tank Effluents to Study the Effect of Cu Particle Transformation on Zebrafish Embryo Hatching. While Cu is efficacious as a bactericide or a fungicide, the environmental impact of Cu on other environmental species that may be inadvertently exposed needs to be considered. Since it has been shown by LCA modeling that nano-Cu gains access to WWTPs,[15] we developed a model septic tank system to simulate an exposure environment in which particles could be introduced to study their transformation and speciation on another

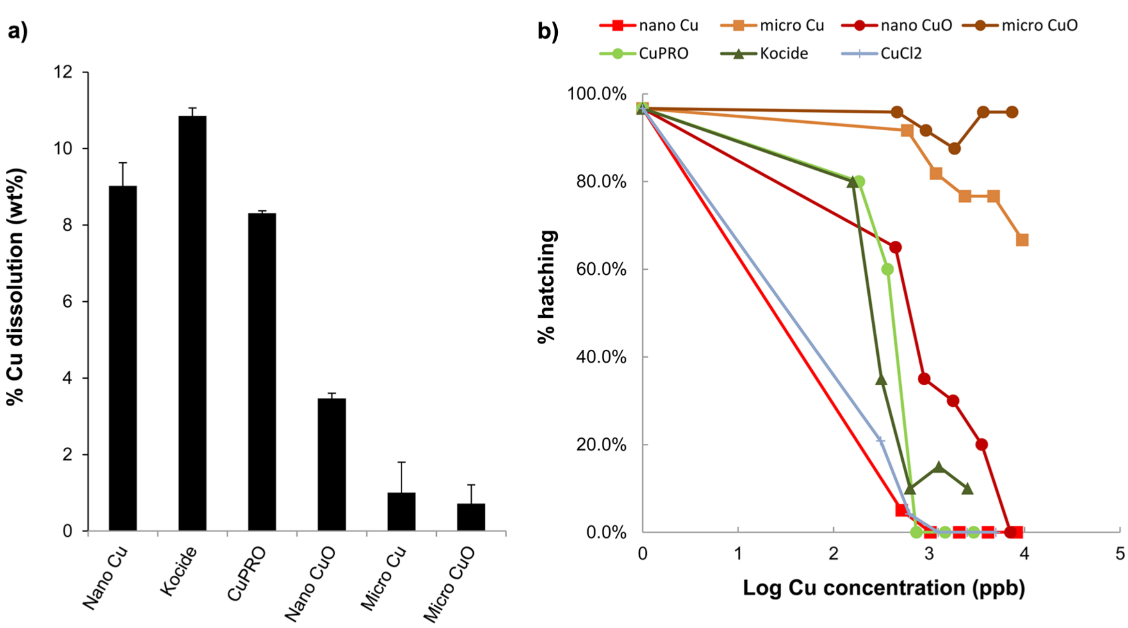

Figure 1. Cu dissolution and hatching interference of as-received Cu particles. (A) Calculation of the weight percent dissolution of nano-Cu, Kocide and CuPRO (highly soluble); nano-CuO (intermediate solubility); and micro-Cu and -CuO (minimally soluble). (B) Percent hatching of zebrafish embryos exposed to as-received Cu particles (0−10 ppm) for 72 h, commencing at 4 h postfertilization. The dose-dependent curve is expressed as % hatching vs log[Cu] (ppb).

aquatic species. The zebrafish was chosen because of their well-studied utility for nanosafety studies, including the demonstration of a high level of sensitivity of the zebrafish embryo to nano-Cu.[38,39] We reasoned that the toxicity profiling of the effluent would be informative for studying Cu speciation as a means of following the particle transformation rather than tracking the particles directly. Figure 2A shows the design of the model septic tank system to generate effluents for assessment of zebrafish toxicity. On the basis of their hazard ranking and dissolution characteristics, nano-Cu, CuPRO, and micro-Cu were selected from the library materials for introduction to the septic tank. Prior to adding the particles, the septic tank underwent 4 weeks of conditioning by introducing simulated wastewater into the primary chamber, followed by weekly collection of effluents from the secondary chamber. These effluents were pooled and regarded as "background" effluent, which served as a control to rule out possible interference of non-Cu materials in the effluent on embryo hatching. Each type of particle was added in individual experiments, daily for 3 weeks, to reach a cumulative dose of 10 ppm by the end of week 3. The effluents were collected from the secondary chamber, weekly for 3 weeks (week 1−3), as well as for an additional 3 weeks (week 4−6) during which no particles were introduced. The "background" as well as the week 1−6 effluents were used to assess the impact on zebrafish embryo hatching. As a positive control, we used 0.5 ppm of nano-Cu in Holtfreter's medium; this dose leads to 50% hatching interference (Figure 2B). Interestingly, all effluents, irrespective of whether they were from "background" origin or collected after the addition of Cu to the tank did not interfere with embryo hatching (Figure 2B). There was also no effect on embryo morphology or the survival rate (Figure S2B). The lack of an effect by the effluents signified that there was either no significant Cu carryover or that the Cu in the effluent was not bioavailable for hatching interference.

Cu Speciation Explains the Lack of Toxicity in the Zebrafish Assay. In order to assess Cu in the effluent, inductively coupled plasma mass spectrometry (ICP-MS) was used to measure the elemental Cu content in the weekly effluent collections, as shown in Figure 3A. This demonstrated a progressive increase in the elemental Cu content over the course of the first 3 weeks, beyond which there was a gradual reduction in Cu content during weeks 4−6. The nano-Cu and CuPRO effluents showed consistently higher Cu concentrations compared to micro-Cu. Interestingly, the Cu content of the effluents collected during weeks 2−4 following the introduction of nano-Cu and CuPRO was higher than the threshold levels (indicated by the dashed lines in Figure 3A) at which the as-received nano-Cu and CuPRO would have caused significant hatching interference. This finding suggests that Cu in the effluent may not be bioavailable as a result of Cu speciation after particle introduction.

In order to assess the Cu particle transformation, XRD analysis was performed on week 3 effluents collected after addition of nano-Cu, CuPRO, and micro-Cu. These results were compared to the XRD peaks of the as-received materials. As shown in Figure 3B, the characteristic XRD profiles of the as-received Cu particles could no longer be seen in the effluents. Instead,

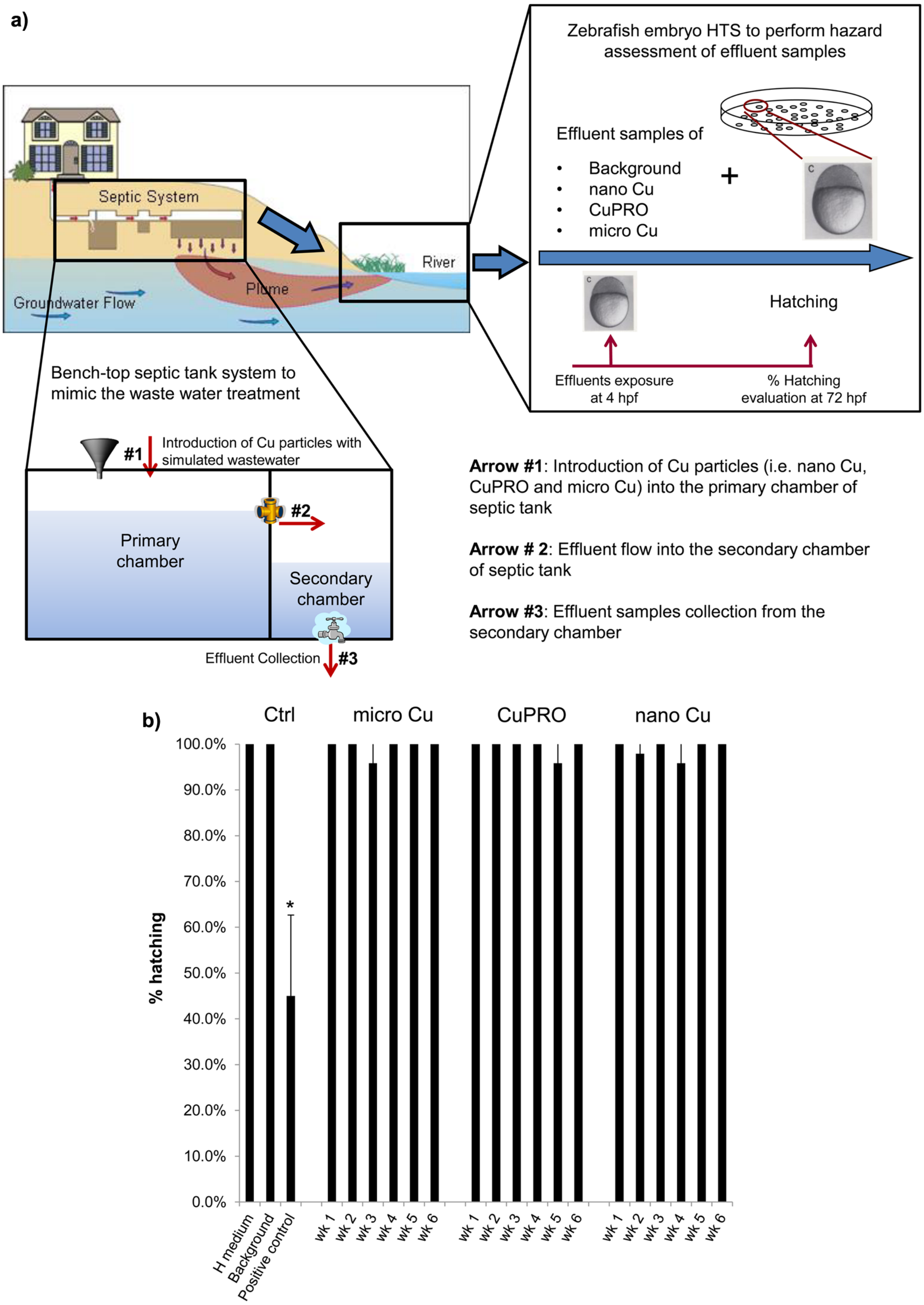

Figure 2. Combined use of a model septic tank and zebrafish embryo high content screening to study the effects of Cu-containing effluents on embryo hatching. (A) Schematic diagram of the model septic system to generate effluents for testing in zebrafish embryos. (B) Percent hatching of zebrafish embryos exposed to the effluents collected weekly from the nano-Cu, CuPRO, and micro-Cu groups for 6 weeks. The introduction of 0.5 ppm of nano-Cu in Holtfreter's medium was used as a positive control. Symbol * denotes statistical significance at $p < 0.05$.

the effluents showed new peaks at ∼12.39° and ∼24.76° (2θ CuKα), which represent water-insoluble inorganic $Cu(H_2PO_2)_2$ and $CuSO_4$, respectively. These Cu species appear in the effluent as early as 1 week after the introduction of the particles (Figure S3). The presence of NaCl crystalline peaks reflect "background" ingredients in the

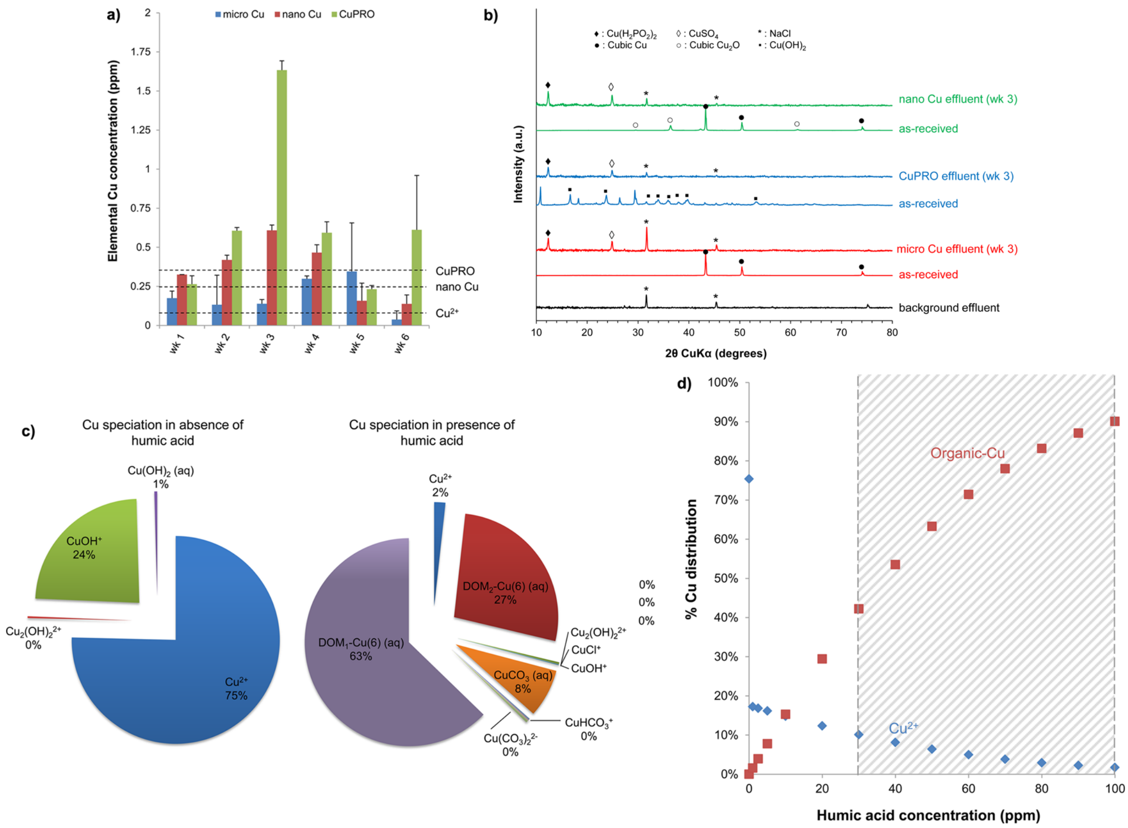

Figure 3. Characterization of the septic effluents. (A) ICP-MS measurements were undertaken to quantify elemental Cu in the effluents collected on a weekly basis following the introduction of the different particle types. The dashed lines represent the as-received Cu^{2+}, nano-Cu, and CuPRO concentrations providing ~50% hatching interference in Holtfreter's medium. (B) XRD analysis on the as-received particulates as well as the corresponding effluents at week 3. (C) Visual MINTEQ modeling to shows Cu speciation in the presence and absence of DOM. HA was used as a form of DOM to perform Visual MINTEQ modeling. Without the presence of DOM, Cu^{2+} is the dominant species, accounting for 75% of the total Cu. The presence of 100 ppm of DOM decreases the Cu^{2+} content precipitously (to 2%) as a result of metal complexation. DOM-bound Cu (DOM_1-Cu(6), 63%; or DOM_2-Cu(6), 27%) accounts for 90% of the total Cu. DOM_1 and DOM_2 are used to describe different humic components. (D) Visual MINTEQ modeling to show the Cu distribution into ionic (Cu^{2+}) and organic Cu following the introduction of incremental amounts of humic acid. The gray area indicates the humic acid concentration range (30–100 ppm) that is expected in the septic tank effluent.

effluent (Figure 3B). To directly identify other potential Cu species, we also performed XPS analysis on the Cu particles and the Cu-containing effluent. However, in most cases, the Cu content was too low to be detected; therefore, no additional information could be obtained with this method (data not shown). While XAS analysis can provide additional information, we unfortunately do not have access to a XAS beamline at present. All considered, these results show that Cu particles undergo transformation in the septic tank, resulting in the formation of new Cu species.

In addition to the detection of inorganic Cu species, it is possible that there could also be the formation of organic Cu, which cannot be detected by XRD. In order to address this possibility, we used Visual MINTEQ software to model the chemical speciation of Cu in the presence of organic material. Our first modeling attempt introduced humic acid (HA) as a source of dissolved organic matter (DOM) in the effluent, which is also known to be able to bind to Cu ions. The predominant Cu species in Holtfreter's medium (devoid of HA) is Cu^{2+}, which comprises ~75% of the total Cu content (Figure 3C). However, upon the introduction of 100 ppm of HA to the aqueous environment, the amount of Cu^{2+} rapidly decreases to ~2% of the total Cu, while the organic-bound Cu increased to ~90% (Figure 3C). This trend is progressive for incremental amounts of HA in the model, as shown in Figure 3D. As indicated by the shaded area, which represents the HA concentration range (30–100 ppm) in the effluent, the organic-bound Cu comprised >65% of the total Cu, while the Cu^{2+} content accounted for <10%.

To provide experimental support for the Visual MINTEQ modeling, we also used methodology for diffusive gradients in thin-films (DGT) to quantify the diffusible Cu^{2+} content of the effluent as a % of the total Cu.[43,44] Soaking of the DGT unit, composed of nitrocellulose membrane filter, diffusion gel, and resin gel layers (Figure 4, inset), in the effluent showed that the % Cu^{2+} (determined by ICP-OES) is <18% of the total Cu in week 3 effluents for all particle types

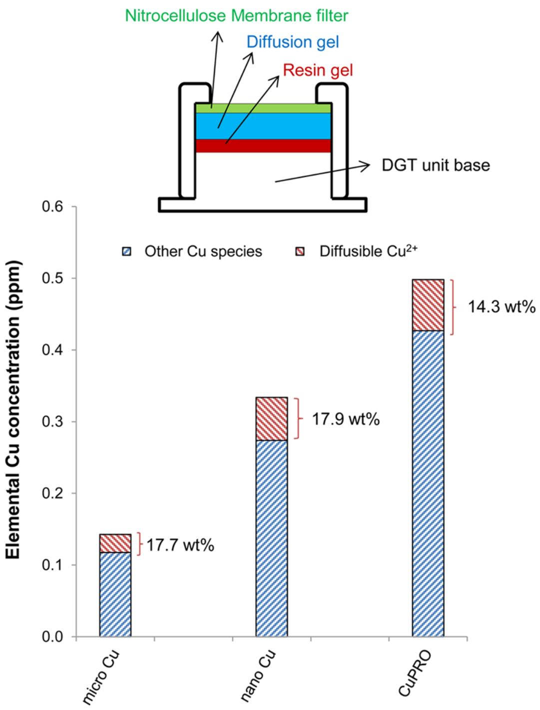

Figure 4. Use of the methodology for diffusive gradients in thin-films (DGT) to quantify diffusible Cu^{2+} in the effluents. Schematic diagram of a DGT unit composed of a nitrocellulose membrane filter, a gel for diffusion, and a resin gel layer (inset). Separation of the diffusible Cu^{2+} and organic Cu in the effluent collected during week 3 (postintroduction of micro-Cu, nano-Cu, and CuPRO) was undertaken by incubation of the DGT unit in the effluents for 3 days at 28 °C. The resin gel was retrieved and digested in nitric acid, before performance of ICP-OES.

introduced into the septic tank (Figure 4). This is in reasonable agreement with the Visual MINTEQ calculation. All considered, the above data suggest that particle transformation in the septic tank results in Cu speciation, with organic nonbioavailable Cu being the dominant species, which lacks the ability to interfere in embryo hatching.

Experimental Addition of Natural Organic Matters Decreases Cu Particle Toxicity. To provide direct experimental evidence that HA impacts Cu toxicity, Holtfreter's medium was spiked with 0.5 or 1 ppm of Cu^{2+} following which HA was added in the amounts of 0–500 ppm. These mixtures were tested for their effects on embryo hatching. As shown in Figure 5A, an HA concentration as low as 7.8 ppm could significantly reverse the Cu^{2+} interference in embryo hatching, with 100% protection at HA concentrations >15 ppm. Similar protective effects were seen using a Suwannee River NOM solution (125 ppm) as well as "background" effluent (Figure 5B). Furthermore, the comparison between "background" effluent and Holtfreter's medium spiked with Cu^{2+} or nano-Cu at 0.125–1 ppm revealed that HA present in the "background" effluent could significantly reverse hatching interference by both ionic and nano-Cu (Figure 5C,D). However, in the absence of HA in Holtfreter's medium, Cu^{2+} and nano-Cu showed significant hatching interference at 0.125 and 0.25 ppm, respectively. Spiking of Holtfreter's with "background" effluent increased the concentrations of hatching interference by Cu^{2+} and nano-Cu to >1 ppm.

DISCUSSION

In this study, we used a model septic tank system and zebrafish hatching interference to investigate the impact of particulate Cu transformation and speciation to obtain information on the inadvertent hazard potential of commercial Cu nanoparticles outside of their immediate scope of use. We demonstrated that while the Cu dissolution played a key role in determining the hazard potential of the as-received particles, the transformation of these materials in a septic tank system rendered the Cu nonbioavailable to the zebrafish embryos in the hatching assay, thereby preventing an effect on hatching interference. The decrease in Cu toxicity was due to Cu speciation to insoluble inorganic and nondiffusive organic Cu species, which does not interfere with the hatching enzyme. We also demonstrated that the addition of humic acid (HA) led to a dose-dependent decrease of Cu toxicity as a result of the organic complexation of Cu. These data demonstrate that it is possible to address the environmental hazard of particulate Cu (including nano-Cu) through the use of simulation studies that can be used for modeling the complexity of the environmental fate and transformation of these materials.

The increased commercialization of nanotechnology is introducing a wave of nanoenabled products to the marketplace. While a great deal of consideration has been given to the safety of workers and consumers, much less is known regarding the environmental implications of nanotechnology. In this study, using the effluent from a model septic tank system, we investigated the hazard potential of Cu particles (including nanoparticles used as fungicides) at different stages of the septic tank operation to project what might happen to a fish embryo at the aquatic disposal sites for the effluent. Our results show a significant decrease in Cu toxicity in the effluent compared to the as-received materials, suggesting that the ingredients in the septic tank are effective for mitigating the hazardous impact of Cu particles in a fish embryo model that is frequently used for nanotoxicology studies.[8,9] The decrease in embryo toxicity was observed at all stages of the wastewater treatment process (Figure 2B), irrespective of the amount of Cu introduced (Figure 3A). The presence of organic matter in the septic tank effluent acts as an effective buffer of the hatching interference effect exerted by the ionic and particulate Cu (Figure 5).

Cu is considered as an environmental toxicant of concern to aquatic invertebrates and vertebrates. Although the total Cu content (of all Cu species) is commonly used to establish national and regional

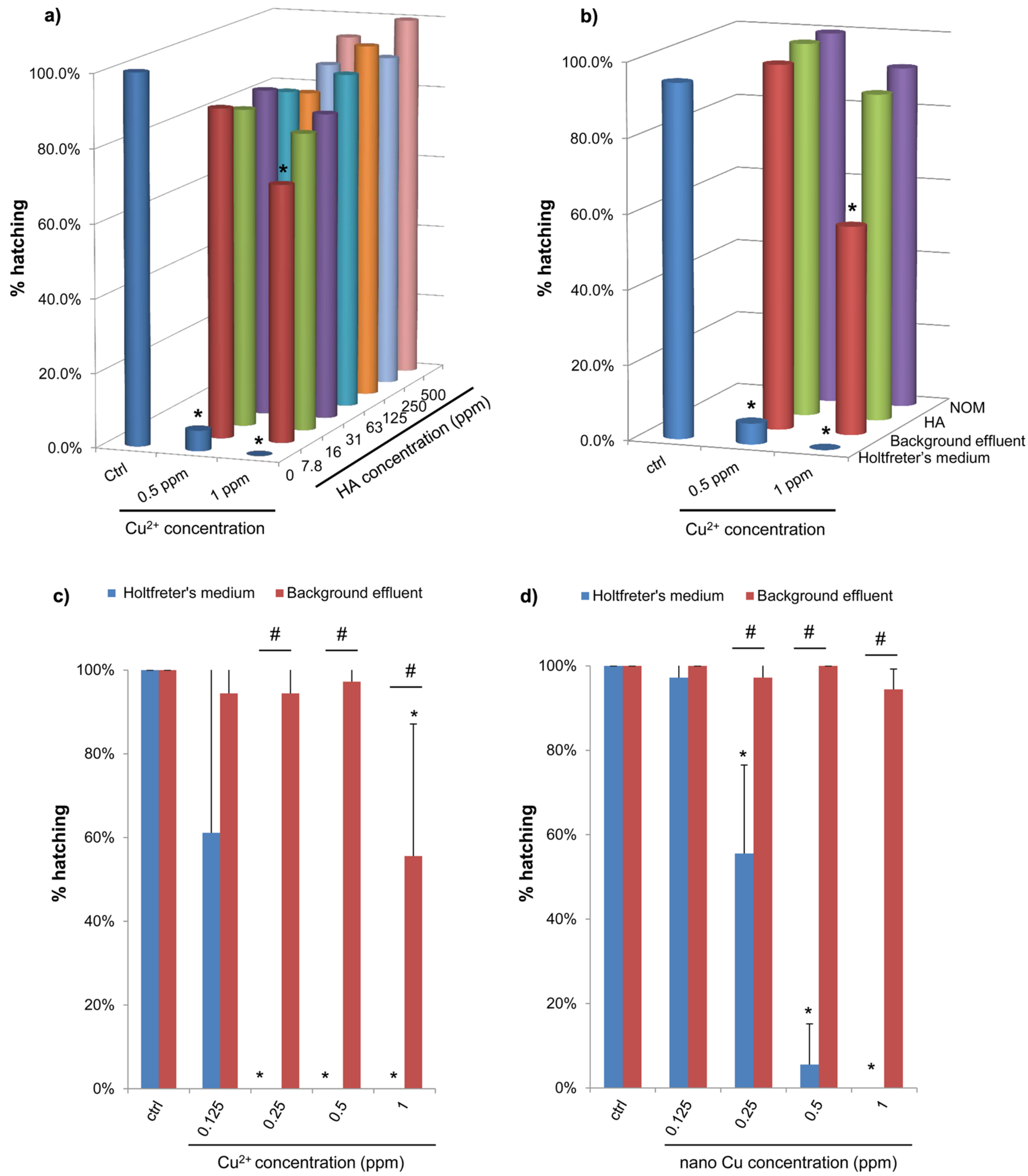

Figure 5. Addition of humic acid (HA) decreased Cu toxicity. (A) The effect of humic acid (0−500 ppm) on hatching interference by 0.5 and 1 ppm of Cu^{2+} in Holtfreter's medium. (B) Comparison of the effect of Suwannee River NOM (100 ppm) with humic acid (100 ppm) and "background" effluent for their effects on embryo hatching in the presence of 0.5 and 1 ppm of Cu^{2+} in Holtfreter's medium. The data in the 3D bar chart represent the average of three individual experiments in which the standard deviation varied less than 6%. (C) Comparison of the effect of known concentrations (0.125, 0.25, 0.5, and 1 ppm) of Cu^{2+} directly spiked into Holtfreter's medium or into "background" effluent. (D) Comparison of the effect of zebrafish embryos exposed to known concentrations (0.125, 0.25, 0.5, and 1 ppm) of nano-Cu directly spiked into Holtfreter's medium or into "background" effluent. The * and # symbols denote statistical significance at $p < 0.05$.

guidelines for water quality, Cu toxicity is generally known to reside in Cu^{2+} being released rather than the total Cu content.[4,45−48] In our study, all Cu particles, irrespective of composition and size, underwent particle transformation in the septic tank, resulting in Cu speciation to Cu^{2+}, insoluble $Cu(H_2PO_2)_2$, and organic Cu species (Figures 3B,C and S3). Thus, although the total Cu content in the effluent was clearly higher than the concentrations at which Cu^{2+}, nano-Cu, or CuPRO interfere in embryo hatching, the chemical complexation in the effluents results in <18 wt % of total Cu being available as Cu^{2+} (Figure 4). These quantities are insufficient for hatching interference. We suggest, therefore, that a more appropriate standard for Cu toxicity should include chemical speciation and a test for bioavailability rather than just relying on total Cu content. Consistent with the literature indicating that Cu toxicity to freshwater organisms could be significantly altered through organic ligands,[44,49−55] our results showed that natural organic matter such as HA provides effective organic complexation of Cu^{2+} to act as a buffer of Cu toxicity (Figure 5). These results corroborate our previous demonstration that interference in embryo hatching and inhibition of ZHE1 activity by Cu^{2+}, Zn^{2+}, Ni^{2+}, or Cr^{3+} can be reversed by the metal chelator, diethylene triamine pentaacetic acid (DTPA).[38,39] It is also worthwhile mentioning that the presence of humic acid in the septic tank system might influence the dissolution characteristics of the Cu particles. According to Adeleye *et al.*, the presence of organic matter (*i.e.*, extracellular polymeric substance, EPS) resulted in a higher dissolution rate of Cu particles.[1] However, since the amount of humic acid in the septic tank system (30−100 ppm) was orders of magnitude higher than the total Cu (Figure 3A), most of the dissolved Cu^{2+} ions from the particles would be organically chelated.

The combined use of a septic tank model and a zebrafish assay to assess Cu toxicity introduces a practical approach to assess the hazard of nanoparticles and nanoenabled products in complex environmental settings. We took advantage of a small-scale domestic septic tank model that provides easy access to effluent that could be sampled and tested at various intervals and stages of the WWT process. We demonstrate that the effluent could be used for hazard assessment and Cu speciation even though it is difficult to monitor the physical presence and physicochemical characteristics of the particles under these exposure conditions. The use of the zebrafish embryo as a screening tool to examine embryo toxicity could be expanded and refined to include other environmentally relevant organisms that could be in harm's way if nanoparticles are introduced into the environment. While for proof-of-principle testing, a fixed amount of particles (10 ppm) were used, which could be orders of magnitude higher than actual environmental exposures, our approach can be easily adapted for a range of metal and metal oxide nanoparticles at different concentrations. These adaptations can be based on LCA, which identifies the hot spots of exposure that can be subsequently modeled to provide information about the amount of exposure and speciation that can be addressed by environmental modeling software being developed by the UC Center for the Environmental Implications of Nanotechnology (UC CEIN).

CONCLUSION

In summary, we have successfully combined the use of a model septic tank system and zebrafish HCS to study the hazard potential of Cu-based particles and fungicides before and after introduction into a WWT system. We demonstrate that the Cu containing effluent has significantly reduced impact on zebrafish embryo hatching. This toxicity decrease is due to particle transformation and Cu speciation to less bioavailable species, among which humic acid was used to show how organic speciation can reduce Cu toxicity to zebrafish.

METHODS

Cu Particle Acquisition and Physicochemical Characterization. Six Cu particles, including nanosized Cu and CuO, micron-sized Cu and CuO, and two nano-$Cu(OH)_2$-based fungicides (CuPRO and Kocide) were used in this study. They were purchased in powder form from commercially available vendors. Nano-Cu was from US Research Nanomaterials, Inc., and nano-CuO, micro-Cu, and micro-CuO were from Sigma-Aldrich. CuPRO 2005 was from SePRO, and Kocide 3000 was from DuPont. All materials were used, as received, without further purification or modification. Physicochemical characterization, including primary particle size, shape, crystal structure, purity, and endotoxin levels were assessed on all materials. TEM and scanning electron microscopy (SEM) were used to measure the primary particle size and shape. XRD was used to determine the crystal structure of each particle. Purity was measured by inductively coupled plasma optical emission spectroscopy (ICP-OES) and presented as weight percentage of each main component per unit mass of Cu particles (*i.e.*, $Cu(OH)_2$ in Kocide and CuPRO; Cu in nano-Cu and micro-Cu; CuO in nano-CuO and micro-CuO, respectively). The hydrodynamic sizes and surface charge of the particles dispersed in deionized water (DI H_2O) and Holtfreter's medium were determined by a high-throughput dynamic light scattering instrument (HT-DLS, Dynapro Plate Reader, Wyatt Technology) and a ZetaPALS instrument (Brookhaven Instruments, Holtsville, NY), respectively. The dissolution characteristics of Cu particles were analyzed by ICP-OES through ultracentrifugation, as described by Lin *et al.* Briefly Cu particle suspensions (in DI H_2O and Holtfreter's medium) were kept at 28.5 °C for 48 h and centrifuged for 1 h at 20 000*g*. The supernatants were collected for quantification of elemental Cu content, using ICP-OES.

Septic Tank Design and Effluent Sample Collection. A septic tank system, comprising primary and secondary chambers,[56] was used to simulate the fate, transport, and transformation of the Cu particles in a decentralized wastewater treatment process. Details of the septic tank construction and function appear in the Supporting Information. Briefly, before dosing of the primary chamber with Cu particles, the tank system underwent 4 weeks of conditioning. The effluent collected from the secondary chamber during this period was considered as "background". Subsequently, nano-Cu, CuPRO, or micro-Cu were introduced into the primary chamber (three individual experiments) daily for 3 weeks, by mixing with synthetic gray water and colon waste from a model colon reactor to mimic household wastewater. The total quantities of Cu particles added to the septic system amounted to 500, 1000, and 1500 mg, at the culmination of weeks 1−3, respectively. This was equivalent to Cu concentrations of 3.33, 6.67, and 10 ppm, respectively. This period was followed by a three week interval during which no new particles were added to the septic tank but during which effluents were collected on a weekly basis. These were labeled week 1−6 effluents for each of the particle types being assessed. The final concentration of 10 ppm was selected based upon predicted concentrations of Cu found in WWTPs.[15,57,58] Moreover, since the system has a three week residence time for liquids, it was anticipated that an effect would not be observed with dosing the system once only based on the amount of liquid that enters the primary chamber on a weekly basis (∼75 L per week). We expect that household waste household would contain a steady stream of a low concentration of nanomaterials from consumer products. Therefore, we selected to dose the system over a three week period with a dose of ∼3.3 ppm per week (or a final total concentration of 10 ppm).

Toxicity Assessment Using Zebrafish Embryo High Content Screening (HCS). Our robotic and automated system for zebrafish embryo hatching was used to assess the impact of as-received Cu particles, "background" effluent, week 1−6 effluents, and "background" effluent spiked with known concentrations (0.125−1 ppm) of Cu^{2+} and nano-Cu. Details of our embryo HCS screening protocol appear in the Supporting Information and ref 38.

Copper Partitioning, Transformation, and Speciation. To quantify the Cu content of the septic system, ICP-MS was used to measure the elemental Cu concentrations in the primary chamber sludge as well as the various effluents. The method for sample digestion and ICP analysis appear in the Supporting Information. Cu particle transformation and inorganic speciation was assessed by XRD analysis. Ten milliliters of each effluent was dried on the sample stub prior to XRD spectral acquisition, using a Panalytical X'Pert Pro diffractometer (Cu Kα radiation). Inorganic Cu species were identified by comparing the diffraction peaks in the effluents with a standard spectral library. However, since this technique cannot be used for organic Cu, organic Cu speciation was modeled by the Windows software program, Visual MINTEQ (version 3.1, 2014, KTH Royal Institute of Technology, Stockholm, Sweden).[59] A "sweep" function was used to determine the organic Cu speciation in the presence of HA as a source of DOM (as appears in the effluent). The percentage distribution of organic-Cu vs Cu^{2+} was calculated and plotted against increasing HA concentrations (0−100 ppm). These results were supplemented by using the diffusive gradients in thin-films method (DGT Research Ltd.) to measure the diffusible effluent content of Cu^{2+}. Each DGT unit is composed of a nitrocellulose membrane filter and diffusion and resin gel layers to isolate the diffusible Cu^{2+} from the nondiffusible organic-Cu complexes in the effluent. After incubation of the DGT unit in the effluent for 3 days at 28 °C, the resin gel was retrieved and digested in nitric acid, before performance of ICP-OES.

Statistical Methods. Results were statistically analyzed using two-side Student's *t* test. The difference is regarded as statistically significant with $p < 0.05$. Data are reported as the mean ± standard deviation from at least three separate experiments.

Conflict of Interest: The authors declare no competing financial interest.

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Supporting Information Available: Additional information on water quality characterizations, SEM and TEM images, XRD spectra of Cu particles, dose−response analysis of percent hatching, XRD spectra of effluents from nano-Cu-treated septic tank and information on characterization of the septic system, including alkalinity, conductivity, and hardness. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES AND NOTES

1. Adeleye, A. S.; Conway, J. R.; Perez, T.; Rutten, P.; Keller, A. A. Influence of Extracellular Polymeric Substances on the Long-term Fate, Dissolution, and Speciation of Copper-based Nanoparticles. *Environ. Sci. Technol.* **2014**, *48*, 12561–12568.
2. Thomas, K.; Raymond, K.; Chadwick, J.; Waldock, M. The Effects of Short-term Changes in Environmental Parameters on the Release of Biocides from Antifouling Coating: Cuprous Oxide and Tributyltin. *Appl. Organomet. Chem.* **1999**, *13*, 453–460.
3. Ribolzi, O.; Valles, V.; Gomez, L.; Voltz, M. Speciation and Origin of Particulate Copper in Runoff Water from a Mediterranean Vineyard Catchment. *Environ. Pollut.* **2002**, *117*, 261–271.
4. Giesy, J. P.; Newell, A.; Leversee, G. J. Copper Speciation in Soft, Acid, Humic Waters: Effects on Copper Bioaccumulation by and Toxicity to *Simocephalus Serrulatus*. *Sci. Total Environ.* **1983**, *28*, 23–36.
5. Arias, M.; Lopez, E.; Fernandez, D.; Soto, B. Copper Distribution and Dynamics in Acid Vineyard Soils Treated with Copper-Based Fungicides. *Soil Sci.* **2004**, *169*, 796–805.
6. Ren, G.; Hu, D.; Cheng, E. W.; Vargas-Reus, M. A.; Reip, P.; Allaker, R. P. Characterisation of Copper Oxide Nanoparticles for Antimicrobial Applications. *Int. J. Antimicrob. Agents* **2009**, *33*, 587–590.
7. Cioffi, N.; Torsi, L.; Ditaranto, N.; Tantillo, G.; Ghibelli, L.; Sabbatini, L.; Bleve-Zacheo, T.; Alessio, M. D.; Zambonin, P. G.; Traversa, E. Copper Nanoparticle/Polymer Composites with Antifungal and Bacteriostatic Properties. *Chem. Mater.* **2005**, *17*, 5255–5262.
8. Lin, S.; Zhao, Y.; Nel, A. E.; Lin, S. Zebrafish: an *in Vivo* Model for Nano EHS Studies. *Small* **2013**, *9*, 1608–1618.
9. Fako, V. E.; Furgeson, D. Y. Zebrafish as a Correlative and Predictive Model for Assessing Biomaterial Nanotoxicity. *Adv. Drug. Delivery Rev.* **2009**, *61*, 478–486.
10. Lowry, G. V.; Gregory, K. B.; Apte, S. C.; Lead, J. R. Transformations of Nanomaterials in the Environment. *Environ. Sci. Technol.* **2012**, *46*, 6893–6899.
11. Klaine, S. J.; Alvarez, P. J. J.; Batley, G. E.; Fernandes, T. F.; Handy, R. D.; Lyon, D. Y.; Mahendra, S.; Mclaughlin, M. J.; Lead, J. R. Nanomaterials in the Environment: Behavior, Fate, Bioavailability, and Effects. *Environ. Toxicol. Chem.* **2008**, *27*, 1825–1851.
12. Mueller, N. C.; Nowack, B. Exposure Modeling of Engineered Nanoparticles in the Environment. *Environ. Sci. Technol.* **2008**, *42*, 4447–4453.
13. Nowack, B. Is Anything out There? *Nano Today* **2009**, *4*, 11–12.
14. Beaudrie, C. E.; Kandlikar, M.; Satterfield, T. From Cradle-to-Grave at the Nanoscale: Gaps in U.S. Regulatory Oversight along the Nanomaterial Life Cycle. *Environ. Sci. Technol.* **2013**, *47*, 5524–5534.
15. Keller, A. A.; Lazareva, A. Predicted Releases of Engineered Nanomaterials: From Global to Regional to Local. *Environ. Sci. Technol. Lett.* **2014**, *1*, 65–70.
16. EPA. Decentralized Systems Technology Fact Sheet Septic Tank - Soil Absorption Systems; Washington DC, **1999**.
17. EPA. Decentralized Wastewater Treatment Systems: Memorandum of Understanding; Washington DC, **2011**.
18. Benn, T. M.; Westerhoff, P. Nanoparticle Silver Released into Water from Commercially Available Sock Fabrics. *Environ. Sci. Technol.* **2008**, *42*, 4133–4139.
19. Choi, O.; Deng, K. K.; Kim, N.-J.; Ross, L., Jr.; Surampalli, R. Y.; Hu, Z. The Inhibitory Effects of Silver Nanoparticles, Silver Ions, and Silver Chloride Colloids on Microbial Growth. *Water Res.* **2008**, *42*, 3066–3074.
20. García, A.; Delgado, L.; Torà, J. A.; Casals, E.; González, E.; Puntes, V.; Font, X.; Carrera, J.; Sánchez, A. Effect of Cerium Dioxide, Titanium Dioxide, Silver, and Gold Nanoparticles on the Activity of Microbial Communities Intended in Wastewater Treatment. *J. Hazard. Mater.* **2012**, *199−200*, 64–72.
21. Jarvie, H. P.; Al-Obaidi, H.; King, S. M.; Bowes, M. J.; Lawrence, M. J.; Drake, A. F.; Green, M. A.; Dobson, P. J. Fate of Silica Nanoparticles in Simulated Primary Wastewater Treatment. *Environ. Sci. Technol.* **2009**, *43*, 8622–8628.
22. Kaegi, R.; Voegelin, A.; Sinnet, B.; Zuleeg, S.; Hagendorfer, H.; Burkhardt, M.; Siegrist, H. Behavior of Metallic Silver Nanoparticles in a Pilot Wastewater Treatment Plant. *Environ. Sci. Technol.* **2011**, *45*, 3902–3908.

23. Kiser, M. A.; Ryu, H.; Jang, H.; Hristovski, K.; Westerhoff, P. Biosorption of Nanoparticles to Heterotrophic Wastewater Biomass. *Water Res.* **2010**, *44*, 4105–4114.
24. Kiser, M. A.; Westerhoff, P.; Benn, T.; Wang, Y.; Pérez-Rivera, J.; Hristovski, K. Titanium Nanomaterial Removal and Release from Wastewater Treatment Plants. *Environ. Sci. Technol.* **2009**, *43*, 6757–6763.
25. Limbach, L. K.; Bereiter, R.; Müller, E.; Krebs, R.; Gälli, R.; Stark, W. J. Removal of Oxide Nanoparticles in a Model Wastewater Treatment Plant: Influence of Agglomeration and Surfactants on Clearing Efficiency. *Environ. Sci. Technol.* **2008**, *42*, 5828–5833.
26. Mu, H.; Chen, Y.; Xiao, N. Effects of Metal Oxide Nanoparticles (TiO_2, Al_2O_3, SiO_2 and ZnO) on Waste Activated Sludge Anaerobic Digestion. *Bioresour. Technol.* **2011**, *102*, 10305–10311.
27. Sheng, Z.; Liu, Y. Effects of Silver Nanoparticles on Wastewater Biofilms. *Water Res.* **2011**, *45*, 6039–6050.
28. Zheng, X.; Wu, R.; Chen, Y. Effects of ZnO Nanoparticles on Wastewater Biological Nitrogen and Phosphorus Removal. *Environ. Sci. Technol.* **2011**, *45*, 2826–2832.
29. Federal Water Pollution Control Act Amendments of 1972. In Pubic Law 92-500, Ed. **1972**.
30. EPA. Manual for Evaluating Public Drinking Water Supplies; Washington, D.C., **1971**.
31. EPA. National Interim Primary Drinking Water Regulations; Washington, D.C., **1976**.
32. EPA. NPDES Permit Writers' Manual. EPA-833-B-96-003; Washington, D.C., **1996**.
33. EPA. Centralized Waste Treatment Effluent Limitations Guidelines and Pretreatment Standards (40 CFR 437); Washington, D.C., **2001**.
34. EPA. Protecting the Nation's Waters Through Effective NPDES Permits A Strategic Plan FY 2001 and Beyond; Washington, D.C., **2001**.
35. EPA. Alternative Waste Management Techniques for Best Practicable Waste Treatment; Washington, D.C, **1976**; Vol. *43*, pp 6190−6191.
36. EPA. The Report to Congress: Waste Disposal Practices and Their Effects on Ground Water EPA 570/9-77-001; Washington, D.C, June **1977**.
37. Canter, L. W.; Knox, R. C. *Septic Tank System Effects on Ground Water Quality*; Lewis Publishers, Inc: Albany, GA, 1985.
38. Lin, S.; Zhao, Y.; Xia, T.; Meng, H.; Ji, Z.; Liu, R.; George, S.; Xiong, S.; Wang, X.; Zhang, H.; *et al.* High Content Screening in Zebrafish Speeds up Hazard Ranking of Transition Metal Oxide Nanoparticles. *ACS Nano* **2011**, *5*, 7284–7295.
39. Lin, S.; Zhao, Y.; Ji, Z.; Ear, J.; Chang, C. H.; Zhang, H.; Low-Kam, C.; Yamada, K.; Meng, H.; Wang, X.; *et al.* Zebrafish High-Throughput Screening to Study the Impact of Dissolvable Metal Oxide Nanoparticles on the Hatching Enzyme, ZHE1. *Small* **2013**, *9*, 1776–1785.
40. Xia, T.; Zhao, Y.; Sager, T.; George, S.; Pokhrel, S.; Li, N.; Schoenfeld, D.; Meng, H.; Lin, S.; Wang, X.; *et al.* Decreased Dissolution of ZnO by Iron Doping Yields Nanoparticles with Reduced Toxicity in the Rodent Lung and Zebrafish Embryos. *ACS Nano* **2011**, *5*, 1223–1235.
41. Wang, X.; Ji, Z.; Chang, C. H.; Zhang, H.; Wang, M.; Liao, Y. P.; Lin, S.; Meng, H.; Li, R.; Sun, B.; *et al.* Use of Coated Silver Nanoparticles to Understand the Relationship of Particle Dissolution and Bioavailability to Cell and Lung Toxicological Potential. *Small* **2014**, *10*, 385–398.
42. Liu, R.; Lin, S.; Rallo, R.; Zhao, Y.; Damoiseaux, R.; Xia, T.; Lin, S.; Nel, A.; Cohen, Y. Automated Phenotype Recognition for Zebrafish Embryo based *in vivo* High Throughput Toxicity Screening of Engineered Nano-materials. *PLoS One* **2012**, *7*, e35014.
43. Amato, E. D.; Simpson, S. L.; Jarolimek, C. V.; Jolley, D. F. Diffusive Gradients in Thin Films Technique Provide Robust Prediction of Metal Bioavailability and Toxicity in Estuarine Sediments. *Environ. Sci. Technol.* **2014**, *48*, 4485–4494.
44. Luider, C. D.; Crusius, J.; Playle, R. C.; Curtis, P. J. Influence of Natural Organic Matter Source on Copper Speciation as Demonstrated by Cu Binding to Fish Gills, by Ion Selective Electrode, and by DGT Gel Sampler. *Environ. Sci. Technol.* **2004**, *38*, 2865–2872.
45. Cuppett, J. D.; Duncan, S. E.; Dietrich, A. M. Evaluation of Copper Speciation and Water Quality Factors That Affect Aqueous Copper Tasting Response. *Chem. Senses* **2006**, *31*, 689–697.
46. Pagenkopf, G. K.; Russo, R. C.; Thurston, R. V. Effect of Complexation on Toxicity of Copper to Fishes. *J. Fish. Res. Board Can.* **1974**, *31*, 462–465.
47. Stauber, J. L.; Florence, T. M. Mechanism of Toxicity of Ionic Copper and Copper Complexes to Algae. *Mar. Biol.* **1987**, *94*, 511–519.
48. Flemming, C. A.; Trevors, J. T. Copper Toxicity and Chemistry in the Environment: A Review. *Water Air Soil Pullut.* **1989**, *44*, 143–158.
49. McIntyre, J. K.; Baldwin, D. H.; Meador, J. P.; Scholz, N. L. Chemosensory Deprivation in Juvenile Coho Salmon Exposed to Dissolved Copper under Varying Water Chemistry Conditions. *Environ. Sci. Technol.* **2008**, *42*, 1352–1358.
50. Breault, R. F.; Colman, J. A. Copper Speciation and Binding by Organic Matter in Copper-Contaminated Streamwater. *Environ. Sci. Technol.* **1996**, *30*, 3477–3486.
51. Linbo, T. L.; Baldwin, D. H.; Mcintyre, J. K.; Scholz, N. L. Effects of Water Hardness, Alkalinity and Dissolved Organic Carbon on the Toxicity of Copper to the Lateral Line of Developing Fish. *Environ. Toxicol. Chem.* **2009**, *28*, 1455–1461.
52. Hoffman, S. R.; Shafer, M. M.; Armstrong, D. E. Strong Colloidal and Dissolved Organic Ligands Binding Copper and Zinc in Rivers. *Environ. Sci. Technol.* **2007**, *41*, 6996–7002.
53. Gaetke, L. Copper Toxicity, Oxidative Stress, and Antioxidant Nutrients. *Toxicology* **2003**, *189*, 147–163.
54. Cooper, C. A.; Tait, T.; Gray, H.; Cimprich, G.; Santore, R. C.; McGeer, J. C.; Wood, C. M.; Smith, D. S. Influence of Salinity and Dissolved Organic Carbon on Acute Cu Toxicity to the Rotifer *Brachionus plicatilis*. *Environ. Sci. Technol.* **2014**, *48*, 1213–1221.
55. Kiaune, L.; Singhasemanon, N. Pesticidal Copper (I) Oxide: Environmental Fate and Aquatic Toxicity. *Rev. Envrion. Contam. Toxicol.* **2011**, *213*, 1–26.
56. Marcus, I. M.; Wilder, H. A.; Quazi, S. J.; Walker, S. L. Linking Microbial Community Structure to Function in Representative Simulated Systems. *Appl. Environ. Microbiol.* **2013**, *79*, 2552–2559.
57. Keller, A.; McFerran, S.; Lazareva, A.; Suh, S. Global Life Cycle Releases of Engineered Nanomaterials. *J. Nanopart. Res.* **2013**, *15*, 1–17.
58. Lazareva, A.; Keller, A. A. Estimating Potential Life Cycle Releases of Engineered Nanomaterials from Wastewater Treatment Plants. *ACS Sustain. Chem. Eng.* **2014**, *2*, 1656–1665.
59. Gustafsson, J. P. *Visual MINTEQ*, ver. 3.0, 2010. http://www2.lwr.kth.se/English/OurSoftware/vminteq/.