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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 nano-sized 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 non-dissolvable inorganic $\text{Cu}(\text{H}_2\text{PO}_2)_2$ and non-diffusible 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 non-bioavailable 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

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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 are provided online free of charge *via* the Internet at <http://pubs.acs.org>. We also provide information on characterization of the septic system, including alkalinity, conductivity, and hardness.

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

Nano-enabled 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 anti-fouling paint on the hulls of boats and ships.¹⁻⁷ For example, CuPRO and Kocide are Cu(OH)₂-based nano-products 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 it is challenging to track the presence and behavior of commercially applied nanoparticles in complex 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 have not been considered on 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 nano-sized 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 non-diffusible 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 nano-sized 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 discernable 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 Cu₂O 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 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 wt% 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 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 hours post fertilization (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: $\text{CuCl}_2 > \text{nano Cu} > \text{CuPRO} = \text{Kocide} > \text{nano CuO} > \text{micro Cu} = \text{micro CuO}$. Statistically significant hatching interference was observed at 0.1 ppm CuCl_2 , 0.25 ppm nano Cu, 0.3 ppm CuPRO and Kocide, and 0.5 ppm 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 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. Based on 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 four 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 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, 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, the effluents showed new peaks at ~ 12.39 and ~ 24.76 degrees (2θ CuK α), which represent water-insoluble inorganic Cu(H₂PO₂)₂, as well as CuSO₄, 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 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 beam line 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 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 total Cu while the Cu^{2+} content accounted < 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, comprised of a nitrocellulose membrane filter, diffusion gel and resin gel layers (Figure 4, insert), 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 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 non-bioavailable 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 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 and 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 non-bioavailable 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 non-diffusive 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 nano-enabled 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 is 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 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 $\text{Cu}_2(\text{PO}_4)_2$, as well as organic Cu species (Figure 3B, C, and Figure 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 liganding^{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 Keller *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 Cu²⁺ 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 nano-enabled 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 exam 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.

MATERIALS AND METHODS

Cu Particle Acquisition and Physicochemical Characterization

Six Cu particles, including nano-sized 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. Transmission electron microscopy (TEM)

and scanning electron microscopy (SEM) were used to measure the primary particle size and shape. X-ray diffraction (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 H₂O) 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 H₂O and Holtfreter's medium) were kept at 28.5 °C for 48 h and centrifuged for 1 h at 20,000g. The supernatants were collected for quantification of elemental Cu content, using ICP-OES.

Septic Tank Design and Effluent Sample Collection

A septic tank system, comprised of 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 SI. Briefly, before dosing of the primary chamber with Cu particles, the tank system underwent four 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 three weeks, by mixing with synthetic grey 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, as well as “background” effluent spiked with known concentrations (0.125 – 1 ppm) of Cu²⁺ and nano Cu. Details of our embryo HCS screening protocol appear in the SI and reference 38.

Copper Partitioning, Transformation and Speciation

To quantify the Cu content of the septic system, inductively coupled plasma mass spectrometry (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 SI. Cu particle transformation and inorganic speciation was assessed by X-ray diffraction (XRD) analysis. Ten mL of each effluent was dried on the sample stub prior to XRD spectral acquisition, using a Panalytical X'Pert Pro diffractometer (Cu K α 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 humic acid (HA) as a source of dissolved organic matters (DOM, as appears in the effluent). The percentage distribution of organic-Cu vs. Cu²⁺ 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 Cu²⁺. Each DGT unit is comprised of a nitrocellulose membrane filter, diffusion and resin gel layers to isolate the diffusible Cu²⁺ from the non-diffusible 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 $\pm$ standard deviation from at least three separate experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A and B.

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 hours, commencing at 4 hours post-fertilization. The dose dependent curve is expressed as % hatching vs. Log [Cu] (ppb).

A.

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 nano Cu in Holtfreter's medium was used as a positive control. Symbol * denotes statistical significance at $p < 0.05$.

A.

B.

C

D.

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 Cu²⁺, 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 show Cu speciation in the presence and absence of Dissolved Organic Matter (DOM). Humic acid (HA) was used as a form of DOM to perform Visual MINTEQ modeling. Without the presence of DOM, Cu²⁺ is the dominant species, accounting for 75% of the total Cu. The presence of 100 ppm DOM decreases the Cu²⁺ content precipitously (to 2%) as a result of metal complexation. DOM-bound Cu (DOM₁-Cu(6): 63%, or DOM₂-Cu(6): 27%) accounts for 90% of the total Cu. DOM₁ and DOM₂ are used to describe different humic components. (D) Visual MINTEQ modeling to show the Cu distribution into ionic (Cu²⁺) and organic Cu following the introduction of incremental

amounts of humic acid. The grey area indicates the humic acid concentration range (30 – 100 ppm) that is expected in the septic tank effluent.

Figure 4.

Use of the methodology for diffusive gradients in thin-films (DGT) to quantify diffusible Cu²⁺ in the effluents. Schematic diagram of a DGT unit comprised of a nitrocellulose membrane filter, a gel for diffusion, and a resin gel layer (insert). Separation of the diffusible Cu²⁺ and organic Cu in the effluent collected during week 3 (post introduction of micro Cu, nano Cu and CuPRO) was undertaken by incubation 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.

A and B.

C and D.

Figure 5.

The 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 Cu²⁺ 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 Cu²⁺ in Holtfreter's medium. The data in the 3D bar chart represent the average of 3 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 Cu²⁺ 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 $p < 0.05$.

Table 1

Physicochemical Characterization of Cu Particles.

Physicochemical characterizations	Technique	Unit	Particles				
			Kocide	CuPRO	micro Cu	micro CuO	nano CuO
Primary Size	TEM	nm	N/A ^a	~10	>10000	200–2000	20–100
Phase & Structure	XRD		Orthorhombic Cu(OH) ₂ , Impurities	Orthorhombic Cu(OH) ₂ , Impurities	Cubic Cu	Monoclinic CuO	Monoclinic CuO
Shape/Morphology	TEM		Irregular	Irregular	Dendritic	Irregular	Irregular
Size in DI H ₂ O	HT-DLS	nm	1397±143	889±156	n/a ^c	1316±176	420±15
Zeta Potential in DI H ₂ O	ZetaPALS	mV	-53.8±0.7	-45.1±0.8	-32.5±2.9	-28.5±0.9	-16.5±0.8
Size in H buffer (w/alginate)	HT-DLS	nm	1172±104	953±88	n/a ^c	1349±62	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	-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	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.