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EFFECTS OF MICRONIZED AND NANO-COPPER AZOLE ON MARINE BENTHIC COMMUNITIES

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Abstract

The widespread use of copper nanomaterials (CuNMs) as antibacterial and antifouling agents in consumer products increases the risk for metal contamination and adverse effects in aquatic environments. Information gaps exist on the potential toxicity of CuNMs in marine environments. We exposed field-collected marine meio- and macrobenthic communities to sediments spiked with micronized copper azole (MCA) using a novel method that brings intact benthic cores into the laboratory and exposes the organisms via surface application of sediments. Treatments included field and laboratory controls, 3 spiked sediments: low-MCA (51.9 mg/kg sediment), high-MCA (519 mg/kg sediment), and CuSO₄ (519 mg/kg sediment). In addition, single-species acute testing was performed with both MCA and CuSO₄. Our results indicate that meio- and macrofaunal assemblages exposed to High-MCA and CuSO₄ treatments differed significantly from both the laboratory control and the low-MCA treatments. Differences in macrofauna were driven by decreases in 3 *Podocopa* ostracod species, the bivalve *Gemma gemma*, and the polychaetes *Exogone verugera* and *Prionospio heterobranchia* relative to the laboratory control. Differences in the meiofaunal community are largely driven by nematodes. The benthic community test results were more sensitive than the single-species test results. Findings of this investigation indicate that CuNMs represent a source of risk to marine benthic communities comparable to that of dissolved Cu.

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This article includes online-only Supplemental Data.

Keywords

Nano-copper; Micronized copper azole; Copper; Benthic community; Sediment; Macrofauna; Meiofauna

Introduction

Copper nanoparticles (CuNPs) are used as an anti-bacterial and anti-fouling agent in numerous commercial and industrial products, including water purifiers, fungicides, algacides, wood and interactive touch surfaces [1]. In the aquatic environment, dissolved Cu has been long used as a biocide in paints to prevent fouling of boats and other submerged surfaces [2]. As a result of the widespread and increasing popularity of CuNPs in many consumer products, over 200 metric tons were estimated to be produced in 2010 [3]. New forms of Cu have been introduced to the aquatic environment increasing the likelihood of contamination and effects in aquatic environments. Copper nanoparticles, nano or micro sized particles of Cu (mixtures of micro- and nano-Cu are classified as nano-copper by the U.S. EPA) have been shown to be effective in extending copper's antimicrobial and conductive properties into traditionally untreated products [4]. While there has been some research performed on pristine CuNP's and pristine nanoparticles in general, there is much less data on nanoparticles embedded in consumer products. This is especially true for the effects of CuNMs on the marine benthos. In this research, we explored the toxicity of micronized copper azole (MCA) which consists of CuNPs (34.5% Cu) with azole (0.62%) used as an anti-fungal agent and insecticide for pressure treating wood. Micronized copper azole (MCA) is a registered CuNP wood treatment used in structural lumber in above-ground, ground contact, and, fresh and marine water applications [5]. Previous characterization studies of MCA indicate that 68% of the copper particles have at least one dimension less than 100 nm [5, 6].

A study on Cu leaching from non-nanomaterial pressure treated wood (e.g., chromated copper arsenate (CCA)) in different salinities indicated that seawater caused more Cu (21%) to be released than did deionized water (14%) [7]. The authors hypothesized that this resulted from the relatively higher levels of ligands in natural seawater which would more effectively bind the dissolved Cu being released relative to de-ionized water [7]. Like many trace metals, dissolved Cu will often become associated with dissolved and suspended particulate organic matter and through diagenetic processes accumulate as particulate Cu in sediments [8]. While relatively little is known about the ecological effects of MCA on marine ecosystems, the accumulation of particulate Cu released from conventional and nano-Cu treated lumber suggests that exposure and adverse effects may occur to marine benthic communities.

In addition, it is yet unknown whether MCA will behave toxicologically differently than dissolved Cu in marine systems. While Cu is an essential micronutrient, high concentrations of bioavailable Cu dissolved from MCA can cause toxic effects on marine organisms [9–11]. Studies on nano-sized particles of copper oxide (CuO) show that nano-CuO is up to 50-fold more toxic in water exposures than bulk CuO to crustaceans [12], algae [13], and

protozoa[14]. A number of studies have also been performed on bivalves. Studies of the clam, *Macoma balthica*, demonstrated bioaccumulation and depuration of Cu is form dependent with nano-CuO uptake intermediate between dissolved and micro-sized Cu [15] but no significant effects on mortality, condition index, or burrowing behavior was observed for up to 35 days of exposure at 200 µg/g. Mussel studies found that the gill and digestive glands are the main target tissues for nano-CuO and that the nano-Cu particles exerted different effects than those of dissolved Cu [16, 17]. In fish, gills appear to be the main target tissue for nanoCu and researchers have determined that nanoparticles can exert a different, if not always more toxic, effect than dissolved Cu [18, 19].

Studies suggest that a decrease in CuNM size (micron- to nano-) increases solubility potential and ultimately, bioavailability [20]. Once deposited within sediments, the bioavailability and toxicity of both dissolved, and nano- to micron-sized particulate forms of Cu will be strongly influenced by the sediment properties [21, 22]. In deeper anoxic sediments, the surface layers of metal-oxide particles, and eventually entire nano-CuO particles, may be converted to Cu sulfides which display considerably lower bioavailability to most benthic invertebrates [23]. In surface sediments, Cu sulfide phases may be readily oxidized [24] and Cu-binding to particulate organic carbon (OC) and iron and manganese oxyhydroxide phases will have a stronger influence on Cu bioavailability [11, 25]. Chemical characterization was performed to ensure the nature of the test material.

Assessing the effects of any contaminant on intact benthic communities has always been problematic. It is impractical to “treat” communities in natural areas with uncontaminated exposure water because of the potential to contaminate adjacent sites. On the other hand, testing single species or even assemblies of a few species together in the laboratory does not duplicate the complexities of community interactions [26]. For this investigation, we used a hybrid approach modified from Chandler et al. (1997) [27] to assess natural community interactions by applying spiked sediments on top of intact benthic cores maintained in the laboratory with natural flowing seawater [27, 28]. This allowed us to start the exposures with a natural community of meio- and macro-benthic organisms. As described in Ho et al. [26], we recognize that communities change even through the simple act of bringing them into the laboratory; however, this approach allows us to maintain some community interactions and complexity, and gain insight into responses of sensitive and tolerant classes of benthic organisms.

Our objectives were to determine the effects of MCA to marine benthic meio- and macro-communities in estuarine environments. We also assessed a relatively novel method for measuring the adverse effects of MCA on intact marine benthic communities using laboratory mesocosms.

Materials and Methods

Single Species Toxicity Testing

MCA and copper sulfate single species sediment toxicity tests were performed as: 1) a range finder for concentrations for the sediment core study, 2) to generate Cu- nanomaterial toxicity information for marine benthic invertebrates, and 3) a comparison point for the

mesocosm studies. Toxicity tests were performed according to standard methods [29, 30]. Briefly, *Ampelisca abdita* (amphipods) were collected from the John H. Chafee National Wildlife Refuge at Pettaquamscutt Cove (Narrow River, Narragansett, RI, USA) (N 41° 26.92', W 71° 27.51'). This National Wildlife Refuge has no known inputs of contaminants and has served as a source of amphipod test organisms for the U.S. EPA for decades. Organisms were brought to the laboratory and sieved to obtain young adults in the size range of 0.71 to 1.0 mm. *Americamysis bahia* (mysid shrimp) were cultured in the laboratory and 48-h-old organisms were used in the study. Copper sulfate and MCA were amended into Long Island Sound (LIS) control sediments to attain nominal concentrations of 200, 500, 1000, 1,500 and 2,000 mg Cu/kg dry weight (DW). These concentrations were chosen based upon literature values bracketing likely effect concentrations [11]. The amended sediments were mixed on a roller mill at 4°C for 16 d. After the 16 d mixing period, 20 g of amended or control sediments and 60 mL of filtered reconstituted 30 ‰ salinity seawater were added to each exposure chamber for each toxicity test replicate. There were three replicates for each concentration. All exposure chambers were allowed to equilibrate for 24 h with gentle aeration prior to introducing ten amphipods and ten mysids to each. During the 7 d exposure period, the amphipods were not fed; mysids were fed brine shrimp *Artemia salina ad lib*. Dissolved oxygen and pH were measured on Day 5 with a Hach HQ30d (Loveland, Colorado, USA) and an Orion pH meter Model 230A (Boston, MA, USA), respectively. All exposure chambers were aerated and the temperature was maintained at 22°C ± 1°C. At test termination, live and dead amphipods and mysids were sieved and counted. Missing organisms were considered mortalities.

Sediment Core Collection and Maintenance

Sediment cores were collected and treated as described in Ho et al. [26]. Briefly, thirty-three sediment cores (15 cm high, 15 cm dia.) were collected at low tide from Narrow River, Narragansett, RI USA (N 41 26.921', W 71 27.515') in April 2013. Three cores (Field Sample) were immediately sacrificed in order to obtain initial meio- and macro-benthos counts and chemistry measures. Thirty remaining cores were brought to the laboratory and maintained with aeration, a 13 h:11 h light:dark cycle and ~17 cm of overlying water replenished with flow-through filtered Narragansett Bay seawater (31‰, 11°C,) (Figure S1.). A live algae mixture (1:1:1 *Isochrysis galbana*, *Dunaliella tertiolecta*, *Tetraselmis suecica*) was added to the cores (~ 1.2 x 10⁸ cells/ core) 3 times a week. Water quality parameters (temperature, pH, dissolved oxygen, and salinity) were measured weekly. Seawater turnover was evaluated from ten cores twice a week and necessary adjustments were made to maintain a flow rate of 12 turnovers/day. Each core's turnover rate was evaluated twice throughout the experiment.

Sediment Core Experimental Design and Treatment

The cores were randomly placed in one of five treatment groups. Each treatment contained six replicate cores: (1) Field Control (FC) - with no sediment surface layer added, (2) Laboratory Control (LC) - with clean reference sediment added, (3) Low-MCA (Low) - 51.9 mg Cu /kg dry sediment, (4) High-MCA (High) - 519 mg Cu/kg dry sediment, and (5) CuSO₄ – dissolved Cu sulfate at the “High-MCA” level (519 mg Cu/kg dry sediment). These nominal concentrations were chosen as they were similar to the results of the single

species LC50 tests with amphipods (Table 1) and are environmentally relevant as levels of Cu in field sediments range between 4 and 1000 mg/kg (http://www.chemet.com/assets/1/6/Copper_and_the_Ocean_Environment.pdf). LIS was used as the spiked sediment layer because it is a chemically well-characterized, non-toxic sediment collected from Long Island Sound (LIS) [31, 32]. In addition to being well characterized, it has had all benthic invertebrates removed through long term storage (over 3 years, in the dark at 4 °C). All MCA and Cu-spiked sediments were mixed by rolling the sediment slurries at 4°C in the dark for a week, then equilibrating at 4°C for one additional week prior to application. The two-week equilibration period for the spiked Cu was considered adequate for achieving suitable partitioning of dissolved Cu to the sediments [33]. Because of the saline buffering system, the pH did not require adjustment to counter the acidity released from the hydrolysis of dissolved Cu. Low- and High-MCA treatments consisted of LIS sediment spiked with a wet MCA formulation (34.5% Cu; 0.62% azoles; and 64.88% inert proprietary ingredients obtained from Philip Evans, University of British Columbia, Vancouver, Canada). We were interested in the effect of the total consumer product with Cu (i.e., MCA) as the primary toxicant in the formulation. We performed a dissolved Cu control (CuSO₄) but were unable to test the other proprietary ingredients separately (e.g., azoles) as we could not extract just the Cu from the MCA. The CuSO₄ spiked-sediment treatment was LIS sediment spiked with Cu sulfate purchased from Sigma-Aldrich (St. Louis, MO, USA). After cores had equilibrated in the laboratory for 24 h, treatment sediments were applied by the addition of a ~1.5 cm sediment layer (420 mL 60:40; sediment:filtered seawater) to the top of the sediment surface in all treatments but the Field Control. This sediment addition was intended to force the migration of the resident benthic sediment community to the oxic treatment layer [27] (Figure S1.).

Two weeks following the application of the treatment layer, a second ~1.5 cm sediment layer of “DNA-free” sediment consisting of unspiked LIS sediment autoclaved (121°C, 10 minutes) in order to denature DNA that may interfere with our genetic analyses [28] was added to the top of all cores except for Field Controls. This second layer was necessary to facilitate the use of a DNA-based molecular diversity technique, as genomic methods cannot distinguish between dead and live DNA. The results of the genomic endpoint will be provided in a companion paper (Chariton et al., unpublished data). This layer again forced the surviving organisms from the treatment layer to migrate to the new oxic DNA-free layer of sediment. The DNA-free layer of sediment was allowed to equilibrate for one week. After the one week equilibration of the DNA-free sediment (three weeks from the addition of the treatment layer- day 21), the exposure was ended and final samples were collected from all cores (Figure S1).

Sampling

Meio- and Macrofauna Sampling—Meio- and macrofauna samples were taken from three cores at the initiation of the study (Field Sample) as well as all cores at the conclusion of the experiment. Sediment for meiofauna samples (2.65 cm³; 1 sample/core) were taken with a 1.5 cm-diameter glass tube, placed 1.5 cm into the sediment surface, and capped to create a vacuum to remove the sediment plug from the core. The remaining top 1.5 cm of sediment was visually identified (lighter in color and a slightly different texture) and

manually removed with a scoop for macrofauna samples and rinsed with seawater through a 500 μm sieve. Organisms were preserved with rose bengal buffered formalin. Macrofauna were identified to the lowest practicable level, generally species. Meiofauna were identified to phylum, class, or order.

Sediment sampling for chemistry: Sediment samples for bulk Cu analysis were taken from two of the three Field Sample cores. Subsamples of the reference LIS, Cu-spiked sediment treatments, and autoclaved LIS were collected for chemical analysis directly before each sediment layer application. Duplicate chemistry samples were taken from approximately half of the cores at each sampling time. Three random cores per treatment (15 samples total) had sediment removed for chemical analysis one day prior to the addition of the autoclaved LIS sediment layer. The same cores were again sampled at the conclusion of the experiment after the top layer of clean 'DNA-free' sediment had been removed for macrofaunal sampling. The top layer of clean 'DNA-free' sediment was determined visually (top oxidized layer was a lighter color and different texture than the lower reduced layer). All sediment samples for bulk Cu analysis (2.65 cm^3) were taken with a 1.5 cm diameter glass tube, placed 1.5 cm into the sediment surface. They were placed into plastic (HDPE) bottles and stored in the dark at 4°C until extraction and analysis.

Duplicate sediment samples for acid-volatile sulfide and simultaneously extractable metals (AVS-SEM) analysis were taken from the Narrow River sediment during core collection, and from the reference LIS and Cu-spiked sediment prior to application to the cores. At the conclusion of the experiment, sediment samples were collected from the Field Control, Lab Control, MCA and CuSO_4 spiked layer of sediment (three cores per treatment from the same cores as were sampled for Cu analysis; 15 samples total) after the DNA-free layer had been removed for macrofauna sampling. The samples (~30 mL) were placed in thick-walled glass jars with as little headspace as possible and stored at -4°C until analysis.

Copper Chemical Analyses

Wet sediment samples were weighed and dried at 60°C overnight to determine wet/dry mass ratios. Aliquots of sediment were weighed (~1-2 g wet weight) into conical vials and digested with 4 mL of aqua regia (3mL HNO_3 , 1 mL of HCl) for 48 h at 20°C. Deionized (DI) water was added and samples were further digested (Digi-Prep Jr (SCP Science, Quebec, Canada)) at 50°C for 2 h, and then 90°C for 4 h. Samples were gravity filtered through Whatman 42 TM filters (ashless 125 mm diameter, CAT No. 1442-125) then brought up to 100mL in volume with DI water. All samples were analyzed for Cu using a Horiba Jobin Yvon Ultima 2 inductively coupled plasma atomic emission spectroscopy (ICP-AES) (Sunnyvale, CA, USA). The ICP-AES was calibrated with five standards over the range of 12-50 mg/L Cu ($r^2 = 0.99$). Analysis of a Standard Reference Material (MESS-3, NRC-CNRC Canada, 33.9 ± 1.6 mg/kg Cu), was performed separately for the mesocosm experiments and the single species toxicity tests as samples were analyzed at different times. Detection limits (DL) were 2 $\mu\text{g/L}$ with a lowest quantifiable limit of 3x to 5x the DL. All Cu concentrations are expressed on a dry weight basis unless otherwise noted.

Acid Volatile Sulfide-Simultaneously Extracted Metal (AVS–SEM) analyzes

Sediment samples were analyzed for acid volatile sulfide (AVS) by a purge and trap method with sulfide specific electrode detection [34–37]. The accuracy of analyses was assessed by use of calibration check samples and spiked blanks; all calibration check results measured between 93% and 129% of the expected values, and recovery of sulfide spikes to blank samples ranged from 72% to 125%. Analysis of simultaneously extracted metals (SEM) in sediment extracts was performed by ICP-AES. Accuracy was assessed by use of calibration verification standards obtained independently from calibration standards; measured concentrations of metals were consistently lower than certificate values, but within the specified QC limits of 10%-15%. Replicate analyses showed less than 3% relative percent difference. Recoveries of standard additions to sample solutions, used to assess matrix effects ranged from 81 to 111%. SEM concentrations are reported as the sum of molar concentrations of Cu, Zn, Pb, Ni and Cd.

Micronized Copper Azole Chemical Characterization

Micronized Cu azole was characterized by the supplier as 34.5% Cu and 0.62% azoles and contained both micro and nano-Cu [38, 39] as well as dissolved Cu owing to dissolution of MCA materials. Previous research shows that approximately 68% of the copper has at least one dimension than 100 nm [6]. Particle size and shape were determined by field emission scanning electron microscopy (SEM) (JEOL JSM-7600F, Tokyo, Japan) conducted in backscatter electron imaging mode. The oxidation state, local bonding environment, and solid phase of Cu technical material were examined using X-ray absorption fine structure (XAFS) and Fourier transformed infrared (FTIR) spectroscopy. The Cu K-edge spectra were collected at beam line 10-BM (Materials Research Collaborative Access Team, Advanced Photon Source, Argonne National Laboratory, Argonne, IL USA). In addition to the MCA, XAFS scans were collected for pure mineral forms of malachite ($\text{Cu}_2\text{CO}_3(\text{OH})_2$), Cu hydroxide ($\text{Cu}(\text{OH})_2$), tenorite (CuO), cuprite (Cu_2O), and pseudomalachite ($\text{Cu}_5(\text{PO}_4)_2(\text{OH})_2$); and aqueous Cu solutions of copper chloride, copper acetate, copper oxalate, and copper EDTA. Absorption spectra were collected at the K-edge energies of 8979 eV and scans were collected from 8779–9979 eV. Data collection was performed in fluorescence mode using a 4-element solid-state Si-detector. The synchrotron was operated at 7.0 GeV at a nominal 100 mA fill current. The energy of a Si (111) double crystal monochromator was calibrated using an elemental Cu foil. All spectra were collected under ambient conditions. A minimum of three scans (and up to 5) were collected for each sample. All spectra were processed using the Athena program in the IFEFFIT software package for analysis of X-ray absorption spectroscopy [40]. Speciation was determined through linear combination fitting (LCF) of the first derivative of the X-ray absorption near edge structure (XANES) spectra following averaging and normalization of spectra. LCF analysis was conducted on both the normalized and first derivative of the normalized data. FTIR spectra of the oven-dried technical material and a malachite reference sample were collected on a Bruker Vertex 80 FTIR (Bruker Optics, Billerica, MA) using a single bounce diamond attenuated total reflectance (ATR) cell. The spectrum presented are the average of 128 scans. The surface chemical composition and surface chemistry of the technical material and a malachite reference were determined by X-ray photoelectron spectroscopy (XPS) (Phi Quanterra II, ULVAC-PHI, Kanagawa, Japan). Broad scans were collected with an Al X-ray

source operating at 15 keV and 50 W with a 200 μm spot size, a 140 eV pass energy, 0.25 eV step size, and a 100 msec count rate. In order to avoid sample beam damage and artificial reduction of Cu(II) to Cu(I or 0), narrow scans for Cu were collected with the X-ray source operating at 15 keV and 12.5 with a 50 mm spot size, 55 eV pass energy a 0.05 eV step size, and a 50 msec count rate. The Cu 2p $3/2$ and $1/2$ energy regions were collected separately to avoid beam damage due to prolonged x-ray exposure. The Cu 2p data presented is the average of 24 individual scans collected from a $1300 \times 1300 \mu\text{m}^2$ area. All scans were collected from a new location within this area.

Statistical analysis

Differences in community indices (e.g., total abundance, taxa richness (S), diversity (H') and evenness (J')) and the abundances of selected meio- and macrofauna taxa were analysed using a balanced, single-factor ANOVA with a post-hoc Student Newman-Kuels (SNK) test. Diversity was calculated as Shannon-Weiner diversity ($-\sum (p_i * \ln(p_i))$). Pielou's evenness $H' / \log(S)$ was also calculated. Variables were transformed as needed to meet normality and homogeneity of variance assumptions [41]. In one case (meiofaunal nematodes), transformation was not adequate to meet the homogeneity of variance assumption so differences among treatments was assessed using Kruskal-Wallis test [40] followed by the Dunnett T3 test [42]. All ANOVAs were performed using IBM SPSS, Version 24.

Multivariate analyses were performed using the Primer 6+ statistical package (Plymouth Marine Laboratory, UK). Ordination of both the meio- and macrobenthic data was performed by non-metric multidimensional scaling (nMDS). The first two nMDS axes were used for visualization. Data was square root transformed prior to computation, with distances between samples measured by Bray-Curtis similarity coefficients [41]. For both the meio- and macrofaunal data, statistical differences among treatments were tested by permutational multivariate analysis of variance (PERMANOVA) on the transformed data using Bray-Curtis dissimilarities between samples [42]. Differences among treatments ($p < 0.05$) were identified by pairwise *a posteriori* tests based on 9,999 random permutations.

Single species mortality data was summarized as point estimates (e.g., LC_{50} s) generated using the U.S. EPA Toxicity Relationship Analysis Program (TRAP) Version 1.30a. To calculate the point estimates, the TRAP program applies a maximum-likelihood tolerance distribution model using a triangular distribution to calculate the specific LC_{50} and standard deviation values. (https://archive.epa.gov/med/med_archive_03/web/html/trap.html).

Comparison of copper concentrations and LC_{50} s throughout the study were performed using two tailed, equal variance t-tests (ExcelTM MSO 2016). Significance values are set at $p < 0.05$ unless otherwise stated.

Results and Discussion

Experimental Parameters Measured in Cores

All weekly measured parameters in sediment cores were relatively stable and remained within acceptable experimental boundaries (Table S2). Mean flow rate was approximately 12.1 ± 3.9 turnovers/day.

Micronized Copper Azole Characterization

Scanning electron microscopy images for the MCA indicated the particle size and shape varied considerably (Figure 1a–c). Particle size of the copper ranged from less than 10 nm to approximately 1 μm . The broad particle size distribution made it difficult to calculate a meaningful average particle size and aspect ratio (L/W). In general, larger particles exhibited a greater aspect ratio compared to smaller particles. Additional characterization of MCA can be found in Platten et al., 2016 and US EPA (2014). Previous research examining the particle size and shape of the technical MCA material and MCA dispersed in treated lumber have reported similar SEM and transmission electron microscope results [6, 43–47]. The spectral data indicate that the MCA consisted primarily of Cu carbonate. Comparison of the EXAFS spectra of MCA and reagent grade malachite (copper carbonate) showed no difference indicating the primary phase present was Cu carbonate (Figure 2a). Comparison of the normalized and first derivative of the XANES spectra showed some differences (Figure 2b and c); linear combination fitting of the normalized and first derivative indicated that between 10 and 20% of the Cu present was in an dissolved aqueous form. The lack of any indication of an aqueous species in the chi distribution function is likely due to the limited scattering that would occur beyond the immediate coordination environment of aqueous Cu. FTIR data between 4000 and 2500 cm^{-1} for MCA and Cu carbonate indicated small differences between the two spectra, namely the absorption bands present between 3000 and 2800 cm^{-1} indicative of aliphatic C-H stretches (Figure 3a). Between 1800 and 400 cm^{-1} there were no visible differences between the two spectra indicating again that Cu carbonate was the primary Cu species (Figure 3b). The XPS broad scan spectra and peak identification also indicated differences between the MCA and pure malachite. In addition to Cu, O, and C, the malachite reference scan showed traces of Si, likely from quartz present in the sample (Figure 4). The MCA scan revealed the presence of Na, Cl, and N in addition to Cu, O, and C. There was also a noticeable increase in the concentration of C on the MCA surface compared to the malachite (Table S1). The presence of N and Cl along with the increase in C was related to the azole fungicide component used in the treatment formulation. Of interest was whether the presence of the azole compound used in the MCA altered the surface chemistry of the Cu carbonate, specifically the formation of Cu(I) sites at the crystal surface. Narrow scans of the Cu 2p $3/2$ and $1/2$ doublet were identical indicating there was no substantial reduction of Cu at the crystal surface (Figure S2).

Copper Chemistry

Duplicate chemical analysis from 22 cores taken throughout the experiment indicated a 10% mean chemical standard error. Standard deviations for Cu concentrations for each treatment were between 1.90 and 29.6 mg/kg. Copper concentrations in the Field Control (Narrow River) and reference sediment (LIS) (8.11 ± 0.16 to 30.2 ± 1.2 mg/kg sediment, respectively), were within the range of background concentrations reported for most uncontaminated sediments [48]. Results from Standard Reference Material (see Methods) analyzes was $71.5\% \pm 2.9\%$ for mesocosm samples, and $76.6\% \pm 0.09\%$ for single species testing. Although the % recovery is relatively low, duplicate analyzed mesocosm samples indicate that the chemistry extraction methods were consistent within the experiment and results were compared between and among samples for mesocosm comparisons.

Copper concentrations were significantly greater in the High-MCA and CuSO₄ spiked sediment treatments than in the Field Control, Laboratory Control and Low-MCA treatments at both the beginning and the end of the two week exposure (Figure 5, Table S3). To determine the exposure concentration of the organisms, we used the mean of the concentrations at the beginning and the end of the two week exposure period. There were no significant differences in total mean Cu exposure concentrations between the High-MCA and CuSO₄ treatments, nor was there any significant difference in exposure concentrations between the Laboratory Control and Low-MCA treatments. Copper concentrations measured in sediments of the Low-MCA, High-MCA and CuSO₄ treatments at the time of application of the treatment layer were 69.2, 427, and 262 mg/kg respectively (Figure 5). These measured concentrations were 50 to 133% of the nominal concentration. At the end of the two-week exposure, Cu concentrations for Low-MCA, High-MCA and CuSO₄ were 31.9, 156, and 208 mg/kg, respectively. Copper concentrations in the spiked surface sediments (top 1.5 cm) decreased by 54 and 64% in the Low and High MCA treatments, respectively, during the 2-week exposure period but only 20% in the CuSO₄ treatment (Figure 5, Table S3). These changes are most likely due to mixing into the deeper layers by active benthic fauna, loss of particles in the flow-through exposure system, and dissolution of Cu or mobilization of particulate Cu into the overlying water. We speculate that the larger decrease in sediment Cu concentrations between the beginning and the end of the study for the particulate MCA treatment is because the Cu in the MCA treatment was CuCO₃, which is a very insoluble (solubility limit- 1.46×10^{-4} g/100g water) and inert form of Cu. These relatively small (10-1000 nm), insoluble, high Cu concentration particles can easily be suspended in the water column. Observed bioturbation in the cores caused a moderate amount of sediment resuspension which could account for the loss of Cu particles in the MCA treatments over the course of two weeks resulting in a greater overall loss of Cu in the MCA treatment. Conversely, Cu in the CuSO₄ treatment was dissolved readily in seawater (solubility limit 32g/100g water), and is a very active form of Cu which rapidly complexes and binds to organic carbon in the sediment. This bound organic carbon-Cu complex would remain in the sediment core system. Determining the fate of Cu in this system was beyond the scope of this study but to truly test this hypothesis, the overlying water would have to be monitored using XAS, or a similar technique to discern Cu speciation. Additionally, Cu concentrations should be measured vertically through the core to determine transport throughout the core.

Measured Cu concentrations in the overlying 1.5 cm DNA-free sediment layer after the one week upward migration period were 22.8, 35.7 and 55.0 mg/kg for the Low-MCA, High-MCA and CuSO₄ treatments, respectively (Table S3). Copper concentrations in the DNA-free layers for the Low-MCA were similar to the Laboratory Control while those in the High-MCA and CuSO₄ treatments were 1.5 - 2.2 times higher. The higher concentrations were most likely due to sediment mixing by benthic fauna causing Cu to migrate up into the oxidized sediment layers.

The results of AVS-SEM analysis of the sediments provide greater insight into the toxic action of Cu in the sediments (Table S4). The Field- and Laboratory Control sediments had low concentrations of both AVS and SEM initially; therefore, the SEM-AVS difference was not substantially greater than 0, which indicates there was enough AVS to bind SEM-Cu in

both sediments. AVS increased in both control sediments during the two-week exposure, while the SEM concentrations remained the same. In the spiked sediments, however, the amount of added Cu exceeded the amount of AVS initially present, and SEM concentrations reflected the excess metal. Large values of excess metals (SEM-AVS) in the Low and High-MCA and CuSO₄ treatments (~2 to 8 $\mu\text{mole/g dry}$) implies non-sulfur bound copper was likely present in the interstitial waters of all three spiked sediment treatments and thus could diffuse into overlying waters. As stated above, ~45% of the Cu was lost from each of the treatments during the two week exposure. The loss of excess Cu and the development of additional AVS in the Low-MCA treatment over the course of the exposure resulted in the binding of the excess Cu (SEM-AVS <0). In the High-MCA and CuSO₄ treatments, however, loss of Cu to overlying waters or to burial deeper in the sediments was not sufficient to eliminate excess Cu (SEM-AVS >0) throughout the exposure period. Furthermore, the Cu concentrations and trends were almost the same in the CuSO₄ and High-MCA treatments. Thus, it could be expected that toxic effects would be seen in the High-MCA and CuSO₄ treatments, with the possibility of a lesser effect in the Low-MCA treatment due to the initial excess Cu exposure. These chemistry results support both the macro- and meio-benthic community results (below).

Single Species Toxicity Testing

Results of single species sediment toxicity testing with amphipods and mysids for CuSO₄ and MCA in this experiment ranged from LC50s of 325 to > 2,400 mg Cu/ kg (Table 1). These numbers may slightly overestimate the toxicity as our SRM recovery was 76.6% and not 80% as is the generally acceptable recovery rate. Literature results for LC50s from dissolved Cu spiked sediment bioassays have a fairly large range. Results range from 426 mg Cu/kg for the midge *Chironomus riparius* [49], to a no-effect concentration of 1,300 mg Cu/kg for the polychaetes *Nephtys australiensis* and *Australonereis ehlersi*, and the bivalve *Mysella anomala*; however the same concentration (1,300 mg Cu/kg) elicited 100% mortality from the bivalve *Tellina deltoidalis* and 77% mortality from the bivalve *Soletellina alba* [50]. Other studies report LC50s in Cu spiked sediment assays from 516 to > 1000 mg Cu/ kg for the worm *Tubifex tubifex* [51]. These experiments were performed under various exposure conditions including different times, types of sediments, levels of sediment organic carbon and other exposure factors so it is not surprising that results vary, however, our results are within a two-fold factor of these concentrations.

MCA is comprised of 35% Cu micro-and nano-particles and 0.65% azole or tebuconazole. In water-only exposures, tebuconazole's LC50s range from 237 – 10,000 $\mu\text{g/L}$. A 2013 ECHA report states that most acute toxicity tests for fish, crustaceans and algae, are above 1,000 $\mu\text{g/L}$ with two exceptions, the estuarine mysid shrimp *Americamysis bahia* (460 $\mu\text{g/L}$) and *Lemna gibba*, the freshwater aquatic plant (237 $\mu\text{g/L}$). (<https://echa.europa.eu/documents/10162/5be48e81-985c-49dc-8e45-159a60b2859b>). Soil exposures of tebuconazole to earthworms indicate the LC50 to be approximately 1,035 mg/kg [52]. By contrast, the median marine Cu LC50s for the 10 most sensitive species is 8.25 $\mu\text{g/l}$ and the marine final acute value for Cu is 2 $\mu\text{g/l}$ (<https://www.epa.gov/sites/production/files/2017-02/documents/copper-estuarine-marine-draft-document.pdf>). These levels indicate that Cu is generally more toxic than tebuconazole.

Based upon the reported percentage of tebuconazole in MCA, estimates of the concentration of tebuconazole in the study sediments ranged from 0.94 to 5.4 mg/Kg. Using these whole sediment concentrations (C_p) ($\mu\text{g/Kg}$), interstitial water concentrations (C_{IW}) ($\mu\text{g/L}$) of the azole were calculated using equilibrium partitioning [53] with the following equation:

$$C_{IW} = \frac{C_p}{K_{OC} + f_{OC}} \quad [1]$$

Where, K_{OC} is the organic carbon normalized partition coefficient (L/Kg OC) and f_{OC} is the fraction organic carbon of the study sediment (Kg OC/Kg). The f_{OC} for the study sediment is 0.021 [31] and the K_{OC} for the azole was calculated using this equation [53]:

$$\log K_{OC} = 0.00028 + 0.983 \log K_{OW} \quad [2]$$

Where, the $\log K_{OW}$ for the azole is 3.70 (<https://pubchem.ncbi.nlm.nih.gov/compound/Tebuconazole#section=Top>, <https://echa.europa.eu/documents/10162/5be48e81-985c-49dc-8e45-159a60b2859b>, http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Evaluation94/tebucona.pdf) resulting in a $\log K_{OC}$ of 3.64. Based on Equation 2, the concentrations of interstitial water azole ranged from 11 to 59 $\mu\text{g/L}$. Several studies have shown that the interstitial water concentration of a given contaminant is a good surrogate for the whole sediment exposure [31, 53]. These interstitial water concentrations are below the known toxic effects concentrations for azole to marine invertebrates (i.e., 237 – 10,000 $\mu\text{g/L}$) and certainly below the soil effects concentration (1,035 mg/kg). Concentrations of azole in the water column above the cores would be much less than the interstitial water concentrations as a result of the spiked sediment exposure and flow-through conditions in the exposure systems. Consequently, while the fungicide tebuconazole was likely present in the interstitial and overlying water, and we cannot rule out any contribution from the azole, we do not expect that the azole, which was a very small component of the MCA, would contribute substantially to the toxicity observed relative to the influence of the more toxic Cu that was present at higher concentrations.

Our toxicity test results for individual species also indicate that MCA did not increase toxicity relative to the CuSO_4 spiked sediments for either organism (Table 1). In fact, MCA (LC 50 > 2, 400 and 526 mg Cu/ kg for mysids and amphipods, respectively) appeared to be less toxic to marine invertebrates than the CuSO_4 spiked sediments (LC50s: 708 and 325 mg/kg for mysids and amphipods, respectively).

Comparing the sensitivity of the two species, the amphipod, *A. abdita*, is more sensitive to MCA and CuSO_4 than the mysid, *A. bahia*, in a sediment exposure. This may be due to the different exposure routes of the two organisms: the mysid is an epibenthic carnivore, and was fed non-contaminated food and therefore it received a large part of its exposure from the overlying water, whereas the amphipod is a filter feeder with closer proximity to the sediment and most likely ingested sediment particles that may have contained adsorbed dissolved Cu as well as nanocopper. Also, the amphipod forms a burrow in the sediment and

consequently received more of a sediment/porewater exposure. These results, in addition to no increased toxicity due to the fungicide in the MCA in the mesocosm exposures implies the fungicide tebuconazole and other proprietary inert ingredients may have limited acute toxicity to marine invertebrates; however, this experiment was not designed to estimate an effect threshold for the fungicide.

Benthic Community Results

Macrofauna—The most abundant macrofauna in the Field and Laboratory Controls were polychaetes (31-33%), bivalves (26-33%) and ostracods (29%) (Figure 6). In the High-MCA and the CuSO₄ treatments, the relative percentage of ostracods increased (51-60%) while polychaetes (23-27%) and bivalves (10-14%) decreased. No significant changes in the relative percentages of maxillopoda, malacostraca, gastropoda or nemertea were observed among the LC, Low MCA, High MCA and CuSO₄ treatments. The PERMANOVA results of macrofaunal community analysis indicated differences between the Low and High-MCA treatments, as well as differences between the LC and the High-MCA treatment, and the LC and the CuSO₄ treatments (Figure 7). No differences were detected between the High-MCA and the CuSO₄ treatments ($p = 0.68$). Differences between the LC and high Cu treatments were driven by a relative increase in the three Podocopa ostracods, and decreases in the bivalve *Gemma gemma*, and the polychaetes *Exogone verugera* and *Prionospio heterobranchia*. Univariate measures were less sensitive than macrofaunal assemblage data with only the number of individuals (N) supporting the PERMANOVA results indicating a difference between the LC and both the High-MCA and CuSO₄ treatments. There appears to be no patterns in the feeding habits of the organisms with the greatest differences; *G. gemma* is a filter feeder, *E. verugera* is generally known as an omnivore (<http://eol.org/pages/406981/overview>), *P. heterobranchia* is considered a detritivore, and uses a special appendage to gather particles [54], and ostracods are often considered to be filter feeders or detritivores. The feeding strategy of these particular ostracods is unknown.

Meiofauna

Nematodes (52-61%) were the dominant meiofaunal class in the Laboratory and Field Control treatments (Figure 8). Meiofauna PERMANOVA results (Figure 9) indicated that the LC was different from the Low-MCA ($p = 0.04$), but not the High-MCA ($p = 0.09$) or the CuSO₄ treatments ($p = 0.08$). No differences were detected between the High-MCA and the CuSO₄ treatments ($p = 0.678$). The meiofauna univariate measures were also not sensitive, with only evenness (J') indicating a difference between the LC and High-MCA. No significant changes in the relative percentages of polychaetes, harpacticoids and ostracods were observed among the LC, Low MCA, High MCA and CuSO₄ treatments. Differences in the meiofaunal community seem to be largely driven by decreases in nematodes (Figure 8). Nematodes are often considered predators and their diet includes diatoms, algae, and bacteria. Because little information is known about feeding habits of many meiofauna and feeding modes within classes vary widely we cannot hypothesize about how the ecological niche of specific species may influence the effects of Cu nanoparticles or CuSO₄ spiked sediments. We suspect that because the meiofauna were sorted at a relatively high taxonomic level (class), our measures were less likely to be able to detect any differences among treatments.

Because we used two concentrations in our mesocosm investigations, we cannot determine a benthic community EC50 concentration for either MCA or CuSO₄ spiked sediments; however, we hypothesize that the concentration at which effects are most likely to occur is between the Low-MCA (50.5 mg Cu/ kg) and the High-MCA (291 mg Cu/ kg). The mean concentration between the High and Low-MCA is about 170 mg Cu/ kg, which is within the reported range of LC50s for Cu toxicity to sediment organisms [11, 55]. Gardham et al. [56] also found an effect level between 100 and 400 mg particulate Cu/kg in benthic mesocosms, and Olsgard [57] found an effect on re-colonization of marine benthic communities in sediments spiked with 300 mg Cu/kg. These effect concentrations are within 2-fold of our estimated benthic community toxicity threshold of 170 mg Cu/kg DW. The community toxicity threshold is lower than the EC50s for CuSO₄ and MCA estimated for *A. abdita* and *A. bahia* based on individual species toxicity tests (Table 1). The relatively high LC50s for these two organisms exposed to CuSO₄ and MCA spiked into sediments imply that the community endpoint may be more sensitive than that of the individual marine test species. There were several differences between the tests including exposure time (e.g., seven days for single species tests compared to 21 days for community tests), and the experimental design (e.g., single species compared to multiple species testing, application of toxicant in layered sediment compared to application of toxicant in bedded sediment). However, all of these exposures were performed in LIS sediment, which suggests similar exposure conditions and highlights the greater sensitivity of the benthic community endpoints.

Like the literature on Cu nanoparticles, the broader literature on benthic community effects from nanoparticles, has widely varying effect concentrations depending upon the type of nanoparticle and the exposure test system [58–63]. Standardized test protocols for nanomaterials are in their infancy [64] and standardized test systems to evaluate benthic communities vary widely with type of exposure, dosing parameters, and exposure period [28, 65–70]. Standardization of test procedures is critical for better understanding the fate and effects of nanoparticles on benthic communities.

Differences between Laboratory and Field Controls.

Bringing benthic communities into the laboratory and holding them under flow-through conditions for 4 weeks (Field Control) changed the communities drastically from the meiofaunal communities in the field at the time of collection (Field Sample) ($p < 0.01$) (Figure 8). Similarities were noted in other studies [28] and illustrate the complexity and difficulty in maintaining natural systems in a laboratory setting. In addition to the differences arising from the addition of MCA or CuSO₄ to the sediments, there was a difference in benthic community composition between Field and Laboratory Controls ($p < 0.01$) for both meio- and macrofauna. The addition of two 1.5-cm layers of sediment in the LC changed the system significantly, yet we could still see the adverse effects of MCA and dissolved Cu. These effects included changes in species composition, abundance and evenness among certain classes in the benthic community (Figure 6–9). While it is clearly difficult to mimic natural communities in their entirety, this system of considering community interactions rather than single species toxicity testing moves us towards a more ecological interpretation of the effects of MCA and dissolved Cu. Refinements to our experimental setup would be to decrease the number of sediment layers added and therefore

decrease changes in the benthic community from suffocation rather than toxicity. One way to do this would be to develop a method to use mRNA to identify organisms rather than DNA. mRNA is only found in living organisms and therefore would decrease the need to add the DNA-free layer of sediment. Another refinement would be to evaluate just the meiofauna. This would allow us to collect and maintain smaller and, therefore, more cores which could either increase our sample size and allow the testing of more treatments (toxicant concentrations).

Difference Between Dissolved Cu and MCA Spiked Sediments

No differences ($p=0.68$) were detected between benthic communities exposed to the mean exposure concentrations in the High-MCA (291 mg Cu/kg) and the CuSO₄ (235 mg Cu/kg) spiked sediments. This may be because the MCA treatments exercised most of its toxic effect via dissolved Cu released from the nanoparticles, or that dissolved Cu from MCA along with any toxicity from azole or inert ingredients in the MCA are equally toxic to the CuSO₄, or it may indicate that we needed to identify organisms at a more detailed taxonomic level, and have more specific and sensitive endpoints in this benthic community assay. Bivalves are known to be particularly sensitive to Cu [11, 55]. It has been speculated that CuNP caused toxicity to gills, possibly by lodging within the gill structure of mussels *Mytilus galloprovincialis* and *Mytilus edulis* and causing oxidative stress while dissolved Cu does not [16]. This toxic action is not specific to CuNP as Ag nanoparticles also caused toxicity to gill structures in bivalves [71]. Other filter feeding or particle gathering apparatuses may be susceptible to nanoparticles in the same manner as gills, as nanoparticles may be lodged within these structures and continue to release dissolved Cu close to the site of injury or cause mechanical particulate injury at the site where it is lodged. These differences indicate that CuNPs and dissolved Cu may have different exposure routes and toxic modes of action. Differences between dissolved Cu and CuNP toxicity were also seen in zebrafish [19, 72], and researchers demonstrated that the differences in CuNP and dissolved Cu toxicity were largely due to morphological effects and physiological changes as indicated by gene patterns [73]. Differences in toxicity may also occur due to intake via dissolved and particulate exposure routes [74]. However, other researchers have found that while the uptake and depuration of Cu is size dependent, there were no measurable differences in the effects of Cu (e.g., mortality, condition index or burrowing behavior) for any of the Cu forms (dissolved, nano- and micro-) at measured sediment concentrations (150-200 mg/kg) during a 35 d exposure to the deposit-feeding clam, *Macoma balthica* [15]. In addition, researchers have shown that terrestrial isopods (*Porcellio scaber*) showed similar patterns of assimilated and depurated amounts of Cu, regardless of whether the animals were fed with CuNPs or dissolved Cu spiked food [75]. Targeted research on the toxicological and biochemical effects of bioavailable particulate nano and dissolved Cu are needed to develop a general unifying principal that explains the differences in effects.

Summary

Results of this investigation indicate that the modified benthic mesocosm approach is a sensitive method for testing consumer products containing nanomaterials, and that MCA represents a source of risk to marine benthic communities comparable to dissolved Cu released from CuSO₄. Sediment quality guideline values are frequently used for screening

purposes within early stages of sediments quality assessment frameworks [76, 77]. For marine sediments, the guideline values proposed for total Cu have ranged over approximately one order of magnitude (e.g., 34 to 270 mg/kg), with bioavailability-modifying factors such as AVS and organic carbon having a strong influence on this range [11, 21, 23]. The present study indicates that this guideline value range should also be suitable for screening risk posed by sediments contaminated with MCA. Discrepancies and unknowns among the existing literature, and lack of a paradigm to fully explain the effects of specifically MCA and generally other CuNP in marine systems suggest the need for targeted uptake and mode of action studies as well as a better understanding of benthic community interactions when exposed to nanomaterial stress. Further research is also required of the transformations that CuNP may undergo once present in sediments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1a-c.

FESEM micrographs of the air dried MCA magnified a) 20,000 times, b) 50,000 times and c) 100,000 times. Particle size of the malachite crystals ranged from approximately 1 micron to less than 10 nm.

Figure 2a-c.

X-ray absorption fine structure (XAFS) spectra of the Micronized Copper Azole (MCA): a) k^3 weighted XAFS spectra of MCA, reagent grade malachite ($\text{Cu}_2\text{CO}_3(\text{OH})_2$) and $\text{CuCl}_2(\text{aq})$; b) normalized X-ray absorption near edge structure (XANES) spectra of MCA, reagent grade malachite and $\text{CuCl}_2(\text{aq})$. Solid line with points overlaid is the result of the linear combination fitting (LCF) to MCA.; and c) first derivative of the normalized XANES spectra of MCA, reagent grade malachite and $\text{CuCl}_2(\text{aq})$. Solid line with points overlaid is the result of the LCF to MCA.

Figure 4.
XPS survey scans and elemental peak identification of MCA and malachite ($\text{Cu}_2\text{CO}_3(\text{OH})_2$).

Figure 5.

Copper concentrations measured in sediments at the start and end of the two-week exposure period. Bar height represents the mean concentration, error bars = sd, n=3.

Figure 6.

Macrofauna abundance in the different sediment treatments. Other phyla present but not abundant enough to discern in the figure include Clitellata (3, 2, 4, 1, 0 individuals in the Field Control, Laboratory Control, Low MCA, High MCA, and CuSO₄, respectively) and Anthozoa and Platyhelminths which had either 0 or 1 individuals in a treatment. * Indicates a significant proportional difference from Laboratory Control treatment ($\alpha=0.05$).

Transform: Square root
 Resemblance: S17 Bray Curtis similarity

Figure 7.

Nonmetric multidimensional scaling ordination plot for macrofaunal communities sampled from the four treatments and field controls

Permanova results indicate a difference ($p < 0.05$) between: the Laboratory Control (●) and the High-MCA Copper (△), Lab Control (●) and the CuSO₄ (■), the Low-MCA Copper (▼) and High-MCA Copper (△).

Figure 8. Meiofauna abundance of different treatments. * Indicates a significant proportional difference from Lab Control treatment ($\alpha = 0.05$).

Transform: Square root

Resemblance: S17 Bray Curtis similarity

Figure 9.

Nonmetric multidimensional scaling ordination plot for meiofaunal communities sampled from the four treatments and field controls. Permanova results indicate a difference ($p < 0.05$) between: the Lab Control (●) and the High-MCA Copper (△), Lab Control (●) and the CuSO₄ (■), the Low-MCA Copper (▼) and High-MCA Copper (△).

Table 1.

Mean LC₅₀ values (mg Cu/ kg DW) for the mysid *Americamysis bahia* and amphipod *Ampelisca abdita* exposed to Micronized Copper Azole (MCA) and CuSO₄.

LC ₅₀ Values of Copper and MCA Toxicity for <i>A. bahia</i> and <i>A. abdita</i>		
Spiked copper form	<i>Americamysis bahia</i> LC 50 (SD)	<i>Ampelisca abdita</i> LC 50 (SD)
CuSO ₄	708 (126) *, **	325(114) **
Micronized Copper Azole	> 2, 400 *, **	526 (299) **

* indicates a significant difference in LC₅₀ between the two compounds, CuSO₄ and MCA within the same species.

** indicates the two species responded differently to the test compound