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Assessing the Release of Copper from Nanocopper-treated and Conventional Copper-treated Lumber into Marine Waters: (2) Forms and Bioavailability

Ashley N Parks^{1,2}, Mark G Cantwell³, David R Katz³, Michaela A Cashman⁴, Todd P Luxton⁵, Justin G Clar^{6,7}, Monique M Perron⁸, Lisa Portis⁹, Kay T Ho³, and Robert M Burgess^{3,*}

¹National Research Council c/o U.S. EPA, ORD/NHEERL, Atlantic Ecology Division, Narragansett, RI, USA

³U.S. EPA, ORD/NHEERL, Atlantic Ecology Division, Narragansett, RI, USA

⁴University of Rhode Island, Department of Geosciences, Kingston, RI, USA

⁵U.S. EPA, ORD/NRMRL, Land and Materials Management Division, Cincinnati, OH, USA

⁶Oak Ridge Institute for Science and Education c/o U.S. EPA, ORD/NRMRL/LMMD, Cincinnati, OH, USA

⁸US EPA, OCSPP, Office of Pesticides Programs, Washington, DC, USA

⁹Lifespan Ambulatory Care Center, East Greenwich, RI USA

Abstract

One application of nanocopper is as a wood-preserving pesticide in pressure treated lumber. Recent research has shown that pressure treated lumber amended with micronized copper azole (MCA), which contains nano-sized copper, releases copper under estuarine and marine conditions. The form of copper released (i.e., ionic, nanocopper (1 to 100 nm in size)) is not fully understood but will affect the bioavailability and toxicity of the metal. In this investigation, multiple lines of evidence, including size fractionation, ion selective electrode (ISE) electrochemistry, comparative toxicity, and copper speciation were used to determine the form of copper released from lumber blocks and sawdust. Results of all lines of evidence supported the hypothesis that ionic copper was released from MCA lumber and sawdust with little evidence that nanocopper was released. For example, copper concentrations in size fractionations of lumber block aqueous leachates including unfiltered, 0.1 μ m and 3 kiloDaltons (kDa) were not significantly different suggesting the form of copper released was in the size range operationally-defined as dissolved. These results correlated

*To whom correspondence may be addressed: burgess.robert@epa.gov.

²Current affiliation: Southern California Coastal Water Research Project, Costa Mesa, CA, USA

⁷Current affiliation: Department of Chemistry, Elon University, Elon, NC, USA

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DATA ACCESSIBILITY

with the ISE data which detects only ionic copper. In addition, comparative toxicity testing resulted in a narrow range of LC₅₀s (i.e., 221 to 257 µg/L) for MCA lumber blocks and CuSO₄. This investigation concluded ionic copper was released from the nanocopper pressure treated lumber under estuarine and marine conditions.

Keywords

Nanomaterials; copper nanomaterials; pressure treated lumber; ionic; speciation; bioavailability

Introduction

Amongst the most commonly used engineered nanomaterials (ENMs) in consumer products are the metallic ENMs which include titanium, silver, and copper. Copper ENMs are currently used as pesticides, and as additives in lubricants, polymers, and inks (Wang et al. 2005, Tang et al. 2010, Chapman et al. 2013, Anyaogu et al. 2008, Matsunaga et al. 2011, Evans et al. 2008, Platten et al. 2016, Adeleye et al. 2014, 2016, Conway et al. 2015, Keller et al. 2017). Relative to the use of copper ENMs as a pesticide, there is particular interest in their application in or near aquatic environments, including marine and estuarine systems, as an anti-fouling wood preservative in pressure treated lumber. Pressure treated lumber is often used in marine settings because of the harsh conditions including the presence of wood boring organisms and growth of dense microbial mats. Because copper is notoriously toxic to marine organisms (U.S. EPA 2016), it is an effective additive to protect lumber used in marine environments. The fate and effects of some ENMs in the marine environment have been investigated including silver, titanium, single-walled carbon nanotubes, and quantum dots (Parks et al. 2013, Baker et al. 2014, Parks et al. 2014a,b, Wang et al. 2014a,b, Rocha et al. 2015, Canesi and Corsi 2016, Minetto et al. 2016, Xiao et al. 2017). However, very little data is available on the fate and effects of copper ENMs in the marine environment. Recently, Parks et al. (*resubmitted*) reported the release and release rates of total copper from pressure treated lumber amended with nanocopper in estuarine and marine waters. Specifically, in a functional assay system designed to examine the liberation of nanomaterials from consumer products, copper released from lumber treated with micronized copper azole (MCA) approached 2 mg Cu/L/d under estuarine conditions (i.e., 1‰) (Parks et al. *resubmitted*). The MCA treatment is known to contain copper carbonate in the nano size range (McIntyre 2010, Platten et al. 2015). However, the form of copper released into the estuarine and marine waters (e.g., ionic, nanocopper, organo-copper complexes, or all) was not determined in that investigation (i.e., Parks et al. *resubmitted*). Consequently, the bioavailability of the copper and potential for adverse effects to estuarine and marine organisms remains uncertain.

The geochemistry and toxicity of copper to aquatic organisms has been known for decades (Forstner and Wittmann 1981, Flemming and Trevors 1989, U.S. EPA 2007, U.S. EPA 2016). Like many divalent transition metals, copper, when present in the aquatic environment, is distributed between different environmental phases including ionic, dissolved inorganic ligands, particulate, and colloidal/dissolved organic carbon (COC/DOC) (Forstner and Wittmann 1981, Chester and Jickells 2012). The toxicity of copper most often

correlates with the ionic form (i.e., Cu^{2+}) of the metal (Sunda and Guillard 1976, Campbell 1995), and when copper is strongly associated with sediment particles and different forms of organic carbon like COC or DOC, the metal's bioavailability, and consequently, its toxicity decreases (Kogut and Voelker 2001, U.S. EPA 2016). The toxicity of the nanoform of copper associated with ENMs is far less well understood than ionic copper toxicity. However, in the last decade, the toxicity of several forms of nanocopper including copper carbonate (CuCO_3) and copper oxide (CuO) has been reported for a range of aquatic organisms. For example, the toxicity of nanocopper to bacteria (*Escherichia coli* and *Vibrio fischeri*), fungi (*Rhodonía placenta*), microalgae (*Pseudokirchneriella subcapitata*), duckweed (*Landoltia punctata*), daphnids (*Daphnia magna*), shrimp (*Thamnocephalus platyurus*), zebra fish (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss*), and marine benthic communities has been documented (Griffitt et al. 2007, Heinlaan et al. 2008, Arouja et al. 2009, Gunawan et al. 2011, Shi et al. 2011, Shaw et al. 2012, Civardi et al. 2015, Ho et al. 2018). While still an area of active research, many of these studies, including the evaluations of the toxicity of copper carbonate nanoparticles (i.e., Civardi et al. 2015), the type of nanocopper present in MCA, concluded that the ionic form of copper, not the nanoform, was responsible for the observed adverse biological effects. If this explanation of the toxicity associated with copper ENMs proves accurate and wide-spread, it would be fortuitous, as our understanding of the toxicity of ionic copper is relatively sophisticated and protective measures could be incorporated into existing controls to reduce the adverse effects of this source of copper to the environment (U.S. EPA 2007, 2016). However, there are several studies demonstrating the appearance of substantial toxicity being caused by the nanoform of copper (e.g., Shi et al. 2011, Bielmyer-Fraser 2014, Gonçalves et al. 2017).

As noted above, in Parks et al. earlier investigation, we found pressure treated lumber amended with nanocopper released copper into aquatic systems. That study generated sufficient data to justify a paper on copper release and release rates (Parks et al. *resubmitted*). However, the form of copper released (e.g., ionic versus nanocopper) was not reported in that paper and is addressed here. Given the uncertainties associated with nanocopper versus ionic copper toxicity, it is critical to better understand the form of copper released from MCA treated lumber. Consequently, in this study, based on the findings of Parks et al. (*resubmitted*) and leachate samples from that work, we determined the form and bioavailability of the copper released from MCA treated lumber. Specifically, several lines of evidence were applied including (i) filtration-based fractionation of aqueous leachates to assess the size ranges of copper present, (ii) ion selective electrochemistry to evaluate ionic copper concentrations, (iii) comparative toxicity testing of various forms of copper with the marine mysid *Americamysis bahia*, and (iv) x-ray absorption fine structure (XAFS) analysis to measure the speciation of copper before and after the leachate studies. The types of treatments discussed in this paper are rated primarily for terrestrial use; however, the data collected are intended to provide information about potential worst-case scenarios in which prolonged flooding, unintended home or commercial use, or improper disposal, result in the lumber's presence in the marine environment.

MATERIALS AND METHODS

Pressure treated lumber and parent copper materials

Three types of lumber treated with copper and available commercially from local franchises of national United States distributors (e.g., Ace, Home Depot, Lowes) were evaluated in this study: MCA, chromated copper arsenate (CCA), and alkaline copper quaternary (ACQ). The MCA and ACQ included low and high copper concentration forms. Descriptions of each type of lumber can be found in the supporting information (SI) SI Table S1. Both CCA and ACQ are pressure-treated into lumber as aqueous solutions containing ionic copper (Cu^{2+}). In contrast, MCA is pressure-treated with finely-milled particles of copper salts in the nano- and microparticle size range (McIntyre 2010, Platten et al. 2015). Because the treatment amounts reported by the manufacturers were not copper-specific, each lumber material was sampled and analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-AES) for total copper concentrations. This was performed on segmented cores and sawdust for each material. Results of these analyses are discussed in Parks et al. (*resubmitted*) and are reported in SI Table S1.

Filtration-based fractionation of leachates

Fractionations were conducted on archived MCA and ACQ low and high and CCA lumber block leachate samples performed in 0, 1, 10, and 30 % filtered natural seawater (FSW) from Parks et al. (*resubmitted*). In that study, sampling occurred at 8 hr and 1, 2, 7, 14 and 28 da. Unfiltered and filtered (0.1 micron Minisart® High-Flow syringe filters with a polyethersulfone membrane (Sartorius)) subsamples taken at each time point. In addition, at time points 1, 7, and 28 da, an additional ultrafiltered (3 kDa Amicon Ultra-15 centrifugal filter device, EMD Millipore) water sample was taken to quantify the presence of dissolved copper. According the manufacturer, this ultrafilter has an effective cut-off of 1.5 to 3 nm. These subsamples (8 mL each) were centrifuged for one hour at $4000 \times g$ on a Thermo IEC Centra GP8R centrifuge with an IEC 218 swinging bucket rotor. All filtrate samples were prepared for ICP-AES analysis by acidification with concentrated nitric acid to 2.5% (v/v) after filtration on the day of sampling and stored in metal-free polypropylene conical tubes in the dark at 4°C until analysis. In Parks et al. (*resubmitted*), a second leachate study was performed under the same conditions described above using sawdust produced from the MCA low and MCA high to measure the copper concentrations and form of copper released from that composition of the lumber. Sampling occurred at 8 hr and 1, 2, 7, 17, and 28 da, and included three filtered subsamples (0.45 micron, 0.1 micron, and 3 kDa) for each time point.

Ion selective electrode (ISE) analyses

In order to evaluate the behavior of the ionic copper (Cu^{2+}) during the leaching process, two large-scale leachate studies were performed over 44 hr using 150 gof MCA high lumber blocks and sawdust in 1.8 L Milli-Q water (Millipore, Billerica, MA, USA). This large-scale design was necessary because the ion selective electrode was not sufficiently sensitive at the copper concentrations produced in our standard leachate design (i.e., ~10 mg/L) discussed above (Parks et al. *resubmitted*). As described above, the ionic form of copper is related to toxicity while copper associated with dissolved organic carbon and inorganic ligands is less

bioavailable and less toxic. The ISE measures the ionic form of copper (Cu^{2+}) and does not detect the other forms. The copper selective electrode also demonstrated elevated variability in seawater matrices necessitating the use of Mill-Q water only in this part of the investigation. The copper ion selective electrode (ThermoScientific Orion, Vernon Hills, IL, USA) was placed through the side of a two-liter plastic Erlenmeyer flask just above a teflon-coated stir bar to make continuous copper ion measurements (SI Figure S1). Data were collected by the electrode and relayed to a ThermoScientific Orion meter approximately every 60 seconds. Conventional aqueous subsamples for filtration-based fractionation were also taken periodically during the ion selective electrode study (e.g., 0, 1, 4, 12 hr) following the leachate study design described above for complementary ICP-AES analysis. These subsamples consisted of unfiltered (lumber blocks), 0.45 μm (sawdust) and 3 kDa (lumber blocks and sawdust) treatments.

Leachate toxicity testing

To evaluate the form of aqueous copper released from the low and high MCA-treated lumber leachates, acute toxicity testing was performed following methods described in U.S. EPA (2002). Specifically, exposures were performed in 100 mL glass jars containing 60 mL of test solution, FSW or reconstituted seawater (RS) (prepared from 100‰ brine and deionized water) as a control. Jars were capped with a glass petri dish with a hole in the center for insertion of an airline to provide gentle aeration via a glass pipet. In these aqueous-only exposures to *A. bahia*, survival was determined by visual inspection of the chambers at 48 hours, 96 hours, and 7 days for the lumber block leachate study and at 48 hours and 96 hours for the sawdust leachate study. The toxicity testing media was prepared by filtering the leachate through a cellulose membrane (0.45 μm pore size (Millipore, Billerica, MA, USA)) to remove any large particles. Ten 48 hr old cultured *A. bahia* were added to each exposure chamber and tests were performed without renewal (static). Test organisms were fed newly hatched *Artemia salina* daily (0.14 g (wet)/mL). Physical measurements such as pH, salinity, temperature, and dissolved oxygen were monitored to ensure water quality was maintained. Upon test termination, organisms were sieved from the exposure jars and assessed for mortality (missing organisms were considered mortalities).

To establish the exposure concentrations for testing the leachate, the filtrate was analyzed by ICP-AES to determine the total copper concentration, and subsequently diluted to the target test concentrations using FSW. The lumber control (no copper added), MCA low and MCA high leachates were tested for toxicity in addition to the controls noted above, and a reconstituted brine and deionized water control. An additional treatment with CuSO_4 was also performed to represent toxicity known to be caused by ionic copper. The reagent grade CuSO_4 powder (Sigma-Aldrich, St Louis, MO, USA) was dissolved in FSW or RS and diluted as necessary to prepare the exposure concentrations. The reported acute toxicity of ionic copper to *A. bahia* is approximately 150 $\mu\text{g Cu/L}$ (U.S. EPA 2016). Therefore, the exposure concentrations were prepared with the leachate and CuSO_4 solutions to bracket this known level of toxicity. Consequently, leachate solutions and the CuSO_4 treatment were tested at copper concentrations ranging from 25 to 600 $\mu\text{g Cu/L FSW}$. Test media concentrations were sampled on days 0 and 7 for analysis by graphite furnace atomic absorption (GFAA) spectroscopy to confirm copper concentrations.

X-Ray absorption fine structure (XAFS) analyses

To characterize copper speciation in the lumber and sawdust, oxidation states and local bonding environments of the copper were examined using XAFS spectroscopy. Copper K-edge spectra were collected at beam line 10-BM (Materials Research Collaborative Access Team, Advanced Photon Source, Argonne National Laboratory, Argonne, IL, USA) (Kropf et al., 2010). For the MCA low and high lumber and sawdust samples, XAFS data was collected on samples before and after the leachate studies for the lumber and post-leachate study for the sawdust. For the analysis, if necessary, samples were ground and compressed into 13 mm pellets using a hand press and sealed between two strips of Kapton® tape.

Adsorption spectra were collected at the K-edge energy 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. XAFS data processing and analysis were conducted using the Athena software package in the IFEFFIT computer program (Ravel and Newville, 2005). The collected fluorescence data were averaged and normalized to the edge jump height. The K-edge inflection point was determined using the energy at the maximum of the first derivative of the normalized spectra. Copper speciation in the samples was characterized by linear combination fitting utilizing a least squares procedure to match the reference compounds (e.g., micronized copper azole, copper chloride (surrogate for ionic copper), and copper organic solution complexes) to the sample spectra. During the linear combination fitting (LCF) procedure, the energy shift of the reference compounds were constrained to ± 0.5 eV of the K-edge inflection point. The number of reference compounds to describe samples were kept to a minimum and any reference compound returned with <10% or negative concentrations were omitted from the fitting process. Even with these fitting constraints, it has been suggested that the error in any LCF analysis is approximately 5-10% (Grafe et al. 2014).

ICP-AES and GFAA analyses

Descriptions of the extraction preparations for the lumber blocks and sawdust samples can be found in the Supporting Information. To determine the total copper concentrations in water samples, each acidified subsample was analyzed for copper at the 324.754 nm emission line against an external calibration using a Horiba Jobin Yvon Ultima 2 ICP-AES equipped with an AS500 autosampler (Kyoto, Japan). A single element Cu ICP standard (Ultra Scientific (North Kingstown, RI, USA), certified value of 1001 ± 2 mg/L) was used to make calibration standards at 0.12, 1.26, 5, 20, and 50 mg/L in 0, 1, 10, and 30 ‰ FSW and 2.5% nitric acid. Quality control was maintained by analyzing one of the calibration standards every 10 to 15 samples. These calibration checks did not deviate more than 20% from the reported value with a majority deviating less than 10%. The copper concentration in each sample was based on an average of three individual measurements by the instrument. The instrumental detection limit (dl) for copper was 2 µg/L with the limits of quantitation three to five times the limit of detection. Aqueous samples collected as part of leachate toxicity testing were analyzed for copper at 324.754 nm on a Perkin Elmer PinAAcle 900z

GFAA spectrometer (Branford, CT, USA) to quantify the copper concentrations before and after the exposures. The previously mentioned single element Cu ICP standard was used to make calibration standards at 5, 15, 25, 50, 100, and 200 µg/L in 30 ‰ FSW and 2.5% nitric acid. Repeated sample measurements were the mean of three individual measurements by the instrument. The same quality control parameters were applied to these analyses as previously discussed for the ICP-AES. The instrumental detection limit (dl) for copper was 3 µg/L.

Statistical Analysis

Two-sided one-way analysis of variance (ANOVA, $p < 0.05$) was performed using JMP Pro 10.0.0 (SAS Institute Inc, Cary, NC, USA) to determine if the copper concentration released for each treatment, salinity, and subsample type (e.g., unfiltered, 0.1 µm filter, 3 kDa filter) significantly differed over time. Upon determination of significant differences in ANOVA, the Tukey-Kramer HSD post-hoc test was used to determine the specific significant differences between filtration types for each treatment, time, and salinity. In analyzing the ISE data, correlation analysis was performed in Excel (Microsoft Corp., Redmond, WA, USA, version 2016) and the correlation coefficient (r) and probability of the relationship between the ISE and conventional measures of copper being unrelated (p) are reported. The calculation of median lethal concentrations (LC_{50} s) for the aqueous *A. bahia* and *A. abdita* toxicity tests was performed with Toxicity Relationship Analysis Program (TRAP) version 1.30a (U.S. EPA, Duluth, MN, USA) (https://archive.epa.gov/med/med_archive_03/web/html/trap.html). Unless otherwise noted, data are reported as mean $\pm$ standard deviation for a triplicate sample.

RESULTS AND DISCUSSION

Size fractionation of released copper

As described by Parks et al. (*resubmitted*), over time copper was released from the MCA low and high treatments into the aqueous phase from both the lumber blocks and sawdust treatments. In the current investigation, size fractionation of those same samples from the lumber block treatments found no significant differences in copper concentrations between the unfiltered, 0.1 µm and 3 kDa treatments at each time interval over the 28 days at all salinities (Figure 1) except for one sampling event (Figure 1d). The amount of copper measured in the high MCA was about twice as much as the MCA low with relatively higher concentrations of copper being released in the two estuarine salinities: 1 and 10‰ compared to the 0 and 30 ‰ salinities (see Parks et al. *resubmitted* for a discussion of those results). The consistent size fractionation findings across salinities and low and high MCA treatments suggests the form of copper was the same in each size fraction. Given the smallest size fraction isolated (i.e., 3 kDa) was not significantly different from the size fraction that went unfiltered, the implication is that the predominant form of copper released is very small (i.e., less than 3 kDa (i.e., < 1.5 nm)). The hypothesis behind the behavior of MCA is that either nanocopper or ionic copper (or both) are released when treated lumber is exposed to an aqueous environment. The lack of a significant difference between the 0.1 µm and 3 kDa size fractions, in which the 0.1 µm would isolate larger nanomaterials, suggests the form of copper being released is ionic copper or very small nanocopper particles (< 3 kDa). Further,

the lack of an effect related to salinity, from pure water to nearly full-strength seawater (30 ‰), implies that the presence of other ions is not a major determinant in the form of copper released. There was one exception to the overall statistical trend discussed above. In the MCA low 30‰, 28-day time period, the 3 kDa filtration had significantly lower copper concentrations by about a factor of two compared to the unfiltered treatment (Figure 1d). However, because of the abundance of cases where no differences occurred, we suspect this significant difference resulted from filtration and/or analytical errors rather than actual real differences between size fractions. Similar studies with the other lumber types (i.e., ACQ low, ACQ high, CCA) resulted in nearly identical findings – in 96% of the comparisons of total copper concentrations in the unfiltered and 0.1 µm fractions to the 3 kDa fraction there were no significant statistical differences (SI Figure S2). This lends further support to the hypothesis that ionic copper is being released from the MCA-treated lumber as the ACQ and CCA lumbars are designed to release only ionic copper.

The sawdust leachates demonstrated a pulsed release of copper into the aqueous phase that then decreased as the study continued. They also demonstrated a higher release of copper from the estuarine salinity treatments (i.e., 1 and 10‰) as was observed in the lumber block leachates (Figure 2) (Parks et al. *resubmitted*). As with the lumber blocks, there were not large differences in copper concentrations between the filtration sizes; however, in many instances, because the variance was low, the differences were statistically significant. In most cases where differences existed, the 0.45 µm and 0.1 µm size fractionations had higher copper concentrations than the 3 kDa treatment. In only three of 56 comparisons, the 3 kDa treatment was significantly larger than the 0.45 µm or 0.1 µm treatments. As noted in Parks et al. (*resubmitted*), the sawdust leachates were a more complex sample than the corresponding lumber blocks. For example, it was always necessary to filter (0.45 µm) the leachates as a form of clean-up prior to instrumentally analyze the samples for copper (this was not necessary for the lumber block leachates). In addition, the concentrations of aqueous total organic carbon (TOC) in the sawdust leachates ranged from 100 to 250 mg/L while the lumber block aqueous TOCs were less than 50 mg/L (Parks et al. *resubmitted*). While an aqueous variable like salinity does not seem to be substantially affecting the form of copper in these studies, the behavior of copper in aqueous solution, as discussed earlier, is known to be readily affected by the presence of aqueous TOC. In about 55% of the comparisons, the 3 kDa treatments were significantly less than the 0.45 µm and 0.1 µm size fractionations (Figures 2c, g are good examples), we suspect the presence of abundant TOC in the aqueous samples acted to scavenge ionic copper from the smaller size fractions into the larger size fractions. The increase in the occurrence of statistical differences between 3 kDa and the larger size fractions with increasing salinity (e.g., 100% and 86% of comparisons in the 10‰ and 30‰ salinities, respectively) would support this hypothesis, as the various forms of aqueous TOC are expected to increase in size and undergo reductions in solubility with increasing ionic strength (i.e., the ‘salting-out effect’) (Turner 2003). Despite these observations, in 34% of the comparisons between filtration sizes, there were no statistical differences, especially in the lower salinity treatments (similar to the lumber block results). Identically-conducted studies with the ACQ low, ACQ high and CCA lumber sawdust samples resulted in very similar responses. In 76% of comparisons, the total copper concentration in the 3 kDa fraction was statistically significantly smaller than the 0.45 µm or

0.1 μm fractions or both (SI Figure S3). Further, the occurrence of significant differences increased with salinity. This finding also suggests the ionic copper in the 3 kDa fraction is being scavenged by the aqueous TOC, with concentrations ranging from 150 to 300 mg/L (Parks et al. *resubmitted*), while undergoing the effects of salting-out.

Ion-selective electrode study

Visual inspection of the ISE measurements (ionic copper concentrations) performed in deionized water along with the unfiltered and 3 kDa copper concentrations based on ICP-AES analysis versus time for the lumber blocks demonstrates a remarkable degree of similarity in the data's respective trajectories and concentrations (Figure 3a). As observed in Figure 2 the unfiltered and 3 kDa measurements of copper were very similar. Relative to ionic copper concentrations, as the ion selective measurements show an increase in copper ion concentration from undetected to approximately 7 mg/L with time, both the unfiltered and 3 kDa copper concentrations reflect the same behavior with the concentrations within a factor of two (and very often much less than a factor of two) over the 48-hour study. Like the size fractionation study described above, these data lend further support to the hypothesis that the form of copper released from the lumber is ionic copper (Cu^{2+}) and not some form of nanocopper, since the ion selective electrode would not detect nanocopper. While changes in the salinity of the matrix can affect the speciation of many metals (e.g., cadmium, lead), copper speciation is not strongly affected by changes in salinity over an environmental range (0 to 40‰) (Kester 1986). This understanding of copper speciation suggests that results reported in Figure 3 and generated in a 0‰ matrix would be relevant at higher estuarine and marine salinities (e.g., 30‰).

Regression analysis of the relationship between the ISE and combined unfiltered and 3 kDa total copper concentrations resulted in a moderately linear relationship with a correlation coefficient (r) of 0.66 and a significant relationship between the two variables ($p < 0.001$) (SI Figure S4a). These statistical findings support the conclusion that the filtration studies discussed above were measuring a form of copper similar to the form measured by the ion selective electrode. Given the ISE measures only ionic copper, the data suggest ionic copper is the predominant form released from the lumber blocks into the aqueous phase in this study and not some form of nanocopper.

For the ISE study performed with sawdust, the concentrations of copper measured by the ISE and the two ICP-AES methods are similar especially towards the end of the 48-hours, while the initial trajectories of the measurements are different (Figure 3b). The ISE measurements of the copper ions reflect an increase with time, as might be expected as ionic copper is released from the sawdust particles. In contrast, the 0.45 μm and 3 kDa size fractions appear to show the pulsed release of copper discussed earlier (Figure 2). The differences in the data patterns may reflect the much higher resolution of the ISE measurements which were being made every 1.3 minutes compared to the ICP-AES measurements which were performed, as shown in Figure 3b, on an hourly basis. As noted above, by the second day of the study, all measurements were recording between 8 and 10 mg/L copper concentrations.

Regression analysis of the sawdust data study also generated a moderately linear relationship between the ion selective electrode and combined 0.45 μm filtered and 3 kDa total copper concentrations with an r of 0.54 and a significant relationship between the variables ($p = 0.005$) (SI Figure S4b). These results, like the ion selective electrode measurements from the lumber blocks and size fractionation study, support the hypothesis that the predominant form of copper released from the treated lumber is ionic copper.

Aqueous toxicity testing

Control survival for the mysids ranged from 90 to 100%. The dose-response curves for the lumber block leachates are presented in Figure 4a. The dose-response curves for the three treatments (i.e., MCA low, MCA high, CuSO_4) demonstrate a good degree of similarity in their overall shapes and trajectories with the major inflection reflecting the onset of acute toxicity occurring in a narrow 100 $\mu\text{g/L}$ section between 150 and 250 $\mu\text{g/L}$ for all treatments. In addition, the resulting LC_{50} s range from 221 to 257 $\mu\text{g/L}$ which, from an aquatic toxicological perspective, are essentially identical. Given the toxicity of the CuSO_4 is known to be caused by ionic copper (Sunda and Guillard 1976), the similarity of its dose-response curves to the dose-response curves of the two MCA treatments indicates strongly that the form of copper present in the lumber block leachate, and form of copper causing the resulting toxicity to mysids, is ionic copper.

Figure 4b reports the results of the aqueous toxicity testing performed on the leachates from the sawdust study. Like the results of the aqueous toxicity testing of the lumber blocks in Figure 4a, the MCA high and CuSO_4 treatments demonstrated a high degree of agreement in terms of response curve shape and trajectory, and were also very similar quantitatively to the lumber blocks. The inflection starts, again, at approximately 150 and spans a range of about 100 $\mu\text{g/L}$. The resulting LC_{50} s for the high MCA and CuSO_4 treatments were 238 and 189 $\mu\text{g/L}$, respectively, and very similar to the LC_{50} s (e.g., 221 and 257 $\mu\text{g/L}$) derived for the lumber blocks. Like the lumber blocks, these data suggest that the cause of toxicity in the MCA high is ionic copper and not a nanoform of the metal. An exception to this interpretation is the behavior of the MCA low treatment from the sawdust study. With a response curve deflecting to much higher concentrations, substantial toxic effects on survival are not observed until over 400 $\mu\text{g/L}$ resulting in a very high LC_{50} of 433 $\mu\text{g/L}$. We suspect these results reflect a reduction in the bioavailability of the ionic copper released by the MCA low sawdust treatment. As discussed previously, ionic copper can be readily chelated by dissolved organic carbon resulting in its bioavailability being reduced (U.S. EPA 2016). Because of the design of this study, the total organic carbon in the sawdust treatment was not measured (i.e., all of the 30‰ leachate was needed to prepare the solutions for toxicity testing). Measurements of aqueous TOC reported in Parks et al. (*resubmitted*) found concentrations of approximately 50 mg/L in the low and high MCA lumber block treatments regardless of salinity (i.e., 0 to 30 ‰) while the sawdust MCA low and high treatments ranged from 100 to 250 mg/L in the 0 to 10 ‰ salinities. These data suggest that the sawdust treatment did have elevated aqueous organic carbon potentially causing a reduction in the bioavailability of the ionic copper. This would explain the deflection in the MCA low dose-response curve in Figure 4b. However, the presence of elevated aqueous TOC in the MCA high treatment did not appear to have a similar effect on dissolved copper bioavailability.

Despite the anomalous behavior of the MCA low response curve in the sawdust study, most of the data from the aqueous toxicity testing suggests the leachates contain predominantly ionic copper, which is causing the observed toxicity. Because of the close matching of most of the MCA dose-response curves with those of the CuSO_4 , these results suggest there is little evidence that nanocopper is causing any of the observed toxicity. In addition, MCA, along with its copper content contains organic fungicides like tebuconazole (manufacturer provided information). These fungicides are generally present in the MCA at much lower levels than the copper, suggesting their contribution to any observed toxicity to aquatic organisms would be limited. Further, if they did contribute effects it would be observed as 'extra' toxicity, causing the dose-response curves for the MCA treatments to deflect to the left in Figure 4 – which did not occur. Finally, pH values during the toxicity testing of the leachates ranged from 6.77 to 8.09 and 7.63 to 8.03 for the lumber blocks and sawdust, respectively (SI Table S2). Under these pH conditions in seawater, the dominant inorganic copper species will be copper carbonates (e.g., CuCO_3 , CuHCO_3^+) followed by ionic copper (Kester 1986). In summary, these findings agree with the results from the size fractionation and ion selective electrode studies that the form of copper released from the MCA treated lumber is predominantly ionic copper.

X-Ray Absorption Fine Structure (XAFS) analyses

A final line of evidence in this investigation of the forms of copper in the nanocopper treated lumber leachates is an assessment of the copper speciation present in the lumber before and after the studies. Prior to the leaching studies, the form of copper in the MCA low and high was dominated by micronized copper azole (malachite; $\text{Cu}_2\text{CO}_3(\text{OH})_2$) and organo-copper molecules (Table 1) (representative raw and normalized XAFS data are provided in SI Figure S5 for the samples and reference compounds). For example, 77% and 23% MCA and organo-copper, respectively, in the MCA low lumber blocks and 65% and 35% MCA and organo-copper, respectively, in the MCA high (Table 1). Although not analyzed before the leachate study, the sawdust is expected to share similar distributions as the blocks. This finding is expected, as the blocks, and resulting sawdust, were pressure-treated with a solution of micro- and nanoparticle copper azoles and other organic molecules in the manufacturing process. After the leaching study was performed, there is some indication that the copper azole and organo-copper molecules have been reduced in their proportions in the lumber and sawdust. This is particularly evident in the post-leaching samples from the high salinities where the copper azoles are reduced by about 30 to 50% of the distribution. The organo-copper molecules show little evidence of reductions in the lumber blocks while showing some decreases in their proportions in the sawdust treatments. Interestingly, copper chloride is observed to increase from not being detected in the pre-leachate study samples to dominating 45% of the copper species (e.g., MCA low sawdust post-leachate). Copper chloride is not added to the lumber as part of the pressure treating or manufacturing process but occurred in increasing proportions in the higher salinity treatments. The only way for copper chloride to occur in the lumber is for ionic copper to have been released from the lumber and formed ion pairs with chloride ions in the seawater as the wood samples dried prior to the XAFS analysis. It was not a focus of this investigation but the presence of the copper chloride in the lumber indicates the formation of ionic copper was occurring in the lumber matrix. Unfortunately, with the current design, it is not possible to determine if this

transformation was occurring entirely in the lumber matrix or in combination with ionic copper formation external to the lumber's surface.

While this line of evidence is not as direct as the size filtration, ion selective electrode, or toxicity testing in arguing that ionic copper is the predominant form of copper released from the MCA treated lumber, it does support the argument by showing that ionic copper was generated during these studies. It is also worth noting that as the copper chloride increased in proportion to salinity, the proportion of copper azole decreased, suggesting that as ionic copper is released, copper azole may be transformed into another organo-copper molecule(s) (SI Figure S6).

CONCLUSIONS

Based on three lines of evidence including size fractionation of total copper measurements, ion selective electrode measurements, and aquatic toxicity testing, complemented by determinations of the distribution of copper species via XAFS, we concluded the form of copper released from nanocopper pressure treated lumber (i.e., MCA) into the marine environment is predominantly ionic copper (Cu^{2+}). The release of nanocopper from treated lumber was not supported by the results of this analysis. These findings are in agreement with similar evaluations performed in freshwater and terrestrial studies (e.g., Platten et al. 2015, 2016) where ionic copper was determined to be the predominant form released from nanocopper treated lumber. While ionic copper is known to be toxic to marine organisms and represents a specific hazard, a great deal of data exists about the behavior, fate and effects of ionic copper in marine environments and protective measures are readily available to environmental managers to make informed decisions about how to address any risk (e.g., U.S. EPA 2016).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Copper concentrations released from MCA low lumber blocks in 0 ‰ (a), 1 ‰ (b), 10 ‰ (c), and 30 ‰ (d) samples, and MCA high lumber blocks in 0 ‰ (e), 1 ‰ (f), 10 ‰ (g), and 30 ‰ (h) samples. Within a sampling time, columns with different capitalized letters (e.g., 'A', 'B') are statistically different from one another whereas columns with the same capitalized letter or no capitalized letters are not statistically different from one another.

Figure 2.

Copper concentrations released from MCA low lumber sawdust in 0 ‰ (a), 1 ‰ (b), 10 ‰ (c), and 30 ‰ (d) samples, and MCA high lumber sawdust in 0 ‰ (e), 1 ‰ (f), 10 ‰ (g), and 30 ‰ (h) samples. Within a sampling time, columns with different capitalized letters (e.g., 'A', 'B') are statistically different from one another whereas columns with the same capitalized letter or no capitalized letters are not statistically different from one another.

Figure 3. Results of ion selective electrode measurements from (a) lumber blocks and (b) sawdust treatments using MCA high treated lumber. In addition, results of periodic sampling for ICP-AES analysis on unfiltered (blocks), 0.45 μ m filtered (sawdust), and 3 kDa ultrafiltered samples are presented. Electrode data are culled to report an hourly measurement.

Figure 4.

Comparison of the aqueous toxicity of low and high MCA-pressure treated lumber leachates and a conventional copper salt (CuSO₄) from (a) lumber block and (b) sawdust exposures. Exposure concentrations were designed such that total copper concentrations were identical between treatments (i.e., MCA low, MCA high, CuSO₄). Insets show the LC₅₀s for the different treatments.

Table 1

Proportion of copper species from X-Ray Absorption Fine Structure (XAFS) analyses performed on the lumber blocks (grounded for the analysis) before the leachate studies (Parks et al. *resubmitted*) were initiated and on the lumber blocks and sawdust after the leachate studies were completed. All samples were collected from the outer block edge of the lumber. Data presented is based on the linear combination fitting of the first derivatives of the normalized raw data. Most analyses were performed with one replicate ($n = 1$), in limited cases, the replication was $n = 2$.

Treatment	Wood Form	Salinity (‰)	Copper Species		
			Micronized Copper Azole (MCA)	Organo-copper molecules	Copper chloride (ionic)
MCA Low	Lumber block				
	Pre-Leachate	-	77 ± 2	23 ± 2	nd
	Post-Leachate	0	67	33	0
		1	62	33	5
		10	54	22	24
		30	46	22	32
MCA High	Sawdust				
	Post-Leachate	0	66	34	nd
		30	39	16	45
	Lumber block				
	Pre-Leachate	-	65 ± 1	35 ± 1	nd
	Post-Leachate	0	67	33	nd
		1	64	31	5
		10	57	29	14
		30	23	37	39
	Sawdust				
	Post-Leachate	0	61	34	4
		30	37	24	39

nd Not detected.