**Effect of colloid-size copper-based pesticides and wood preservatives against microbial activities of Gram-positive Bacillus species using five-day biochemical oxygen demand test**

Ayenachew Tegenawa, Thabet M. Tolaymatb, George A. Soriala,\*, Endalkachew Sahle-Demessiec

a Environmental Engineering Program, Department of Chemical and Environmental Engineering, College of Engineering and Applied Science, University of Cincinnati, 701 Engineering Research Center, 2901 Woodside Drive P.O. Box 210012, Cincinnati, OH 45221-0012, United States

b U.S. Environmental Protection Agency, Center for Environmental Solution and Emergency Response, 5995 Center Hill Ave, Cincinnati, OH, 45224, USA

c U.S. Environmental Protection Agency, Office of Research and Development, Center for Environmental Solution and Emergency Response, 26 W. Martin Luther Drive, Cincinnati, OH 45268, United States

\* Corresponding author.

E-mail address: george.sorial@uc.edu, Tel: +1 (513) 556-2987

**ABSTRACT**

Copper-based pesticides and wood preservative fungicides could end up in the environment during production, use, and end-of-life via different pathways that could cause unintended ecological and adverse health effects. This paper provides the effect of colloid-size Cu-based pesticides (CuPRO and Kocide), micronized Cu azole (MCA-1 and MCA-2) and alkaline Cu quaternary (ACQ) treated woods, ionic Cu, ionic Cu spiked untreated wood (UTW), and CuCO3 solutions against Gram-positive Bacillus species using five-day biochemical oxygen demand (BOD5) standard test. CuPRO and Kocide contained about ~350 and ~300 g kg-1 of the product as Cu, respectively, whereas MCA-1, MCA-2, and ACQ comprised about ~1.3, ~1.5, and ~4.0 g kg-1 of wood as Cu, respectively. The amount of Cu leached from MCA-1, MCA-2, and ACQ in Milli-Q water after 5 days were 0.07, 0.08, and 0.27 g kg-1 of wood, respectively. The form of Cu leached from MCA was mostly ionic (> 90%). The dissolved oxygen (DO) consumption value in the presence of Cu ions, CuCO3 solution, CuPRO, and Kocide was directly related to Cu particles/ions. However, the DO consumption values in the presence of UTW spike, MCA-1, MCA-2, and ACQ was directly related to the organics leached out of the woods. On the other hand, the DO consumption of MCA sawdust was much greater than (300%) of MCA pieces/blocks. The findings of this study provide more insight into how organics leached from woods significantly reduce the toxic effects of Cu ions against Gram-positive Bacillus species.

***Keywords****:* *Bacillus species*; *BOD5; Colloid-size Cu-based pesticides; Leaching; Wood preservatives*

1. **Introduction**

Copper-based pesticides and wood preservatives play essential roles to enhance agricultural food production and prolong the service life of general construction woods, respectively by protecting or controlling pests, pathogens, and plant diseases or wood decay ([Freeman and McIntyre, 2008](#_ENREF_14); [Kanhed et al., 2014](#_ENREF_29); [Matsunaga et al., 2009](#_ENREF_43); [Pose-Juan et al., 2009](#_ENREF_53)). Currently, colloid-size Cu-based commercial pesticides and wood preservatives have largely replaced the bulk and ionic formulations due to superior physicochemical properties, antimicrobial applications, and economic advantages ([Civardi et al., 2015](#_ENREF_9); [Kah, 2015](#_ENREF_27); [Lankone et al., 2019](#_ENREF_34); [Pantano et al., 2018](#_ENREF_48)). However, the vast majority of these pesticides and wood preservatives end up in the biotic and abiotic ecosystems and water and wastewater treatment facilities during production, application, and end-of-life management via different routes to cause unintended adverse effects ([Civardi et al., 2016](#_ENREF_8); [Gavrilescu, 2005](#_ENREF_16); [Griggs et al., 2017](#_ENREF_18); [Larsbo et al., 2016](#_ENREF_35); [Pimentel, 1995](#_ENREF_51)). Moreover, nanoscale particles are potentially more reactive, bioaccumulative, and toxic than their larger counterparts and may lead to complicated effects that are not yet fully known ([Heinlaan et al., 2008](#_ENREF_20); [Wang et al., 2016](#_ENREF_62)).

Copper is essential to all organisms to carry out fundamental biological functions for healthy growth and development ([Hordyjewska et al., 2014](#_ENREF_22); [Kuo et al., 2001](#_ENREF_32); [Tapiero et al., 2003](#_ENREF_58); [Yruela, 2009](#_ENREF_66)). However, at higher doses, Cu oxidizes important biomolecules such as lipids, proteins, deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and alters molecular oxygen to yield reactive oxygen species (ROS) that cause oxidative stress/damage mainly through the Haber-Weiss and Fenton reactions ([Díaz et al., 2009](#_ENREF_11); [Drążkiewicz et al., 2004](#_ENREF_12); [Galano et al., 2015](#_ENREF_15)). Thus, a higher concentration of Cu in agricultural soils and aquatic ecosystems is an emerging concern ([Dewey et al., 2012](#_ENREF_10); [Komárek et al., 2010](#_ENREF_31); [Wightwick et al., 2013](#_ENREF_63)).

Various studies have shown the hepatotoxic, nephrotoxic, cytotoxic, phytotoxic, genotoxic, and carcinogenic effects of Cu-based NPs ([Abudayyak et al., 2016](#_ENREF_1); [Alarifi et al., 2013](#_ENREF_3); [Hejazy et al., 2018](#_ENREF_21); [Ingle et al., 2014](#_ENREF_25); [Lei et al., 2008](#_ENREF_37); [Magaye et al., 2012](#_ENREF_39); [Mosa et al., 2018](#_ENREF_44); [Prasad et al., 2016](#_ENREF_54); [Shafagh et al., 2015](#_ENREF_56)). The performance of key wastewater treatment processes such as nitrification, denitrification, anaerobic digestion and enhanced biological phosphorus removal has been impacted by Cu-based NPs ([Mu et al., 2014](#_ENREF_45); [Yang et al., 2013](#_ENREF_65)). The microbial degradation of several pesticides has been inhibited by Cu ([Gaw et al., 2006](#_ENREF_17); [Gunasekara et al., 2005](#_ENREF_19); [Jindal et al., 2000](#_ENREF_26); [Kim et al., 2011](#_ENREF_30); [Liu et al., 2007](#_ENREF_38)). Cu and its compounds are also listed as toxic and priority pollutants by the United States Environmental Protection Agency (U.S. EPA) under the Clean Water Act (CWA) and Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) at the Code of Federal Regulations 40 CFR 401.15 and 40 CFR 302, respectively. Moreover, Cu-based pesticides are among the most toxic pesticides to soil microorganisms and aquatic species ([Bünemann et al., 2006](#_ENREF_4)).

Although nanopesticides and wood preservatives have yielded an overall improved efficiency than their conventional counterparts, researchers have suggested large-scale critical evaluation of nano-agrochemicals and their conventional agrochemicals in realistic field conditions to avoid unjustified expectations or associated fears/uncertainties ([Kah, 2015](#_ENREF_27); [Kah et al., 2018](#_ENREF_28)). The 5-day biochemical oxygen demand (BOD5) test is the most common and widely used standardized method to determine the relative oxygen requirements for aqueous microbes to consume organic materials in wastewaters and surface waters in which Cu-based pesticides and wood preservatives be transported ([Hsieh and Chung, 2014](#_ENREF_23); [Hur and Kong, 2008](#_ENREF_24); [Kwak et al., 2012](#_ENREF_33); [Nataraja et al., 2006](#_ENREF_46); [Pasco et al., 2000](#_ENREF_50); [Udeigwe and Wang, 2010](#_ENREF_61)). Various studies were conducted to indicate the impact of NPs on microbial activities using standard BOD5 test method ([Cervantes-Avilés et al., 2016](#_ENREF_5); [Cervantes-Avilés et al., 2017](#_ENREF_6); [Mahlalela et al., 2017](#_ENREF_40); [Mallevre et al., 2016](#_ENREF_42); [Svartz et al., 2017](#_ENREF_57); [Taylor and Walker, 2016](#_ENREF_59)). Thus, the current research presents a detailed effect of colloid-size Cu-based pesticides and micronized Cu pressure treated wood products against Gram-positive Bacillus species using BOD5 standardized test method.

1. **Materials and methods**
	1. *Copper-based pesticides and pressure treated woods*

Two copper-based pesticides (CuPRO 2005 and Kocide 3000) and four wood types (two micronized copper azole (MCA-1 and MCA-2) pressure treated lumbers from two different companies, one alkaline copper quaternary (ACQ) pressure treated lumber, and the other untreated wood (UTW) lumber) were selected for this study. As per the manufacturers, CuPRO 2005 and Kocide 3000 consist of 538 g (53.8%) and 461 g (46.1%) of Cu(OH)2 as an active ingredient and 462 g (46.2%) and 539 g (53.9%) of inert ingredients per kg of product, respectively. The wood preservative contains inert ingredients such as wetting, antifoam, dispersing, disintegrating, binding, and acid-base neutralizing agents to give high retention, better efficacy, and improved shelf life to the active ingredient ([Chen and Lin, 2005](#_ENREF_7); [Lefiles et al., 1995](#_ENREF_36)). The metallic Cu equivalents of CuPRO 2005 and Kocide 3000 are 350 g (35%) and 300 g (30%) per kg of product, respectively. The granules and suspensions characterizations of the two pesticides (CuPRO 2005 as Product A and Kocide 3000 as Product B) are shown in previously published work ([Tegenaw et al., 2015](#_ENREF_60)). Both pesticides are used against fungal and bacterial diseases on a wide range of crops/trees using ground and areal applications. The size of the four lumber samples is 5.08 cm x 20.32 cm x 243.84 cm. The retention of MCA-1 and MCA-2 lumbers is 2.4 kg m-3 while that of ACQ lumber is 9.6 kg m-3 ([Parks et al., 2018](#_ENREF_49)). The four lumbers were from the Southern Yellow Pine woods and were obtained from local retailers and wood suppliers within 50 miles of Cincinnati, OH. All lumbers were treated for above ground applications.

* 1. *Elemental compositions*

The total elemental compositions of the two pesticides (CuPRO 2005 and Kocide 3000) and the four lumbers (MCA-1, MCA-2, ACQ, and UTW) were analyzed using Thermo Scientific iCAP 6000 Series inductively coupled plasma atomic emission spectroscopy (ICP-AES) after triplicate samples were acid digested following the EPA Method 3051 ([Agazzi and Pirola, 2000](#_ENREF_2)).

* 1. *Leaching experiment of the four lumbers*

The MCA-1, MCA-2, ACQ, and UTW lumbers were cut into a size of 1.5 cm x1.5 cm x2.5 cm plates with a power saw. Each piece of lumber plates was cleaned with pressurized air before conducting the leaching experiment. Care was also taken to avoid the contamination of untreated lumber during cutting/sawing and cleaning. A total of ~11 g (4 pieces) of each lumber was soaked in 50 mL Milli-Q water (deionized ultra-pure water, Millipore Corporation, "Type 1", as defined by ISO 3696 (resistivity (> 18 MΩ-cm) and conductivity (< 0.056 µS cm-1) at 25 °C) in a 125 mL sealed tubes. The as prepared samples were put into a rotary tumbler at 30 + 1 revolution per minute (rpm) for 5 days. All leachates were filtered with 0.45 m membrane filter to avoid wooden strips. The total Cu concentrations leached from MCA-1, MCA-2, ACQ, and UTW were then analyzed using atomic absorption spectrophotometer (AAS: PerkinElmer AAnalyst 800) after triplicate leachate samples of each wooden lumber were digested following EPA Method 3015 ([Agazzi and Pirola, 2000](#_ENREF_2)). The total, inorganic, and organic carbon contents of each wood leachate were also analyzed using total organic carbon analyzer (TOC-L CSH, Shimadzu). The pH of each wood leachate was also measured using Accumet Research AR50 pH meter/probe (Fisher scientific).

* 1. *Ionic and colloidal fractions of MCA-1 wood*

Triplicate samples of MCA-1 leachates after 1, 2, and 3 days were analyzed using AAS after acid digestion following EPA method 3015 to determine the total Cu. The free Cu ions of MCA-1 leachates samples after 1, 2, and 3 days were also measured using Thermo Scientific Orion AQUAfast IV AQ4000 colorimeter following Method 8506 (Bicinchoninate Method, Powder pillows) in order to determine the ionic and colloidal fractions of MCA-1. The experimental steps of this colorimeter method are shown in the Supplementary Data Figure S1a, S1b. Scanning electron microscope (SEM: FEI XL-30) equipped with energy dispersive X-ray (EDX) was also used to analyze imaging/morphology of micronized Cu particles impregnated in MCA-1 lumber by taking a very thin layer of samples.

* 1. *Standard BOD5 experiment*

A standard BOD5 test procedure, method 5210B*,* was used as a microbial activity indicator by measuring the dissolved oxygen (DO) consumption used by Gram-positive Bacillus species (PolySeed). The test microorganisms were obtained from Interlab Supply. Before and after each experimental run, all containers and accessories used throughout the BOD5 test procedures were cleaned with detergents (soap and then acid), rinsed with deionized water, and sterilized by autoclave to avoid contamination. The BOD5 test media (Milli-Q water, phosphate buffer, and nutrients) was prepared by adding 1 mL of each of phosphate buffer (8.5 g of KH2PO4, 21.75 g of K2HPO4, 33.4 g of Na2HPO4, and 1.7 g of NH4Cl in 1000 mL of Milli-Q water) and nutrients such as MgSO4 (22.5 g in 1000 mL of Milli-Q water), CaCl2 (27.5 g in 1000 mL of Milli-Q water), and FeCl3 (0.25 g in 1000 mL of Milli-Q water) solutions. The as-prepared BOD5 test media was aerated for about 90 min with pressurized air until saturation with oxygen is reached. CuPRO 2005 and Kocide 3000 suspensions and wood leachates (MCA-1, MCA-2, and ACQ) at concentrations of 0.1, 1, 2.5, 5, and 10 mg L-1 as Cu in triplicates was added to separate BOD glass bottles (300 mL). The same ionic Cu (from CuSO4.5H2O) concentrations (0.1, 1, 2.5, 5, and 10 mg L-1 as Cu) in triplicates were used as a sample control. Micronized Cu preservative solution in the form of CuCO3 was also used at the same concentrations as above. The same concentrations of Cu ions were spiked into UTW leachates to see the effect of wooden organics in comparison to ionic Cu solution alone. BOD5 test was also conducted on sawdust (0.5, 1, 2, 3, and 4 g) of MCA-1; 2 g of UTW sawdust and 2 g of MCA-1 wooden pieces for comparison. The suspension characterizations (particle size and ζ-potential) of the two pesticides and the CuCO3 solution are shown in Table S1 of the Supplementary data. The pesticides suspensions, wooden wafer leachates, ionic Cu, and CuCO3 samples outside of pH 6-8 range were adjusted to pH 7.0-7.2 using ≤ 0.1 M H2SO4 or NaOH solution.

The BOD5 test media was then added into the 300 mL BOD glass bottles. A mixture of glucose (1.5 mg) and glutamic acid (1.5 mg) in 1000 mL of Milli-Q water was used as a positive control and the same amount of glucose and glutamic acid mixture was added to each bottle as a source of food for the bacteria before inoculating the PolySeed into the BOD bottles. The PolySeed was then introduced into the bottles (1 capsule in 500 mL Milli-Q water, stirred, aerated, and settled). The DO concentrations of the blank (BOD test media), control (a mixture of BOD test media, glucose and glutamine, and PolySeed), and each sample (a mixture of BOD test media, glucose and glutamine, PolySeed, and pesticides/wood/ionic Cu) in mg L-1 were measured using a DO probe (Thermo Scientific Orion) immediately after the introduction of the PolySeed treatments. The BOD bottles are then sealed to ensure airtight and put in an incubator at 20 oC. The DO values of all initially measured samples were then measured after 5 days. The DO consumption was calculated as the difference between the two measurements values. The pH of the BOD matrix was also measured during the five-day period.

1. **Results and discussion**
	1. *Elemental compositions*

The elemental compositions of CuPRO 2005, Kocide 3000, UTW, MCA-1, MCA-2, and ACQ after ICP-AES total metals analysis are shown in Figure 1. CuPRO 2005 and Kocide 3000 pesticides contained 360 ± 4 g (36 ± 0.4%) and 310 ± 4 g (31 ± 0.4%) of Cu per kg of product, respectively with trace amounts of Al, Ca, Fe, K, Mg, Na, P, Pb, S, Si, Ti, and Zn (Figure 1a, b). The ICP-AES analysis was also conducted for other elements, but their measured concentrations were below the method detection limit (MDL). The UTW, MCA-1, MCA-2, and ACQ lumbers contained about 3.72\*10-3 ± 4\*10-4 g (3.72\*10-4 ± 4\*10-5), 1.47 ± 0.1 g (0.147 ± 0.01%), 1.52 ± 0.2 g (0.152 ± 0.02%), and 3.83 ± 0.13 g (0.383 ± 0.0013%) of Cu per kg of lumber, respectively with trace amounts of B, Ba, Ca, Fe, K, Mg, Mn, Na, Pb, S, Si, Sr, and Zn (Figure 1c-f). The amount of each trace element was less than 1 g (0.1%) per kg of wood. The ICP-AES analysis was also conducted for other elements, but their concentrations were below the MDL value. ACQ lumber contained more Cu (by 250% weight) than MCA-1 and MCA-2 lumbers; however, MCA-1 and MCA-2 lumbers contained very comparable amount of Cu within 3% weight marginal error. Thus, micronized Cu pressure treated woods are impregnated with much less Cu than ionic Cu pressure treated woods. This implies the economic advantage of micronized Cu pressure treated woods. The intensive, widespread, and ever-increasing use of such and related inorganic commercial pesticides, fertilizers, wood preservatives, antifouling agents, and miscellaneous products could also increase the amount of Cu and other heavy metals in the environment that may cause unintended adverse effects to soil microorganisms, aquatic species, human health, water and wastewater treatment processes, and landfills ([Dewey et al., 2012](#_ENREF_10); [El Hadri et al., 2012](#_ENREF_13); [Mahmood and Malik, 2014](#_ENREF_41); [Niyogi et al., 2008](#_ENREF_47); [Sanches et al., 2010](#_ENREF_55); [Wyszkowska et al., 2013](#_ENREF_64); [Yang et al., 2013](#_ENREF_65)).

* 1. *Leaching of Cu from wooden lumbers*

The leaching of Cu from UTW, MCA-1, MCA-2, and ACQ lumbers after AAS total Cu analysis is shown in Figure 2a. The amounts of Cu leached from UTW, MCA-1, MCA-2, and ACQ wooden lumbers in 5 days were 0, 70, 80, and 270 mg kg-1 of wood (i.e., 0%, 0.007%, 0.008%, and 0.027% weight of wood), respectively (Figure 2a). The amounts of Cu leached from MCA-1 and MCA-2 lumbers were 26% and 30% of Cu leached from ACQ lumber, respectively. The amount of Cu leached from MCA-1 wooden lumber was 88% of Cu leached from MCA-2 wooden lumber. Thus, the amount of Cu leached from the evaluated pressure treated wooden lumbers was in the order of MCA-1 < MCA-2 < ACQ. This result showed that ionic Cu pressure treated woods release much more Cu than micronized Cu pressure treated woods in aqueous ecosystems.

The total and ionic Cu amounts of MCA-1 triplicate leachate samples after AAS and AQ4000 colorimeter analyses are shown in Figure 2b. The total Cu amounts of MCA-1 at day 1, 2, and 3 after AAS analysis were 52 ± 1, 19.3 ± 1.2, and 8.9 ± 0.8 mg kg-1 of wood, respectively (Figure 2b). The ionic Cu amounts of MCA-1 after AQ4000 colorimeter analysis at day 1, 2, and 3 were 50 ± 1.4, 17 ± 0.8, and 8.0 ± 0.5 mg kg-1 of wood, respectively (Figure 2b). The ionic Cu portion of MCA-1 leachates at day 1, 2, and 3 were 96-99%, 85-91%, and 85-94%, respectively. Generally, the Cu leached from MCA-1 lumber is mostly ionic (> 90%). A leaching experiment conducted by Platten et al., was also showed more than 95% ionic Cu release from MCA wood ([Platten et al., 2014](#_ENREF_52)). Thus, the vast majority of Cu released from the investigated micronized Cu pressure treated woods was in the form of ionic Cu. This implies that the micronized Cu particles were dissolved inside the wood itself. The SEM images and the corresponding elemental spectra of MCA-1 wooden lumber are shown in Figure 2c, d. The micronized Cu particles were observed as polydispersed irregular particles with sizes less than 300 nm and agglomerated clusters with sizes less than 4000 nm (Figure 2c). The EDX spectra shown in Figure 2d confirmed the particles and the agglomerated clusters as Cu-based. The intense/strong spectra peak of C and O were from the wood itself.

* 1. *Effect of Cu-based pesticides and wood preservatives on microbial activities of Gram positive Bacillus species using BOD5 test method*

The DO consumptions by Gram positive Bacillus species (PolySeed) in the presence of Cu ions, UTW spike, MCA-1, MCA-2, ACQ, CuCO3 solution, CuPRO 2005, and Kocide 3000 solution samples at concentrations of 0.1, 1, 2.5, 5, and 10 mg L-1 as Cu after BOD5 standard test method are shown in Figure 3. The DO consumptions in the presence of Cu ions at concentrations of 0.1, 1, 2.5, 5, and 10 mg L-1 were 4.06 ± 0.2, 1.33 ± 0.18, 1.14 ± 0.37, 1.10 ± 0.11, and 0.84 ± 0.15 mg L-1, respectively (Figure 3a). All the DO consumption values in the presence of Cu ions at all concentrations were below the DO consumption values of the BOD control (Figure 3a). The increased in Cu ions concentration from 0.1 to 10 mg L-1 resulted in ~80% reduction in DO consumption. The Cu ions followed a normal trend, i.e., increasing Cu ions concentrations decreased the DO consumption which may be due to a reduction in the microbial activities of Gram-positive Bacillus species. On the other hand, increasing Cu concentration from 0.1 to 10 mg L-1 resulted in ~77%, ~72%, and ~110% increase in DO consumption in the presence of MCA-1, MCA-2, and ACQ, respectively (Figure 3b-d). The increased in DO consumptions in the case of MCA-1, MCA-2, and ACQ may be due to the fact that Cu ions and/or particles were bounded by organics that leached out from the wood ([Platten et al., 2014](#_ENREF_52)). This fact was also explained by injecting/spiking the same concentration of Cu ions into UTW leachates. The result showed a ~73% increase in DO consumption in the presence of UTW spike at concentrations from 0.1 to 10 mg L-1 (Figure 3e). This result is in the very close range of MCA-1 and MCA-2 DO consumption values. Thus, organics that leach out from woods (both treated and untreated) play a critical role on the effect of Cu ions against microbial activities and DO consumptions of Gram-positive Bacillus species. This implies that the toxicity of free Cu ions against Gram positive Bacillus species was greatly reduced by the presence of organics that leach out from wood. The presence of CuCO3 solution (a solution used to treat MCA pressure treated woods) at Cu concentrations from 0.1 mg L-1 to 10 mg L-1 resulted in a ~6% reduction in DO consumption (Figure 3f). However, in the presence of CuPRO 2005 and Kocide 3000 the DO consumption was decreased by ~75% and ~81%, respectively (Figure 3g, h). This showed that CuCO3 is less toxic than CuPRO 2005 and Kocide 3000 (Cu(OH)2: the active ingredient) in the tested range of 0.1 to 10 mg L-1 concentration as Cu.

In the case of Cu ions, CuCO3 solution, CuPRO 2005, and Kocide 3000 the DO consumption values were directly related to Cu ions/particles, whereas in the case of UTW spike, MCA-1, MCA-2, and ACQ the DO consumption values were directly related to the organics in the wood. The organics served as food for the Gram-positive Bacillus species and/or a coating for the cupric ions. The total, inorganic, and organic carbon contents of UTW (366, 10, and 356 mg L-1), MCA-1 (540, 2, and 538 mg L-1), MCA-2 (600, 3, and 597 mg L-1), and ACQ (750, 10, and 740 mg L-1) wood leachates, respectively are listed in Figure 4a. The change in pH of all BOD matrixes over the 5 days was 0.07 ± 0.05. This change was insignificant and did not influence DO consumption.

On the other hand, the DO consumptions in the presence of 0.5, 1, 2, 3, and 4 g of MCA-1 sawdust were 4.64, 4.91, 5.56, 7.85, and 9.28 mg L-1, respectively (Figure 4b). This result showed that the more organic matters present, the higher the BOD value is. Thus, there was a linear relationship between the amount of sawdust and the DO consumption with R2 value of 0.952. This result also supports the DO consumption results of wooden leachates shown in Figure 3. However, the DO consumption in the presence of 2 g of MCA-1 wood pieces was 1.75 ± 0.54 mg L-1 (Figure 4b). The DO consumption of 2 g MCA-1 sawdust was 317% more than that of the 2 g MCA-1 pieces. The surface interaction of MCA-1 wood pieces by the Bacillus species may be contributed to the reduction of DO consumption and/or Bacillus species activity.

1. **Conclusion**

The amount of Cu leached from MCA-1, MCA-2, and ACQ woods in an aqueous solution of Milli-Q water was in the order of MCA-1 < MCA-2 < ACQ. The form of Cu leached from MCA was mostly ionic (> 90% weight). The DO consumption values in the presence of Cu ions, CuCO3 solution, CuPRO 2005, and Kocide 3000 was directly related to Cu ions or particles. However, the DO consumption values in the presence of UTW spike, MCA-1, MCA-2, and ACQ was directly related to the organics leached out from woods. The organics may serve as food for the Gram-positive Bacillus species and/or a coating for the cupric ions. On the other hand, the DO consumption of MCA sawdust was much more than (300%) that of MCA pieces. The findings in this study provide an insight into how organics that leached out from woods significantly reduce the toxicity of Cu against Gram-positive Bacillus species. However, BOD5 test at laboratory and field levels in the presence of Cu-based (Cu, Cu2O, and CuO) NPs with and without wood leachates and HAs need to be done to get comprehensive ecotoxicity information.

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**Conflicts of interest**

The authors declare no conflicts of interest regarding the publication of this manuscript.

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Figure 3. The dissolved oxygen consumption of (a) Cu ion, (b) MCA-1, (c) MCA-2, (d) ACQ, (e) UTW spike, (f) CuCO3 solution, (g) CuPRO 2005, and (h) Kocide 3000.

Figure 4. Carbon content of UTW, MCA-1, MCA-2, and ACQ woods leachates (a) and DO consumption of MCA-1 wood sawdust and pieces/blocks (b).