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Section 3 **Wood protecting chemicals**

The Form of Copper: Does It Really Matter?

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ABSTRACT

In recent years, several micronized copper formulations for lumber treatment have supplanted the solubilized copper formulations that in turn replaced CCA after its voluntary relabeling in 2004. The micronized or dispersed copper systems use finely ground but solid copper particles and deposit those particles within the wood framework. In contrast, copper in the soluble formulations is relatively mobile and available to react with various wood sites. However, this mobility can also lead to depletion from the wood. This paper explores whether or not the different "forms" of copper affect efficacy of the copper based systems.

Keywords: micronized copper, copper tolerant fungi, wood pH, copper solubility, copper MIC

1. INTRODUCTION

A comprehensive review published late last year summarized the available information on both solubilized and micronized copper-based wood preservatives (Freeman and McIntyre 2008a). Portions of the review were also presented at the IRG Americas meeting in Costa Rica in December 2008 (McIntyre and Freeman 2008, Freeman and McIntyre 2008b). Other papers on micronized copper at the IRG Americas meeting presented data from Australasian field tests (Cookson *et al.* 2008), stake tests underway in Hawaii (Larkin *et al.* 2008) and leaching tests (Cooper *et al.* 2008). These papers conclude that micronized formulations perform similarly or better than amine solubilized formulations in standardized tests.

In contrast, Jin *et al.* (2008) presented data that suggested there may be insufficient ionic copper available from the micronized formulations to prevent attack. This work used small cubes treated with various formulations and measured the soluble copper in expressates taken at various times. Understandably, there was significantly less soluble copper in expressates from micronized formulations than expressates from the solubilized comparators. The question remains as to whether the levels found for the micronized products are sufficient to protect wood in ground contact applications. This paper further explores this question.

2. SOLUBILITY OF FORMULATIONS

As part of the overall question, it is instructive to briefly review the various copper based wood preservation systems. A large grouping of products in use today can be simply described as copper plus cobiocide. These include copper azole, copper quaternary, copper HDO, copper betaine and others that have yet to make it to market. Both amine solubilized and micronized versions of copper azole and copper quaternary are available.

A second grouping would be the copper plus organic ligand types. These include copper naphthenate and oxine copper. Both of these rely on the organic ligand to impart some degree of solubility in various solvents although oxine copper is difficultly soluble at best.

The third group consists of the reacted copper salts such as the copper chrome arsenates (CCA) and copper dimethyldithiocarbamate (CDDC). Other reacted salts are ammoniacal copper zinc arsenate (ACZA) and acid copper chromate (ACC).

These various copper formulations naturally have a wide range of water solubilities.

2.1. General Considerations

As noted above, Jin *et al.* (2008) have questioned if a lack of cupric ions in solution or ionized in wood will yield ineffective wood preservatives. In other words, if Cu^{++} ions are not in high enough concentrations in wood, then decay organisms have the ability to degrade the wood. This thought has been carried further to state that acidic copper like that in CCA that's fully ionized in solution or solubulized copper like the d9 co-ordination complex formed by amines (eg., 2-aminoethanol) or ammonia are the only systems capable of long term protection of wood.

These arguments run counter to considerable data on reactive, fixed wood preservatives, which will be further explained below. Many of the half-dozen plus water insoluble complexes formed in wood from the CCA fixation reaction have very low solubility product (K_{sp}) values.

2.2. Solubility Products

Solubility product constants are used to describe saturated solutions of ionic compounds of relatively low solubility. A saturated solution is in a state of dynamic equilibrium between the dissolved, dissociated, ionic compound and the undissolved solid:

$$M_aA_b(s) \text{ -----> } a\ M^+(aq) + b\ A^-(aq) \quad (1)$$

The general equilibrium constant for such processes can generally be written as:

$$K = [M+]^a * [A-]^b \quad (2)$$

Since the equilibrium constant refers to the product of the concentration of the ions that are present in a saturated solution of an ionic compound, it is given the name solubility product constant, and given the symbol K_{sp}. Solubility product constants can be calculated, and used in a variety of applications.

2.3. Basic Copper Carbonate

Most of the micronized or dispersed copper based wood preservative systems utilize basic copper carbonate (BCC) as the copper fraction of the multi-biocicidal wood preservative formulation. Since this compound is relatively hard and fracture mechanics work well for it to undergo milling into sub-micron particle size (Schilling and Freeman 2009), it is the major component of most residential lumber systems being used for residential lumber protection in the USA today. Basically, micronized copper has replaced the amine based products due to better leaching, mold growth and metal corrosion characteristics. Since BCC is essentially water-insoluble, it is instructive to investigate the water solubility of this copper complex at various pH's, especially those found in wood under both normal and under brown rot conditions, as done later.

2.4. Other Solubilities

Kaars Sijpesteijn and Janssen (1958) attribute the differences in toxicity of oxine copper, CDDC and copper pyridine-N-oxide-2-thiol to the relative solubilities of the complexes. Respectively, the water solubilities are 1, 0.01 and 0.20 mg/L.

Arsenault *et al.* (1991) commented that the MIC for CDDC was about 0.2 mg/L if the 1:1 mono-dentate complex is formed but it is about 1 mg/L if the more thermodynamically stable 1:2 complex is formed. The water solubility of the 1:2 complex is only 0.01 mg/L which is considerably less than the MIC concentrations. Although the fungitoxicity is largely due to the dithiocarbamate availability, the copper availability certainly plays a role.

Copper arsenate appears to be the most fungitoxic species formed of all the fixation products of CCA-C. However the published K_{sp} values of this complex is 7.95×10^{-36} which is extraordinarily low. Copper oxalate, which is proposed to be the principle precipitation product in the attack by copper tolerant fungi (which will be discussed in detail later), has a K_{sp} of 4.43×10^{-10} at pH 7.0.

Some other copper species, typically used in solvent-borne systems, contain a highly complex ligand, which may influence their solubility, but are not discussed here and will be reviewed in future work.

3. WOOD PH

3.1 Southern Pine

Southern pine (*Pinus spp.*) is the species of choice for the N. American micronized formulations. Part of the reason is that the large pore structure of southern pine allows for penetration of the micronized particles and part of the reason is that southern pine is easily the dominant species for lumber treatment in the US. In terms of lumber, southern pine accounts for about 80-90% of all treated material produced.

Stamm (1964) discusses the subtleties of accurately measuring the pH of wood and notes that the slope intercept method gave a pH of 4.7 for southern pine. Use of a pH electrode procedure gave a similar pH of 4.8. Koch (1972) notes that ovendried southern pine gave a pH of 4.7 by the Stamm slope intercept while use of the method of McNamara *et al.* (1970) gave a pH of 4.4 for ovendried southern pine. For the purposes of this paper, using a pH value of 4.75 as typical for southern pine is a reasonable compromise.

3.2 Other Species

There is a broad range of pH values reported for wood. Stamm notes that some work has given a range from 2.5 to 6.7 while the more general range is 3.0 to 5.5.

4. COPPER TOLERANT FUNGI

4.1 Metal Toxicity Mechanisms

High levels of copper uptake are required for fungicidal action and it is suggested that there are different sites of action for the fungistatic and fungicidal processes (Somers 1963). The initial uptake of copper in cells is by ion-exchange followed by permeation throughout the cell. Copper is accumulated passively in the spores by unspecific reactions with cell constituents. Increased copper uptake by spores is observed under anaerobic conditions and with temperature increase. The uptake at 35^{o}C is almost three times that at 4^{o}C. The distribution of accumulated copper in conidia varies with fungal species and cell walls have a varying avidity for copper.

Because of the wide spectrum of potentially toxic interactions between metals and fungi, almost every aspect of their metabolism, growth and differentiation may be affected (Gadd 1992). Toxic metals exert harmful effects in many ways but principally as a result of their strong coordinating abilities. Toxic effects include blocking of functional groups of biologically important molecules (e.g. enzymes and transport systems for essential nutrients and ions), the displacement and/or substitution of essential metal ions from biomolecules and functional cellular units, conformational modification, denaturation and inactivation of enzymes and disruption of cellular and organellar membrane integrity (Gadd 1992).

For copper in particular, the fungicidal property is dependent on its affinity to different groups on fungal proteins, particularly thiol groups. Copper causes damage through oxidizing proteins, enzymes, lipids and therefore interrupting enzymatic processes (Eaton and Hale 1993; Rui and Morrell 1994). Copper prevents spore germination, inhibits intracellular enzymes responsible for destruction of lignocellulosic materials, binds to the cell wall and interrupts transport of nutrients into and out of the cell. If enough copper enters the cell it can lead to denaturation of proteins and enzymes leading to cell death (Archer and Preston 2006). The cell membrane is an initial site of action for a toxic metal species and membrane damage can result in loss of mobile cellular solutes, e.g. K^{+} and increased permeability of the cell to external materials.

Another indirect mechanism proposed is through the interaction of Cu^{++} with hydrogen peroxide resulting in reduction to Cu^{+} and production of free radicals from hydrogen peroxide and oxygen. Free radicals result in uncontrolled oxidation process via the Haber-Weiss cycle (Simpson et al. 1988; Gunther et al. 1995). Free radicals are deleterious to cells as they take part in chain reactions which involve the breakdown of biological macromolecules. Major targets in cells are membranes whereby the alkyl chains of lipids are converted to peroxyalkyl radicals and fatty acid hydroperoxides. Lipid-soluble complexes of transition elements such as Fe^{++} may undergo the Fenton reaction with the hydroperoxides and accelerate this process. Organometallics are generally more toxic towards fungi than corresponding free metal ions and the toxicity varies with the number and with the identity of the organic groups. Organometallics may also damage membranes by the production of free radicals since the carbon-metal bond

readily reacts with available radicals to produce peroxyalkyl radicals which can result in lipid peroxidation. As well as the mitochondrial membrane, organometallic compounds may also exert a disruptive effect on cell membranes and cause a loss of K^+ (Gadd 1992).

4.2 Environmental influence on metal toxicity towards fungi

Metal toxicity towards fungi is reduced in complex media compared to simple defined media. Dissolved and particulate organic matter in the environment, and in growth media, can influence metal toxicity by complexation and binding, generally reducing toxicity. The physico-chemical properties of a given environment (such as growth medium) determine metal speciation and therefore biological availability, toxicity, and metal-organism interactions. Where such factors as pH, the presence of other anions and cations, the presence of particulate and soluble organic matter and clay minerals decrease biological availability, toxicity may be reduced. Acidity (pH) affects metal-fungal responses directly by influencing metal speciation and mobility and indirectly by influencing other aspects of cell physiology and metabolism. Increasing pH can result in the formation and precipitation of metal hydroxides or oxides (Gadd 1992). Typically, divalent metal cations form hydroxylated species in aqueous solution and the pH values at which hydroxylated species form varies between different metals. The different hydroxylated forms possess differing toxicities. Gadd and White (1985) observed an intracellular Cu accumulation with increasing pH in *Penicillium ochrochloron* and at pH 6 and above, this fungus was sensitive to quite low concentrations of Cu. Low pH increases concentration of free metal ions in solution and H^+ may compete with metal ions for cellular binding sites and reduce potential interactions with cells hence reducing toxicity.

4.3 Resistance and tolerance

The terms 'resistance' and 'tolerance' are often used interchangeably in the literature. ‘Resistance’ is the ability of an organism to survive metal toxicity by means of a mechanism produced in direct response to the metal species concerned. 'Metal tolerance' may be defined as the ability of an organism to survive metal toxicity by means of intrinsic properties and/or environmental modification of toxicity (Gadd 1992). Copper tolerance is the ability of an organism to grow and thrive in the presence of copper ions. Compared with other microbes, fungi can be extremely tolerant of toxic metals at high concentrations. Microbes possess a range of tolerance mechanisms, most featuring some kind of detoxification. Copper tolerance by fungi has been extensively investigated, and the ability to prevent cellular entry or reducing cellular accumulation of copper has been reported as the main mechanism for tolerance (Gadd and White 1989).

4.4 Brown rot tolerant fungi

Brown-rot fungi are unique in that they can degrade holocellulose in wood without removing the lignin and cause a rapid decrease in degree of polymerization at low weight loss. Understanding how brown-rot fungi degrade wood has received increasing attention because of a need to identify novel targets that can be inhibited for the next generation of wood preservatives (Green and Highley 1997). Tolerant fungi are particularly interesting for investigations related to new wood protectants since one of the many requirements for these systems is effectiveness against brown-rot fungi. Brown rot fungi in general present a greater tolerance to copper based preservatives and they are much more important in structural conifers which dominate the building market (De Groot and Woodward 1999).

Antrodia vaillantii and related species form a group of interior brown-rot fungi associated with the decay of softwoods in buildings. In central Europe, these fungi are ranked below the dry rot fungus *Serpula lacrymans* and equal to *Coniophora* spp. as the most common internal decay fungi. In laboratory studies, *A. vaillantii* has been found the most copper-tolerant fungus and produces the highest amount of oxalic acid on copper treated wood (Schmidt 1995; Green and Clausen 2003). In laboratory studies Pohleven et al (2002) evaluated Norway spruce with commercial copper-based preservatives common to the European market. When wood treated with copper based biocides was challenged with several copper tolerant *Antrodia* spp., individual strains exhibited varying degrees of copper tolerance.

Wolfiporia cocos has also been shown to be copper tolerant (Woodward and DeGroot, 1999). The *W. cocos* strain (106R) that caused the most weight loss was a prolific accumulator of oxalic acid and is usually included in evaluations for replacement of copper organic preservative formulations (Green and Clausen 2003). DeGroot and Woodward (1999) showed that two *W. cocos* isolates caused weight losses with CC and ACQ-B that were significantly greater than in untreated SYP at retentions less than 1% a.i. Several decay fungi in the genera *Serpula* and some species that were once included within the genus *Poria* (now *Postia*) are also tolerant of copper (De Groot and Woodward 1999). *Poria placenta*, *Poria cocos* and *Poria vaillantii* have exhibited extreme resistance to copper and exhibited high weight losses at retentions up to 1.6 kg/m^3 (0.1 pcf) of CCA and 16 kg/m^3 (1 pcf) of copper chrome (Morrell 1991).

4.5 Mechanisms of copper tolerance

Copper tolerance has been ascribed to diverse chemical, biochemical, and physiological mechanisms involving changes in the pH of media, change in mycelial growth and formation of mucilaginous sheaths trapping of the metal by cell-wall components, altered uptake of copper when the plasma membrane of mycelium is less permeable to copper, extracellular chelation, complexation and crystallization or precipitation by secreted metabolites and intracellular complexing by metallothioneins and phytochelatins (Cervantes and Gutierrez-Corona 1994). Other mechanisms may include transformation of metal species by oxidation, reduction, methylation and dealkylation, biosorption to cell walls, pigments and extracellular polysaccharides, decreased transport or impermeability and intracellular compartmentation. A particular organism may directly and/or indirectly rely on several survival strategies. These strategies are discussed in the following sections.

4.5.1. Change in pH of media

Tolerance of the brown-rot species is strikingly increased by lowering the pH of the substratum from pH 6 to 2. The brown-rot fungus *Postia placenta* decreases the pH very rapidly to 1.6 at the inner sites of the decayed wood. Several mechanisms for fungus tolerance to copper have been suggested and all require a decrease in the pH of the medium (Young 1961). Isolates of *Gloeophyllum trabeum* that cause extensive decay lower the pH of wood whereas isolates that do not lower wood pH result in low amounts of decay (Espejo and Agosin 1991). DaCosta and Kerruish (1964) showed that copper tolerant Poria vaillantii strains reduced agar media pH to <3.0 from the initial 5.3. Other fungi gave final pH of 3.0 to 6.2 and did not correlate with fungal tolerance.

A reduction in pH of the medium leads to a reduction in the toxicity of copper due to a decrease in the amount of copper taken up by the cell. Decreasing pH increases concentration of free metal ions in solution and H^+ may compete with metal ions for

cellular binding sites and reduce potential interactions with cells. External pH also affects the speciation of metal-binding ligands and therefore metal complexation. Many toxic interactions of heavy metals with fungal cells can be interpreted in terms of pH effects on metal speciation and availability (Gadd *et al.* 1984). The nature and amount of organic acids excreted by fungi are mainly influenced by the pH and buffering capacity of the environment, the carbon, phosphorus, and nitrogen sources, and the presence of certain metals. External pH has been shown to be the main factor governing oxalic acid production by *Aspergillus niger* with production of oxalate reported to be optimal in the pH range of 5–8 (Ruijter *et al.* 1999).

4.5.2 Formation of mucilaginous sheaths and change in mycelial growth

One aspect of toxic metal tolerance, which is easily observed, is change in mycelial growth. Mycelial aggregation represented by the phalanx (or slow dense) strategy is well known for its protective function and has been observed for fungi colonizing toxic metal-contaminated domains. Fomina *et al* (2005) observed that *Beauveria caledonica* adopted the phalanx (slow dense) strategy and cord-forming (reallocation) strategy, with the result that it tolerated the toxic metal stress but maintained a high biomass yield (Fomina *et al.* 2005). Fungal reactions triggered by the presence of copper include the formation of a thicker cell wall and increased proportion of extracellular mucilaginous material (ECMM). Increased copper sulfate concentration in the culture media has been shown to result in decrease in the length of mycelia peripheral growth unit (PGU) and increased proportion of (ECMM) in *Trametes versicolor* (Vesentini *et al.* 2006b). Decreased PGU may result in larger amount of branched morphology and hyphal tips. Decreased PGU and increased ECMM increase tolerance in fungi to copper. The existence of mucilaginous sheaths around hyphae has been reported for a wide range of fungi, and the functions of these sheaths include protection and attachment. Fomina *et al.* (2005) observed that the sheath provided a matrix for fungus-mineral interactions and metal transformations, harboring metal-chelating agents and resulting in crystal growth and deposition of secondary mycogenic minerals, such as calcium and copper oxalates in *Beauveria caledonica*. All the processes of metal diffusion and precipitation of metal oxalates occurred in this well-hydrated mucilaginous microenvironment.

Vesentini *et al.* (2006a) exposed copper sulfate treated Scots pine blocks to *Coriolus versicolor* and *Gloeophyllum trabeum and* observed an increase in *N*-acetyl glucosamine in fungal mycelium. The amount of *N*-acetyl glucosamine is correlated with the amount of chitin present. The increase of chitin deposition leads to the development of a thick cell wall.

4.5.3. Metal binding to cell walls

Metabolism dependent association of metal species to fungal walls may include ion exchange, adsorption, complexation, precipitation and crystallization. Metal biosorption in fungal cell walls involves different mechanisms and variables depending on wall structure and composition. Potential sites involved in metal sequestration include carboxyl, amine, hydroxyl, phosphate and sulphydryl groups. Biomass concentration may affect fungal biosorption. The uptake of metals when expressed on a unit dry weight or cell basis is generally greater at lower cell densities than at high ones. Growth conditions affect the structure and composition of fungal cell walls and their manipulation offers potential for biosorbency for specific purposes. Chitin and chitosan are significant metal-biosorbing substances in cell walls of fungi and chitin-chitosan content of fungal walls varies between species. Insoluble chitosan-glucan complexes and glucans possessing amino- or sugar acid groups from *Aspergillus niger* exhibit

biosorptive properties and may efficiently remove transition metal ions from solution. Fungal melanins are located in and/or exterior to cell walls and may be released into the external medium. Melanins in cell walls of basidiomycetes are derived from 7-glutaminyl-3, 4-dihydroxybenzene (GDHB) or catechol and contain oxygen-containing groups including carboxyl, phenolic and alcoholic hydroxyl, carbonyl and methoxyl groups may be particularly important in metal binding (Gadd 1999).

4.5.4. Metal-binding proteins and peptides of filamentous fungi

The super-family of proteins called metallothioneins bind metal ions such as copper and zinc as well as inessential metals such as cadmium to cysteine thiolate groups (Hall 2002). Metallothioneins are polypeptides that have low molecular mass, high metal content, high cysteine (Cys) content, lack aromatic amino acids and histidine and possess abundant Cys-XCys sequences (where X is an amino acid other than Cys). Metallothionein synthesis has been shown to be is a mechanism of Cu^{++} resistance in *S. cerevisiae.*

4.5.5. Vacuolar compartmentation

Functions of the fungal vacuole include macromolecular degradation, storage of metabolites and cytosolic ion and pH homeostasis. A vacuolar ATPase has been identified in several fungi and the ATPase utilizes the energy arising from ATP hydrolysis to pump H^+ into the vacuole, resulting in an electrochemical proton gradient. This gradient energizes transport of monovalent and divalent cations, as well as other substances including basic amino acids, into the vacuole. Localization of metal ions in the vacuole enables low cytosolic concentrations to be maintained relatively constant, even under considerable environmental perturbations. Compartmentation of the metal ions in the vacuole may contribute to fungal tolerant behaviour. It is known that polyphosphate granules, which are localized in fungal vacuoles, may be important in intracellular homeostasis and can incorporate metal cations, including potentially toxic metals (Fomina *et al.* 2005).

4.5.6 Metal transformations

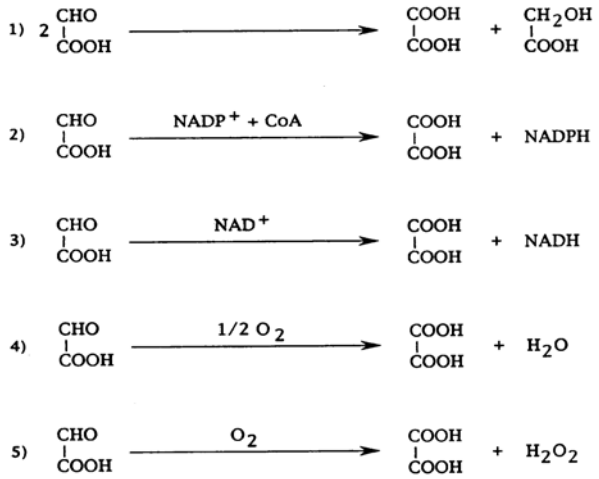
Fungi, as well as other microorganisms, can effect chemical transformations of metals by a number of mechanisms: oxidation, reduction, methylation and dealkylation (Gadd 1992*)* although, in contrast to bacteria, detailed information is not available in several areas for fungi. Enzymatic metal transformations may be involved in survival since certain transformed metal species are less toxic and/or more volatile than the original species. A copper-reducing enzyme, copper reductase located in the cell wall catalyses Cu^{++} reduction to Cu^{+} with NADH or NADPH as electron donor. Methylation of mercury and other metals and metalloids can be catalyzed by several fungi and may be viewed as a detoxification mechanism since methylated species are more volatile. In organometallic compounds, degradation involves breaking of the metal-C bonds. Degradation of tributyltin oxide and tributyltin naphthenate, or copper naphthenate used as wood preservatives can be achieved by fungal action. Early failure of copper naphthenate treated wood has been attributed to presence of copper tolerant fungi which detoxify the preservative (Morrell 1991). Non wood decay fungi or bacteria which are part of soil or wood microflora may transform a chemical allowing less tolerant basidiomycetes to colonize and decay the wood (Morrell 1991).

4.6 Oxalic acid and its formation in fungi

Oxalic acid is an organic acid commonly occurring in plants, fungi, and animals. It seems to play different roles in different living organisms. In contrast to other low-

molecular-weight carboxylic acids with low complexing abilities that erode minerals in acid solution by protonolysis (e.g., acetic and lactic acids), oxalic acid is able to mobilize metals very efficiently at neutral pH and even in basic solutions (Fomina et al., 2005). Recently, the biochemical roles of oxalic acid have been receiving much attention in relation to lignin and cellulose biodegradation by basidiomycetes (Green et al., 1991, 1992, 1995; Green and Clausen 2003, 2005) and bioremediation of xenobiotic pollutants. Among non wood decay fungi, *Aspergillus niger* has been studied for enzymatic formation of oxalate (Shimada et al., 1997). In *A. niger*, the role of oxalic acid may be related to mobilizing substrates from cell wall polysaccharides, e.g. pectin (Ruijter et al., 1999).

Production of oxalic acid is an early event in the decay and potent inducers of oxalic acid include xylan, glucomannan, chitin, uronic acids, pectin and compounds containing acetyl sidechains. Although cellobiose also induces oxalic acid, utilization of cellulose is considered a relatively late event, therefore not influencing incipient decay (Green et al., 1994). Biosynthesis of oxalic acid from glucose occurs by hydrolysis of oxaloacetate to oxalate and acetate catalysed by cytosolic oxaloacetase. Cell-free extracts, obtained from mycelia of the white-rots *Phanerochaete chrysosporium*, *Coriolus versicolor* and the brown-rot fungus *Tyromyces palustris*, have been demonstrated to contain oxaloacetase and glyoxylate oxidase (Akamatsu et al., 1994). Glyoxylate oxidase purified from *T. palustris* extracts catalyzes oxidation of glyoxylate, yielding oxalate and hydrogen peroxide in the presence of oxygen. Different types of the oxalic acid producing reactions are summarized below:



4.7 Role of oxalic acid in wood biodegradation

Oxalic acid interacts with metal ions to form insoluble oxalate crystals around cell walls and in the external medium. Oxalate secretion by fungi provides many advantages for their growth and colonization of substrates. There is good correlation between pathogenesis, virulence, and oxalic acid secretion. The functions of oxalic acid in brown rot fungi colonization include:

i. Lowering the pH of the media: Fungi acidify their environment via an extracellular process involving glucose oxidase, by secreting organic acids which first accumulate intracellularly (Ruijter et al., 1999). Brown-rot fungus *Postia placenta* MAD-698 initiates a 2-fold decrease in wood pH within 7 days of colonization which is mediated by production of oxalic acid. (Green et al., 1994). *Postia placenta* Strain ME-20, a non-decay isolate does not accumulate oxalic acid when colonizing wood. Lowering of the pH by oxalic acid contributes more to copper tolerance than does the low solubility of copper oxalate. Young (1961)

studied *W. cocos* isolated from failed fence posts that had been treated with copper naphthenate. He demonstrated a striking increase in tolerance to copper when the pH of agar medium supplemented with copper sulfate was lowered from 6 to 2.

ii. Contributing in the depolymerization of holocellulose during incipient decay:- Enzymes produced by brown-rot fungi are too large to penetrate sound wood structures, even after decay begins. Thus, nonenzymatic metabolites have been proposed to initiate brown-rot decay. Oxalic acid is capable of diffusing long distance through the cell wall (Morrell 1991) and plays a unique role in lignocellulose degradation by wood-rotting basidiomycetes, acting as a low molecular mass agent initiating decay. Oxalic acid is involved in nonenzymatic initiation of wood cell wall depolymerization through the production of free radical species in the early stages of both lignin and cellulose degradation by fungi (Fomina et al. 2005). During attack of cellulose by *Postia placenta* MAD-698, the degree of polymerization (DP) decreases over a period of 5-6 weeks with relatively low weight loss. Oxalic acid likely contributes to depolarization without effecting a measurable reduction in percent crystallinity of the cellulose (Green et al., 1993). The oxalate also plays an important role in lignocellulose degradation by affecting activities of key enzymes and metal oxido-reduction reactions (Gadd 1999).

iii. Metal complexation, chelating and precipitation:-The production of oxalic acid provides both protons and an organic anion, the latter capable of forming a complex with the metal cation, and the subsequent formation of an insoluble less toxic oxalate resulting in metal immobilization (Jarosz-Wilkolazka and Gadd 2003; Morrell 1991)**.** The formation of calcium oxalate crystals weakens the cell walls, thereby allowing polygalacturonase to effect degradation more rapidly in a synergistic response. Tolerance of brown-rot fungi may be linked to oxalic acid which precipitates copper into the insoluble form of the oxalate around the hyphae, rendering the copper metabolite inert (Murphy and Levy 1983; Sutter et al., 1983; Sayer and Gadd 1997). Insoluble metal oxalate formation is a process of marked environmental significance both regarding fungal survival, biodeterioration, pathogenesis, soil weathering, mineral formation and metal detoxification (Dutton and Evans 1996; Gadd 1999). Immobilizing soluble metal complexes, as insoluble oxalates decreases bioavailability and confers tolerance. Most simple metal oxalates [except those of alkali metals, Fe(III) and Al] are sparingly soluble and precipitate as crystalline or amorphous solids. Calcium oxalate is the most important manifestation of this in the environment. During the growth of wood-rotting fungi, the production of oxalate can also chelate calcium from the pectate component of cell walls to form calcium oxalate (Dutton et al., 1996).

4.8 Correlation between oxalic acid production and copper tolerance

Several workers including Murphy and Levi (1983), Green and Clausen (2003; 2005) have suggested that formation of copper oxalate crystals resulted in a higher copper tolerance for several fungi. *Aspergillus niger* has been shown to produce metal oxalates after 1–2 days when grown on medium amended with a wide range of metal compounds e.g., Co, Zn, Cu, and Mn, or metal-bearing minerals (Sayer and Gadd 1997) and is tolerant to the fungicide copper oxychloride investigation due to immobilization by oxalate formation (Gharieb et al., 2004). In the fungus *Beauveria caledonica* a non wood destroying fungi, the induction of an oxalic acid efflux correlated closely with copper tolerance and overexcretion of organic acids with strong metal-

chelating properties (oxalic and citric acids), suggested that a ligand-promoted mechanism was the main mechanism rendering it highly tolerant to toxic metals (Fomina et al., 2005). Overexcretion of oxalic acid by the fungus led to the transformation of all metal minerals into metal oxalates. The presence of toxic metal minerals led to the formation of mycelial cords, and in the presence of copper-containing minerals, these cords exhibited enhanced excretion of oxalic acid resulting in considerable encrustation of the cords by copper oxalate hydrate. Jennings and Kamdem (2000) showed that the ratio of oxalic acid to copper can be important. The greatest tolerance was obtained with a 1:1 ratio while either 0.5:1 or 2:1 mixtures gave more inhibition.

Copper induces rapid oxalic acid production by copper-tolerant brown-rot fungi (Clausen et al., 2000; Clausen and Green 2003; Green and Clausen 2003). In studies, involving copper (II) sulfate and copper naphthenate, Clausen et al (1994) concluded that copper tolerance in *Postia* species is a function of copper oxalate precipitation. These studies examined decay capacity and microscopic copper oxalate crystal formation. Using thin transverse sections of pine sapwood impregnated with copper sulphate pentahydrate or copper naphthenate, Sutter et al. (1983, 1984) reported that the woodrotting fungi *Poria placenta* and *Poria vaillantii* could immobilize copper in wood by the formation of insoluble and hence nontoxic copper oxalate. Both fungi were able to immobilize copper by precipitating copper oxalate, which is either deposited in the lumen or transported to the wood surface. Up to 90% of the original copper present in solution was removed from the copper sulphate treated sections. Copper oxalate crystals were deposited in large crystal aggregates and also as microcrystalline deposits around the hyphae, forming tube-like envelopes together with extracellular fungal material. Copper oxalate precipitation was assumed to be the most important mechanism of copper tolerance in the *Poria* species.

Similarly, using X-ray absorption spectroscopy, Fomina et al.(2005) showed that copper within the *B. caledonica* mycelium were coordinated by carboxylic groups, a considerable proportion of which were derived from oxalate and were either crystalline or amorphous. Other species in which copper tolerance has been linked to oxalic acid production include *Wolfiporia cocos* (Sutter et al., 1983; Clausen et al., 2000; Green and Clausen 2005). In a study on 19 isolates of *Wolfiporia cocos*, the isolates showed considerable variability in their ability to tolerate ammoniacal CC, ranging from very to barely tolerant. This variability led to the conclusion that there is no direct statistical linear correlation between oxalic acid production and copper tolerance for this group of fungi. Murphy and Levy (1983) reported the formation of numerous copper oxalate crystals in developing colonies of *A. niger*, *Penicillium spinulosum*, *Verticillium psalliotae* and *Poria placenta* grown in agar media containing copper sulphate. Gharieb (2002) found that adsorption of the fungicide copper oxychloride onto the fungal cells induces excretion of acidic complexing agents, which are implicated in the fungicide solubilization and tolerance.

Copper in waterborne preservatives ACQ, copper citrate (CC), and CCA has been shown to stimulate 66-93% more oxalic acid compared to untreated controls within 2 weeks of exposure of blocks to test fungi (Clausen and Green 2003). Calcium oxalate and copper oxalate crystals are commonly observed in decayed wood that has been treated with these preservatives. In a test to estimate the potential of known copper tolerant fungi to produce oxalic acid in SYP timber treated with arsenic-free preservative formulations, brown-rot fungi produced 2-17 times more oxalic acid in CC treated blocks

than in untreated controls (Clausen and Green 2003). Sub-lethal concentrations of Cu based preservatives induced rapid oxalic acid production; 66–93% more oxalic acid was produced in 4 weeks in blocks treated with CCA, the ammoniacal copper quats ACQ-B and ACQ-D, and ammoniacal copper citrate (CC) than in untreated controls. The presence of a nitrogen source e.g., increased ammoniacal ratio in ACQ stimulates oxalic acid production hence reducing copper toxicity in some copper tolerant fungal species (Ruddick and Xie 1994; Humar et al., 2005). Williams and Fox (1994) also commented that higher levels of copper were required to counteract the effect of additional nitrogen in tests of amine solubilized formulations.

Green and Clausen (2003) evaluated the relationship of oxalic acid production with the decay capacity of known copper-tolerant and copper-sensitive brown-rots. Fifteen brown-rot fungi representing the genera *Postia*, *Wolfiporia*, *Meruliporia*, *Gloeophyllum*, *Laetiporus*, *Coniophora*, *Antrodia*, *Serpula*, and *Tyromyces* were evaluated for oxalic acid production in SP blocks treated with 1.2% ammoniacal copper citrate (CC). After 2 weeks, these fungi produced 2–17 times more oxalic acid in CC-treated blocks than in untreated blocks. After 10 weeks, weight loss ranged from 32% to 57% in CC-treated SP. Four fungi were copper sensitive, producing low levels of oxalic acid and minimal weight loss in CC treated blocks. Rapid induction of oxalic acid correlated closely with copper tolerance. The brown-rot fungi that were able to exceed and maintain an oxalic acid concentration of ≥ 600 μmol/g effectively decayed SP treated with CC. Table 2 shows the mean maximum values of oxalic acid produced for each fungus and weight loss of control and CC-treated SP over the 2–10 weeks. Maximum weight loss was achieved at 10 weeks. Higher levels of oxalic acid were achieved in 2 weeks in the CC-treated blocks exposed to copper-tolerant fungi. In contrast in copper-sensitive fungi (*C. puteana* and *S. lacrymans*), oxalic acid production on a dry weight basis was strikingly lower and increased at 4–10 weeks.

Table 1: Mean maximum values of oxalic acid produced and weight loss for each fungi

Fungus	1.2% ammoniacal copper citrate treated		Copper tolerance	Control	
	Oxalic acid [mM]	Weight loss [%]		Oxalic acid [mM])	Weight loss [%]
Antrodia vaillantii FP90077	624±16	32±5	+	380±31	38±4
Antrodia radiculosa FP90848T	580±19	49±9	+	490±7	24±3
Lactiporus sulphurcus Boat 206	518±66	32±5	+	438±26	54±4
Meruliporia Incrassata TFFH-294	486±20	45±4	+	426±24	37±5
Wolfiporia cocos MD106R	474±115	57±10	+	126±8	61±6
Postia placenta MAD 698	468±21	39±7	+	403±22	65±3
Meruliporia Incrassata MAD 563	466±46	51±11	+	148±62	59±6
Tyromyces palustris Typ 6137	442±44	51±4	+	581±25	42±3
Postia placenta TRL 2556	429±14	55±3	+	386±27	63±3
Wolfiporia cocos FP97438sp	405±40	32±11	+	99±61	50±7
Tyromyces palustris L15755sp	373±57	39±5	+	45±8	35±3
Coniophora puteana MAD 515	239±150	6±5	-	291±43	34±3
Serpula lacrymans Bam Ebers 315	178±67	2±1	-	169±22	53±4
Gloeophyllum trabeum MAD 617	73±15	0±0	-	36±6	50±3
Serpula lacrymans Harm 888-R	49±16	5±1	-	27±3	15±2

Green III and Clausen (2003)

The four fungi including *Coniophora puteana* MAD-515 designated as not copper tolerant failed to achieve oxalic acid levels above 600 μmol/g in the presence of CC and maintained low levels of oxalic acid during the course of study. Copper-tolerant brown-rot fungi showed rapid production of oxalic acid in CC-treated SP compared to untreated blocks. Copper tolerance correlated with the capacity of decay fungi to initiate early and sustained oxalic acid levels over the 10-week incubation period. Oxalic acid is clearly a key component in the successful colonization and degradation of treated wood by copper-tolerant fungi. Failure to exhibit copper tolerance appears linked to lack of oxalic acid accumulation in *G. trabeum* MAD 617. *C. puteana* is purported to produce both oxalic and acetic acid, apparently neutralizing or buffering the oxalic acid effect. Micales (1995) demonstrated that a non wood destroying isolate of *Postia placenta* (ME-20) produced oxalate decarboxylase, which rapidly broke down oxalic acid.

5. MIC

5.1 Copper

Although the above discussion suggests that the cobiocide would be the dominant factor in controlling copper tolerant fungi, there is considerable literature giving the minimum inhibitory concentration (MIC) or a similar index value for control of various fungal species with copper.

DaCosta and Kerruish (1964) surveyed 32 different isolates of *Poria* brown rot species. In agar plate tests, good control was exerted over most isolates with between 0.51 and 2.0% copper in the agar medium. However, 11 strains of *P. vaillantii* were not controlled at this level although the mycelia growth appeared abnormal. Soil block tests with 6 of the *P. vaillantii* strains against CCA showed no control at loadings of 6.4 kg/m^3 (0.40 pcf) of formulation. This corresponds to 1.76 kg/m^3 (0.11 pcf) of copper as metal.

Interestingly, in the DaCosta paper, there was considerable discussion regarding the field performance of the then-new CCA preservative system. The authors noted that the laboratory results needed perspective since no early failures of CCA had been reported. In other words, *P. vaillantii* attacked CCA in the laboratory tests but it did not seem to "constitute an actual service hazard in this type of preservative." Morrell (1991) echoed this sentiment in his discussion of copper tolerant fungi.

Archer *et al.* (1995) compared soil block, agar block and agar plate tests. In their work, the IC_{50} for copper was about 170 mg/kg for *Postea* (previously *Poria*) *placenta* and about 70 mg/kg for *G.trabeum* and 130 mg/kg for *Trametes versicolor*. The MIC was seemingly about 300 mg/kg where less than 3% growth occurred but total inhibition required about 1000 mg/kg for the three fungi. Since the co-authors of the paper seemingly used the same data in their patent (Nicholas and Schultz, 1996), presumably the work was done with the readily soluble copper (II) chloride listed in the patent.

Nonetheless, it seems a reasonable conclusion that concentrations of about 300 ppm of copper are capable of controlling most fungi without a cobiocide being present. In a previous paper, Archer *et al.* (1993) explored synergisms and found that copper:DDAC mixture exhibit synergism with an MIC of about 100 ppm. Depending on the formulation then, one would expect that 100-300 mg/kg of copper in solution would be sufficient to control most fungi.

5.2 Cobiocides

5.2.1 DDAC

In 2001, Walker presented Agar Plate MIC data for DDAC alone against 2 brown rot and 2 white fungi in comparison to either ACQ or CCA (Table 2). This showed that the MIC for ACQ was about one-third that of DDAC alone and that 100 mg/kg was the maximum for the four fungi tested. Interestingly, the copper tolerant *P. placenta* had the greatest sensitivity to the ACQ formulation.

Table 2: MIC of various preservatives

	MIC (mg/kg)			
	G. trabeum(BR)	*P. placenta(BR)*	*T. versicolor(WR)*	*I. lacteus(WR)*
DDAC	>300	100	>300	>300
CCA-C	1000	300	300	300
ACQ-B	100	30	100	100

5.2.2 Azoles

Obanda (2008) measured the MIC of tebuconazole against the brown rot *Meruliporia incrassata* and found that 6 mg/kg gave complete control. Thus, low levels of azoles appear to be very effective against this copper tolerant fungus.

Tseng and Walker (2003) reported MIC data for common azoles against two brown rot, one white rot and one soft rot fungi (Table3). In this case, the soft rot fungi had the greatest tolerance.

Table 3: MIC of various azoles

	MIC (mg/kg)			
	G. trabeum(BR)	*P. placenta(BR)*	*T. versicolor(WR)*	*C. globosum(SR)*
Cyproconazole	2.5	2.5	2.5	25
Propiconazole	>50	50	50	>50
Tebuconazole	10	50	25	50-500

Other azole data of interest came from Berg and Buschhaus (1993) who presented data to the AWPA for tebuconazole that showed 1 kg/m^3 was sufficient to limit attack in soil block tests with several brown and white rot fungi. Tebuconazole has a solubility of 0.0032% in water.

Also, in 1994, Goodwine *et al.* presented data to the AWPA for propiconazole that showed 1 kg/m^3 was sufficient to limit attack in soil block tests with several brown and white rot fungi. Propiconazole has a solubility of 0.01% in water.

6. SPECIATION

Cui (1999) investigated both cuprous (Cu^+) and cupric (Cu^{++}) systems to determine the relative efficacy of both forms against *P. placenta* and *G. trabeum*. Cui generated the cuprous form *in-situ* by post treatment steaming of cupric forms. For southern pine,

treatment to 1.6, 2.6 5.1 kg/m^3 (0.10, 0.16 and 0.32 pcf) with amine copper (Cu-MEA) complex and then post steaming for up to 4 hours gave 27, 54 and 29% conversion to cuprous copper. Subsequent soil block testing showed that within a retention group, the weight losses increased as the cuprous content increased. The author concluded that the efficacy of the copper formulation lies with just the cupric form of copper and that the cuprous ion is relatively ineffective as a fungicide.

Recently, Tascioglu *et al.* (2008) investigated the copper speciation of ACQ treated red pine (*Pinus resinosa* Alt.) conditioned at different temperatures under dry and wet conditions. At lower concentrations, essentially 100% conversion to the monovalent form could be achieved if sufficient steaming is used. Although this raises the possibility that post-steaming may significant reduce the activity of copper as a biocide, it was found that rewetting or leaching converts most of the monovalent copper back to the divalent, more active form.

7. EXPERIMENTAL RESULTS

7.1 Solubility Measurements

Various copper preservative formulations were made and stirred in buffered water for a minimum of 6 hours. The copper systems were BCC, micronized copper alone (Micro C) and then Micro C with appropriate cobiocides added in the correct ratio to make either Micro CQ or Micro CA. After double filtration, the filtrate was analyzed for soluble copper (Figure 1). Our results disagree with literature values (Scaife 1957) but our results are reproducible. Further work may be done regarding this.

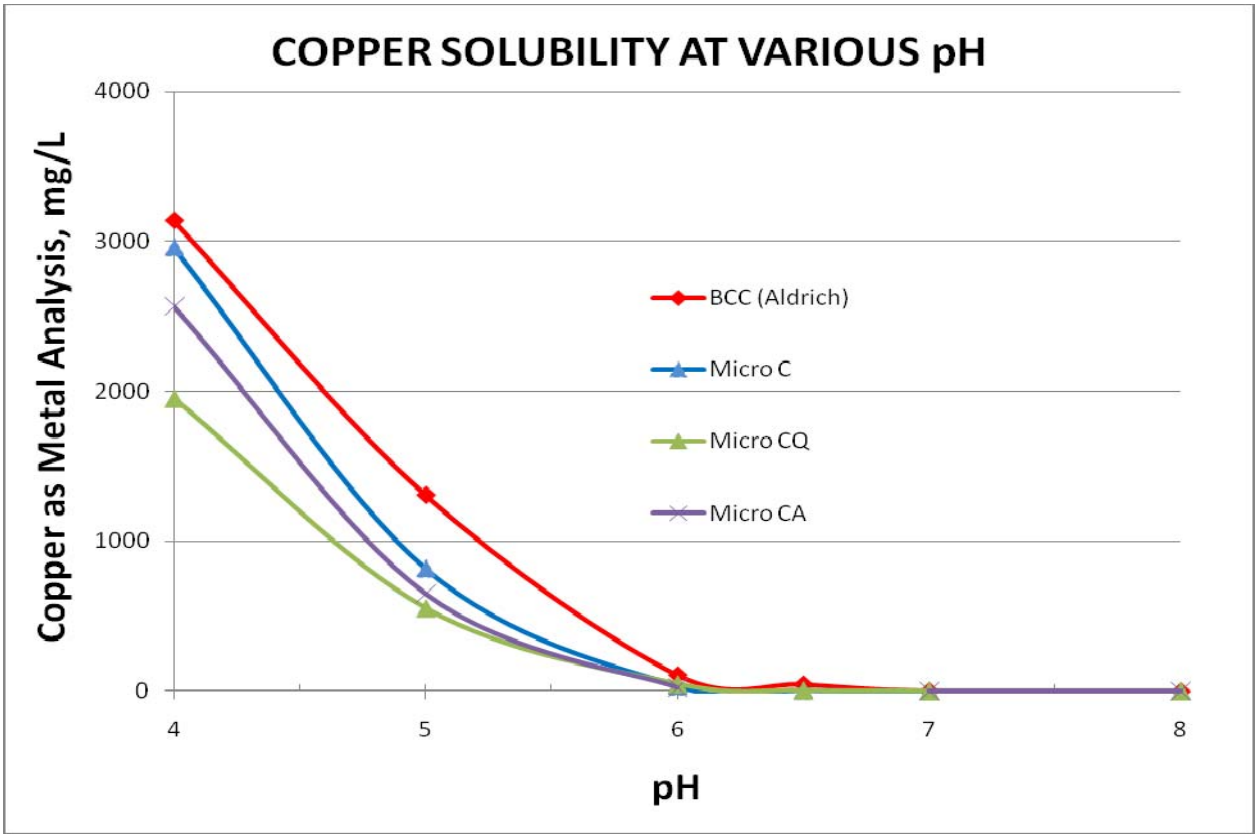


Figure 1: Copper solubility of various systems at different pH

7.2 Gradient Measurements

7.2.1 Posts (102 mm) Gradient

Another aspect of the copper "form" is that of the gradient produced during the actual treatment may be of a completely different character than that of the solubilized products. For example, one could speculate that the micronized would be very steep due to a build-up of particular matter in the outer zones.

To assess the nature of such gradients, posts (nominal 102 x 102 x 2438 mm (4 x 4 x 96 in.) commercially treated with ACQ-D and eight similar sized posts treated with micronized CQ were purchased. The ACQ material was labelled as 6.4 kg/m^3 (0.40 pcf) while the Micro CQ was labelled as 5.4 kg/m^3 (0.34 pcf).

The material was selected on the basis that as much sapwood as possible was included and further selection was done to ensure that sapwood would be available throughout the piece in the gradient area of interest. This resulted in 5 pieces of ACQ-D material and 6 pieces of Micro CQ material being used.

Starting at the end, 13 mm (0.5 in.) wafers were cut at the 152, 610, 1219, 1829 and 2286 mm (6, 24, 48, 72 and 90 in.) mark. Each wafer was then cut into 0-13, 13-25, 25-38 mm (0-0.5, 0.5-1, 1-1.5 in.) zones with the zone orientation carefully maintained. Thus, a gradient profile could be obtained that would reflect both the longitudinal dimension and the transverse dimension. Each zone was analyzed for copper and quaternary content and the results are shown in Figure 2.

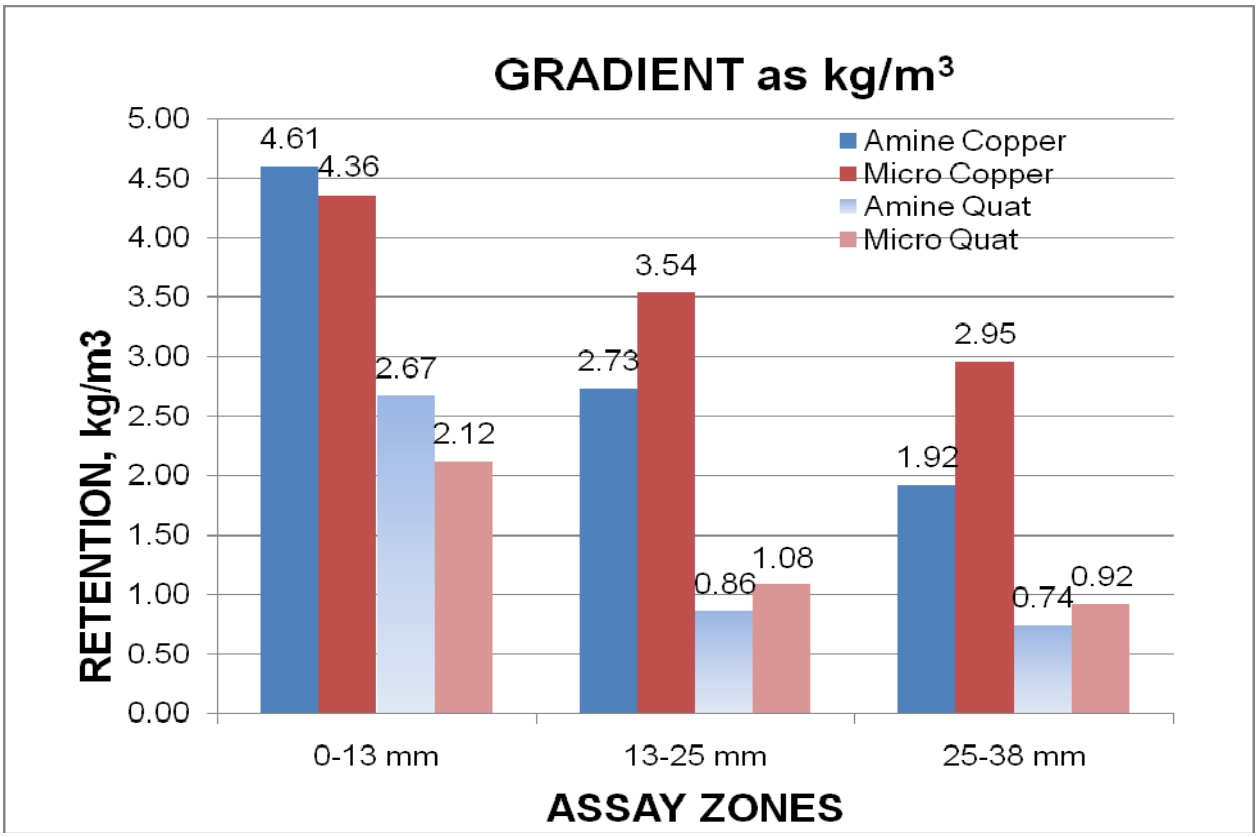


Figure 2: Gradient Comparison

Other gradient data was available on ACQ-D treated posts of similar size. This material was treated at different times, used different sources of wood and so it would reflect a second case for measuring the ACQ gradient. For purposes of comparison, the retentions of the inner zones are normalized to that of the outer zone. Interestingly, the completely separate measurements of the ACQ gradients resulted in essentially the identical pattern (Figure 3). Compared to these, the gradient for Micro CQ is much flatter than those for ACQ-D.

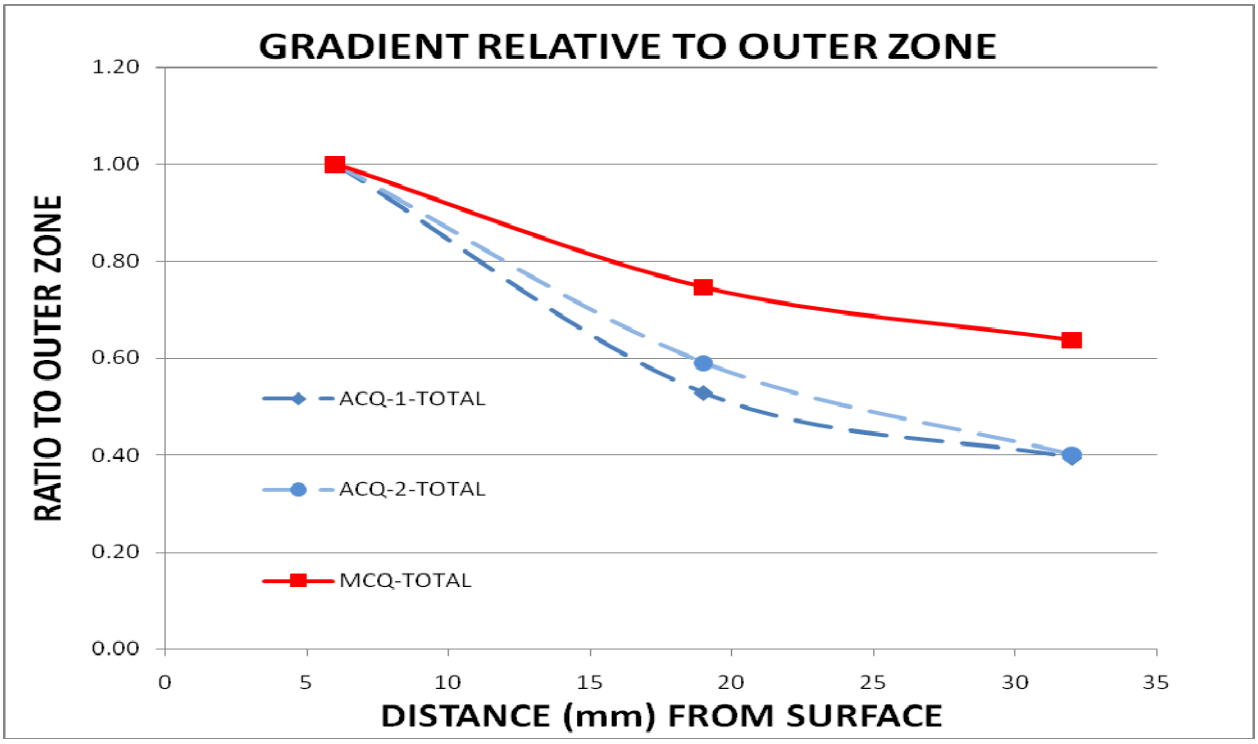


Figure 3: Second Gradient Comparison of 102 mm Posts

7.2.2 Lumber (51 mm) Gradient

Work with 51 x 102 mm (2 x 4 in.) lumber shows the same gradient phenomenon as the larger posts. In that work, end-matched (but double epoxy end-coated) sections of the same mother pieces were treated with either ACQ-D or Micro CQ and the gradients determined in 3 mm (1/8 in.) slices taken from the "top" of the wide face (Figure 4). This work suggests a retention effect in that the higher retentions have a flatter gradient.

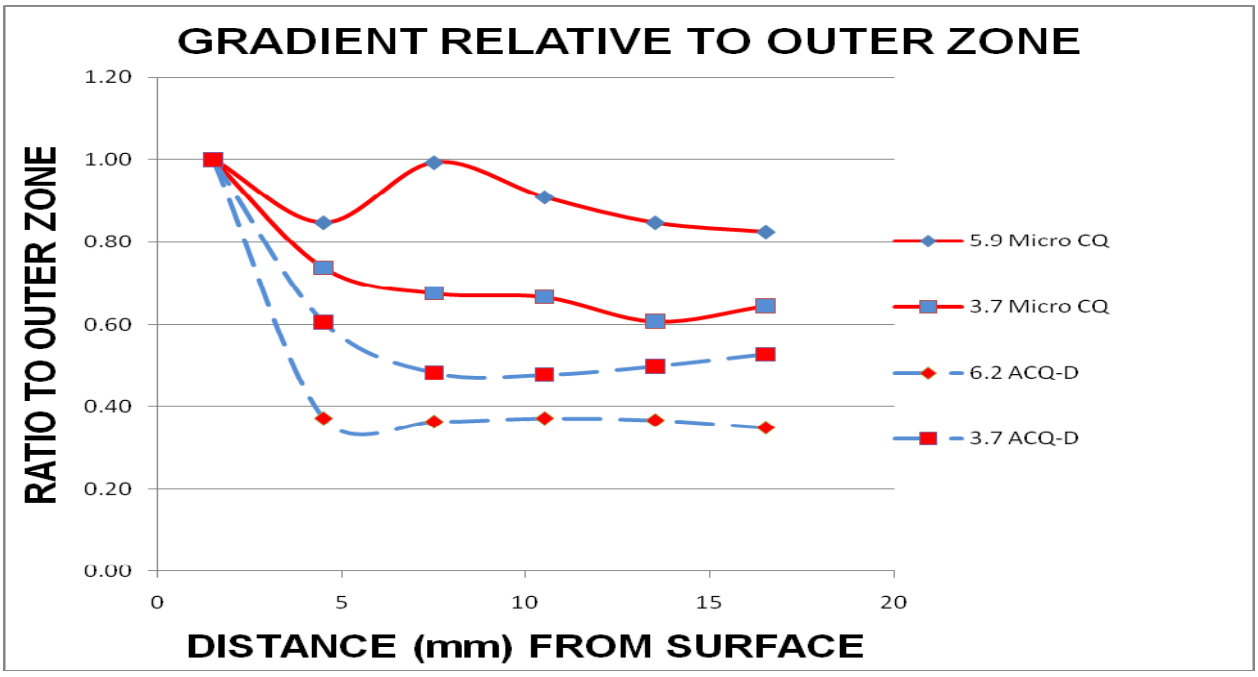


Figure 4: Gradient Comparison of 51 mm Lumber

7.2.3 Critical Use Post (152 mm) Gradient

The end-matching procedures of 7.2.2 were used to determine the gradients for large posts typically used in critical applications. The posts were 152 x 152 mm (6 x 6 in.) in cross section and treated to higher retentions. The gradients were determined by measuring retentions in 13 mm (1/2 in.) slices from an all sapwood side (Figure 5).

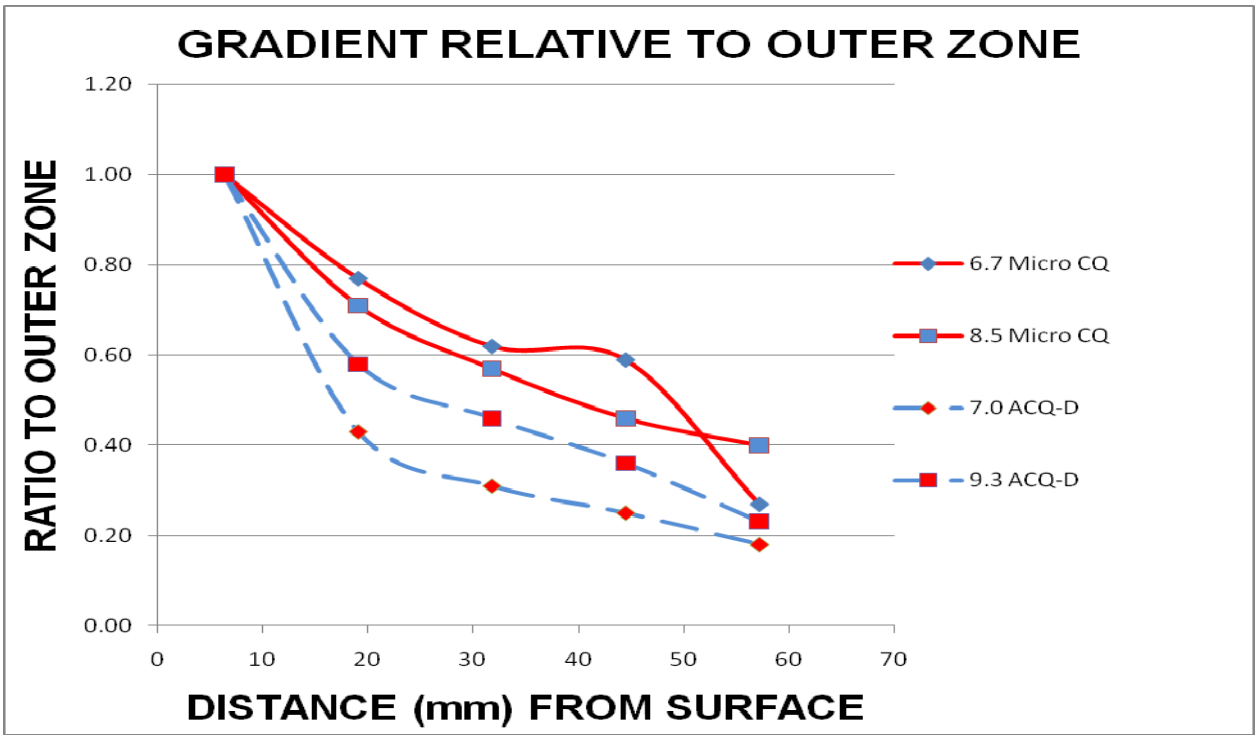


Figure 5: Gradient Comparison of 6x6s

8. SUMMARY AND CONCLUSIONS

The literature and experimental data discussed above suggests that an excess of soluble copper exists for micronized copper quaternary in wood relative to that required to inhibit fungi. The generally accepted pH of southern pine is 4.75 and both micronized copper quaternary and micronized copper azole have solubilities of 1000 mg/L or more of copper ions at that pH. Fungal tests show that common wood decay fungi are controlled by copper quaternary formulations at 100 mg/L. Thus, there appears that a 10-fold excess of copper quaternary would be available at the wood pH.

As well, the flatter gradients obtained with micronized formulations reflect higher more uniform concentrations of the biocides in the inner zones of the wood. This is presumably due to the well-known selective absorption of the quaternary component from amine solutions and its preferential binding to wood cell walls. There is no build-up of particulate copper in the outer zones of the wood.

Copper tolerance, copper speciation and the solubilities of several highly insoluble copper preservative systems that are very effective in protecting wood were also discussed. Consideration of all of this suggests strongly that the "form" of copper in these systems does not materially impact the performance.

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