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INCORPORATION OF ORGANIC FUNGICIDES IN BINDERED ANTHRACITE BRICKS FOR IMPROVED STORAGE AND PERFORMANCE IN IRON FOUNDRIES

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by

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ABSTRACT

Anthracite bricks bound with collagen, lignin, and silicon metals have the potential to be used as an alternative fuel source for metal production in iron foundries. However, humid conditions and prolonged storage may promote fungal growth on the organic binder materials decreasing the strength and performance of the bricks in a cupola furnace. The organic fungicidal neem compounds, azadirachtin, crude neem oil (NO), and clarified neem oil extract (CNO), were examined for their effective growth inhibition of ectomycorrhizal soft-rot fungus *Chaetomium globosum*. When the most effective neem compound, CNO, was combined with a low dose of copper, a synergistic fungicidal interaction was observed on nutrient media (two-factor ANOVA with triplicate replication: $P < 0.05$). Interaction was confirmed on lab-scale anthracite briquettes, with identical composition of full-scale foundry bricks, by measuring unconfined compressive (UC) strength at room temperature to primarily quantify collagen degradation. Effective collagen strength was enhanced by applying CNO to the surface of the briquettes. Effective collagen strength increased $28 \pm 4.6\%$ when CNO (0.4 mg/cm^2) was surface-applied or $43 \pm 3.0\%$ when CNO and copper (0.14 $\mu g/cm^2$) were surface-applied, even with fungus present. Effective lignin strength was measured by UC strength after pyrolysis of the briquettes and revealed a decrease in strength; however, pyrolyzed samples still retained an average of $76 \pm 13\%$ of the effective lignin strength when CNO was surface-applied. These results indicate that clarified neem oil extract and copper can be combined to prevent fungal growth on bindered anthracite bricks and improve the storage and performance of the bricks in iron foundry metal production.

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NOTATION

CNO	Clarified Neem Oil Extract
NO	Crude, or Cold-Pressed Neem Oil
AZ	Pure Lab-Grade Azadirachtin
AS	Commercial Azadirachtin Compound - Azasol™
AM	Commercial Azadirachtin Compoud - Azamax®
CuAz	Lumber Preservative Copper Azole
AKNM	Antibiotic (Ampicillin/Kanamycin) Nutrient Media
NMC	Nutrient Media Control – AKNM with no Antibiotics
LCM	Lignin-Collagen Media
Cu/LC	Copper to Lignin-Collagen Ratio (mg/g)
TEA	Total Esterase Activity – Enzyme Assay
SPB	Sodium Phosphate Buffer – used in TEA

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Chapter 1

Introduction

High–grade carbon based fuels have become a limited and expensive resource sparking exploration into new resources and technologies. Foundry coke, a fuel source for cupola furnaces in metal foundries, is no exception. As high-grade bituminous coal is depleted and prices rise (U.S. EIA, 2013), investigation into alternative fuels has led to the production of bindered anthracite bricks. Waste components (anthracite fines, collagen, and lignin) have been combined with silicon materials to produce a coke-equivalent fuel source (Huang et al. 2011a, 2011b, Lumadue et al. 2012) with 15-20% less life-cycle energy than coke production (Avallone et al., 2006).

While bindered anthracite bricks may be produced and effectively utilized in foundry cupolas, biological activity stimulated by organic binder materials may reduce the physical strength of the bricks during long-term storage or transportation. Organic collagen and lignin combined with residual moisture from brick manufacturing can provide a suitable environment for the activity and growth of ectomycorrhizal fungi, which are generally observed in a symbiotic relationship with plant roots. Intensive post-production drying of the anthracite bricks reduces the moisture content, but also increases energy consumption and production costs. Conventional fungicides such as copper azole (wood preservative), lime sulfur (agricultural additive), and chlorinated compounds are expensive, undesirable, and potentially toxic. Copper alone may provide some antifungal activity, but is typically combined with a co-biocide: the dangerous and/or expensive portion of the antifungal product. Therefore, a safe, inexpensive fungicide is required to assist or replace copper.

Neem oil, produced from the fruit of *Azadirachta indica* (neem tree), is commercially available and possesses natural biocides which have been used in medicinal and agricultural practices (Biswas et al., 2002). Active ingredients in the oil may be variable and difficult to identify (Kumar and Parmar, 1996) and oil production yields different characteristics of resulting products: crude neem oil (NO) or clarified neem oil extract (CNO). However, neem oil is a safe alternative to possible endocrine disrupting compounds like azoles (wood preservative).

The overall objective of this research is to prevent fungal growth on bindered anthracite bricks, even when they contain residual production moisture or are stored in humid environments; and maintain strength of the bricks for optimal performance in cupola furnaces. It was projected that neem compounds, alone or in combination with copper, could prevent biological growth on bindered anthracite bricks and preserve the structural integrity of the bricks. In this work, the isolated wild fungus, *Chaetomium globosum*, was used to test the fungicidal effects of various neem, azadirachtin, and copper compounds. Interaction effects between fungicides were examined and applied at the lab-scale to quantify antifungal activity and study application techniques for future full-scale anthracite brick production.

Chapter 2

Literature Review

2.1 Anthracite Bricks: Energy Saving Fuel Replacement for Coke in Cupola Furnaces

While coal ranks as the second largest energy source worldwide, high-grade coking coal has become a limited and expensive resource with severe increases in price over the last few years. Premium coking coals have an estimated heat content of 26.3 million BTU per ton, while low-grade coals range from 4.9 to 12.9 BTU per ton (U.S. EIA, 2013), making coal selection the most important factor in coke quality. Foundry coke, produced from high quality bituminous coal, is prepared through extensive pyrolysis that requires significant amounts of energy with furnaces operating at 900-1100 ^{o}C for 14-36 hours (Mussatti, 1998). The process also produces pollutant emissions such as carbon dioxide, volatile organic compounds (VOCs), methane, and nitrogen and sulfur oxides (World Bank Group, 1998). Consequently, an energy and cost efficient alternative to foundry coke has become a topic of great interest and importance.

To reduce cost and meet environmental regulations, research has been directed toward alternative and complementary fuel sources to be used in place of foundry coke. Manufactured replacement fuels comprised of anthracite or coke breeze (fines) was suggested for the replacement of large coke in ferroalloy production due to low ash content, few mineral impurities, and high electrical resistivity (Strakhov, 2008). However, these manufactured alternative fuels must retain the strength and combustion properties of conventional coke. Using anthracite and coke breeze bonded with coal tar acid resin, a potential energy saving alternative, was discovered; however, the release of volatile compounds upon combustion and insufficient strength rendered

the briquettes inadequate for foundry cupolas (Thoms et al., 1999). More recently, lignin has been proposed as a carbon reducing agent and fusing binder at high temperatures (Strakhov, 2009; Lumadue et al., 2012). Ongoing investigation by the Penn State-Furness Newburge team has led to complete production of bindered anthracite bricks to be used as an alternative to foundry coke. Waste or underutilized components such as anthracite fines (coal production), collagen (animal production), and lignin (paper production) have been combined with silicon materials to produce a coke-equivalent fuel source with 15-20% less life cycle energy than the energy required to produce coke. The combination of binder materials creates strength throughout the various temperature regions of the foundry cupola furnace (Huang et al., 2011a; Huang et al. 2011b; Lumadue et al., 2012). The use of recycled materials and decreased energy and air emissions from coke production make bindered anthracite bricks an environmentally and financially conscious product.

2.2 Ectomycorrhizal Fungal Degradation of Lignin and Collagen Reduces Binder Strength

Organic binder materials used in the production of bindered anthracite bricks, lignin and collagen, provide a possible substrate to induce fungal growth. Mycorrhizal fungi, in particular ectomycorrhizal fungi, encompass many wood-rotting fungal species of the Ascomycota and Basidiomycota phyla. Ectomycorrhizal fungi have been largely studied due to their unique relationship with the root systems of host plants (Dighton et al., 1987; Haselwandter et al., 1990; Trojanowski et al., 1984). Furthermore, the relationship has been carefully investigated to determine whether these wood-rotting fungi can attack and degrade lignocellulose—cellulose, hemicellulose, and lignin—from their plant host. While it is generally accepted that brown-rot (solely Basidiomycota), white-rot (mostly Basidiomycota), and soft-rot (mostly Ascomycota) fungi easily degrade cellulose and hemicellulose, the attack on lignin varies between the three

wood-rotting fungal groups. Haselwandter et al. (1990) confirmed that the ectomycorrhizal fungus, *Paxillus involutus* (Basidiomycota), had lower ligninolytic capabilities than other fungal species, but still possessed biologically significant lignin degrading enzyme activity. Bending and Read (1997) concurred that ectomycorrhizal fungi (Basidiomycota and Ascomycota) have significant ligninolytic ability to degrade cell wall lignin. In studies comparing soft-rot and brown-rot fungi, both Ascomycota and Basidiomycota fungi showed the ability to degrade major carbon portions of plant material, including recalcitrant lignin (Trojanowski et al., 1984). Early investigation by T.K. Kirk (1971) summarized that *C. globosum,* an Ascomycota soft-rot fungus, can indeed cause degradation of lignin in wood. Soon after, Haider and Trojanowski (1975) concluded that soft-rot fungi can carry out significant degradation of lignin despite no laccase activity and low peroxidase activity, two important enzymes involved in the degradation of lignin (Perez et al., 2002; Martinez et al., 2005). Whether complete degradation or slight alteration of lignin, it is evident that ectomycorrhizal fungi of the Ascomycota and Basidiomycota phyla do possess the ability to break down complex carbon structures such as lignin.

While ectomycorrhizal degradation of wood has been extensively studied, research on the ability of these organisms to degrade other organics, such as protein, is not as immense. Mycorrhizal fungi, similar to the species used by Bending and Read (1997), have shown proteolytic activity by degrading and utilizing nitrogen from peptide and protein sources. However, these ectomycorrhizal fungi required an additional carbon source for substantial growth (Abuzinadah and Read, 1986). Further evidence suggests that Ascomycota species, *Cochliobolus carbonum*, produces an extracellular protease induced by collagen (Walton, 1994), one of the key ingredients in anthracite brick production required for strength at room temperature. While still unknown, protein degradation may depend on ‘early’ or ‘late-stage’ fungal growth; however, some ‘early-stage’ mycorrhizal fungi have almost no proteolytic ability while others degrade protein without difficulty (Read, 1991). The cellulose degrader, *Chaetomium globosum*

(Ascomycota), also displayed proteolytic activity in the degradation of collagen in leather when accompanied by *Aspergillus* and *Trichoderma* species; *C. globosum* being the dominant microorganism (Strzelczyk et al., 1989). Evidence suggests that ectomycorrhizal soft rot fungi, such as *C. globosum*, have the ability to degrade or alter proteins such as collagen.

The genus *Chaetomium,* one of the largest genera of Ascomycota, is primarily soil-borne, found in warmer climatic regions, and exhibits cellulolytic activity—some species even requiring a cellulose substrate for abundant growth and reproduction (Guarro et al., 1995). Water damaged, aged, organic materials comprised of cellulose have been identified as the most susceptible to *Chaetomium* colonization (Gravesen et al., 1998). *Chaetomium globosum* is one of the most common species in the *Chaetomium* genus, found in various environments from water damaged buildings and structures (Gravesen et al., 1998; Fogle et al., 2007) to infected humans (Guarro et al., 1995; Aru et al., 1997). Early investigation into the degradation of timber in water cooling towers suggests *Chaetomium globosum* is a primary soft-rot degrader of water-laden and soil-contacted hardwoods (Savory, 1954). Soft-rot fungi like *C. globosum* have been known to withstand extreme conditions and can therefore outlast other fungi in harsh environments (Martinez et al., 2005). Many Basidiomycota, brown and white rot fungi, cannot withstand the low oxygen content of water saturated organic substrates, whereas *C. globosum* may tolerate it and still stimulate soft-rot decay (Savory, 1954). The cellulose degradation and associated enzymes of *C. globosum* have been heavily reported and reviewed (Lakshmikant and Mathur, 1990; Langston et al., 2011; Longoni et al., 2012). The attack on lignin and collagen by *C. globosum* is not well known, but literature indicates that the combination of water and organic compounds create an environment suited for soft-rot fungi like *C. globosum*.

2.3 The Neem Tree (*Azadirachta indica*) is a Natural Biocide

A promising organic fungicide, neem oil, is commercially available and has been documented to possess components that naturally inhibit biological activity (Biswas et al., 2002). Neem oil is a product of the neem seed, the fruit of the neem tree, *Azadirachta indica*, which is a member of the mahogany family, and is primarily found in tropical and semi-tropical regions of the Middle East. The tree and its products are historically known to exhibit antibacterial, antimalarial, and antifungal properties, and have been used for medicinal and agricultural purposes (Biswas et al., 2002). The most widely studied neem products are azadirachtin and neem oils, which are readily available for residential gardening applications. Still, the concentrations of active ingredients in the oil may be variable and difficult to identify, sometimes spanning several orders of magnitude (Kumar and Parmar, 1996). Neem oil characterization largely depends, but still varies, on the production method. Three main production methods of neem oil from the neem seed include: i) Expulsion; ii) Water extraction; and iii) Solvent extraction. Each production method yields a different type of neem oil with different biologically-active components.

Expulsion production compresses the neem seeds under reduced temperatures to prevent the degradation of biologically-active compounds and produces crude neem oil, commonly referred to as cold-pressed neem oil (Locke et al., 1994). Expulsion production generates viscous neem oil which contains water and azadirachtin, the most commonly studied active ingredient of neem oil. A less favorable method, water extraction, uses crushed neem seeds steeped in water to dissolve the active ingredients into solution. Due to the low water solubility of neem oil and the desired compounds, a large amount of water is required. However, in developing countries with minimal technology, this extraction method still proves beneficial for crop protection (NRC, 1992). Solvent extraction utilizes non-polar hydrophobic solvents with high neem oil solubility

and close to zero azadirachtin solubility to obtain a neem oil extract. The solvent is then stripped at a low temperature and the extract is separated and filtered to yield a solid wax, or neem cake, and a clarified neem oil extract (Locke et al., 1994). Nimbidin is a primary component of clarified neem oil extract and comprises about 2% of the seed kernel (NRC, 1992). Uncertainty in production of neem oil used in previous studies has resulted in debate over the fungicidal effect that neem oil has on various organisms.

Literature that clearly distinguishes between clarified neem oil (CNO) and crude neem oil (NO) gives valuable insight into the efficacy of neem oil as an antifungal product. Recent investigation of foliar and fruit scab on "Empire" apple trees in Vermont showed promise for CNO as an organic fungicide. For example, CNO reduced foliar and fruit scab of apples compared to the alternative fungicides potassium carbonate and *Bacillus subtilis* (Cromwell et al., 2011). A similar CNO was also tested against various postharvest apple pathogens. Studies concluded it was as effective as conventional calcium chloride at preventing postharvest apple decay (Moline and Locke, 1993). Due to the location of anthracite brick manufacturing (central US), soil-borne plant pathogens from surrounding agriculture are a possible cause of the binder degradation and reduced strength in anthracite bricks. Solvent extracted CNO has shown inhibitory effects on soil-borne plant pathogens such as *Sclerotinia sclerotiorum* (Singh et al., 1980), *Rhizoctonia solani*, and *Fusarium oxysporum* (Locke, 1986). These findings reveal that CNO may serve as a possible organic fungicide to assist or replace copper in anthracite bricks and minimize fungal activity and binder consumption.

2.4 Traditional Copper Fungicides

The effective use of metals as pesticides is widely recognized as evidenced by the continued use of Bordeaux mixtures on crops, especially grapes. Copper, an ingredient in

Bordeaux mixtures, is one of the most widely used metals in biocide applications. Several factors may be associated with the toxicity and pesticidal effects of copper including bioavailability, distribution, and tolerance. Insoluble forms of copper make bioavailability and distribution difficult. Tolerance may be nutritionally dependent on organic substrate availability (Gadd et al., 2001) or metabolically dependent on oxalic acid production (Green and Clausen, 2003). Copper tolerant fungi, including many mycorrhizal brown-rot fungi, have created a necessity for pairing copper with co-biocides in antifungal applications. Traditional lumber preservatives such as chromated copper arsenate (CCA), alkaline copper quaternary (ACQ), and copper azole employ copper as their primary fungicide with arsenate, quaternary ammonium, or azole compounds as co-biocides. However, many of these compounds are expensive, highly regulated, and possibly dangerous as recent investigations have revealed endocrine-disrupting properties of azole-based fungicides (Taxvig et al., 2008; Kjaerstad et al., 2010). With this knowledge, full-scale production of anthracite bricks using similar compounds may be difficult and/or unwarranted.

Neem, a bio-pesticide discussed previously, naturally contains some metals including copper. However, most metals are removed during the extraction of clarified neem oil when they complex with natural ligands in the waste product, neem oil cake (NOC). Complexation of metals by NOC has been studied as a possible treatment for industrial strength wastewaters that contain copper (II), cadmium (II), or lead (II) (Rao and Khan, 2007 & 2009). However, those authors did not discuss synergistic interactions (possibly fungicidal) between clarified neem oil extract and metals such as copper. Active ingredients within neem products, including nimbin, nimbidin, salanin, gedunin, and many fatty acids (oleic, stearic, palmitic, and linoleic), have been proposed to be antifungal compounds associated with the reduction or elimination of fungal biomass and activity (Kumar and Parmar, 1996; Sundarasivarao and Madhusudhanarao, 1977; Biswas et al., 2002; NRC, 1992). Some of these compounds also contain hydroxyl (OH) and carboxyl (COOH) functional groups, which are possible binding and sorption sites for metals like

Cu(II). By combining copper co-biocide concepts with an organic bio-pesticide such as neem oil, an alternative to conventional copper-based fungicides was investigated in this work.

Chapter 3

Materials and Methods

3.1 Fungal Isolation and Culture Maintenance

Full-scale anthracite bricks (14.6 cm diameter x 5.7 cm height) (Figure 3-1) that were produced and stored in Furness-Newburge, Inc. (Versailles, KY), were shipped to Penn State University (PSU). In the laboratory, fungal growth on the outside of the bricks was aseptically removed and applied to an antibiotic (Ampicillin, Sodium Salt - OmniPur® EMD Millipore Chemicals; Kanamycin Sulfate – IBI Scientific) nutrient agar (AKNM) to isolate fungi without bacterial contamination (Atlas, 2004). Spread plates and plate streaking were performed to isolate fungal colonies. After 10-15 transfers, the fungal colony with the fastest growth was selected for further experimentation and identification. DNA was extracted from the fastest growing colony using a PowerSoil® DNA Isolation Kit (Mo Bio Laboratories, Inc.), and the 18S rRNA amplified using two sets of fungal primer pairs (FF390 & FR1, and nu-SSU-0817-5' & nu-SSU-1536-3') (Hagn et al., 2003, Borneman and Hartin, 2000). Sequencing and BLAST alignment revealed a probable 100% match with ectomycorrhizal soft-rot fungus *Chaetomium globosum*.

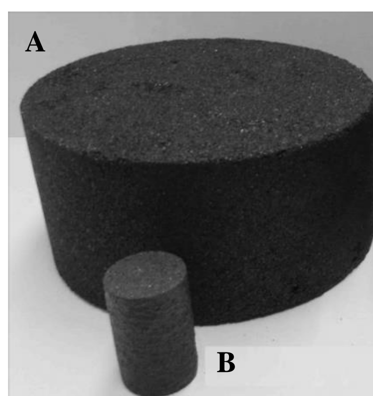

Figure 3-1. Size comparison of (A) full-scale anthracite brick (14.6 cm diameter x 5.7 cm height, 2.21 kg/brick) and (B) lab-scale briquette (2.5 cm diameter x 4.5 cm height, 29.4 g/briquette).

Plate cultures were grown on AKNM for 5-7 days at 30 ^oC and were then maintained at 5 ^oC for one month before being transferred. To transition from AKNM to a lignin-collagen media, fungi were weaned off the nutrient agar and onto a media comprised of only lignin and collagen. Liquid cultures were grown on identical media without agar by inoculating with agar plugs. Liquid cultures were shaken at 125 rpm at 30 ^oC for 7-10 days before liquid media was aseptically decanted, and the fungi rinsed and drained (3x) with sterile water. The resulting fungal biomass was pulse blended on high with an emersion blender (Model No. WSB33, Waring, USA) and stored at 5 ^oC for up to one month for future inoculum of liquid cultures and lab-scale briquettes.

3.2 Media Preparation

Three media were used throughout the experimentation: antibiotic nutrient media (AKNM), nutrient media (NMC), and lignin-collagen media (LCM). Media was prepared in distilled deionized water as follows: AKNM (5 g/L Peptone, 5 g/L NaCl, 2 g/L Yeast Extract, 1

g/L Beef Extract, 50 mg/L Ampicillin, 25 mg/L Kanamycin, 15 g/L R2A Agar); NMC (5 g/L Peptone, 5 g/L NaCl, 2 g/L Yeast Extract, 1 g/L Beef Extract, 15 g/L R2A Agar); LCM (2 g/L Lignin, 0.8 g/L Collagen, and 15 g/L Agar-Agar). All chemicals were reagent-grade or better quality.

3.3 Agar Diffusion Plate Method

Fungicide efficacy was determined using the agar diffusion plate method or in vitro contact assay (Feng and Zheng, 2007) with the isolated fungus *C. globosum*. Nutrient media (AKNM, NMC, and LCM) were autoclave sterilized (120 ^{o}C) and poured into 8 cm petri dishes. Antibiotics, when used, were prepared and added through filter sterilization (0.2 μm cellulose acetate membrane, VWR) directly to cooling media before agar solidification. All fungicides were also added following media sterilization while cooling before agar solidification: Azadiractin (AZ - Powdered Azadirachtin, Chem Service, West Chester, PA); azadirachtin-based compounds (AS - Azasol™, Arborjet, Inc.; AM - Azamax®, General Hydroponics®); crude neem oil (NO - TheraNeem™, Organix® South, Inc.); clarified neem oil extract (CNO - Neem Oil Extract Concentrate, Garden Safe®); copper (copper (II) carbonate basic: Sigma Aldrich, St. Louis MO); and azole compound (Miconazole 99%, Alfa Aesar, Ward Hill, MA). TheraNeem™ reportedly contained a minimum of 1500 ppm azadirachtin, while the Garden Safe® Neem Oil Extract contained no azadirachtin. Copper carbonate, insoluble in the agar media, was continuously mixed during plating to keep copper particles in suspension and create a uniform distribution of copper. Copper azole was applied at the concentrations used in actual lumber preservation (0.13-0.15 lb/ft^3).

Agar media plates containing fungicide, and control plates without fungicide, were prepared in triplicate, inoculated with *C. globosum* circular plugs (1 cm diameter), and incubated

at 30 °C. All plates were sealed with Parafilm (Pechiney Plastic Packaging, Inc., Chicago, IL) to prevent external contamination, and inverted to prevent condensation on the agar during incubation. Radial measurements were taken to calculate average fungal mat growth (cm^2). Treatments were discontinued when mats reached the edge of the plates and growth could no longer be measured, or when growth reached a finite area (plateau in graph).

3.4 Lab-Scale Briquette Production

Lab-scale briquettes (2.5 cm diameter x 4.5 cm height, SA ~ 45 cm^2) (Figure 3-1) were prepared in batches of ten by combining anthracite fines #4 (Jeddo Coal Company, Hazelton, PA), low sulfur softwood lignin (Innnventia, Stockholm, Sweden), silicon metal (Sigma Aldrich, St. Louis, MO), and granular collagen (Hormel Foods Co. via Entelechy Co., Austin, MN) similar to previously reported compositions (Lumadue et al., 2012). Prior to production, the briquette materials were processed as follows: a fraction of the anthracite fines were crushed to obtain tighter packing; silicon lumps were crushed and sieved to pass a #100 mesh (0.146 mm); lignin was crushed and sieved to pass a #40 mesh (0.42 mm); and collagen was denatured via water hydrolysis at approximately 70 °C. To form each briquette, the mixture was placed into a cylindrical briquette die, compacted in a hydraulic press (Model #3912, Carver Inc., USA), and removed for drying at 60 °C for 24 hours to ensure all briquettes maintained similar moisture content.

Briquettes treated with fungicides included batches 3 to 12 (Table 3-2). Internal copper (batch 3,4,7, & 8) was dosed into the briquette mixture using a copper to organic (lignin and collagen) ratio of 5 mg Cu/g LC due to findings by Gadd et al. (2001) which showed an increase in fungal tolerance to copper with increased levels of organic substrate. A 15 mg of copper per gram of lignin-collagen was used for internal copper when CNO was not present within the

briquettes (batch 9, 10, 11, & 12). External or surface applied copper (batch 11 & 12) was prepared in solution (14 mg/L) with a corresponding mass of CNO, stirred and heated to 75 ºC to maintain a homogeneous mixture, and applied by brushing the solution onto the surface of the briquettes. Liquid fungicide CNO is traditionally applied in practice by surface area loading. Therefore, internal CNO concentrations (batch 5, 6, 7, & 8) were applied based on the surface area of the anthracite grains where 40 g/L is equivalent to 0.4 mg/cm^2 (Appendix A.2). The volume of water for collagen denaturing was reduced for all briquettes with internal applied CNO so that the final liquid volume was constant. Surface applied CNO was prepared in solution (40 g/L CNO), stirred and heated to maintain a homogenous mixture, and applied by brushing the solution onto the surface of the briquettes. All surface applied fungicide briquettes were oven dried at 60 ºC for one hour before inoculation and incubation to ensure compound adherence to the surface of the briquettes.

Table 3-2. Experimental design for briquette batch experiments with total mass of lignin, collagen, and fungicide applied per briquette (2.5 cm diameter x 4.5 cm height; 29.4 g/briquette; ~45 cm^2 surface area).

Batch	Lignin (g)	Collagen (g)	Internal Copper (mg)	Surface Applied Copper (mg)	Internal CNO (g)	Surface Applied CNO (mg)	Fungus
Blank	1.27	0.56	---	---	---	---	-
Blank-F	1.27	0.56	---	---	---	---	+
Cu	1.27	0.56	9.15	---	---	---	-
Cu-F	1.27	0.56	9.15	---	---	---	+
CNO	1.27	0.56	---	---	1.25	---	-
CNO-F	1.27	0.56	---	---	1.25	---	+
CuCNO	1.27	0.56	9.15	---	1.25	---	-
CuCNO-F	1.27	0.56	9.15	---	1.25	---	+
Cu(CNO’)	1.27	0.56	27.45	---	---	18	-
Cu(CNO’)-F	1.27	0.56	27.45	---	---	18	+
Cu(CuCNO’)	1.27	0.56	27.45	0.006	---	18	-
Cu(CuCNO’)-F	1.27	0.56	27.45	0.006	---	18	+

CNO: Clarified Neem Oil Extract
+ Fungus Applied
- No Fungus applied

3.5 Fungal Inoculation of Lab-Scale Briquettes

Growth chambers for each briquette were constructed from a 150 mL glass beaker (Kimax: Kimble Chase Life Scientific, Rockwood, TN), partially filled with 60 g (~50 mL) of 5 mm solid soda lime glass beads (#3000: Walter Stern, Inc.) and covered with aluminum foil. All chambers were gravity autoclave sterilized (15 min at 120 ºC, 30 min drying/cooling), then aseptically filled with 10 mL of sterile de-ionized distilled water to humidify the air before the briquettes were inserted. Sterile fungal inoculation was done with one milliliter of liquid mycelia biomass (3.75 mg DM/mL) cultured as previously described (Section 3-1). Briquettes were incubated at 30 ºC for three weeks (21 days) in the absence of light, then removed from the chambers and dried at 60 ºC for 24 hours to remove any absorbed moisture. Following drying, the presence or lack of effective collagen was estimated by testing half of each batch (5 briquettes) for unconfined compressive (UC) strength at room temperature in an electronic universal sand strength machine (Simpson-Gerosa Model #42104, Simpson Tech. Corp.), while the other half was pyrolyzed at 900 ºC for 5 min under a nitrogen atmosphere in a vertical quartz tube furnace (Series 3219, Applied Test Systems, Inc.) and then tested for UC strength to determine the presence or lack of effective lignin. These strength measurements can be used to estimate the effective collagen and lignin remaining after fungal inoculation and incubation by comparison with control briquettes with no fungal inoculation.

3.6 Protease Enzyme Activity of Biomass from Liquid Media

Lignin-collagen liquid media (2.0 g lignin/L, 0.8 g collagen/L) was prepared, autoclave sterilized, and added to 30 mL sterile glass vials. Media and fungicides were combined to a total volume of 25 mL (Cu/LC = 10 mg/g; CNO = 12 g/L) followed by the addition of 5 mL of liquid

mycelia biomass (3.75 mg DM/mL) to reach a final volume of 30 mL. Following seven day growth on a shaker table (30 ºC; 200 rpm), vials were centrifuged (7500 rpm, 10 min) to isolate biomass, the liquid media decanted, biomass rinsed with buffer solution (SPB: sodium phosphate buffer), centrifuged again (8000 rpm, 10 min), supernatant decanted, and filled with SPB to a final volume of 5 mL. Biomass in the resulting 5 mL SPB was added to 95 mL SPB to achieve a final volume of 100 mL. Total esterase activity (TEA) was measured according to Schnurer and Rosswall (1982) and similar to Verma et al. (2008) with sodium fluorescein salt standards prepared according to Adam & Duncan (2001).

Chapter 4

Results

4.1 Fungicidal Effects of Neem, Neem Compounds, and Copper

Radial growth measurements of fungal mycelia on agar media provided a rapid analysis of fungicides against *C. globosum*. Fungicides could be applied alone or in combination on different media (AKNM, NMC, and LCM) without labor intensive briquette production. Results from radial growth plots were ultimately used to determine fungicidal loadings to be applied to lab-scale briquettes.

4.1.1 Azadirachtin Effect on Fungal Growth

Preliminary review of neem oil led to the investigation of azadirachtin, the most studied component of neem and neem products. Analysis of azadirachtin-based compounds Azasol™ (AS), Azamax® (AM), and pure lab grade azadirachtin (AZ) at a typical gardening dose (50 ppm

azadirachtin) showed little biological control of *C. globosum* compared to control growth on antibiotic (AKNM) and nutrient media (NMC) controls as seen in Figure 4-1. Azamax® revealed the largest fungal inhibition, limiting radial growth for an additional two days before reaching maximum growth.

The concentration of azadirachtin in AS and AM was then increased by five and ten times the recommended dose to observe the resulting effect on fungal growth. Due to the high cost and instability of AZ, no further analysis was conducted with this compound. Copper azole (CuAz) was also examined at typical wood preservative concentrations (0.13-0.15 lb Cu/ft^3 lumber = 3.5 g Cu/L water; Appendix A.1). Azasol™ exhibited no change in fungal inhibition from increased concentrations and treatments closely mimicked the control (Figure 4-2). With an increase in concentration, AM did exhibit more fungal inhibition by delaying maximum growth for up to seven days. The wood preservative CuAz showed little to no growth at seven days and the fungus never achieved maximum growth on the surface area available.

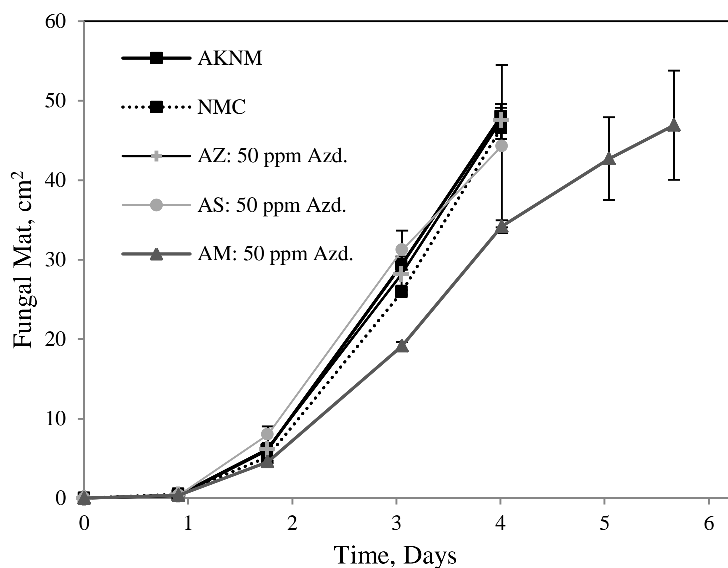

Figure 4-1. The effect of pure lab-grade azadirachtin (AZ) and azadirachtin-based compounds Azasol™ (AS) and Azamax® (AM) on the radial plate growth of *C. globosum*. Values represent the average of triplicate samples; error bars equal one standard deviation; Azd.=Azadirachtin.

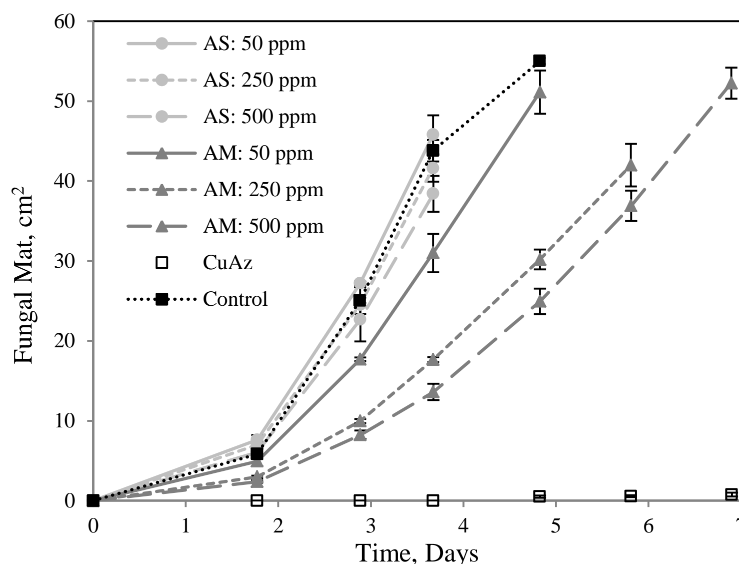

Figure 4-2. Antifungal effects of azadirachtin-based compounds Azasol™ (AS) and Azamax® (AM) at one, five, and ten times the residential gardening dose (50 ppm azadirachtin) on radial plate growth of *C. globosum*. Concentrations are as azadirachtin (ppm); values represent the average of triplicate samples; error bars equal one standard deviation.

4.1.2 Crude Neem Oil and Clarified Neem Oil Extract Fungicide Efficacy

Crude neem oil (NO) and solvent extracted clarified neem oil (CNO) were examined at traditional gardening concentrations as well as five and ten times larger doses. The concentrations of oil and the corresponding azadirachtin concentrations are provided in Table 4-1. Figure 4-3 shows similar trends of increasing antifungal effects with respect to increased doses of NO and CNO. After eight days, NO 10 reached maximum growth but displayed large standard deviations. For 12 days, seven days longer than the controls, CNO 10 mimicked the CuAz treatment and outperformed all other neem treatments. Figure 4-4 summarizes the most inhibitory dose of each compound tested; all doses were ten times larger than the recommended dose. Antibiotics in the antibiotic control (AKNM) had no effect on the radial mycelia growth compared to nutrient media without antibiotics (NMC). Therefore, all future controls were done on nutrient media alone (NMC). Azadirachtin compounds AS and AM showed the least amount

of antifungal activity compared to the oil-based fungicides NO and CNO and the lumber preservative CuAz. Clarified neem oil extract was determined to be the most effective neem compound for inhibiting the growth of ectomycorrhizal fungus *C. globosum*, and was therefore carried forward for future co-biocide analyses.

Table 4-1. Fungicidal neem oil and azadirachtin concentrations in crude neem oil (NO) and clarified neem oil (CNO) petri dish treatments.

Notation	Crude Neem Oil, NO (mg/L media)	Clarified Neem Oil Extract, CNO (mg/L media)	Azadirachtin (mg/L)
*NO	4,750	--	7.8
NO 5	23,750	--	39
NO 10	47,500	--	78
*CNO	--	5,000	--
CNO 5	--	25,000	--
CNO 10	--	50,000	--

* Traditional gardening concentrations

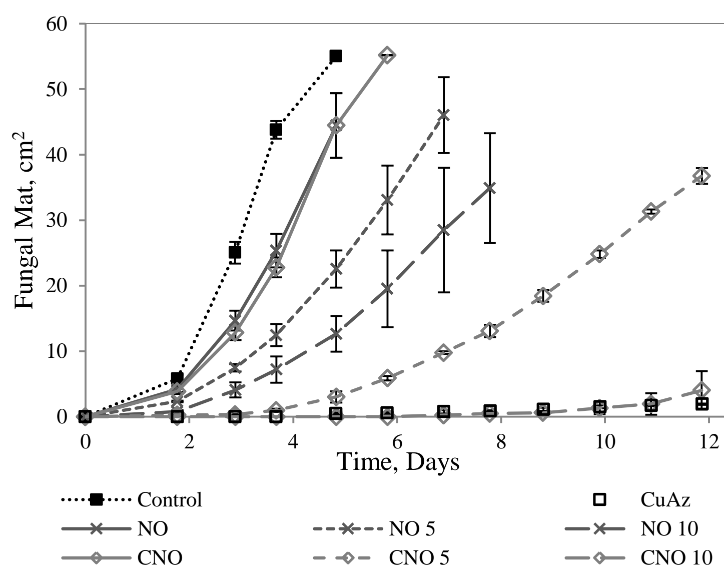

Figure 4-3. The effect on radial growth of *C. globosum* with crude (NO) and clarified (CNO) neem oils compared to lumber preservative copper azole (CuAz). Neem and azadirachtin concentrations from Table 4-1; CuAz: 3.5 g Cu/L, 190 mg miconazole/L. Values represent the average of triplicate samples; error bars equal one standard deviation.

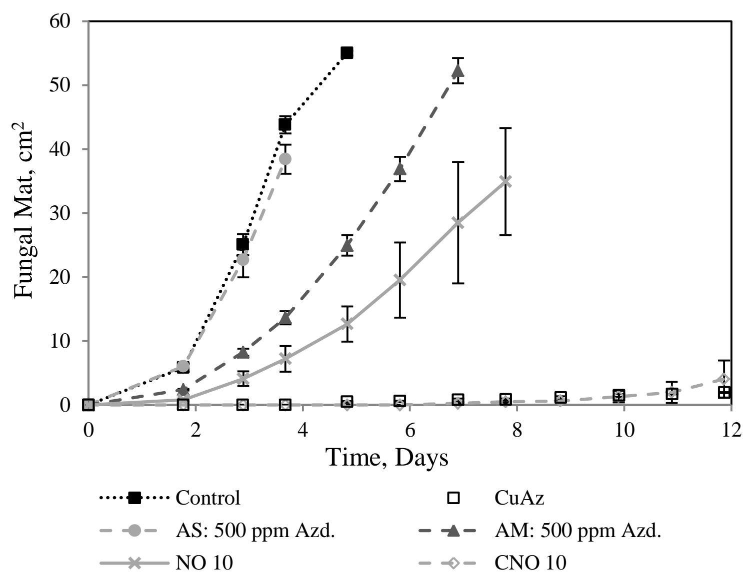

Figure 4-4. Summary of largest antifungal effects of azadirachtin (Azd.) compounds Azamax®(AM) and Azasol™(AS), crude neem oil (NO), and clarified neem oil (CNO) compared to copper azole (CuAz). Values represent the average of triplicate samples; error bars represent one standard deviation.

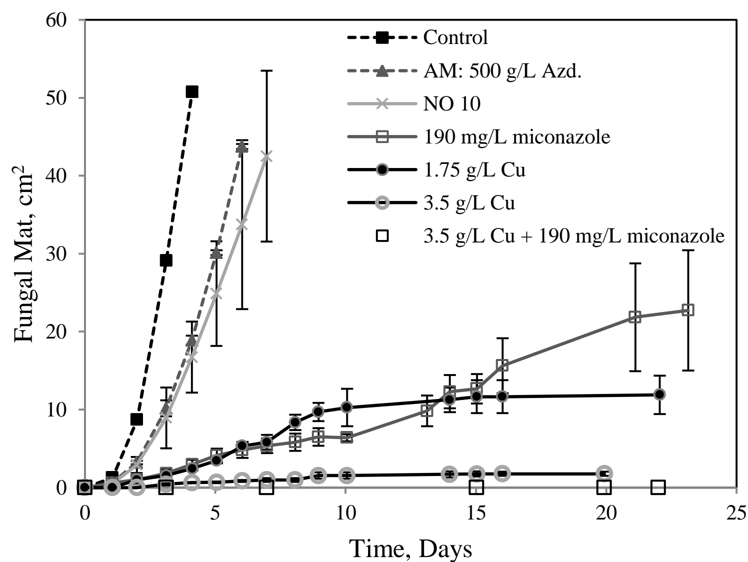

Figure 4-5. Fungal inhibition of copper azole (CuAz) and individual components copper and miconazole compared to the control and reference treatments Azamax™(AM) and crude neem oil (NO 10). Values represent the average of triplicate samples; error bars represent one standard deviation.

4.1.3 Examination of Copper with Co-Biocides Miconazole & CNO

Copper azole and its components, copper carbonate and miconazle, were examined to determine the antifungal effects that each provide. Figure 4-5 reveals a limit or maximum radial growth that can be achieved by the fungal mycelia on nutrient media when grown in the presence of copper at one half (1.75 g/L Cu) and full (3.5 g/L Cu) wood preservative concentrations. Miconazole was able to slow mycelia growth but never terminated growth completely. The combination of copper and miconazole resisted fungal growth for the observed 22 days.

Evidence of the limiting effect of copper on the maximum radial growth of *C. globosum* led to the investigation of clarified neem oil (CNO), deemed the most effective neem compound, to be used as a co-biocide to copper. At a low dose of copper (one half of the wood preservative loading), fungal growth was erratic with little correlation between clarified neem oil concentrations and maximum growth (Figure 4-6 A). As the copper concentration was increased to the full wood preservative loading (3.5 g/L), there was an overall reduction in the maximum radial growth observed (Figure 4-6 B). A noticeable decrease in the maximum growth was also observed when CNO was increased from 7.5 g/L to 10 g/L (Figure 4-6 B). Copper alone (3.5 g/L) showed minimal growth before reaching a maximum mycelia area of 2 cm^2. The only treatments to completely inhibit new fungal growth were 25 g CNO/L at both high and low copper loadings (3.5 and 1.75 g Cu/L).

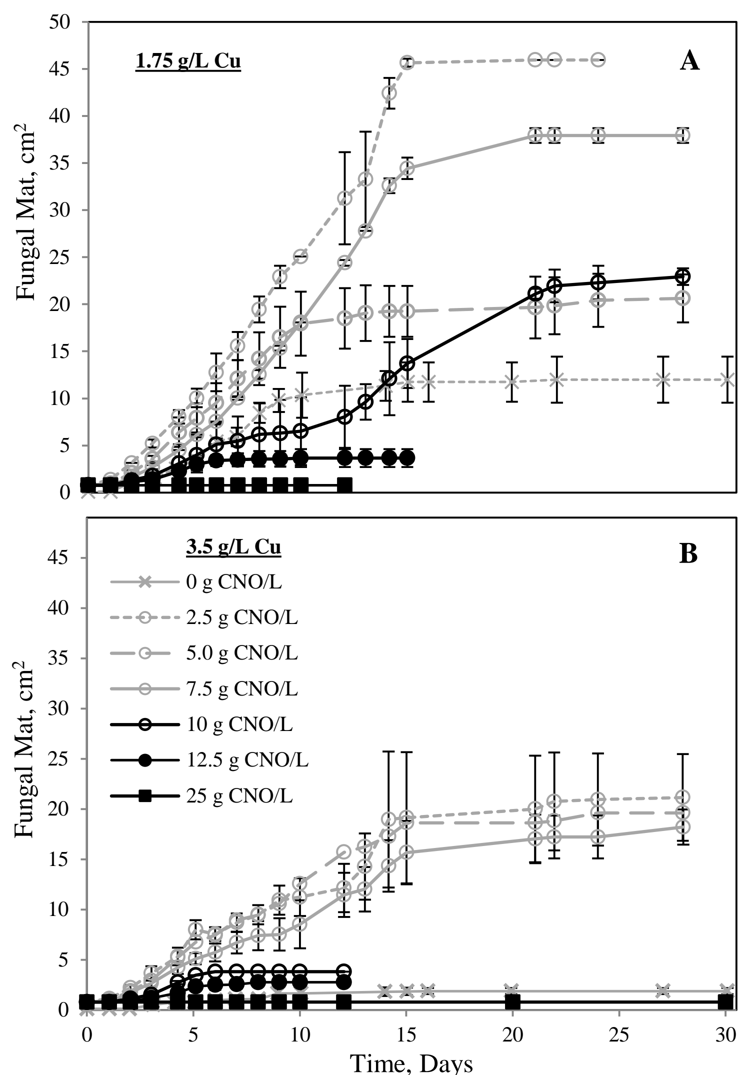

Figure 4-6. The effect of (A) one half the wood preservative copper loading and (B) full wood preservative copper loading with clarified neem oil (CNO) as a co-biocide on the radial growth of *C. globosum*. Values represent the average of triplicate samples that fell within a 95% CI (n=2-3); error bars represent one standard deviation.

4.1.4 Copper and CNO Preservation of Lignin and Collagen

While complete fungal inhibition was achieved on the nutrient based media (NMC), the preservation of the anthracite bricks requires inhibition of fungal degradation of their organic components: lignin and collagen. The fungus *C. globosum* was therefore weaned from NMC to a collagen and lignin based media with a lignin to collagen mass ratio (L:C) identical to full-scale brick production (Table 4-2). Although fungal growth was observed on both collagen and lignin alone, as well as combined lignin and collagen (Figure 4-7), it was most rapid in the presence of collagen alone, revealing this fungus' affinity for collagen.

The final radial growth experiments were completed using the lignin-collagen media (LCM) as copper and clarified neem oil was gradually increased to form a fungicidal interaction matrix (Table 4-3). Radial growth experiments were completed for each copper and clarified neem oil combination in triplicate. The interaction matrix was completed in two segments: (1) copper to lignin-collagen ratio (Cu/LC) ranging from 0–10 mg Cu/g LC; and (2) a Cu/LC ranging from 15–25 mg/g. Controls between segments confirmed that fungal activity did not affect the results. After seven days, the control (0 g CNO/L media & 0 mg Cu/L media) reached maximum growth, and all seven day values were plotted (Figure 4-8). Growth measurements were recorded for an additional seven days, or a total of two weeks, for the first segment (Cu/LC 0-10 mg/g) (Appendix B). Measurements for the second segment were only recorded at day seven. Results show a decrease in fungal growth as copper is increased (0-15 mg Cu/g LC) and as CNO is increased (0-40 g CNO/L media). The addition of CNO also inhibits growth compared to copper alone below a Cu/LC of 10 mg/g for all CNO doses tested. At 10 mg Cu/ g lignin-collagen, the addition of five and ten g CNO/ L media may slightly promote growth compared to copper alone. The importance of Figure 4-8 is to reveal where the addition of CNO either promotes (above 0 g CNO/L media) or inhibits (below 0 g CNO/L media) fungal growth compared to copper alone.

Table 4-2. Composition of lignin and collagen in media to transition the fungus from liquid-based media to anthracite binder paste in bricks.

Source	Lignin, g	Collagen, g	Total Organics, g	L:C
Nutrient Media (NMC)	--	--	10.8	--
Bricks (Full-scale Batch)	375	150	525	2.5
Lignin-Collagen Media (LCM)	2	0.8	2.8	2.5

L:C Lignin to Collagen Ratio

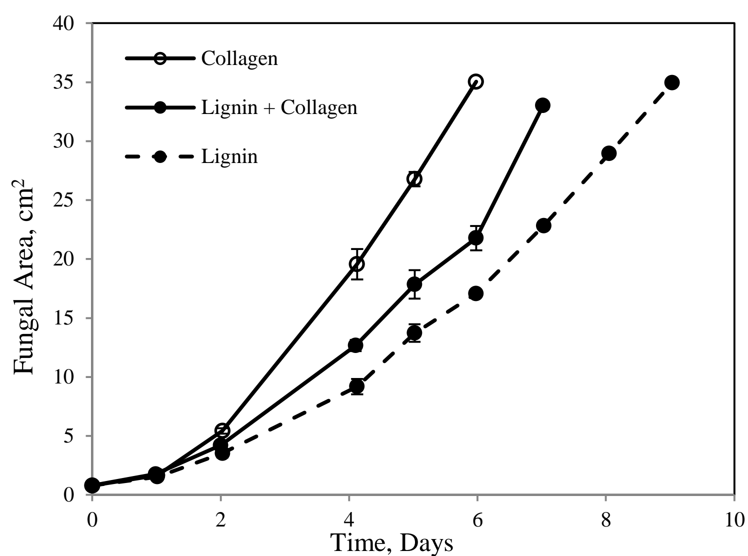

Figure 4-7. Radial growth of *C. globosum* in the presence of collagen (0.8 g/L) and lignin (2 g/L) alone, compared to the combination of lignin and collagen. Values represent the average of triplicate samples; error bars represent one standard deviation.

Table 4-3. Seven day radial growth (cm^2) of *C. globosum* with increased concentrations of copper and clarified neem oil (CNO) within media containing lignin and collagen (LC).

Clarified Neem Oil		**Copper, mg/L (Cu/LC, mg/g)**					
C_{CNO}, g CNO/L media	**CNO/LC, mg/g**	**0 (0)**	**14 (5)**	**28 (10)**	**42 (15)**	**56 (20)**	**70 (25)**
0	**0**	33.0	24.8	9.53	1.15	0.785	0.785
5	**1,786**	13.3	15.7	13.6	2.43	0.785	0.785
10	**3,571**	10.8	11.9	12.3	1.63	0.785	0.785
20	**7,143**	7.89	8.73	7.37	3.43	0.970	0.785
40	**14,286**	4.67	1.18	0.785	1.27	0.813	0.785

Copper to lignin-collagen ratio (Cu/LC) based on 2.8 g lignin + collagen/L media
Radial growth of 0.785 cm^2 = No growth (Inoculation plug size)
Values represent the average of triplicate samples

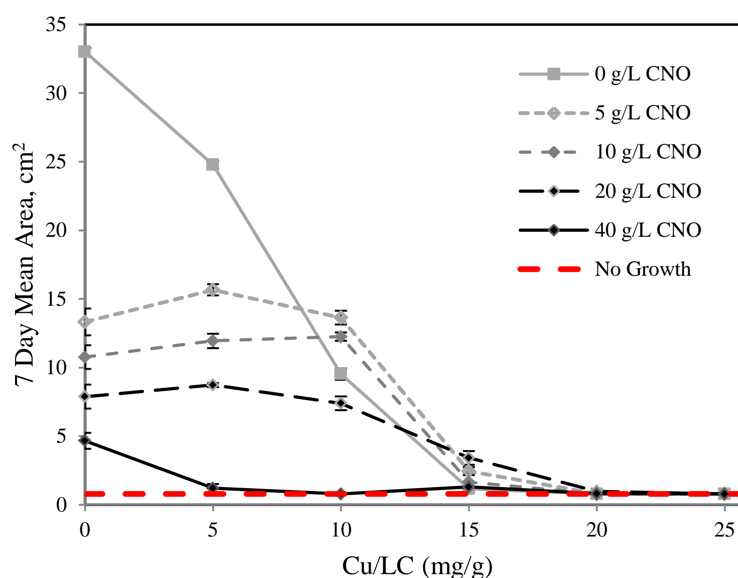

Figure 4-8. Synergistic inhibitory effects of copper and clarified neem oil (CNO) on the seven day radial growth of *C. globosum* reveal the effect of CNO on growth. Experiment conducted in two segments: (1) Cu/LC 0-10; and (2) Cu/LC 15-25. Values represent the average of triplicate samples; error bars represent one standard deviation.

The data from Table 4-3 and Figure 4-8 was analyzed for interaction effects by interaction residuals (Figure 4-9) and two-factor analysis of variance (ANOVA) with replication (Appendix A.4). The response in the interaction residual graph (Figure 4-9) is growth, therefore a lower interaction residual (negative numbers) correlates to reduced growth compared to other treatments. Interaction residuals reveal a similar response to using 40 g CNO/L at a Cu/LC of 5 mg/g or copper alone above 15 mg/g. ANOVA results show a significant difference in the effect of increasing copper or increasing CNO, as well as a statistically significant interaction between the two fungicides with P-values less than 0.05 (Appendix A.4).

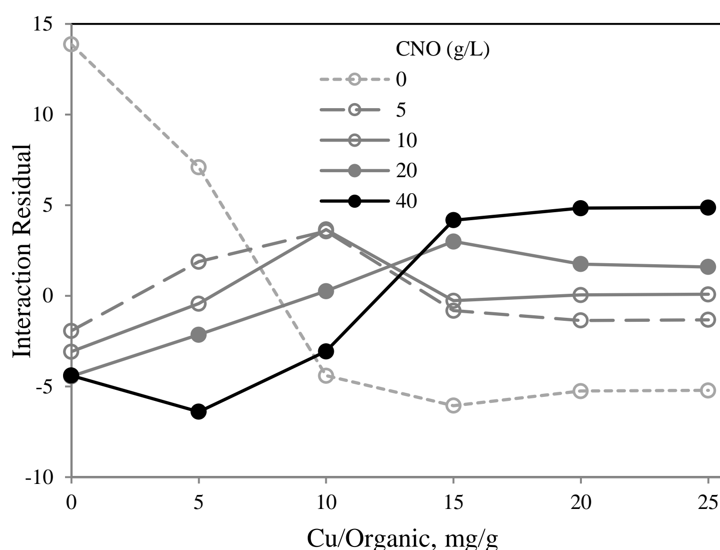

Figure 4-9. Interaction residuals from the seven day radial growth of *C. globosum* on lignin-collagen media reveal interactions for translation from media plates to lab-scale briquettes. Values represent average of triplicate samples adjusted by removing overall, copper, and clarified neem oil averages from the data set (Appendix A.3).

To better understand the mechanisms and relationship between copper and CNO, enzyme analysis of protease (collagen degradation) as well as laccase and peroxidase (lignin degradation) is required. Previous radial growth analysis indicated an affinity for fungal growth on collagen. Enzyme analysis of protease was performed on mycelia biomass grown in liquid lignin-collagen

media for seven days with various copper compounds combined with CNO (Figure 4-10). Results indicate that CNO may have the ability to stimulate growth but suppress the production of protease enzymes. Different variations of activity and mass are provided in Appendix B, Figures B-1 and B-2. Further research should be done to better quantify protease activity and also explore lignin degrading enzymes.

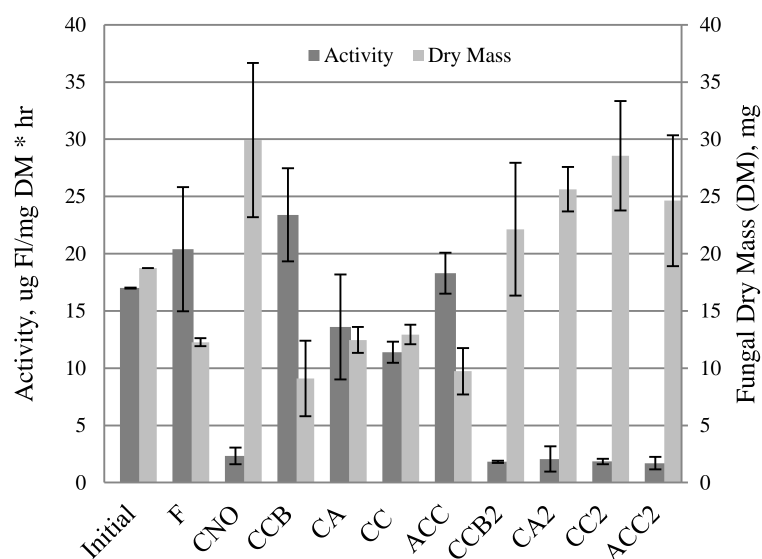

Figure 4-10. Biomass and biological activity of *C. globosum* after seven days on liquid lignin-collagen media with four different copper compounds with and without CNO measured by fluorescein diacetate (FDA) hydrolysis. Activity expressed as micrograms of fluorescein hydrolyzed per milligram of dry mass per hour (ug Fl/mg DM * hr). Copper concentrations: 10 mg Cu/g LC. CNO concentration: 12 g/L. F: Final Measurement; CNO: Clarified Neem Oil; CCB: Copper Carbonate Basic; CA: Copper Acetate; CC: Copper Chloride; ACC: Ammonium Copper Chloride; 2: With CNO.

4.2 Fungicidal Treatment of Lab-Scale Briquettes

Based on the results of the plate growth experiments, the best fungicide combinations were applied to lab-scale briquettes, incubated for 21 days at 30 ^{o}C under 100% humidity, and then tested for unconfined compressive strength. Incubation mimicked harsh, unrealistic conditions for briquette storage to ensure that the organic binders can be preserved under the worst environmental conditions. Following the 21 day incubation, strength tests of the briquettes were conducted at room temperature (to determine primarily collagen strength), after pyrolyzation (to determine primarily pyrolyzed lignin strength), and compared to non-incubated control briquettes (no fungicide or applied fungus). Control briquettes at room temperature had an average UC strength of 314.8 ± 10.3 psi. Pyrolzyed control briquettes had an average UC strength of 161.7 ± 16.3 psi. All briquettes displayed fungal growth (Figure 4-10); however, some briquettes displayed minimal growth while others had extensive growth (Figure 4-11). By measuring UC strength at room temperature and after 900 ºC pyrolysis, the fungal degradation of binder (collagen and lignin) can be estimated, and the effects of the applied fungicide on the strength of the briquettes can be quantified.

Figure 4-10. Lab-scale briquettes following 21 day incubation at 30 ºC and 100% humidity reveals fungal growth on all briquettes with (A) minimal growth on some and (B) extensive growth on others. Fungicides were applied internally to briquette batches unless otherwise noted. Cu=Copper; CNO=Clarified Neem Oil; CNO'=External CNO Fungicide; F=Fungus applied.

Figure 4-11. Lab-scale briquettes following 21 day incubation at 30 ºC and 100% humidity reveals significant difference in the amount of fungal growth observed: (A) minimal growth on some non-inoculated bricks (B) extensive growth on some inoculated bricks.

The room temperature UC strength of briquettes incubated with and without fungus is provided in Figure 4-12. Left to right, the first eight measurements (internal incorporation of fungicides) were used to validate the antifungal interaction between copper (Cu) and clarified neem oil (CNO). Interaction was validated as the inoculation of fungus decreased strength in the Blank (39%), Cu (31%), and CNO (73%) treatments, while the addition of fungus had almost no effect on the room temperature strength of the briquettes treated with Cu and CNO (CuCNO). However, the internal incorporation of CNO (CNO and CuCNO) significantly reduced the room temperature strength of briquettes compared to treatments without CNO (Blank and Cu). As an alternative application method, CNO was applied to the surface of briquettes alone (Cu(CNO')) and combined with copper (Cu(CuCNO')) with an additional internal copper fungicide in both treatments. All treatments with surface applied CNO exhibited an increase in room temperature strength even when inoculated with fungus. A 38% increase in room temperature strength was observed with surface applied CNO (Cu(CNO')), and a 47% increase in room temperature strength was observed when copper and CNO were applied together (Cu(CuCNO')). Collagen degradation from fungal inoculation correlated to a 39% reduction in room temperature strength by comparing Blank and Blank-F briquette batches. Overall, the room temperature strength and preservation of collagen binder was maintained or enhanced through the incorporation of CNO to the surface of anthracite briquettes.

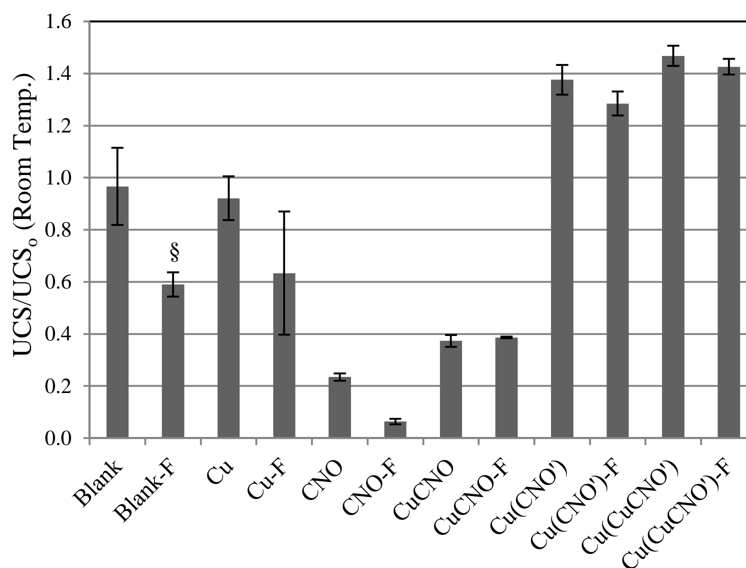

Figure 4-12. Unconfined compressive strength (UCS) of lab-scale anthracite briquettes following 21 day incubation in humidified chambers compared to the strength of non-incubated briquettes without fungicides/fungus (UCS_o) at room temperature (collagen strength). UCS/UCS_o: 1.0 = 314.8 ± 10.3 psi. Average of values within a 99% CI (n= 3-5; §: n=2); error bars represent one standard deviation of values within 99% CI. Mass of fungicides applied to each briquette batch is provided in Table 3-2. Cu=Copper; CNO=Clarified Neem Oil; CNO'=External CNO Fungicide; F=Fungus applied.

The preservation of lignin was determined by UC strength of the briquettes after collagen was removed through pyrolysis, similar to cupola conditions (Figure 4-13). Fungicidal interaction effects of copper and CNO was not observed by briquette strength following pyrolysis. As a whole, briquettes internally treated with copper, CNO, or a combination of the two maintained 73% of the lignin binder strength compared to only 44% of the room temperature collagen strength. Contrary to room temperature strength analysis, there was no distinct difference in strength following pyrolysis between internally applied CNO and externally applied CNO. Lignin degradation from fungal inoculation was correlated to a 26% reduction in pryolyzed strength by comparing Blank and Blank-F briquette batches.

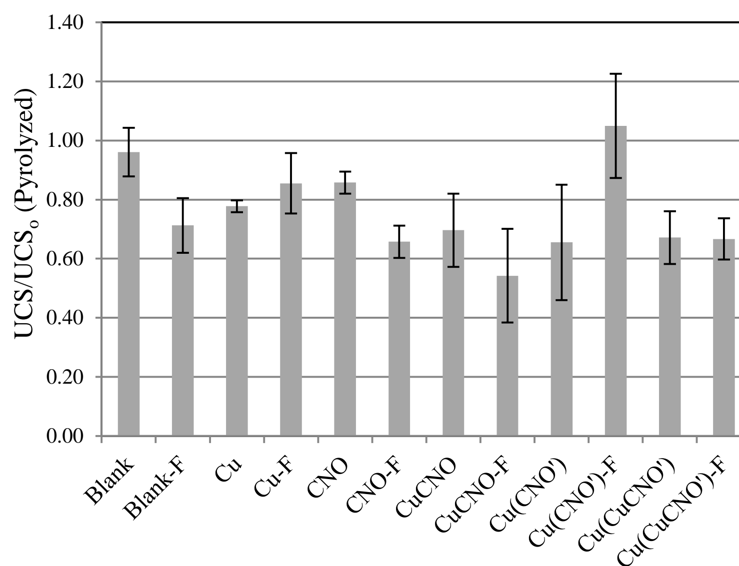

Figure 4-13. Unconfined compressive strength (UCS) of lab-scale anthracite briquettes following 21 day incubation in humidified chambers compared to the strength of non-incubated briquettes without fungicides/fungus (UCS_o) after pyrolyzing (lignin strength). UCS/UCS_o: 1.0 = 161.7 ± 16.3 psi. Average of values within a 99% CI (n= 3-5); error bars represent one standard deviation of values within 99% CI. Mass of fungicides applied to each briquette batch is provided in Table 3-2. Cu=Copper; CNO=Clarified Neem Oil; CNO'=External CNO Fungicide; F=Fungus applied.

Chapter 5

Discussion

5.1 Organic Neem and Traditional Copper Fungicides

5.1.1 Azadirachtin

Results showed that azadirachtin, the most researched active ingredient from the neem tree, and azadirachtin-based compounds alone exhibited little effect on the growth of *Chaetomium globosum*. This evidence is supported by a large volume of literature and literature reviews on azadirachtin as a primary insecticide and not a strong fungal deterrent (Schmutterer, 1990; Biswas et al., 2002; Rembold, 1989; Koul et al., 1990). Azadirachtin has been shown to be relatively unstable with degradation kinetics increasing with increased temperature and decreased pH (Barrek et al., 2004). Authors also indicated a possible increase in degradation of azadirachtin in sunlight. To incorporate azadirachtin and related compounds into agar based media, the compounds were added following autoclave sterilization (120 ^{o}C) before the media could solidify, possibly degrading the compound before it could be tested as a fungicidal option. The most effective azadirachtin compound was Azamax® (AM) at ten times the recommended dose. However, all azadirachtin compounds eventually reached maximum growth within one week, and before copper azole (CuAz) showed any significant growth. With an indication of more insecticidal properties and increased degradation at high temperatures, azadirachtin was deemed a weak fungicide and a poor option for the treatment of anthracite bricks.

5.1.2 Crude Neem Oil and Clarified Neem Oil

The largest fungi-toxic effects of neem compounds were observed with crude neem oil (NO) and clarified neem oil (CNO). The recommended dose of NO mimicked treatment with the recommended concentration of AM, but larger concentrations of NO prevented the spread of *C. globosum* and outlasted elevated AM concentrations (Figure 4-2 and 4-3). The use of NO in radial plate growth experiments yielded large variations within the data, likely due to the lack of homogeneity in the agar plates treated with NO. The cold-pressed neem oil is insoluble in water and created noticeable pockets of NO dispersed randomly across the agar surface (Figure 5-2). With no azadirachtin, CNO was comparable to the lumber preservative copper azole (CuAz) at ten times traditional gardening doses. As CNO was increased from 5,000 to 25,000 ppm, fungal growth inhibition increased 50%, lasting an additional 6 days before reaching maximum growth (Figure 4-3). After twelve days, CNO at ten times the recommended dose experienced a slight increase in fungal growth, but was not significantly larger than the CuAz treatment. The CNO results showed less variation than NO, likely due to emulsifiable properties of the product. The fatty acids and waxes found in NO may act as a lubricant in anthracite brick production, decreasing strength. However, the majority of these compounds are removed during the production of CNO, making it a reasonable addition to the current anthracite bricks. The use of clarified neem oil alone only prevents initial fungal colonization, as larger doses only increase the initial lag time before growth proliferates (Figure 5-3). Similarly, copper alone initially allows growth to occur before plateauing to a finite maximum area. As copper concentrations increase, the maximum growth of *C. globosum* decreases (Figure 4-5 and 4-6). These data indicate that CNO as a co-biocide to copper could provide a fungicidal option for the prevention of fungal growth on anthracite bricks.

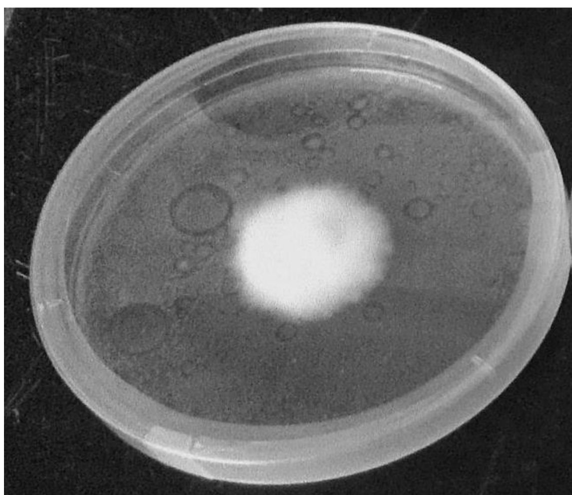

Figure 5-2. Insoluble cold-pressed neem oil, or crude neem oil (NO), creates pockets of oil throughout the surface of the agar leading to variability in fungal inhibition and larger standard deviations in the data.

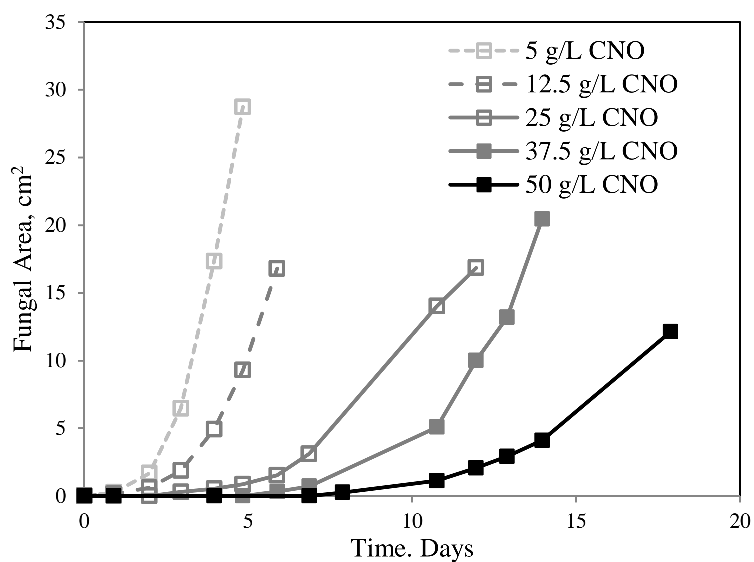

Figure 5-3. Clarified neem oil extract (CNO) exhibits increased initial growth inhibition with increased concentrations, but ultimately allows growth to occur. Data represent average *C. globosum* growth of triplicate samples on NMC media.

5.1.3 Co-Biocide Application of Clarified Neem Oil

The efficacy of clarified neem oil as a co-biocide to copper on lignin-collagen media was confirmed from Figure 4-8 and ANOVA analysis of the resulting data. A statistically significant interaction was confirmed between copper and clarified neem oil. Inhibition of growth using copper alone could be achieved at a Cu/LC of 15 mg/g; however, the addition of CNO significantly reduced seven day fungal growth at 5 mg/g (below copper alone with 0 g/L CNO) indicating that the fungi-toxic combination of copper and CNO is stronger than the effect of copper alone. Two important observations from the experiment, displayed in Figure 5-4, are: (1) copper up to 28 mg/L (or Cu/LC 10 mg/g) does not inhibit growth when CNO is present; and (2) copper alone and the combination of copper and CNO above a Cu/LC of 10 mg/g exhibit similar fungal growth inhibition.

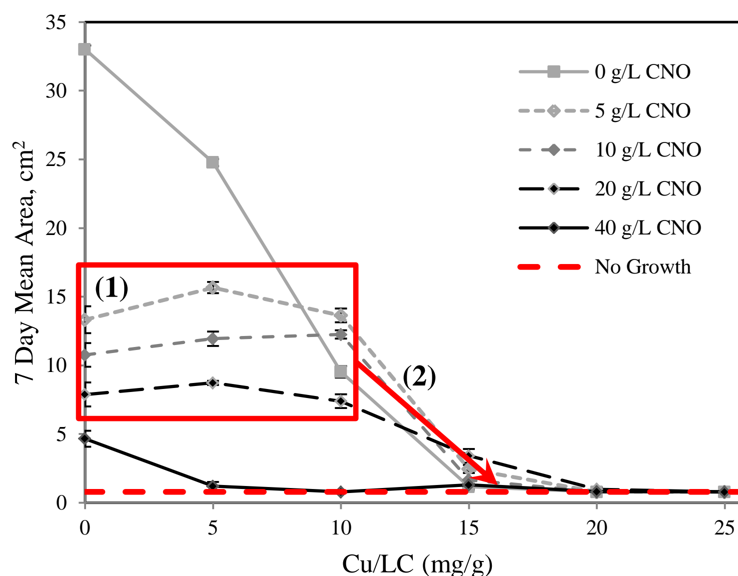

Figure 5-4. Key findings from copper and CNO interaction experiment reveal (1) stable growth in the presence of CNO with increasing copper and (2) a point where stable treatments follow the trend of copper alone.

With copper alone, a consistent decrease in growth is observed as copper concentrations are increased. Alternately, in the presence of 5, 10, and 20 g/L CNO, growth is not significantly reduced until a Cu/LC of 15 mg/g. Due to the organic nature of CNO, it is possible that the fungus can withstand small concentrations of copper by using the CNO as a carbon source, or substrate. However, there is a point at which the copper to lignin-collagen ratio becomes too large and growth is inhibited even with CNO present (15 mg/g). Similarly, there is also a point at which the concentration of CNO (40 g/L) becomes too large and growth is almost completely inhibited with the addition of copper. It is possible that clarified neem oil provides functional groups that bind or chelate with copper, allowing the fungus to tolerate small amounts of copper remaining in the media. Further analysis is required to accurately determine the fungicidal mechanisms and chemical interaction between copper and CNO.

The second important observation from this experiment is the transition from sustained growth in the presence of CNO to a decrease in growth that follows a decreasing trend established by the addition of copper alone. Above 10 mg/g (Cu/LC) the CNO treatments (5, 10, and 20 g/L) are no longer able to sustain growth and decrease to complete inhibition similar to 0 g/L CNO. In this region of Figure 5-4, the addition of CNO has little effect on the growth compared to copper alone and copper appears to be the dominant fungicide.

5.2 Lab-Scale Briquette Treatments

Treatment of anthracite briquettes through the internal incorporation of fungicides confirmed a fungicidal interaction between copper and CNO based on the preservation of collagen, correlated from room temperature strength. However, the fungicide itself compromised the overall strength of the briquettes when applied internally. Therefore, external application of CNO was determined to be the most effective treatment for fungal inhibition and preservation of

briquette strength. A brief cost analysis was completed to assess the most reasonable application method. Internal incorporation of CNO based on coating all the surfaces of the anthracite grains throughout the brick correlated to $2000-$2500 per ton of full-scale anthracite bricks, whereas external surface application was only $5-$10 per ton (Appendix A.5). Strength and cost analysis justify external application of CNO. Fungal growth was observed on all briquettes following incubation, and higher concentrations of surface applied CNO should be investigated to ensure complete inhibition.

Chapter 6

Conclusions, Engineering Significance, and Future Work

6.1 Conclusions

Results from radial growth measurements and the lab-scale briquette experiment reveal the following conclusions related to improving the storage and performance of anthracite bricks:

1. Clarified neem oil alone cannot provide extended fungal inhibition on agar-media or lab-scale anthracite bricks containing lignin and collagen binder materials;
2. Copper alone at low doses can be tolerated by the *C. globosum* fungus, making it ineffective for complete inhibition of growth on lignin and collagen in media and on anthracite briquettes;
3. Co-biocide application of low-dose copper and clarified neem oil can completely inhibit growth on lignin and collagen media at large enough concentrations, as well as inhibit binder degrading growth on anthracite briquettes in extended humidified storage (21 days);
4. Applying clarified neem oil to the surface of anthracite briquettes can enhance the strength of collagen alone at room temperature by 28% and 38% with and without intentional fungal inoculation, respectively, after extended humidified storage. The surface application of copper and clarified neem oil increased room temperature collagen strength by over 40% with and without intentional fungal inoculation. Surface-applied soluble copper in solution may require additional pollution prevention techniques to prevent further mobilization or release of copper.

6.2 Engineering Significance

Results from laboratory experimentation provide the following guidance on fungi and fungicide application at full-scale production of bindered anthracite bricks:

1. Fungal identification helps shape standard operating procedures for full-scale brick production. *Chaetomium globosum* is soil-borne, thermo-tolerant, widespread, and an organic material degrader. Therefore, raw materials going into the production of anthracite bricks (anthracite fines, lignin, collagen, and silicon metals) should avoid contact with water-laden grounds, especially soils. Manufactured anthracite bricks should also avoid storage in wet locations or near soils;
2. External, surface applied CNO is a financially realistic option for the preservation of brick strength. Applying a 40 g/L CNO solution to the surface of the full-scale bricks (identical to concentration applied to the briquettes in this work), will cost less than $10/ton of full-scale bricks.

Results also reveal a potential organic fungicide to be used in new co-biocide applications. Azole and arsenic compounds, conventionally used in wood preservation, are both reported to have dangerous effects on the human body. Clarified neem oil is a less harmful co-biocide that may be used to replace potential endocrine disrupting compounds (azoles) in a variety of fungicidal applications.

6.3 Future Work

Future research will be necessary to transition from lab-scale to full-scale production of anthracite bricks and to further clarify co-biocide interactions between copper and clarified neem oil:

1. Analysis of interaction effects should be replicated to validate and further explore the two important observations discussed in Section 5.2.3;
2. Enzyme activity (protease, laccase, and peroxidase) of fungus in the presence of clarified neem oil should be examined to characterize the mechanisms of fungal prevention with and without copper as a co-biocide.
3. Surface applied clarified neem oil should be optimized to completely inhibit visually observable fungal growth and examined for full-scale performance before a final fungicidal strategy is recommended.

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Appendix A Calculations

A.1 Lumber Preservatives in Lumber Compared to Preservatives in Solution (Mass Basis)

Average Lumber Density: ~ 40-45 lb/ft^3

Average Copper in Preserved Lumber: 0.13 lb/ft^3 – 0.15 lb/ft^3

$$\frac{0.15\ lb\ Cu}{ft^3\ wood} \times \frac{ft^3\ wood}{40-45\ lb\ wood} = \frac{0.00375-0.00333\ lb\ Cu}{lb\ wood}$$

$$\frac{0.0035\ lb\ Cu}{lb\ water} = \frac{0.0035\ g\ Cu}{1\ g\ water} \times \frac{1\ g}{cm^3\ water} \times \frac{1\ cm^3}{1\ mL} \times \frac{1000\ mL}{1\ L} = \frac{3.5\ g\ Cu}{L\ water}$$

A.2 Briquetted Clarified Neem Oil Calculations

Table A-1. Mass of clarified neem oil extract (CNO) on the surface of petri dish plates based on a media concentration of 40 g/L.

CNO, g/L	40
$D_{Petri\ Plate}$, cm	8.5
SA, cm^2	56.74
Height, mm	0.1
Volume of mass, cm^3	0.567
M_{CNO}, g	0.023
M_{CNO}/SA, g/cm^2	0.0004
M_{CNO}/SA, mg/cm^2	**0.40**

Table A-2. Anthracite surface area calculation based on sieve size fractions (Anthracite Density = 1.1 g/cm^3).

Mesh Size	Anthracite Mass, g	Screen Width (in)		Average Grain Diameter		Grain vol, cm^3	Grain SA, cm^2	Total Volume, cm^3	No. Grains	Total SA, cm^2
				in	cm					
6-20	101.25	0.132	0.0331	0.0826	0.2097	4.83E-03	1.38E-01	92.05	1.91E+04	2.63E+03
20-40	96.19	0.0331	0.0165	0.0248	0.0630	1.31E-04	1.25E-02	87.45	6.68E+05	8.33E+03
40-100	37.97	0.0165	0.0059	0.0112	0.0284	1.21E-05	2.54E-03	34.52	2.86E+06	7.28E+03
pass 100	17.72	0.0059	0.000	0.0030	0.0075	2.20E-07	1.76E-04	16.11	7.31E+07	1.29E+04
									Total	**3.11E+04**

Table A-3. Volume of clarified neem oil extract (CNO) required to cover the surface area of anthracite fines.

Parameter	Value
M_{CNO}/SA, mg/cm^2	0.40
Anthracite SA, cm^2	3.11E+04
M_{CNO}, mg	12457.08
Vol CNO_{70}, mL	19.5

CNO_{70}: 70% (m) Clarified Neem oil Extract Concentrate

A.3 Interaction Residual Calculation

Table A-4. Interaction residual calculation table - A) Subtract out overall mean, B) Subtract out column mean, C) Subtract out row mean.

CNO, g/L	Cu/Org, mg/g (Cu, mg/L)						Mean
	0 (0)	5 (14)	10 (28)	15 (42)	20 (56)	25 (70)	
0	33.0	24.8	9.53	1.15	0.785	0.785	11.67
5	13.3	15.7	13.6	2.43	0.785	0.785	7.77
10	10.8	12.0	12.3	1.63	0.785	0.785	6.37
20	7.90	8.73	7.37	3.43	0.970	0.785	4.86
40	4.67	1.18	0.790	1.27	0.813	0.785	1.58
Mean	13.9	12.5	8.72	1.98	0.828	0.785	6.45
A	0 (0)	5 (14)	10 (28)	15 (42)	20 (56)	25 (70)	
0	26.5	18.3	3.08	-5.30	-5.67	-5.67	5.22
5	6.88	9.21	7.18	-4.02	-5.67	-5.67	1.32
10	4.31	5.51	5.81	-4.82	-5.67	-5.67	-0.085
20	1.45	2.28	0.915	-3.02	-5.48	-5.67	-1.59
40	-1.79	-5.27	-5.66	-5.19	-5.64	-5.67	-4.87
	7.48	6.01	2.27	-4.47	-5.62	-5.67	0.00
B	0 (0)	5 (14)	10 (28)	15 (42)	20 (56)	25 (70)	
0	19.1	12.3	0.8	-0.8	0.0	0.00	5.22
5	-0.600	3.2	4.9	0.5	0.0	0.00	1.32
10	-3.17	-0.5	3.5	-0.4	0.0	0.00	-0.085
20	-6.03	-3.7	-1.4	1.5	0.1	0.00	-1.59
40	-9.27	-11.3	-7.9	-0.7	0.0	0.00	-4.87
	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C	0 (0)	5 (14)	10 (28)	15 (42)	20 (56)	25 (70)	
0	13.8	7.1	-4.4	-6.1	-5.3	-5.2	0.00
5	-1.9	1.9	3.6	-0.9	-1.4	-1.3	0.00
10	-3.1	-0.4	3.6	-0.3	0.0	0.1	0.00
20	-4.4	-2.1	0.2	3.0	1.7	1.6	0.00
40	-4.4	-6.4	-3.1	4.2	4.9	4.9	0.00
	0.00	0.00	0.00	0.00	0.00	0.00	0.00

A.4 Two-Factor Analysis of Variance (ANOVA) with Replication

Table A-5. Individual seven day radial growth (cm^2) of *C. globosum* with increased concentrations of copper and clarified neem oil for ANOVA analysis.

	Cu/Org (mg/g)	Clarified Neem Oil (g/L)				
		0	5	10	20	40
Sample	0	33.2	12.3	11.6	8.8	5.3
		33.2	14.2	9.9	7.1	4.5
		32.6	13.5	10.8	7.8	4.2
	5	24.6	15.9	11.3	8.6	1.1
		24.6	15.9	12.3	8.8	1.5
		25.1	15.2	12.3	8.8	0.95
	10	9.1	14.2	12.3	7.5	0.79
		9.9	13.2	12.6	6.8	0.79
		9.6	13.5	11.9	7.8	0.79
	15	1.0	2.7	1.5	3.3	1.3
		1.3	2.5	1.0	3.0	1.3
		1.2	2.1	2.4	4.0	1.2
	20	0.79	0.79	0.79	1.1	0.86
		0.79	0.79	0.79	0.95	0.79
		0.79	0.79	0.79	0.86	0.79
	25	0.79	0.79	0.79	0.79	0.79
		0.79	0.79	0.79	0.79	0.79
		0.79	0.79	0.79	0.79	0.79

Table A-6. ANOVA Data Analysis of Table A-5.

SUMMARY	CNO (g/L) 0	CNO (g/L) 5	CNO (g/L) 10	CNO (g/L) 20	CNO (g/L) 40	Total
Cu/Org (mg/g) 0						
Count	3	3	3	3	3	15
Sum	99	40	32.3	23.7	14	209
Average	33	13.33	10.77	7.9	4.67	13.93
Variance	0.12	0.92	0.72	0.73	0.32	106.73
Cu/Org (mg/g) 5						
Count	3	3	3	3	3	15
Sum	74.3	47	35.9	26.2	3.55	186.95
Average	24.767	15.667	11.967	8.733	1.183	12.463
Variance	0.083	0.163	0.333	0.013	0.081	65.032
Cu/Org (mg/g) 10						
Count	3	3	3	3	3	15
Sum	28.6	40.9	36.8	22.1	2.37	130.77
Average	9.533	13.633	12.267	7.367	0.790	8.718
Variance	0.163	0.263	0.123	0.263	0.000	21.994
Cu/Org (mg/g) 15						
Count	3	3	3	3	3	15
Sum	3.45	7.3	4.9	10.3	3.8	29.75
Average	1.150	2.433	1.633	3.433	1.267	1.983
Variance	0.033	0.093	0.503	0.263	0.003	0.907
Cu/Org (mg/g) 20						
Count	3	3	3	3	3	15
Sum	2.37	2.37	2.37	2.91	2.44	12.46
Average	0.790	0.790	0.790	0.970	0.813	0.831
Variance	0.000	0.000	0.000	0.015	0.002	0.008
Cu/Org (mg/g) 25						
Count	3	3	3	3	3	15
Sum	2.37	2.37	2.37	2.37	2.37	11.85
Average	0.790	0.790	0.790	0.790	0.790	0.790
Variance	0.00	0.00	0.00	0.00	0.00	5.28E-32
Total						
Count	18	18	18	18	18	
Sum	210.09	139.94	114.64	87.58	28.53	
Average	11.672	7.774	6.369	4.866	1.585	
Variance	172.719	44.924	30.221	11.491	2.099	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	2713.018	5	542.604	3118.61	4.195E-71	2.368
Columns	993.693	4	248.423	1427.81	9.850E-59	2.525
Interaction	1721.268	20	86.063	494.65	5.120E-59	1.748
Within	10.439	60	0.174			
Total	5438.418	89				

A.5 Clarified Neem Oil Cost Analysis

A.5.1Clarified Neem Oil applied internally based on the surface area of the anthracite grains

Trilogy® - 70% (mass) Clarified Neem Oil Extract (Certis USA)

Density: 0.914 g/mL

Bulk Price: $26/gallon (Damico, 2013)

$$\frac{\$26}{gallon\ Trilogy} \times \frac{3.785\ gal}{1\ L} \times \frac{1\ L}{1000\ mL} \times \frac{1\ mL}{0.914\ g\ Trilogy} \times \frac{1\ g\ Trilogy}{0.7\ g\ CNO} = \frac{\$0.06}{g\ CNO}$$

Batch of Ten Briquettes:

Weight = 293.71 g

Anthracite SA = 3.11E+4 cm^2 (Table A-2)

Mass of CNO to cover SA at 0.4E-3 g/cm^2 = 12.46 g (Table A-1)

$$\frac{12.46\ g\ CNO}{293.71\ g\ Bricks} \times \frac{\$0.06}{g\ CNO} \times \frac{453.6\ g}{1\ lb} \times \frac{2000\ lb}{1\ ton} = \frac{\$2{,}309.16}{Ton\ Bricks}$$

A.5.2 Clarified Neem Oil applied externally based on the surface area of the full-scale bricks

Trilogy® - 70% (mass) Clarified Neem Oil Extract (Certis USA)

Density: 0.914 g/mL

Bulk Price: $26/gallon (Damico, 2013)

$$\frac{\$26}{gallon\ Trilogy} \times \frac{3.785\ gal}{1\ L} \times \frac{1\ L}{1000\ mL} \times \frac{1\ mL}{0.914\ g\ Trilogy} \times \frac{1\ g\ Trilogy}{0.7\ g\ CNO} = \frac{\$0.06}{g\ CNO}$$

Full-Size Brick:

R=7.3 cm

H=5.7 cm

Weight=2.21 kg = 4.87 lb

CNO: SA Loading = 0.4E-3 g/cm^2 (Table A-1)

$$SA_{Brick} = 2\pi R^2 + 2\pi RH$$

$$SA_{Brick} = 2\pi(7.3cm)^2 + 2\pi(7.3cm)(5.7cm) = 596.27\ cm^2$$

$$\frac{SA_{Brick}}{Mass} = \frac{596.27\ cm^2}{4.87\ lb} \times \frac{2000\ lb}{1\ ton} = \frac{244{,}874.74\ cm^2}{Ton\ Bricks}$$

$$\frac{M_{CNO}}{Ton} = \frac{244{,}874.74\ cm^2}{Ton\ Bricks} \times \frac{0.4 \times 10^{-3}\ g\ CNO}{cm^2} = \frac{97.9\ g\ CNO}{Ton\ Bricks}$$

$$\frac{Cost}{Ton} = \frac{97.9\ g\ CNO}{Ton\ Bricks} \times \frac{\$0.06}{g\ CNO} = \frac{\$5.87}{Ton}$$

Appendix B Supplementary Figures

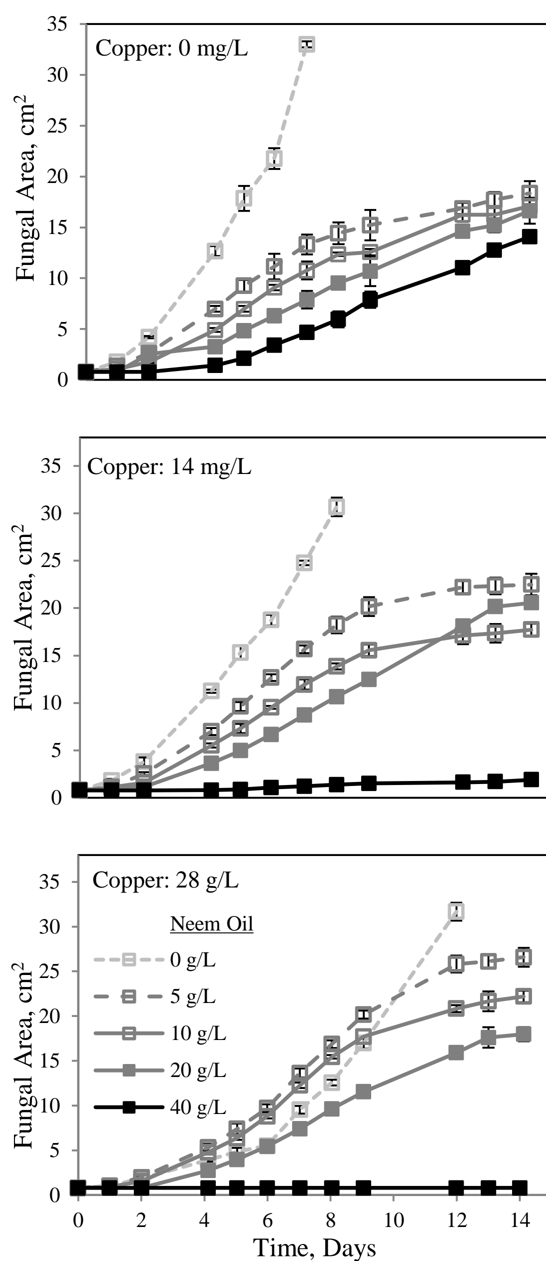

Figure B-1. First segment of the interaction experiment used to determine interaction effects of copper and clarified neem oil (CNO): Figure 4-8. Values are average of triplicate samples; error bars equal one standard deviation.

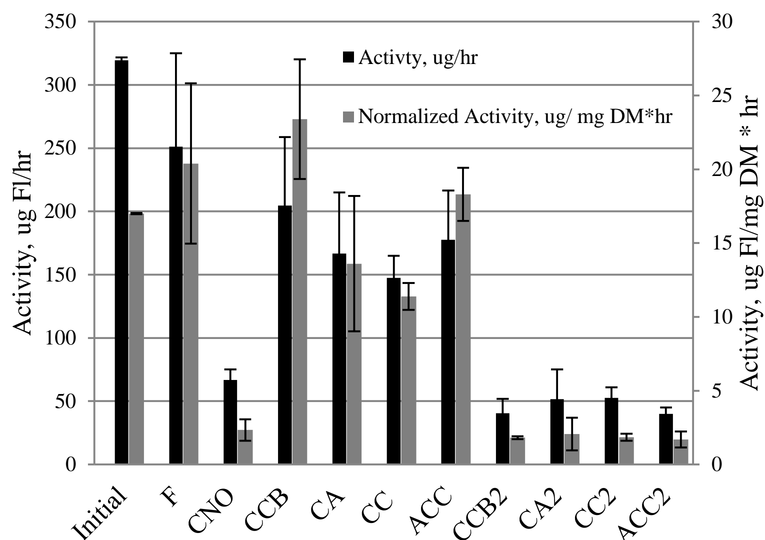

Figure B-2. Biological protease activity (and mass normalized activity) measured by FDA hydrolysis of *C. globosum* grown on liquid lignin-collagen media with four different copper compounds with and without CNO after seven days. Activity expressed as micrograms of fluorescein hydrolyzed per milligram of dry mass per hour (ug Fl/mg DM * hr) or micrograms of fluorescein hydrolyzed per hour (ug Fl/hr). Copper concentrations: 10 mg Cu/g LC. CNO concentration: 12 g/L. CNO: Clarified Neem Oil; CCB: Copper Carbonate Basic; CA: Copper Acetate; CC: Copper Chloride; ACC: Ammonium Copper Chloride; 2: With CNO.

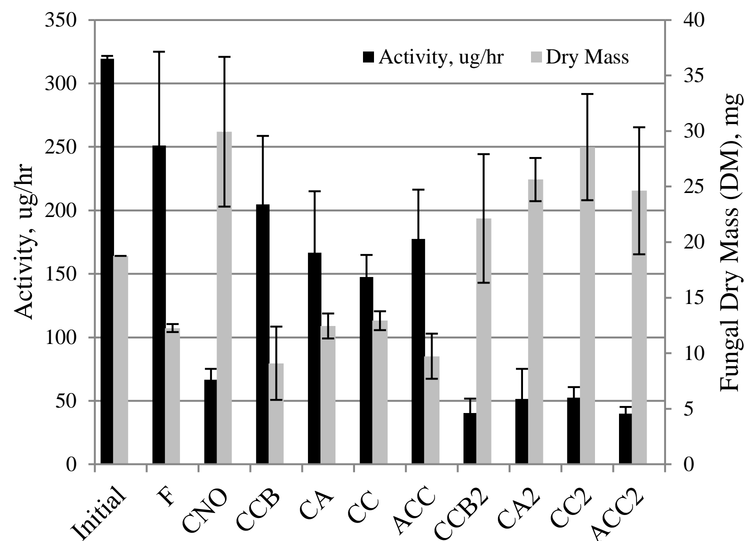

Figure B-3. Fungal growth and biological protease activity measured by FDA hydrolysis of *C. globosum* grown on liquid lignin-collagen media with four different copper compounds with and without CNO after seven days. Activity expressed as micrograms of fluorescein hydrolyzed per hour (ug Fl/hr). Copper concentrations: 10 mg Cu/g LC. CNO concentration: 12 g/L. CNO: Clarified Neem Oil; CCB: Copper Carbonate Basic; CA: Copper Acetate; CC: Copper Chloride; ACC: Ammonium Copper Chloride; 2: With CNO.