THE EFFECTS OF COPPER ON THE SOIL BACTERIAL COMMUNITY IN AN AGRICULTURAL SOIL

by

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ABSTRACT OF THE THESIS

The Effects of Copper on the Soil Bacterial Community of an Agricultural Soil

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Copper is an essential trace element needed for microbial growth and development; however, it is also toxic in higher concentrations. Typical background levels of copper in U.S. soils range from 5 to 50 µg/g dry weight. Elevated copper levels are hypothesized to cause a shift in the soil microbial community resulting in more copper resistant microbes. With the objective of evaluating major shifts in soil bacterial community composition as a result of elevated copper concentrations, three sets of soil microcosms were constructed using an agricultural soil collected from the Adelphia Freehold Research Center. The shifts in the bacterial community within the microcosms were investigated through denaturing gradient gel electrophoresis (DGGE). These data revealed the emergence of a variety of bacteria in the presence of elevated copper. Results indicated that a bacterium most similar to the order Sphingobacteriales was present in 500 µg/g copper amended microcosms, with no additional carbon supplementation after 91 days of incubation. A Chloroflexi-like bacterium was shown to be tolerant of copper in the 250 and 500 µg/g copper amendments after 14 days of incubation. A bacterium from the phylum Actinobacteria was present in highly copper contaminated microcosms (2,000 µg/g). In addition, a microorganism most closely

related to the *Arthrobacter* sp. was shown to be tolerant of 500 µg/g copper, however only while in the presence of an added simple carbon source. Lastly, a bacterium belonging to the Rubrobacteraceae family of Actinobacteria was revealed in copper amended soils regardless of whether a carbon source was supplemented. The presence of these copper tolerant bacteria in a previously uncontaminated agricultural soil suggested a native copper resilience within the bacterial community.

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1.0 INTRODUCTION

Copper is a naturally occurring element present in all environmental media including soil, sediment, air and water (EPA, 2007). It is an essential micronutrient critical for cell function, playing a vital role in processes including but not limited to: respiration, photosynthesis and some superoxide dismutases (Madigan *et al.*, 2003; Prescott et al., 1996). Therefore copper must be present in the environment for biological activity to occur. In elevated concentrations, copper is highly toxic making it of special interest (Pais & Jones, 1997). For instance, agricultural soils in particular are commonly found to contain high levels of copper. This is due to common applications of substances enriched with copper to these sites. Accumulation of copper in soil typically occurs from repeated fertilizer and pesticide application (Iskandar & Kirkham, 2001), land deposition of industrial wastes and sewage, and irrigation with reclaimed sewage water (Pais & Jones, 1997).

Typical background levels of copper in U.S. soils range from approximately 10-50 mg/kg dry weight (dw) (EPA, 2007). However, contaminated soils have been reported with concentrations as high as 3,000 mg/kg dw copper (Kiikkila *et al.*, 2000). Copper has a high bioaccumulation index, and therefore, it accumulates in plant biomass in high concentrations (Pais & Jones, 1997). These elevated levels of copper cause plant phytotoxicity, which in turn facilitates lateral dispersion of metal particles through wind erosion (Vangronsveld *et al.*, 1996). Thus studying the effects of copper in an agricultural soil is critical.

The investigation of the role of the bacterial community and how it responds to elevated concentrations of copper will bring insight into how bacterial communities may

change in agricultural soils. These studies will reveal potential copper resistant soil bacteria as well as the copper sensitive portion of the bacterial community. Studies investigating soil bacterial composition are of high importance because bacteria within the soil decompose organically bound forms of nitrogen, sulfur and phosphorus. They convert these nutrients into plant available forms and hence are vital in nutrient cycling and soil fertility (Brady & Weil, 2000).

2.0 LITERATURE REVIEW

In soil, copper concentrations tend to be relatively uniform in profile distribution, although there can be considerable accumulation at the soil surface. Additionally, copper in soils strongly sorbs to particles of organic matter, clay and sand (Miller & Gardiner, 1998; Pais & Jones, 1997). The dominant form of copper in soil is the cupric cation (Cu^{2+}); this form is most soluble and available under acidic conditions (Brady & Weil, 2000). Humus strongly binds micronutrient metals such as copper to its negatively charged sites (surface chelation), providing a major reserve in top soils (Singer & Munns, 1987). Therefore, this fraction of the copper is not bioavailable; acidic soils reverse this thus, making copper bioavailable.

There are many anthropogenic fluxes of copper into soil. Some include mining and smelting activities, other industrial emissions and effluents, traffic, fly ash, dumped waste material, and contaminated dust and rainfall. Other fluxes include sewage and sludge from wastewater treatment plants, pig slurry, composted refuse, along with the use of agricultural pesticides, fungicides and fertilizers (Prasad, 2008).

The Massachusetts Department of Environmental Protection has identified copper background levels in "natural soils" as 40 mg kg^{-1}, whereas the concentration in soil containing coal ash or wood ash associated with fill material is 200 mg/kg (MassDEP, 2002). The mean concentration of soil collected from two transects in North America was 14.3 mg kg^{-1} of copper in dry soil (from a total of 254 soil samples). The maximum and 90th percentile were 81.9 and 21.8 mg kg^{-1} dry soil, respectively (USGS, 2005). Thus, the MassDEP's definition of contaminated soil contains about five times more copper than expected background levels.

The USEPA has defined the Ecological Soil Screening Level (The Eco-SSL) for copper as the concentration in soil that is protective of ecological receptors that commonly come into contact with and/or consume biota that live in or on soil. The Eco-SSL values for copper range from 28 mg/kg dw for avian wildlife to 80 mg/kg dw for soil invertebrates. Although typical copper concentrations in U.S. soils range from 5-50 mg/kg in the Eastern part of the nation and 15-55 mg/kg in the West (EPA, 2007), contaminated soils have been reported with concentrations as high as 3,000 mg/kg (Kiikkila *et al.*, 2000).

Bacteria play an immense role in all the organic transformations vital to support higher plants. Bacteria within the soil decompose organically bound forms of nitrogen, sulfur and phosphorus and convert them into plant available forms. The fixation of elemental nitrogen, for example, is one of the most important microbial driven processes in soil since this form of nitrogen cannot be used directly by higher plants (Brady & Weil, 2000). Hence, shifts of the soil bacterial community are very important and greatly impact soil fertility.

A recent study by Lejoin *et al.* (2010) evaluated the effects of copper contamination on the abundance, diversity and adaptation of the bacterial community in relation to different soil organic status by targeting the culturable populations. Soil organic status was defined as the amount and type of soil organic matter. Three organic matter managements were used: not amended, containing 12.9 g kg^{-1} organic carbon (NA); amended with straw containing 19.7 g kg^{-1} organic carbon (S); and amended with conifer compost containing 45.9 g kg^{-1} organic carbon (CC). Bioavailable copper concentrations in each amendment were: NA, 460 ng ± 110 ng Cu g^{-1} soil; S, 148 ng ±

60 ng Cu g^{-1} soil; CC, 129 ng ± 51 ng Cu g^{-1} soil. Soil microcosms were artificially contaminated with 250 mg Cu kg^{-1} of soil and incubated for 35 days. Results of the bacterial enumeration experiment indicated that the heterotrophic bacteria in the S and CC soil microcosms did not experience a change, while in the NA microcosms there was a significant decrease of the bacterial members ($p < 0.05$) from 2.8×10^7 to 4.0×10^6 CFU g^{-1} of soil. The results suggested that the copper impact on viable heterotrophic bacteria depends on the biochemical quality of soil organic matter since no modification was observed in either S or CC soil microcosms. As to the diversity of copper resistant isolates, bacterial 16S rRNA gene amplification compared with sequences found in the GenBank database revealed no significant difference in the diversity of copper resistant bacteria between the three organic matter managements of the uncontaminated soils. Bacteria belonging mainly to the Alphaproteobacteria class (53-57%) and to the Firmicutes class (38-43%) were isolated in all soils. The α-Proteobacteria were mainly composed of the *Methylobacterium* genus (47-53%) and the Firmicutes of the *Staphylococcus* (17-37%) and *Bacillus* (6-17%) genera. Phylogenetic trees revealed that the copper resistant isolates belonged to α-Proteobacteria, Firmicutes, Bacteriodetes and Actinobacteria. This group demonstrated that copper amendment did induce an enrichment of particular populations, which varied between soil microcosms. *Methylobacteria*, *Ralstonia* and *Staphylococcus* became dominant in NA, S and CC soil treatments, respectively.

Torch Lake in Houghton County, Michigan (Konstantindis *et al.*, 2003), has been contaminated with copper over the course of 100 years from 1868 to 1968 when copper mining byproducts were dumped into the lake. The copper concentration found in the

lake sediment was as high as 5,500 µg/g copper. Other metals, such as arsenic, chromium and lead, along with organics and residual explosives, have been detected in the sediment. The authors described the sediments of the lake as an extreme environment for the development and evolution of microbial communities. T-RFLP analyses of the microbial communities at these sites indicated a reasonably complex community with up to 20 detectable phylotypes. Direct isolation of copper resistant strains from Torch Lake sediment identified two genera of bacteria, *Arthrobacter* and *Ralstonia*. *Arthrobacter* was isolated from only the top layers of the sediment whereas *Ralstonia* spp. were the only aerobically cultivated copper resistant strains isolated below 6 cm. Although both genera exhibited resistance to at least 200 µg/mL of copper sulfate ($CuSO_4$), *Ralstonia* were resistant up to 1200 µg/mL $CuSO_4$ and produced green colonies when grown with $CuSO_4$, suggesting copper sequestration as a resistance mechanism. The authors did note that additional diversity may reside in the anaerobic sector of the community.

Two highly heavy metal resistant bacterial strains were isolated from a long-term heavy metal contaminated copper mine tailing site in China (Xie *et al.*, 2010). Through 16S rRNA amplification and sequencing, these two strains shared 99% similarity to a *Ralstonia picketti* and a *Sphingomonas* sp. In liquid medium, the strain most similar to *Ralstonia picketti* had tolerance to zinc (10 mM), copper (3.0 mM), lead (0.75 mM) and nickel (1.0 mM), whereas the strain sharing most similarity to *Sphingomonas* sp. had tolerance to cadmium (2 mM), copper (1.5 mM), lead (1.0 mM) and nickel (1.0 mM). The *Ralstonia picketti* like strain could grow on solid medium supplemented with cadmium concentrations as high as 10 mM after a 7 day incubation period. The *Sphingomonas* sp. like strain was shown to grow on solid medium supplemented with 35

mM of zinc after 3 days and 50 mM zinc after 10 days. The authors concluded that biosorption of metals with the use of metal resistant bacteria is efficient and ecofriendly for its immobilization; thus the two isolated bacterial strains had potential for bacterial bioremediation of heavy metal pollution from tailing piles in the future.

A study (Bernard, 2009) constructed to evaluate the influence of copper contamination on the dynamics and diversity of bacterial communities actively involved in wheat residue decomposition was conducted using stable isotope probing. Higher portions of Alphaproteobacteria and Actinobacteria were present in the active fraction of the copper-contaminated soils with 214 mg Cu per kg soil as compared with the unamended soils. In the unamended soils (referred to as pristine in the particular article) Delta- and Gammaproteobacteria were present in higher portions in the active fraction of the population. The article stated that copper pollution seemed to have inhibited certain populations known to be fast growing organisms that feed on readily available carbon such as the Gammaproteobacteria. Oppositely, populations able to degrade polymerized or aromatic compounds, such as Sphingomonadaceae and Actinobacteria, were apparently actively involved in residue degradation in copper contaminated soils.

A literature review of relevant studies pertaining to the effects of copper on the soil microbial community has identified several phyla of bacteria that demonstrated copper resistance. This current study was conducted in hopes of expanding these findings by looking at bacterial groups affected by artificial contamination with copper on an agricultural soil.

3.0 HYPOTHESIS AND OBJECTIVES

It was hypothesized that elevated copper concentration would cause a shift in the soil bacterial community. In addition, the organisms found in the copper amended soils are apt to be copper resistant. This is expected because high levels of copper are toxic and may cause cell death or growth inhibition to copper sensitive bacteria, while increasing the growth of the copper resistant bacterial community. With the objective of evaluating major shifts in soil bacterial community composition as a result of elevated copper concentrations, three sets of soil microcosms were constructed using an agricultural soil. The shifts in the bacterial community within the microcosms were investigated through community analysis. The first microcosms were amended with moderately elevated concentrations of copper over a span of 91 days. The second microcosm experiment was conducted with high concentrations of copper and investigated the effects on the viable cell count as well as the shift in the overall bacterial community after 3 and 8 days. The third and last copper microcosm experiment investigated the effects of moderately elevated copper concentrations on the soil bacterial community over a span of two weeks with and without the addition of a carbon source.

4.0 METHODS

4.1 *Soil Collection*

The top 10 cm of an untilled sandy loam soil was collected from the Adelphia Freehold Research Center in Freehold, New Jersey, on September 3 of 2008. Grab samples in parallel transects stretching the length of the plot were placed in two five-gallon plastic containers. The soil was thoroughly mixed and sieved through a 2 mm sieve (#10) and placed at 4°C before the soil microcosms were constructed.

4.2 *Soil Testing Lab Analyses and pH*

The soil collected from the Adelphia Freehold Research Center was used in all three microcosm experiments. After collection, triplicate samples representative of the bulk soil were sent to the Rutgers University Soil Testing Laboratory for electrical conductivity, mechanical analysis, soil organic matter, micronutrients, and pH analysis. Additionally, the pH of each microcosm was measured at each sampling time point for the 91 moderate copper microcosms experiment as well as the 14 day moderately elevated copper and glucose microcosms. A 10 g sample of soil from each microcosm was dried overnight at 105°C, and mixed with equal amounts of distilled water. The slurry was mixed vigorously and left to sit for 30 minutes. A pH electrode was used to measure the pH after the settling period.

4.3 *Soil Microcosm Set-up and Descriptions*

This study includes three separate microcosm experiments. Since the downstream analyses varied slightly, each experiment will be described in detail separately.

4.3.1 *91 Day Moderately Elevated Copper Microcosms*

Soil microcosms were constructed in acid washed and autoclaved plastic containers to ensure no copper addition through the plastic containers. A total of four amendment and control containers were made, where the copper was diluted in the amount of water needed to bring the soil to a gravimetric water content (GWC) of 15%. Each bucket was mixed thoroughly to allow for a homogenous concentration of both water and copper. Then, 60 grams of soil were placed in each plastic microcosm; the bulk density was adjusted to 1.25 g/cm^3. The microcosm container was covered with aluminum foil with holes punched into it to facilitate aerobic conditions. Microcosms were placed randomly in a drawer beneath the laboratory bench top; the moisture content was adjusted weekly to maintain 15% GWC.

Two sets of controls and two sets of amendments were prepared for this 91 day experiment. One control included a microcosm with no copper sulfate addition (Cu_0). The second control, a pH control (pH) served to mimic the effect on pH caused by the addition of copper sulfate which is acidic. This was done by using sulfuric acid. Amendments with copper sulfate concentrations of 250 µg/g and a 500 µg/g copper microcosm were labeled Cu_1 and Cu_2, respectively. There was a total of 7 sampling time points, where triplicates of all 4 microcosms were sampled destructively. These time points were day 0, 3, 7, 14, 28, 42, and 91.

4.3.2 *8 Day Highly Elevated Copper Microcosms*

Soil microcosms were constructed with the objective of tracking the change in colony forming units (CFU) with the amendment of copper. The construction of the microcosms took place in October of 2009, approximately 13 months after soil collection.

The soil was taken out of 4°C three weeks prior to the start of the microcosms. During these three weeks the soil was brought up to a moisture content of 10% and mixed thoroughly at least once a week. This time was to allow the soil microbial community to acclimate to room temperature. Before the start of the microcosm set up, the soil was thoroughly mixed once again. Copper amendments were created in the volume of water needed to bring the soil up to a moisture content of 12%, and 20 g were placed in each microcosm. Aluminum foil with holes allowing for aeration was placed over the top of each plastic microcosm. The microcosms were adjusted to 12% moisture content every 2-3 days.

The following concentrations of copper where used in the amendments: 0, 125, 250, 500, 1,000 and 2,000 µg/g. The microcosms were sampled destructively during the three sampling time points: day 0, 3 and 8. All samples were spread on soil extract (SE) agar plates in triplicates. The soil extract was made from the soil sampled from Adelphia Freehold Research Center.

4.3.2.1 *Viable Cell Count Analysis*

A viable cell count analysis was performed on the 8 day highly elevated copper microcosms. A soil slurry was made by placing 10 g of soil from the microcosm in 100 mL of a 0.9% NaCl solution making, the 10^{-1} dilution. This slurry was mixed on a horizontal shaker for 5 minutes after which dilutions were made in sterile test tubes. One milliliter of the soil slurry was further diluted to final dilutions of 10^{-5}, 10^{-6} and 10^{-7}. Then 0.1 mL was aseptically plated onto SE agar. The SE agar plates were incubated at room temperature (approximately 25°C) in the dark for 7-10 days. CFU were quantified at the end of the incubation period.

4.3.3 *14 Day Moderately Elevated Copper and Glucose Microcosms*

Soil microcosms amended with copper and glucose were constructed on April 12, 2010. The soil was taken from 4°C and brought to and maintained at a GWC of 12% for approximately a month before the microcosm start date. Each amendment bucket was mixed for 5 minutes at which time all visible aggregates were broken up and the water, copper and glucose was evenly distributed throughout. As in the first microcosm experiment where moderate concentrations of copper were added, 60 g of soil was placed into each plastic microcosm. These microcosms were previously acid washed and autoclaved to ensure that no extraneous copper, nutrients nor microorganisms were introduced into the microcosms. The soil was packed to a bulk density of 1.25 g/cm^3. Each labeled microcosm was covered with aluminum foil that had several holes punctured through it to allow for air circulation. All the microcosms were placed beneath the bench top in the laboratory where they were incubated for up to 14 days. The moisture content was adjusted weekly to 13.5% GWC.

There were a total of six control and amendment containers constructed on day 0. They were: 0 µg/g copper addition along with no addition of glucose, which served as the carbon source (Cu_0dx_0); 500 µg/g of copper and no glucose addition ($Cu_{500}dx_0$) ; two sets of 0 µg/g of copper addition and 1% (d/w) of glucose addition (Cu_0dx_1); and two sets of 500 µg/g of copper addition along with 1% (d/w) of glucose addition ($Cu_{500}dx_1$). All of these samples were collected on day 0 and day 7. On Day 7, in one set of microcosms Cu_0dx_1 and $Cu_{500}dx_1$, a second addition of 1% dw of glucose was added to replenish the carbon source, thus making them into a new treatment called Cu_0dx_2 and $Cu_{500}dx_2$. The second addition of the 1% glucose was added in the volume of water needed to bring the

microcosms back to a GWC of 13.5%. These microcosms were incubated at room temperature for an additional 7 days.

4.4 *Molecular Methods*

After the appropriate incubation period of each microcosm, DNA was extracted from each microcosm using 0.6 g of soil. Soil within the microcosm containers were mixed thoroughly to ensure that the 0.6 g sample used for DNA extraction was representative of the whole microcosm. The MoBio Ultra Clean™ Soil DNA Isolation Kit was utilized for the DNA extraction. The "Experienced User Protocol" was used with the exception of the last step which called for the elution of the DNA with Solution S5. Instead of elution with this reagent, a 10 mM Tris Solution adjusted to a pH of 8 was used. This is the same solution as the S5 included in the kit; however, it was made in the lab due to prior contamination of the MoBio reagent. To ensure that the extracted DNA was approximately 23,130 base pairs, agarose gel electrophoresis was performed on all samples. Each sample was loaded onto a 1% agarose gel with a λHindIII standard (invitrogen™) to determine the appropriate size of DNA extracted from each soil sample. The gel was placed in an ethidium bromide stain and visualized using a Kodak Imaging System.

Polymerase chain reaction (PCR) was used to amplify the highly conserved 16S rRNA region of the DNA using the universal bacterial primers 27F and 519R, which yields a product of approximately 500 base pairs (bp). A master mix solution was made for each PCR reaction that included the Supermix (invitrogen™), bovine serum albumin (BSA)(Roche), forward primer and reverse primer. DNA template extracted from each

sample was mixed with the master mix. Sterile autoclaved water was used as the negative control and DNA isolated from *Eschericia coli* was used as the DNA template for the positive control. To ensure that the forward and reverse primers did not bind to one another before being placed in the thermal cycler, the molecular experiments were conducted on ice and the reverse primer was added into the master mix as the last reagent before being aliquoted into each reaction tube. The PCR product was run on 1% agarose gel and visualized using an ethidium bromide stain and the Kodak Imaging system. Typically, each sample yielded a similar intensity of PCR product.

The PCR products from the various microcosms were loaded onto a denaturing gradient gel (DGGE) to separate the sample into a profile of bands. Each band was assumed to represent an operational taxonomic unit (OTU). Each DGGE gel was run at 55 volts for 17.5 hours at 60°C. Various denaturing gradients were used to capture different portions of the bacterial community. Following electrophoresis, the gel was submerged in an acetic acid fixing bath for 10 minutes followed by 15 to 30 minutes in a SYBR® Green stain. An image of the DGGE gel was taken using the Kodak imaging System. Bands were excised from the polyacrylamide gels, placed in sterile microcentrifuge tubes and stored at -20˚C for later analyses.

Excised bands were defrosted before being completely submerged in sterile autoclaved q-di water for 24 hours at 4°C. This submersion of the bands allowed for the PCR template to elute out of the gel slice. Five µl of this template was transferred to a new PCR tube and reamplified using the 16S rRNA program on the thermal cycler. This PCR product was run an agarose gel to ensure the amplification of PCR product from excised bands occurred without the amplification of the negative control.

Before being sequenced, the reamplified product from each excised band was cleaned with ExoSAP-It® PCR product clean up. The cleaned product was loaded onto a 96 well plate with the 27F and 519R primers and sent into GeneWiz® for sequencing. Results from GeneWiz® where exported into the DNASTAR Lasergene ® SeqMan program where each peak was visualized to ensure that the selected peak was correctly matched to the appropriate nucleotide. The sequence was entered into the GenBank database which compared the experimental sequence to sequences of known organisms deposited in the database. Closest relatives in the GenBank database were found for each excised band.

5.0 RESULTS & DISCUSSION

5.1 *Soil Testing Lab Analysis*

Physical and chemical analyses of the bulk soil collected were conducted in triplicates by the Soil Testing Lab. Electrical conductivity, soil particle size distribution, soil organic matter content and micronutrient concentrations were reported. The results are shown in **Table 1** as averages ± standard deviations. The pH ranged from 5.40 to 5.45. The report concluded that the soil was suitable for growth of blueberry, potato, azalea, rhododendron, and holly, but too acidic for most other plants. The micronutrient concentrations within the soil were reported as being adequate. Salt concentration in soil extracts was directly related to electrical conductivity (EC). High levels of salts, greater than 4 mmho/cm, are known to inhibit the growth and development of plants (Singer & Munns, 1987). The soluble salt content of the soil samples were deemed satisfactory. Typical organic matter found in surface soils ranges from 1-6%, while the organic carbon fraction in the Southern New Jersey region is between 0.9-3.3% (Brady & Weil, 2000). Soil organic matter analyses of the soil used in the microcosm experiments fell into the lower portion of these two ranges.

5.2 *The 91 Day Moderately Elevated Copper Microcosms*

5.2.1 *pH Analysis*

With the objective of evaluating major shifts in soil bacterial community composition, a microcosm experiment was constructed with two moderately elevated concentrations of copper (Cu_{250} and Cu_{500}) over a span of 91 days. A pH and a Cu_0 control were also maintained over the 91 day period.

The pH analysis was conducted on all four treatment types; the averages and standard deviations were calculated by combining the pH results of each triplicate treatment type. Results indicated that the pH in the pH control samples was not different from the pH in the Cu_0 samples and hence does not serve as an effective pH control (**Fig. 1**). The pH control was created to mimic the effects of a decrease in pH due to copper sulfate addition. Nevertheless, these samples were still included in later microbial community analyses as they served as additional Cu_0 controls. The average pH of Cu_0 and pH control samples fluctuated within the range of 5.16-5.24 and 5.13-5.23, respectively. The average pH of Cu_1 samples started at 4.79 on day 0 and increased to 4.94 on day 91. The highest pH was recorded on day 42. In comparison, the average pH of Cu_2 samples started at 4.58 and increased to 4.78 on day 42; the day 91 sample had a slightly lower pH of 4.75. The increasing trends of pH in both copper amended treatments can be attributed to the soil's buffering capacity, which is the ability of soils to resist changes in pH (Brady &Weil, 2000). In addition, Cu_{500} samples were more acidic than the Cu_{250} because more copper was added to the former.

5.2.2 *Molecular Community Analysis*

5.2.2.1 *Day 0 DGGE*

A polyacrylamide gel comparing day 0 samples was cast using a denaturing gradient of 40 to 80% (**Fig. 2**). It should be noted that the pH control samples were included in all day 91 gels; however, they serve as a no copper control since the addition of sulfuric acid did not lower the pH of the soil. Visual analysis of the banding patterns did not indicate the presence of unambiguous discrete bands. Although the gel was adequately stained, the intensity of the bands throughout the gel was low. In the middle

to the bottom portion of the DGGE gel, there seemed to be a complex banding pattern present in all lanes. Although no differences are expected on day 0, due to low band intensity nothing could be concluded about presence or absence of bands differing between treatments within this region. The general banding pattern in all treatments was complex and indicated many types of bacteria were present within the soil microcosms in both the controls and the treatments.

5.2.2.2 *Day 91 DGGE*

A DGGE gel of all day 91 samples, including the pH control, unamended control, 250 and 500 µg/g copper, was cast using a 50 to 90% denaturing gradient. A wide gradient emphasized an overall snapshot of the bacterial community (**Fig. 3**). Visual analysis of the banding pattern indicated at least two discrete bands present in the copper amended microcosms. These bands were excised and compared to known sequences deposited into the GenBank database; results are shown in **Table 2**.

Bands 1 and 2 were present in all of the 250 µg/g and 500 µg/g copper amended microcosms. These bands are complementary to one another and were taken to ensure that each one represented the same organism even though they were found in different samples. Their presence in all triplicate microcosms of the copper amendments indicated that this organism was enriched due to copper addition to the soil. The selected bands were most closely related to a bacterium from the order Sphingobacteriales within the phylum Bacteroidetes. Although the sequences of bands 1 and 2 had well defined peaks that were consistent with both forward and reverse primers, the closest match within the GenBank database was only 90% similar (91% most closely related to a Bacteroidetes bacterium).

Band 3 was only present in one of the triplicate 250 µg/g copper microcosms, and therefore a complementary band was not analyzed. Although the presence of this band was not as consistent as the band related to the Sphingobacteriales bacterium, the organisms related to this band were still of interest due to its absence in the control samples. The sequence of band 3 was approximately 480 base pairs and had well defined peaks in both the forward and reverse primers. This band was most closely related to *Roseiflexus* sp., a genus of the Chloroflexi phylum. Again, the closest match to known sequences was not very high (88% similar) even though a clean sequence was compared with the GenBank database. It is apparent that an organism more closely related to the one excised from the 250 µg/g copper microcosm sample has not yet been added to the database.

These results indicated that while the agricultural soil was not known to previously be exposed to elevated copper concentrations (background levels of 1.37 µg/g), it still harbored bacteria able to tolerate and possibly resistant to copper. There may be a background tolerance of copper by bacteria in agricultural soils.

5.2.2.3 *Cu_{250} and Cu_{500}, All Time Points DGGE*

Lastly, for the 91 day moderate copper microcosm experiment, a polyacrylamide gel comparing all time points treated with 250 and 500 µg/g copper was cast. Equal volumes of DNA from each triplicate copper amended microcosm were added as the DNA template for the PCR reaction. A denaturing gradient of 50-85% was used to visualize an overall snapshot of the bacterial community **(Fig. 4)**. Nine bands of interest were excised, re-amplified, and analyzed through sequencing. Closest relatives of these bands are listed in **Table 3**.

Complementary bands 1 and 2 were only present in 250 and 500 µg/g copper amended microcosms in day 91. A possible band in the location of band 3 was excised to ensure that bands 1 and 2 were discrete. Re-amplification and sequencing was not successful, which suggests that bands 1 and 2 were indeed discrete. The closest relative to these bands was a bacterium from the order Sphingobacteriales within the phylum Bacteroidetes (sharing 92% similarity). As in the sequencing of bands 1 and 2 of day 91 samples (**Table 2**), the closest relative did not match very highly although the sequence was approximately 440 base pairs with little background and discrete peaks representing the four nucleotides. The presence of this organism in the copper amended microcosms indicated it was selected for under elevated copper concentrations, low soil pH, and that the selective nature of this organism is highly reproducible within the microcosms.

When the sequence from complementary bands 4 and 5 were input into the GenBank database, the resultant closest relatives grouped into more than one phylum of bacteria. These results can be found in **Table 3**, numbers 4 and 5 without the asterisk. Due to inconsistencies in closest relatives, the GenBank search was refined to exclude all uncultured/environmental sequences. This search found the bands to be most closely related to an *Acidobacterium* sp. (95-98%). Since the soil is acidic, ranging from 4.79 to 4.94 in the 250 µg/g copper amendment and 4.58 and 4.78 in the 500 µg/g copper amendment, this finding was consistent with expectations. The band most closely related to an *Acidobacterium* sp. excised from the 500 µg/g microcosm treatment was only detected in the last sampling time point (day 91) while the one from the 250 µg/g copper treatment was detected in the samples from day 14. The pH in the 500 µg/g copper microcosms was lower than the 250 µg/g copper microcosms and might explain why the

Acidobacterium sp. like organism was not found in the higher concentration microcosms until a later sampling time point.

When the sequences from bands 6 and 7 were input into the GenBank database, the closest relatives once again grouped into more than one phylum of bacteria. The GenBank search was refined to exclude all uncultured/environmental sequences in hopes of eliminating the inconsistencies within closest relatives. These results (bands 6* and 7*) did not result in high similarity matches, 81-80%, however they did all fall into the Betaproteobacteria with the exception of *Azospirillum*, which is a genus within the Alphaproteobacteria. *Azohydromonas australica*, is from a genus within the family Alcaligenaceae. All remaining genera were within the Comamonadaceae family. The presence of this organism was only apparent in the 500 µg/g copper amended soil during the sampling times of day 42 and 91. The appearance of this organism in the 500 µg/g copper microcosm showed that it is selected for in higher copper concentrations and was tolerant of highly acidic soil conditions. The absence of a complementary band in the 250 µg/g copper microcosms indicated that the bacterium was out-competed by other organisms. It is also possible that it was present in such small numbers that it was not detected by the method used.

Bands 8 and 9 appeared in both the 250 and 500 µg/g copper amended microcosms after the fourteenth day. This delayed period before detection of the organism may be associated with elevated copper concentrations, the lower pH of the soils due to the copper addition, or the slow growing nature of the organism. Sequence analysis found this band as most closely related to a Chloroflexi bacterium clone,

commonly known as the green nonsulfur bacteria, a photoheterotrophic bacterium (Madigen *et al.*, 2003).

As demonstrated by the emergence of several discrete bands within the DGGE gels cast for the 91 day moderately elevated copper microcosm experiment, if this agricultural soil was contaminated with elevated levels of copper, a shift in the bacterial community would be expected. The native bacterial community was found to be resilient to copper amendments of 500 µg/g of copper. Closest relatives of these copper tolerant bacteria belonged to the following phyla: Bacteroidetes, Acidobacteria, Chloroflexi and Proteobacteria.

5.3 *The 8 Day Highly Elevated Copper Microcosms*

5.3.1 *Viable Cell Count Analysis*

To track the change in CFU with the amendment of copper, microcosms were constructed with incremental copper concentrations of 0, 125, 250, 500, 1,000 and 2,000 µg/g in a short term experiment. CFU analysis was used to complement the molecular analysis of the bacterial community in microcosms subject to varying copper concentrations. The analysis elucidated if the culturable bacteria counts decreased with increasing copper concentrations. Soil extracts from day 0, 3 and 8 microcosm treatment soils were spread on soil extract agar plates in triplicate; the results of the enumeration are presented in **Figure 5**.

Equation **(1)** was used to calculate the percent difference in CFU at a given time for the samples containing added copper versus those with no added copper. The results indicated that the there was a 34 to 72% decline in the culturable bacterial community in soil amended with 2,000 µg/g copper as compared with the unamended samples in day 0,

3 and 8 samples. Day 0 samples showed a 34% decrease in viable cell counts in soils exposed to 2,000 µg/g copper. This decrease was most likely caused by the time elapsed on day 0 as the microcosms were being constructed, approximately 4 hours, and shows an immediate toxic effect on the culturable bacteria. After 3 days of incubation, there was a 72% decrease in viable cell counts. Eight days of incubation showed a 64% decrease in viable cell counts of the soils receiving 2,000 µg/g copper. As hypothesized, highly elevated copper concentrations caused a decrease in the viable cell counts of the native bacterial community. At higher copper concentrations, fewer culturable bacteria were able to survive. Therefore, in sites where the copper concentration is in the range of 2,000 µg/g, there may be an inhibitory effect on a large portion of the bacterial community.

$$\frac{\left(CFU_{t,C} - CFU_{t,0}\right)}{CFU_{t,0}} * 100\% = \%\ decrease\ in\ CFU\ in\ a\ given\ copper\ concentration\ (c)\ with\ time\ (t) \qquad \textbf{(1)}$$

5.3.2 *Molecular Community Analysis- Day 3 and 8 DGGE*

Day 3 and 8 soil microcosm samples amended with 2,000 µg/g copper used to quantify the change in bacterial cell counts were also used to resolve the entire bacterial community on a wide gradient DGGE gel (**Fig. 6**). Due to the increased copper addition, the bacterial community was expected to change more drastically than the prior 250 and 500 µg/g copper experiment. Results of sequence analyses can be found in **Table 4**.

Out of all the successfully sequenced bands, all but one discernable pair of discrete bands was present in the unamended copper treatments. Bands 1 and 2, 4, 6, along with 10 and 11 were found in control Cu_0 samples, indicated on the DGGE gel with

black arrows (**Fig. 6**). These bands were not detected in the elevated copper amendments, implying that copper was inhibitory to their growth. Sequence analyses of bands 1 and 2 resulted in closest relatives from three phyla. When the database search was narrowed to exclude all uncultured/environmental sequences, 88% similarity was found with a bacterium from the Acidobacteriaceae family. Band 6 confirmed this finding by also sharing similarity with an Acidobacteriaceae bacterium (99%). Band 4 was found to be 98% similar to a *Bacillus* sp., which was a consistent with finds of bands 10 and 11. They were related to bacteria from the Firmicutes phylum (95 and 94% similarity).

Bands 8 and 9 were detected in all of day 3 and 8 highly copper amended soil samples. This organism was the only one found to be resistant to such a highly elevated copper concentration within this gel. Sequence analysis found both sequences as sharing 97-98% similarity with an uncultured bacterium from the phylum Actinobacteria. A more specific match to an organism belonging to this phylum was not found within the sequences deposited into the GenBank database. This bacterium was consistently found in the 2,000 µg/g copper amendments and may indicate a high resistance to the metal.

In conclusion, the CFU analysis complimentary to DGGE analysis demonstrated an inhibitory effect of a 2,000 µg/g copper amendment on the soil bacterial community. The culturable bacterial counts seemed to decreased when incubated for 8 days in copper amended soils as compared with unamended soils. Closest relative of copper sensitive bacteria belonged to the Acidobacteria and Firmicutes phyla. One organism, most closely related to a bacterium from the phylum Actinobacteria was tolerant of a 2,000 µg/g copper amendment.

5.4 *The 14 Day Moderately Elevated Copper and Glucose Microcosms*

5.4.1 *pH Analysis*

The final microcosm experiment was constructed to investigate the effects of moderately elevated copper concentrations on the soil bacterial community over a span of two weeks with and without the addition of a carbon source. Because highly elevated copper concentrations did not show a greater selection of copper tolerant bacteria in the previous two microcosm studies, it was hypothesized that the soil might be limiting in a readily available carbon source. Glucose is a simple carbon source for bacteria. In hopes of stimulating the growth of the bacterial community within the soil, glucose was added as a readily available carbon source for native bacterial species within the soil.

The analysis for pH was conducted on all six treatments. The average and standard deviation was calculated by combining the pH results of each triplicate treatment type. Results from treatments Cu_0dx_0, Cu_0dx_1, $Cu_{500}dx_0$ and $Cu_{500}dx_0$ are shown in **Figure 7**. The average pH for day 14 Cu_0dx_2 and $Cu_{500}dx_2$ samples was 4.85 (± 0.036) and 4.42 (± 0.035), respectively.

With regard to pH, the copper treatment with the addition of glucose showed an increasing trend within the 14 day incubation period. As seen in the 91 day microcosms amended with copper, it is likely that the soil buffering capacity played a role in raising the soil pH at the final sampling time point. The copper amended microcosms with the addition of glucose were more acidic than the copper amended microcosms without glucose.

On day 14, small filamentous organisms appeared throughout the microcosms in treatments Cu_0dx_1 and Cu_0dx_2. It is unclear whether or not these organisms were fungi, or

bacteria similar to *Streptomyces*. A side and top view of day 14, triplicate number 3, of the Cu_0dx_1 microcosm is shown in **Figure 8**. These filamentous growths were not present in any of the copper treated microcosms, suggesting that these microbes were either copper or pH sensitive. The pH of treatment Cu_0dx_1 was similar to the pH of the Cu_0dx_0 samples until day 14, when the pH rose to 5.23 (± 0.085). The increase in pH of Cu_0dx_1 is possibly due to this yellow filamentous infestation observed in the day 14 samples. However, this same pH increase was not evident in the Cu_0dx_2 samples, which contained the filamentous growths as well.

5.4.2 *Molecular Community Analysis*

5.4.2.1 *Day 0 DGGE*

A smaller denaturing gradient (55-75%) was used to visualize discrete differences in the banding patterns of copper and glucose treatments in the day 0 samples (**Fig. 9**). As in the polyacrylamide gel of day 0 samples from the 91 day moderately elevated copper microcosms experiment, the intensity of the bands throughout the gel was low, even after adequate SYBR® Green staining. A complex banding pattern was visible; however, no discrete bands could be discerned due to the low intensity of the bands. As expected, no differences in banding patterns were discerned within differing copper treatments. This was a similar finding to the day 0 polyacrylamide gel from the 91 day moderately elevated copper amendment experiment without the addition of glucose. Day 0 gels in both experiments appeared to display a complex banding pattern even though they were set up approximately 19 months apart.

A total of 12 bands that appeared in all treatments were excised from the day 0 DGGE gel, represented by red arrows in **Figure 9**. Since no discrete bands were

observed between treatments, it was assumed that all treatments started the experiment with similar community members. Each band was excised with a complementary band from a different treatment, except bands 1 and 2 which were cut within the same treatment. Several bands including 3, 4 and 9 through 12 were not sequenced successfully. Complementary bands 1 and 2 were excised from the $Cu_{500}dx_1$ treatment lanes (**Table 5**). Only the reverse primers for these bands were sequenced successfully; however, the sequences were of poor quality. These sequences were not only short, with a total of about 200 to 210 nucleotides, but they also contained high background. The closest relatives in the GenBank database for band 1 were a *Bradyrhizobium* sp., a genus within the class Alphaproteobacteria with 87% match and a *Halomonas* sp., a genus within the class Gammaproteobacteria. Complementary band 2 had similar results with closest relatives similar to *Rhizobium* sp. and Phyllobacteriaceae, a family of Alphaproteobacteria.

Bands 5 and 6 were very bright in intensity and located at the bottom of the polyacrylamide gel, where bands rich in G+C content migrate. The forward and reverse primers were compared with the GenBank database separately due to their difference in lengths; the forward sequence was about 100 nucleotides longer than the reverse. Band 5 sequence matched an Actinobacterium clone and *Solirubrobacter* sp., a genus within the phylum, with 94% and 93% similarity, respectively. The shorter reverse sequence shared 96% similarity with a Rubrobacteridae bacterium, a subclass of the Actinobacteria. Band 6 resulted in separate findings with regard to the forward and reverse reads. Due to the discrepancies with the closest relatives, the GenBank search was refined to exclude all uncultured/environmental sequences. The results (band 6* in

Table 5) showed 92% similarity with an unknown Actinomycete, as well as 86% similarity to a genus within the family Rubrobacteridae. This was consistent with the results of complementary band 5 analysis.

For complementary bands 7 and 8, only the respective reverse primers yielded successful sequences. Band 7 had shared 95% similarity to three different families within the Rhizobiales order of Alphaproteobacteria including: Hyphomicrobiaceae, Methylocystaceae and Bradyrhizobiaceae. *Afipia* sp. and *Bradyrhizobium* sp. belong in the family Bradyrhizobiaceae, while *Methylosinus trichosporium* belongs to the Methylocystaceae family. Band 8 consisted of a shorter nucleotide sequence, but still shared 97% similarity to several species within the Rhizobiales order. *Rhizobium* sp. is a genus within the Rhizobiaceae family, *Salinarimonas* sp. is within Bradyrhizobiaceae, *Methylocystis* sp. and *Methylosinus* sp. are within the Methylocystaceae family, *Xanthobacter agilis* is within Xanthobacteracea, *Methylocella* sp. belongs to family Beijerinckiaceae while *Ancylobacter* sp. is within the Hyphomicrobiaceae family. The *Magnetospirillum sp*., matched 97% similarity to the 8 reverse sequence, is a genus within the Rhodospirillales order.

All the bands sequenced from the day 0 DGGE gel were present throughout the treatment profiles. The most closely related bacteria to the excised bands included a genus from Gammaproteobacteria, a family of the Actinobacteria, and genera representing six different families within the Rhizobiales order of Alphaproteobacteria. All these organisms are common soil inhabitants (Madigan *et al*., 2003). Although only a small portion of bands within the DGGE profile were analyzed, they revealed the diversity of the existing community within the Adelphia Freehold soil.

5.4.2.2 *Day 7 DGGE*

A DGGE gel comparing the four amendments of day 7 samples was prepared using a denaturing gradient of 55 to 75% (**Fig. 10**). This gel was analyzed to monitor the bacterial community shifts at the midpoint of the microcosms experiment, right before another addition of glucose was added to one control and one treatment. A total of 18 bands were excised and the results of the sequencing analysis found closest relatives belonging to several phyla including Actinobacteria, Firmicutes, and Proteobacteria (**Table 6**).

Bands 14, 15, and 17 appeared in all of the samples regardless of the amendment. Band 14 was most closely related (98%) to the *Bacillus* sp., while band 15 was most closely related to *Janibacter limosus* and *Terrabacter* sp. (84% similarity) from the Intrasporangiaceae family of the Actinobacteria. When band 17 was input into the GenBank database, the closest relatives were from two different phyla; to eliminate this discrepancy, the search results were narrowed to exclude uncultured/environmental sequences. The closest relatives were 91-92% similar to four genera of the Alphaproteobacteria class including *Rhodospirillum* sp., *Phaeospirillum chandramohanii*, *Blastochloris sulfoviridis* and *Azospirillum* sp. The resultant closest relatives to bands present in all of the samples were from three large phyla including Actinobacteria, Firmicutes and the Proteobacteria. These may be the most dominant and resilient populations of the bacterial community within the soil that were not affected by elevated copper concentrations. These populations were exposed to the elevated copper concentration; however, this exposure did not affect their presence in the community. On the other hand, these organisms may have also represented a group of the community that

were not exposed to the elevated copper and hence remained unchanged throughout the experiment. These bacteria may have been in a resting state or spatially separate from the copper in the soil.

Bands 7, 8 and 9 were only present in the Cu_0dx_1 treatment. From the absence of this organism in the Cu_0dx_0 treatment, it can be concluded that a carbon source was limiting to this bacterium's growth within a 7 day incubation period. In addition, these bands were absent in the copper amended microcosms, which indicates that their growth was impeded by the presence of copper in the soil. The most closely related organism from GenBank to these bands was the *Bacillus sp.*, sharing 98% similarity. This is an interesting result because *Bacillus* is a known spore former.

Complementary bands 1 and 2, along with 5 and 6, were excised from two $Cu_{500}dx_1$ treated samples as well as the Cu_0dx_1 samples. The organisms represented by these bands favored the glucose-amended soils, and were present in the elevated copper conditions only if glucose was added, within a 7 day incubation period. These bands were present in the glucose amended microcosms regardless of the copper addition. Sequencing showed that bands 1 and 2 were most closely related (93% of a sequence approximately 195-215 nucleotides long) to members of the genera *Arthrobacter* sp., *Micrococcus* sp., and *Kocuria* sp., within the family Micrococcaceae of Actinobacteria. Band 5 and 6 had longer sequences and confirmed the results of bands 1 and 2 with 98% similarity to an *Arthrobacter* sp.

Bands 3, 4, 11, 12 and 13 were only present in the copper and glucose amended microcosms ($Cu_{500}dx_1$). Bands 11 and 12 were cut from the same exact band. The organisms represented by these bands appeared in elevated copper concentrations only in

the presence of an added carbon source after 7 days of icubation. As shown in Table 7, the highest match in similarity was with the reverse primer of band 3 (99%), to *Arthrobacter* sp. Bands 4, 11 and 12 also related to the *Arthrobacter* sp., however with lower identity percentages ranging from 89% to 94%. Sequences from band 11 were also 88-89% closely related to *Rothia amarae, Micrococcus* sp., *Janibacter* sp. and *Rhodococcus* sp. These are all genera within different families of the Actinobacteria. Bacteria relatives of band 12 also included *Kocuria* sp. Band 13 was only excised from one sample; the closest relatives were the *Micrococcus luteus* and C*itrococcus* sp, sharing 93% similarity. These genera are from the same family as the *Arthrobacter* sp., Micrococcineae family.

In summary, the closest relatives to all of the excised bands from day 7 samples clustered into four groups by their presence in differential treatments (**Table 7**). These bacteria are all well known soil inhabitants (Madigan et al, 2003; Prescott *et al.*, 1996; Lejoin, 2010). The *Arthrobacter* sp., *Micrococcus* sp., and the *Kocuria* sp.-like bacteria seem to be common bacterial players resistant to artificially amended copper when glucose was also present.

5.4.2.3 *Day 14 DGGE Gels*

Two DGGE gels were prepared of the six treatments from the day 14 samples. The initial gel was cast with a denaturing gradient of 60-85%. Due to the lack of clarity within many regions of this gel, a subsequent gel with a smaller denaturing gradient (55-75%) was prepared with the same samples. The smaller gradient clearly captured changes in the banding patterns between lanes. Sequencing analyses of several excised bands were conducted on both gels and the results are reported below.

5.4.2.3.1 *Day 14 DGGE: denaturing gradient 60-85%*

A total of 18 bands were excised within the 60-85% gradient of the day 14 DGGE gel, identified by red arrows in **Figure 11**. Tabulated sequencing results are shown in **Table 8**. Bands present in the microcosms amended with glucose, but without copper (Cu_0dx_1 and Cu_0dx_2) included complementary bands 3 and 4, 5 and 6, 9 and 10, 11 and 12, 13 and 14, 15 and 16 along with bands 17 and 18. These bands were most closely related to *Streptomyces* sp. and/or *Kitasatospora* sp. with similarities ranging from 99-95% with one exception of 88% (15 forward sequence results). These genera lie within the Streptomycetaceae family of Actinobacteria. These organisms were selected for in the glucose amended soils; their presence was impeded with the amendment of copper.

Bands 7 and 8 were found in the $Cu_{500}dx_1$ and $Cu_{500}dx_2$ treatments. The presence of these organisms within those two treatments signified that they are copper tolerate but only detectable within a 14 day period when glucose was supplemented. Their absence in the carbon starved environment, but presence in the carbon rich environment, even in the presence of elevated copper indicated that they were actively growing. These organisms shared 98% similarity to the genus *Arthrobacter* sp.

Bands 1 and 2 were present in all $Cu_{500}dx_0$ and $Cu_{500}dx_1$ samples. The forward sequences of both bands were much longer than the reverses, approximately 330 nucleotides long and were most closely related to an Actinobacterium with 90% similarity. Band 1 and 2 reverse sequences were also most closely related to Actinobacteria, but more specifically from the family Rubrobacteraceae, with 82% and 91% similarity for band 1 and 2 reverse sequences, respectively. The presence of these

bands in copper amended microcosms showed that they were resilient to elevated copper concentrations and did not require a higher availability of a carbon source to survive.

5.4.2.3.2 *Day 14 DGGE: denaturing gradient 55-75%*

To cast a gel with better resolution, day 14 samples from the copper and glucose microcosms were run on a 55-75% denaturing gradient. As expected the bands were better resolved on the DGGE gel with a smaller gradient (**Fig. 12**). Complementary bands 4 and 5, 6 and 7, 12 and 13, 14 and 15, 16 and 17 as well as band 11 (10 was not sequenced successfully) were present in glucose amended microcosms in the absence of copper amendment. These bacteria grew with the addition of glucose as their carbon substrate in the absence of elevated levels of copper. The closest relatives of these bands (**Table 9**) sharing 98-97% similarity were *Streptomyces* sp. and/or *Kitasatospora* sp. This finding is consistent with results from the larger gradient gel of day 14 samples. Therefore it can be concluded that these *Streptomyces* sp. and *Kitasatospora* sp. strains were copper sensitive since their growth could not be detected in the presence of elevated copper.

Bands 18 and 19 were thought to be complementary bands present in all glucose amended microcosms; however, sequencing analyses resolved the bands in two different suborders of the Actinobacteria. Therefore, each band was found only in the treatment from which it was excised. Band 18 was excised from the $Cu_{500}dx_2$ amendment; sequence analysis of this band showed it was 96% similar to an *Arthrobacter nitroguajacolicus*. This finding demonstrated that the *Arthrobacter* sp. is copper tolerant; however, its growth was not detected within a 14 day period in the absence of glucose. Band 19 excised from the Cu_0dx_2 amendment was 97% similar to a *Streptomyces* sp. and

Kitasatospora sp., genera from the Streptomycineae suborder of the Actinobacteria. Again, it can be concluded that these bacteria were copper sensitive since their growth was not detected in the presence of elevated copper.

Bands 1 and 2, 8 and 9 along with band 3 were found only in $Cu_{500}dx_1$ and $Cu_{500}dx_2$ treatments. The presence of these bands in elevated copper concentrations showed that they were copper tolerant. They were not detectable within a 14 day incubation period in the absence of glucose, therefore they were likely in a resting form until a readily available carbon source was added to the environment. Sequencing of these bands found the most closely related to *Arthrobacter* sp. with 96-99% similarity. These were sequences up to 412 nucleotides long with distinctive peaks. The same findings were determined in the larger gradient DGGE gel.

The closest relatives to all of the bands excised from both day 14 DGGE gels were broken down into five groups by their presence in differential treatments (**Table 10**). Only the *Streptomyces* sp. and *Kitasatospora* sp. like bacteria were detected in the absence of copper in both gradient gels. The *Arthrobacter* sp.-like bacterium was detected in copper and glucose amended samples in both gradients. Lastly, the 60-85% polyacrylamide gel identified a bacterium closely related to the Rubrobacteracea as copper resistant, regardless of whether a carbon source was provided.

6.0 CONCLUSIONS

It was hypothesized that elevated copper concentrations would cause a shift in the native soil bacterial communities in an agricultural soil. In addition, it was hypothesized that the organisms found in the copper amended soils would be copper resistant. Through DGGE analysis and subsequent sequencing of bands present in copper amended microcosms, the closest relatives of these copper resistant organisms were discerned.

The first microcosm experiment was incubated for 91 days and included two copper amended treatments of 250 and 500 µg/g. Day 0 DGGE analysis indicated that the starting population was similar within all treatment samples. Over the course of 91 days, several bands emerged in the copper treated soils; these bands were representative of the copper tolerant organisms. A bacterium most similar to the order Sphingobacteriales within the phylum Bacteroidetes was found to be copper resistant to a 500 µg/g copper amendment after 91 days of incubation. Within the same microcosm study, a Chloroflexi-like bacterium was tolerant of 250 and 500 µg/g copper amendments after 14 days of incubation.

The second microcosm experiment was constructed to investigate the short term effects of highly elevated copper concentrations on the soil bacterial community. A copper amendment of 2,000 µg/g was investigated; this is 40 to 200 times the typical copper background concentration in U.S. soils. After 3 and 8 days of incubation, there were less culturable bacteria in the 2,000 µg/g treatment as compared with the unamended copper control. Additionally, DGGE analysis found a bacterium tolerant of a 2,000 µg/g copper amendment after 8 days of incubation. The closest relative to this copper tolerant bacterium was from the phylum Actinobacteria.

In hopes of stimulating the growth of the native bacterial population, the third set of copper amended microcosms was constructed with the addition of glucose as a carbon source. There appeared to be a more complex banding pattern in the glucose amended microcosms as compared with the unamended. An organism most closely related to the *Arthrobacter* sp. was shown to be tolerant of a 500 µg/g copper amendment after 7 and 14 days of incubation with the addition of a simple carbon source. Several genera from the same family as the *Arthrobacter* sp. were also found to be close relatives to this copper tolerant bacterium. Lastly, a band most closely related to a bacterium belonging to the Rubrobacteraceae family of Actinobacteria, was shown to be copper tolerant regardless of whether a carbon source was added after 14 days of incubation.

It can be concluded that elevated copper concentrations within an agricultural soil selected for bacteria within the Bacteroidetes, Chloroflexi and Actinobacteria phyla, shifting the native bacterial community. The enrichments of bacteria within these phyla were related to the copper amendment concentration, carbon supplementation, and incubation time. The presence of these copper tolerant bacteria in an agricultural soil not elevated in background copper concentrations suggests a native copper resilience within the bacterial population. These bacteria should be further investigated to determine what type of copper resistance mechanisms they utilize, as the bacteria might be of importance in bioremediation-based remedies at copper contaminated sites. Additionally, functional analyses of these bacteria would allow for further understanding in the overall role of these bacteria within the microbial population.

APPENDIX A. Figures

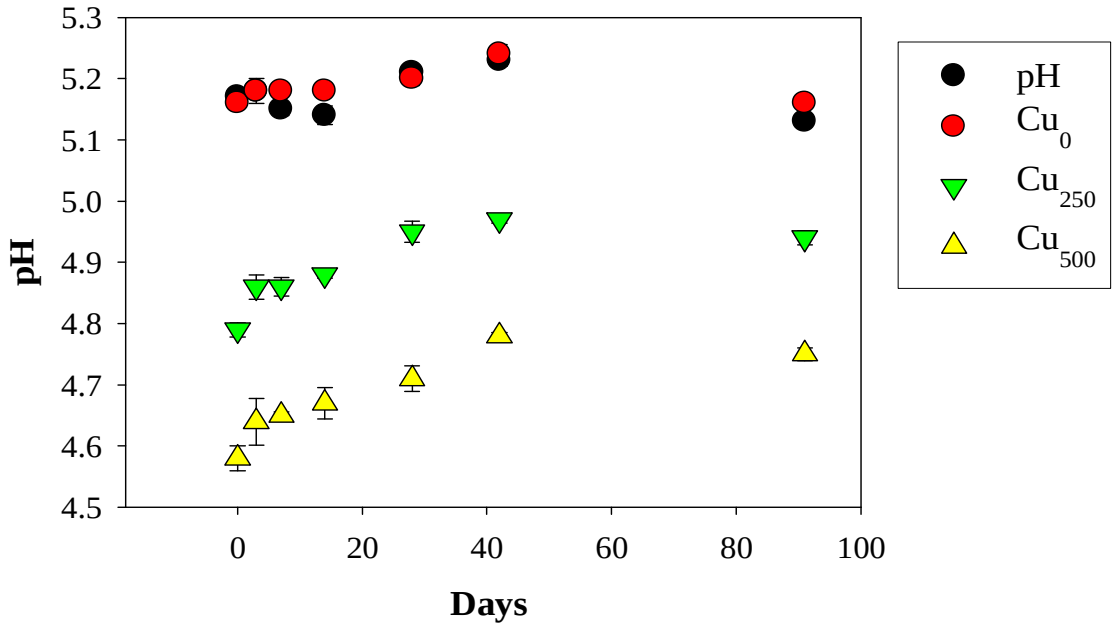

Figure 1: pH of all Moderately Elevated Copper Microcosm samples at each sampling time. Triplicate values for the four treatments were averaged; the standard deviation is shown by bars.

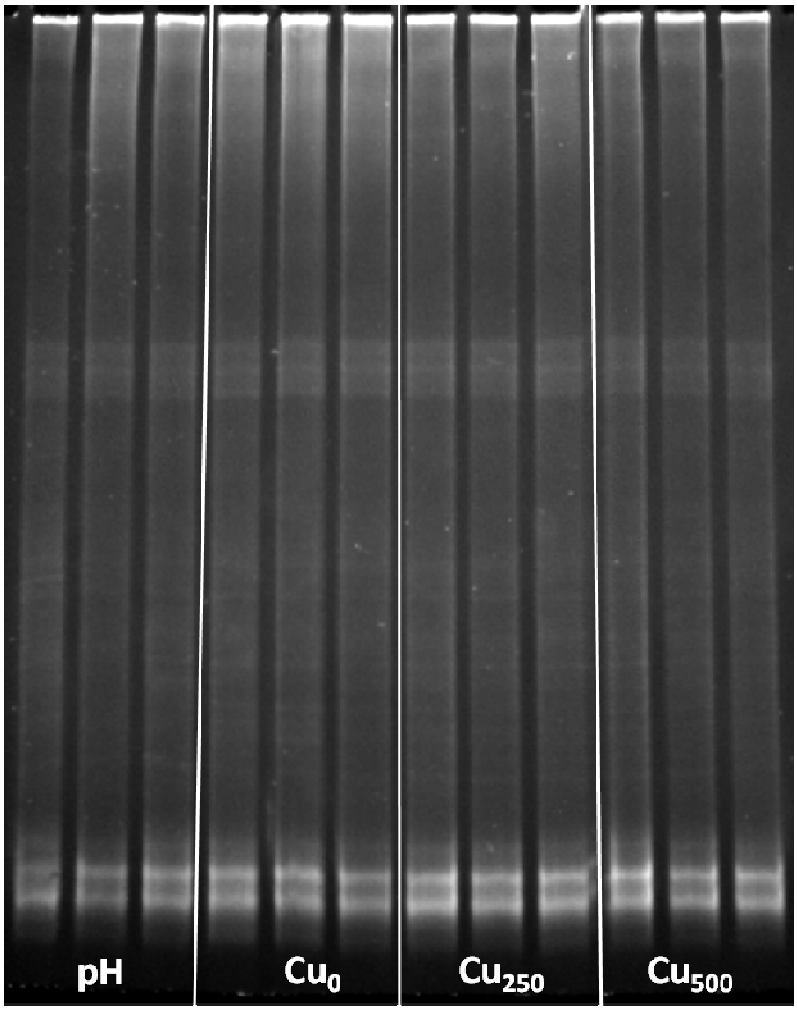

Figure 2: Day 0 DGGE gel of the 91 Day Moderately Elevated Copper Microcosms with a 40-80% denaturing gradient.

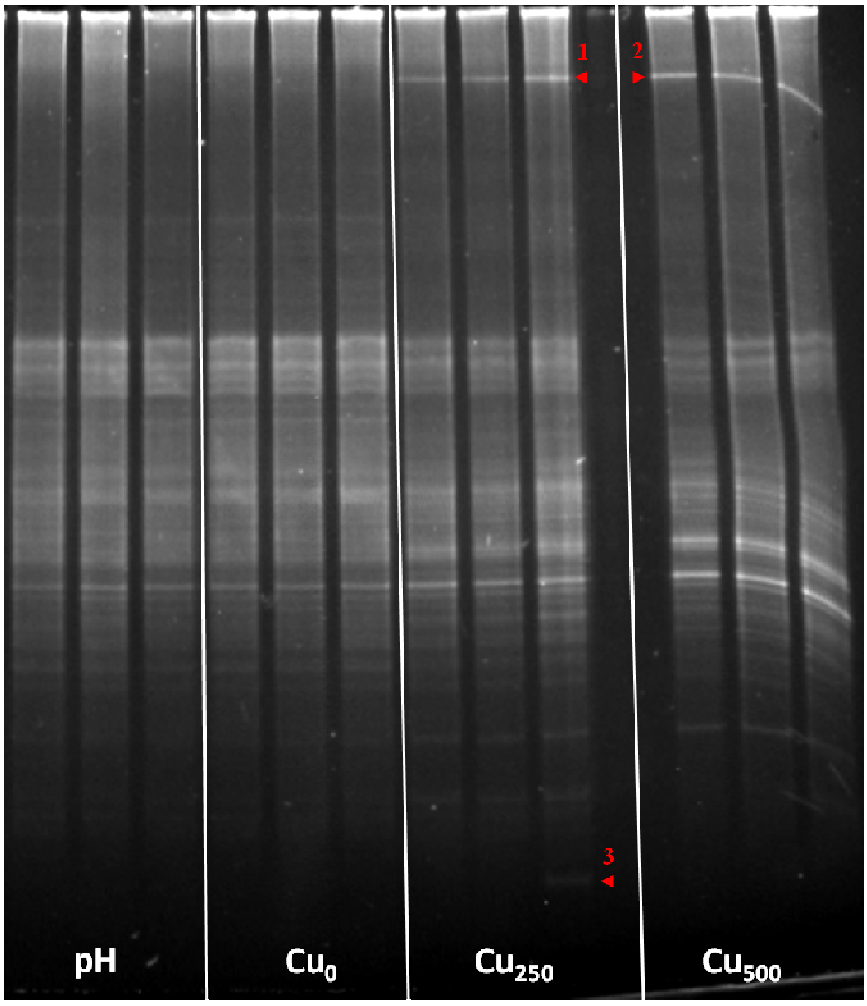

Figure 3: Day 91 DGGE gel of the 91 Day Moderately Elevated Copper Microcosms with a 50-90% denaturing gradient. Red arrows indicate excised discrete bands.

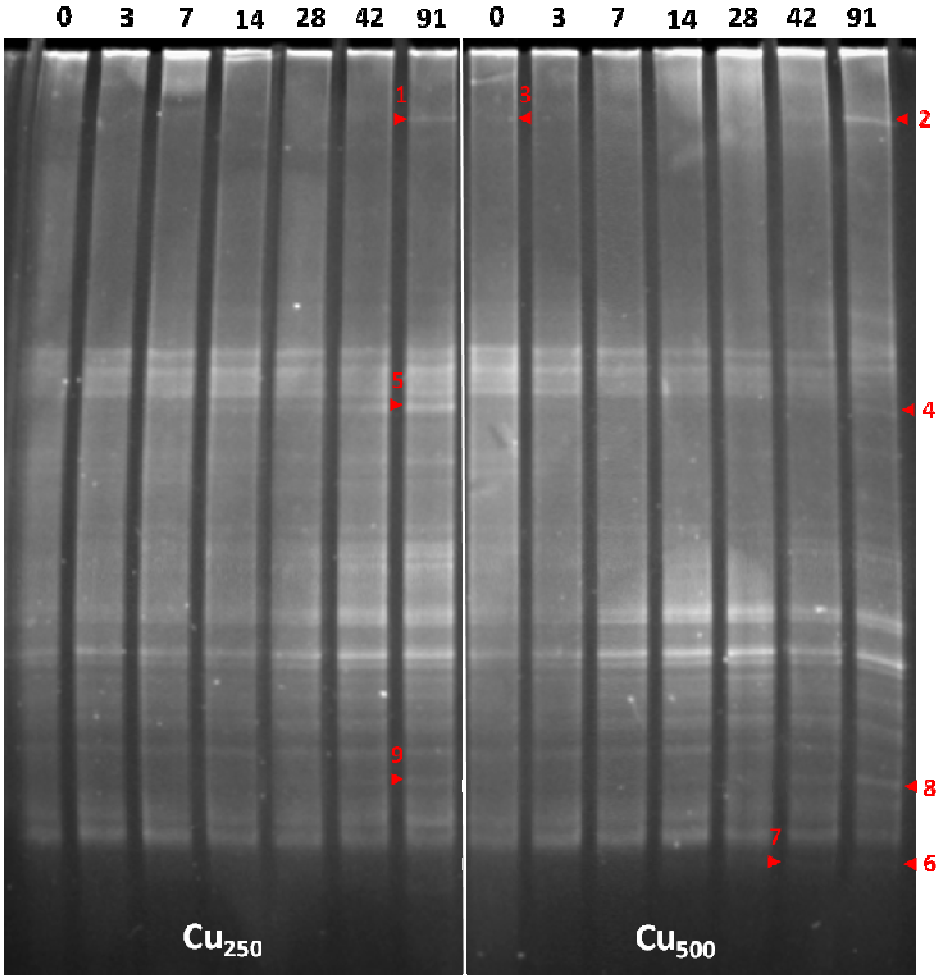

Figure 4: Day 91 DGGE gel (denaturing gradient 50-85%) of all time points of the 250 and 500 µg/g copper amendments within the 91 Day Moderately Elevated Copper Microcosm Experiment. The numbers in bolded black font at the top of the wells indicate sampling time (days). Red arrows indicate discrete excised bands.

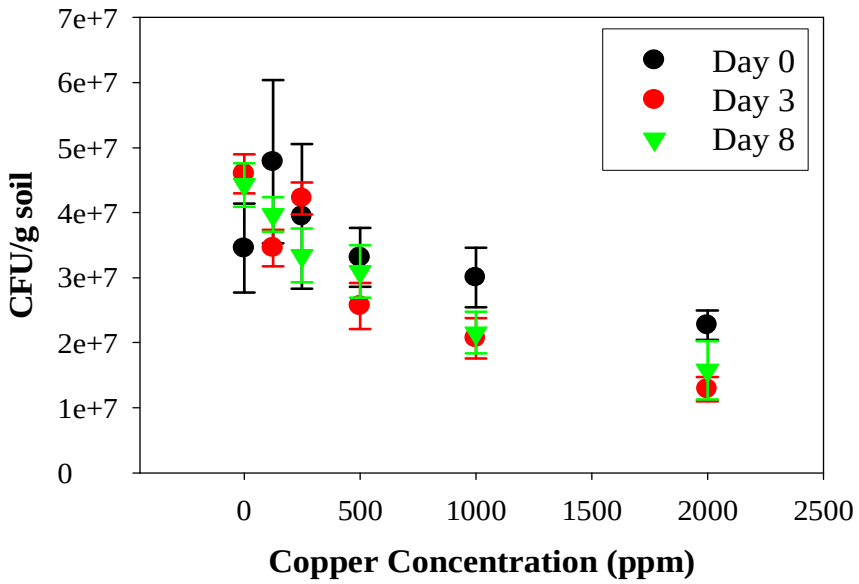

Figure 5: CFU analysis results of day 0, 3 and 8 with varying copper concentrations. Triplicate values for the six treatments were averaged; the standard deviation is shown by the bars.

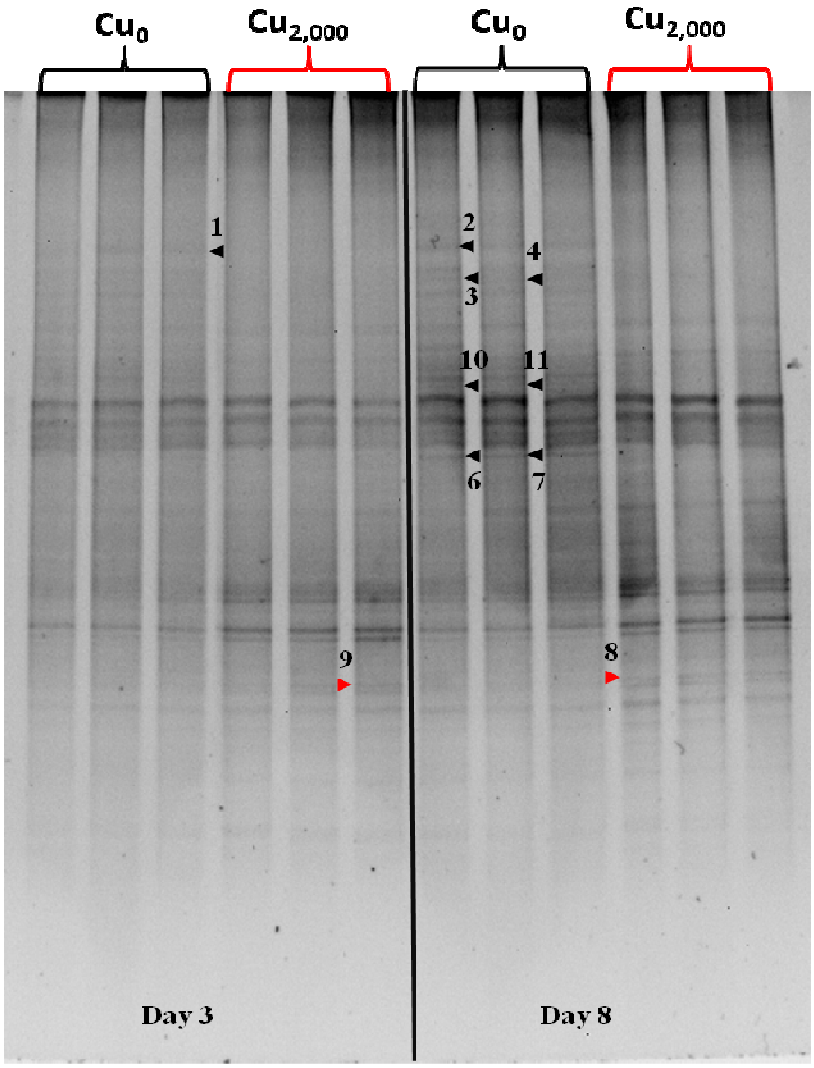

Figure 6: Day 3 & 8 DGGE gel (denaturing gradient 50-85%) of all 0 µg/g and 2,000 µg/g copper amendments within the Viable Cell Count Experiment of Highly Elevated Copper Microcosms. Treatments are labeled at the top of the gel, while time can be found at the bottom of the gel. Red and black arrows indicate discrete excised bands from copper amended and control samples, respectively. The image was inverted.

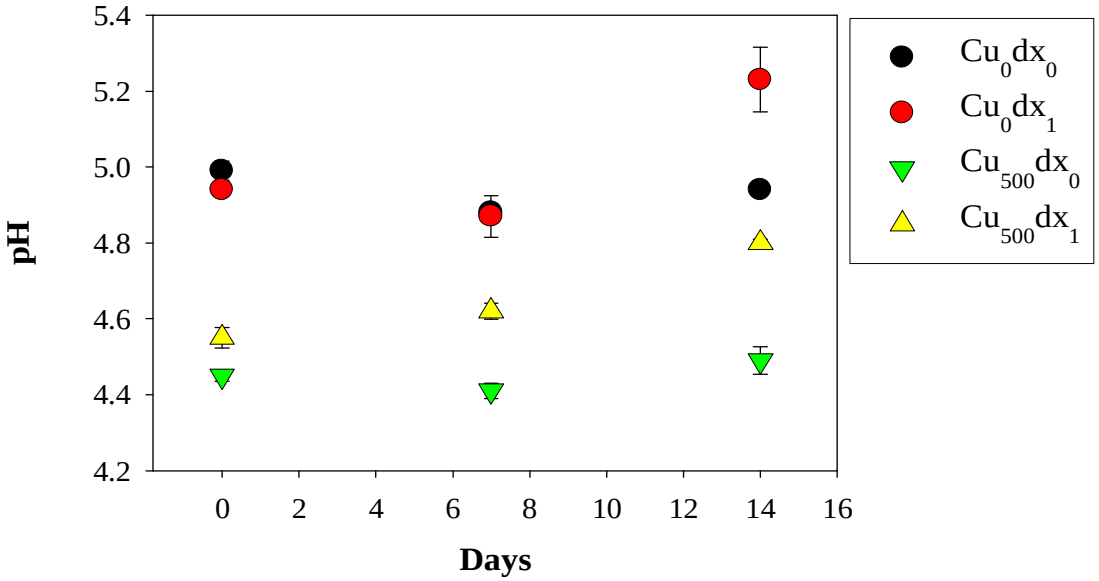

Figure 7: The pH of Moderately Elevated Copper and Glucose Microcosm samples at each sampling time. Triplicate values for the four treatments were averaged; the standard deviation is shown as error bars.

Figure 8: Side view of a microcosm contaminated with fungal like growth (above left); top view of the same microcosms (above right).

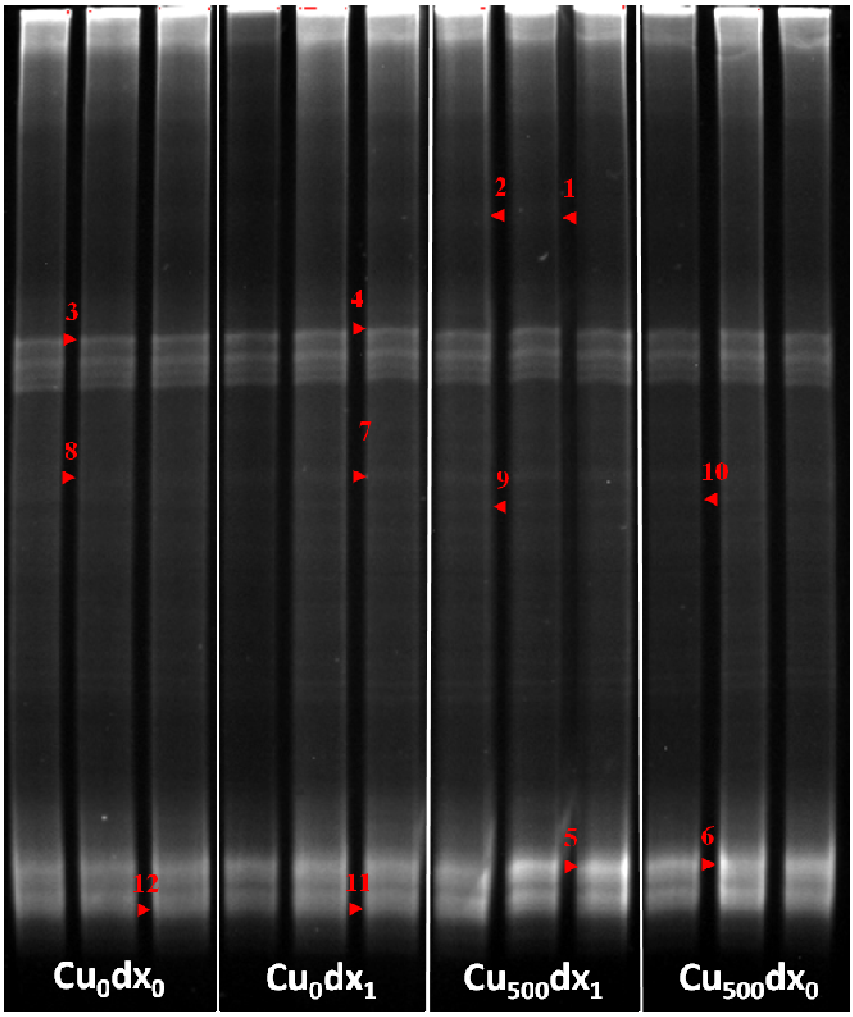

Figure 9: Day 0 DGGE gel (denaturing gradient 55-75%) of 14 Day Moderately Elevated Copper and Glucose Microcosms. Red arrows indicate excised bands.

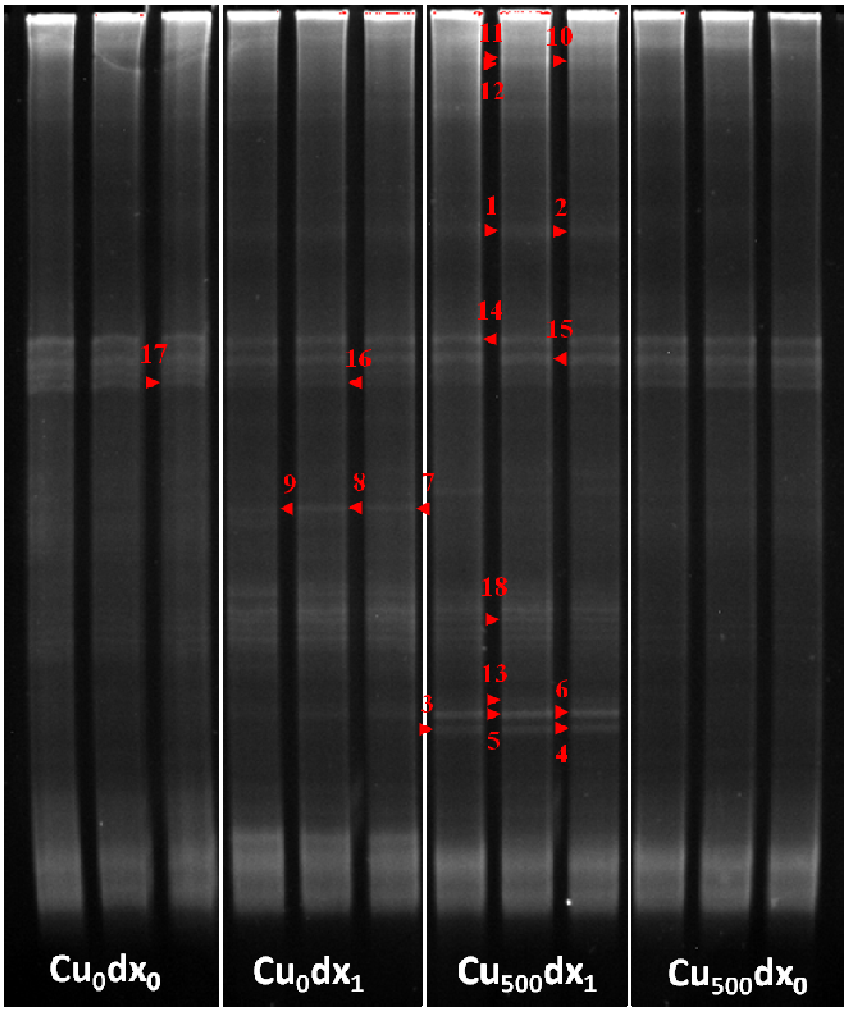

Figure 10: Day 7 DGGE gel (denaturing gradient 55-75%) of 14 Day Moderately Elevated Copper and Glucose Microcosms. Red arrows indicate excised bands.

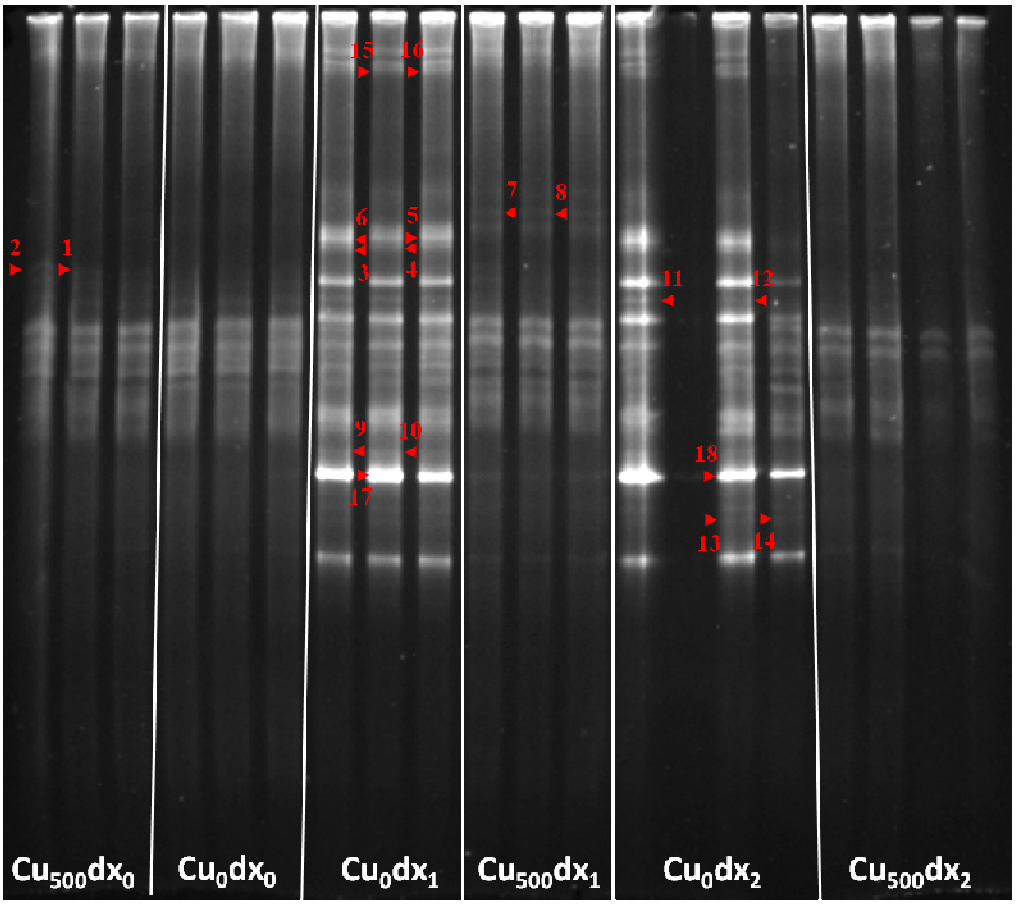

Figure 11: Day 14 DGGE gel (denaturing gradient 60-85%) of 14 Day Moderately Elevated Copper and Glucose Microcosms. Red arrows indicate excised bands.

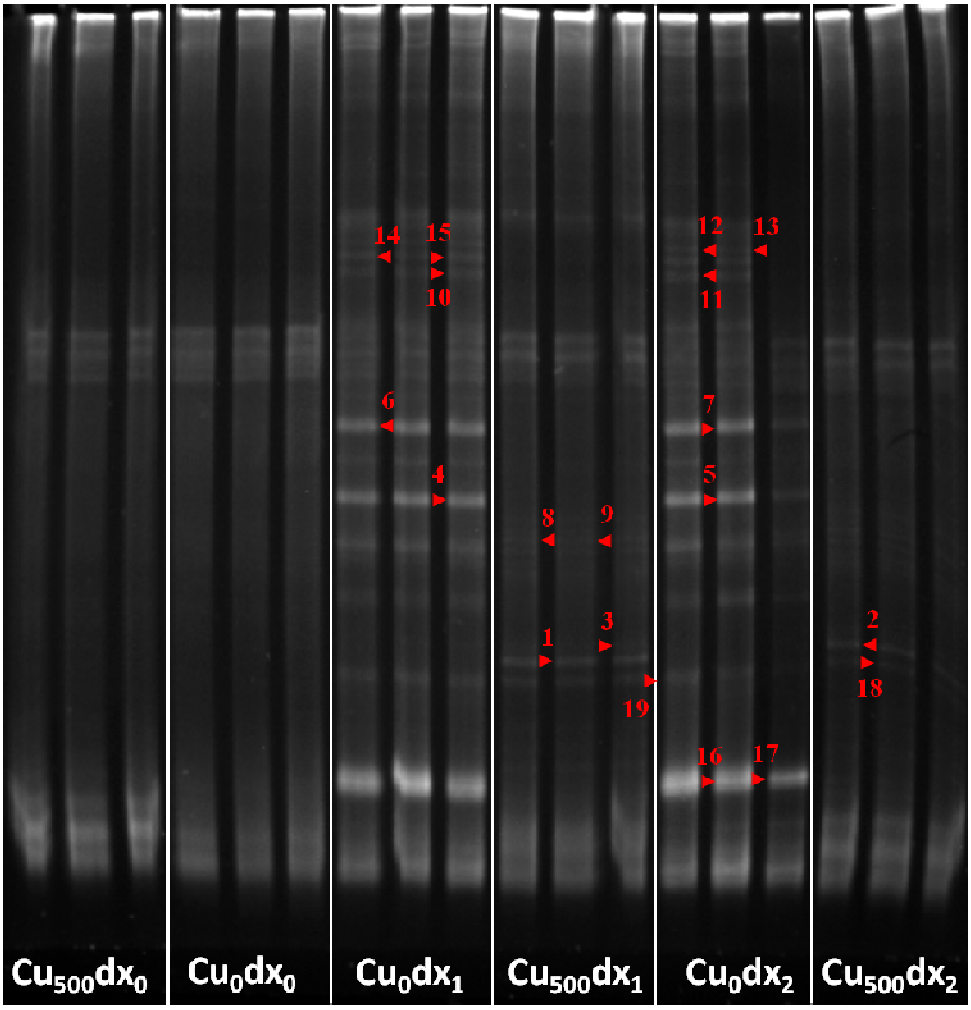

Figure 12: Day 14 DGGE gel (denaturing gradient 55-75%) of 14 Day Moderately Elevated Copper and Glucose Microcosms. Red arrows indicate excised bands.

APPENDIX B. TABLES

Table 1: Soil testing lab results from the bulk soil used in all three microcosms. Triplicate values were averaged ± standard deviation.

	Electrical Conductivity	Mechanical Analysis			Soil Organic Matter	
	Soluble Salt Level (mmho/cm)	Sand (%)	Silt (%)	Clay (%)	Organic Matter (%)	Organic Carbon (%)
Avg. ± st. dev.	0.13 ± 0.00	48 ± 1.2	34 ± 1.2	17 ± 1.2	1.67 ± 0.083	0.970 ± 0.046

	Micronutrients (µg/g)				
	Zinc	Copper	Manganese	Boron	Iron
Avg. ± st. dev.	1.63 ± 0.058	1.37 ± 0.058	5.27 ± 0.058	1.30 ± 0.000	108 ± 0.6

Table 2: Closest relative in GenBank to excised bands of Day 91 Moderately Elevated Copper Microcosms. Bands correlate to red arrows in **Figure 3**. Closest relatives were found using sequences from both forward and reverse primers.

Band	**Closest Relative in GenBank**	**Identity**
1 & 2	u. Bacteroidetes bacterium	466/509 (91%)
	u. Sphingobacteriales bacterium	450/496 (90%)
3	*Roseiflexus* sp.	426/480 (88%)

u indicates uncultured organisms.

Table 3: Closest relative in the GenBank database to excised bands of DGGE gel representative of all time points of the 250 and 500 µg/g copper amendments within the 91 Day Moderately Elevated Copper Microcosms. Bands correlate with the red arrows of **Figure 4.** Band analysis included both forward and reverse primer sequences, unless otherwise noted.

Band	Closest Relative in GenBank	Identities
1(1)	u. Bacteroidetes bacterium clone	407/442 (92%)
	u. Sphingobacteriales bacterium clone	390/428 (91%)
2	u. Bacteroidetes bacterium clone	404/444 (90%)
	u. Sphingobacteriales bacterium clone	399/438 (91%)
3	Not Sequenced Successfully	
4(1)	u. Acidobacteria bacterium clone	236/246 (95%)
	u. *Rhizobium sp*. clone	236/246 (95%)
	u. *Rhodanobacter* sp. clone	236/246 (95%)
4*(1)	*Acidobacterium* sp.	235/246 (95%)
5	u. Acidobacteria bacterium clone	378/378 (100%)
	u. *Rhodanobacter* sp. clone	378/378 (100%)
	u. Acidobacteriaceae bacterium clone	378/378 (100%)
5*	*Acidobacterium* sp.	371/375 (98%)
6	u.Firmicutes bacterium clone	346/411 (84%)
	u. eubacterium 16S rRNA gene	293/351 (83%)
	u. actinobacterium clone	334/408 (81%)
6*	*Rubrivivax* sp.	293/358 (81%)
	Azohydromonas australica gene	293/358 (81%)
	Variovorax sp.	301/369 (81%)
	Caenimonas sp.	291/356 (81%)
	Ramlibacter henchirensis	291/356 (81%)
7	u. Firmicutes bacterium clon	346/411 (84%)
	u. eubacterium 16S rRNA gene	293/351 (83%)
7*	*Azospirillum zeae*	324/403 (80%)
	Rubrivivax sp.	293/358 (81%)
	Azohydromonas australica	293/358 (81%)
	Variovorax sp.	301/369 (81%)
	Caenimonas sp.	291/356 (81%)
	Ramlibacter henchirensis	291/356 (81%)
8	u. Chloroflexi bacterium clone	285/309 (92%)
	u. Chloroflexi bacterium clone	220/234 (94%)
9	u. Chloroflexi bacterium clone	281/309 (90%)

* Search excluded uncultured/environmental sequences
(1) Only the reverse primer amplified successfully
u. indicates uncultured organism

Table 4: Closest relative in the GenBank database to excised bands of DGGE gel representative of day 3 and 8 samples from the 8 Day Highly Elevated Copper Microcosms. Bands correlate to the arrows in **Figure 6**. Closest relatives were found using sequences from both forward and reverse primer, unless otherwise noted.

Band	Closest Relative in GenBank	Identities
1[(1)]	u. Acidobacteria bacterium clone	345/356 (96%)
	u. actinobacterium clone	345/356 (96%)
	u. Firmicutes bacterium clone	345/356 (96%)
1*[(1)]	Acidobacteriaceae bacterium	270/304 (88%)
2[(1)]	u. Acidobacteria bacterium clone	345/356 (96%)
	u. actinobacterium clone	345/356 (96%)
	u. Firmicutes bacterium clone	345/356 (96%)
2*[(1)]	Acidobacteriaceae bacterium	270/304 (88%)
3	Not Sequences Successfully	
4	*Bacillus* sp.	495/505 (98%)
5	Not Sequenced Successfully	
6	u. Acidobacteriaceae bacterium clone	433/436 (99%)
7	Not Sequenced Successfully	
8	u. actinobacterium clone	466/475 (98%)
9	u. actinobacterium clone[(1)]	454/465 (97%)
	u. actinobacterium clone[(2)]	472/481 (98%)
10	u. Firmicutes bacterium clone	427/448 (95%)
11	u. Firmicutes bacterium clone	422/447 (94%)

* Search excluded uncultured/environmental sequences

(1) Only the forward primer amplified successfully

(2) Only the reverse primer amplified successfully

u. indicates uncultured organism

Table 5: Closest relative in the GenBank database to excised bands of DGGE gel representative of day 0 from the 14 Day Moderately Elevated Copper and Glucose Microcosms. Bands correlate to the red arrows in **Figure 9**. Closest relatives were found using sequences from both forward and reverse primer, unless otherwise noted.

Band	Closest Relative in GenBank	Identities
1[(2)]	*Bradyrhizobium* sp.	136/156 (87%)
	Halomonas sp.	129/147 (87%)
2 [(2)]	u. *Rhizobium* sp.	160/195 (82%)
	Phyllobacteriaceae bacterium	159/195 (81%)
4	Not Sequenced Successfully	
5	u. actinobacterium clone[(1)]	359/380 (94%)
	u. *Solirubrobacter* sp. clone[(1)]	357/380 (93%)
	u. actinobacterium clone[(2)]	274/284 (96%)
	u. Rubrobacteridae bacterium clone[(2)]	274/285 (96%)
6	u. actinobacterium clone[(1)]	360/380 (94%)
	u. Rubrobacterineae bacterium clone[(1)]	357/380 (93%)
	u. Bradyrhizobiaceae bacterium clone[(1)]	357/379 (94%)
	u. actinobacterium clone[(2)]	275/283 (97%)
	u. Rubrobacteridae bacterium clone[(2)]	275/284 (96%)
6*[(1)]	u. Actinomycete	353/383 (92%)
	Thermoleiphilum album	334/385 (86%)
	Rubrobacteridae bacterium	332/384 (86%)
	Solirubrobacter sp.	326/377 (86%)
7[(2)]	u. *Afipia* sp. clone	315/330 (95%)
	u. Hyphomicrobiaceae bacterium	310/322 (96%)
	Bradyrhizobium sp.	314/330 (95%)
	Methylosinus trichosporium	313/329 (95%)
8[(2)]	u. *Rhizobium* sp. clone	191/195 (97%)
	Salinarimonas sp.	190/195 (97%)
	u. *Methylocystis* sp.	190/195 (97%)
	Methylosinus sp.	190/195 (97%)
	Magnetospirillum sp.	190/195 (97%)
	Xanthobacter agilis	189/194 (97%)
	Methylocella sp.	190/195 (97%)
	Ancylobacter sp.	189/194 (97%)
9 - 12	Not Sequenced Successfully	

* Search excluded u./environmental sequences

(1) Only the forward primer amplified successfully
(2) Only the reverse primer amplified successfully
u. indicates uncultured organisms

Table 6: Closest relative in the GenBank database to excised bands of DGGE gel representative of day 7 from the 14 Day Moderately Elevated Copper and Glucose Microcosms. Bands correlate to the red arrows in **Figure 10.** Closest relatives were found using sequences from both forward and reverse primer, unless otherwise noted.

Band	Closest Relative in GenBank	Identities
1(2)	*Arthrobacter* sp.	203/217 (93%)
	Micrococcus sp.	202/217 (93%)
	Kocuria sp.	202/217 (93%)
2(2)	*Arthrobacter* sp.	182/194 (93%)
3	*Arthrobacter* sp. (1)	408/411 (99%)
	Arthrobacter sp. (2)	377/397 (94%)
4(2)	*Arthrobacter* sp.	351/378 (92%)
5	*Arthrobacter* sp.	451/459 (98%)
6	*Arthrobacter* sp.	488/493 (98%)
7	*Bacillus funiculus*(1)	457/468 (97%)
	Bacillus funiculus(2)	383/390 (98%)
8	*Bacillus* sp. (1)	365/394 (92%)
	Bacillus sp. (2)	380/384 (98%)
9(2)	*Bacillus* sp.	164/187 (87%)
10	Not Sequenced Successfully	
11(2)	*Rothia amarae*	217/242 (89%)
	Arthrobacter aurescens	217/243 (89%)
	Micrococcus sp.	216/243 (88%)
	Janibacter sp.	216/243 (88%)
	Rhodococcus sp.	216/243 (88%)
12(2)	*Arthrobacter nicotianae*	225/245 (91%)
	Kocuria sp.	217/237 (91%)
13(2)	*Micrococcus luteus*	227/244 (93%)
	Citrococcus sp.	227/244 (93%)
14	u. *Bacillus* sp. clone	380/387 (98%)
15(2)	*Janibacter limosus*	200/238 (84%)
	Terrabacter sp.	200/238 (84%)
16	Not Sequenced Successfully	
17(2)	u. Acidobacteria bacterium clone	190/208 (91%)
	u. *Acidobacterium sp.* clone	176/188 (93%)
	u. Firmicutes bacterium clone	183/198 (92%)
17*(2)	*Rhodospirillum* sp.	180/196 (91%)
	Phaeospirillum chandramohanii	178/192 (92%)
	Blastochloris sulfoviridis	184/202 (91%)
	Azospirillum sp.	182/200 (91%)
18	Not Sequenced Successfully	

* Search excluded u./environmental sequences;

(1) Only the forward primer amplified successfully

(2) Only the reverse primer amplified successfully

u. indicates uncultured organisms

Table 7: Summary of closest relatives in the GenBank database to excised bands of DGGE gel representative of day 7 samples from the 14 Day Moderately Elevated Copper and Glucose Microcosms, tabulated by presence in treatments.

Treatment(s)	Closest Relative in GenBank
Cu_0dx_1	***Bacillus* sp.**
$Cu_{0,500}dx_1$	***Arthrobacter* sp.** ***Micrococcus* sp.** ***Kocuria* sp.**
$Cu_{500}dx_1$	***Arthrobacter* sp.** ***Rothia* sp.** ***Kocuria* sp.** ***Micrococcus* sp.**
ALL	***Bacillus* sp.** ***Janibacter* sp.** ***Rhodospirillum* sp.** ***Phaeospirillum chandramohanii*** ***Blastochloris sulfoviridis*** ***Azospirillum* sp.**

Table 8: Closest relative in the GenBank database to excised bands of DGGE gel representative of day 14 from the 14 Day Moderately Elevated Copper and Glucose Microcosms. Bands correlate to the red arrows in **Figure 11.** Closest relatives were found using sequences from both forward and reverse primer, unless otherwise noted.

Band	Closest Relative in GenBank	Identities
1	u. actinobacterium clone [1]	303/333 (90%)
	u. Rubrobacteraceae clone[2]	123/150 (82%)
2	u. actinobacterium[1]	305/338 (90%)
	u. Rubrobacteraceae [2]	112/122 (91%)
3	*Streptomyces paucisporeus* [1]	408/425 (96%)
	Streptomyces sp. [2]	399/406 (98%)
	Kitasatospora sp. [2]	398/407 (97%)
4	*Streptomyces* sp.	454/472 (96%)
	Kitasatospora sp.	453/473 (95%)
5	Streptomyces sp.	416/424 (98%)
	Kitasatospora sp.	415/425 (97%)
6	Streptomycetaceae	355-358 (99%)
	Kitasatospora sp.	352-358 (98%)
7	*Arthrobacter* sp.	291-296 (98%)
8	*Arthrobacter aurescens*	269/274 (98%)
	Arthrobacter sp.	269/274 (98%)
9	*Streptomyces* sp.	470/482 (97%)
	Kitasatospora sp.	469/482 (97%)
10	*Streptomyces* sp.	462/473 (97%)
	Kitasatospora sp.	461/474 (97%)
11	*Streptomyces* sp.	461/471 (97%)
	Kitasatospora sp.	460/472 (97%)
12	*Streptomyces* sp.	468/482 (97%)
	Kitasatospora sp.	467/483 (96%)
13	*Streptomyces* sp.	476/491 (96%)
	Kitasatospora sp.	478/496 (96%)
14[2]	Streptomycetaceae	387/395 (97%)
	Streptomyces sp.	384/394 (97%)
	Kitasatospora sp.	383/395 (96%)

Table 8 (cont.): Closest relative in the GenBank database to excised bands of DGGE gel (denaturing gradient 60-85%) representative of day 14 from the 14 Day Moderately Elevated Copper and Glucose Microcosms. Bands correlate to the red arrows in **Figure 11.** Closest relatives were found using sequences from both forward and reverse primer, unless otherwise noted.

Band	Closest Relative in GenBank	Identities
15	*Streptomyces* sp. (1)	345/392 (88%)
	Streptomyces paucisporeus (2)	282/287 (98%)
	Kitasatospora sp. (2)	274/279 (98%)
16 (2)	*Streptomyces paucisporeus*	340/346 (98%)
	Kitasatospora arboriphila	339/347(97%)
17	*Streptomyces* sp.	466/477 (97%)
	Kitasatospora sp.	465/478 (97%)
18	*Streptomyces* sp.	466/478 (97%)
	Kitasatospora sp.	465/479 (97%)

(1) Only the forward primer amplified successfully

(2) Only the reverse primer amplified successfully

u. indicates uncultured organisms

Table 9: Closest relative in the GenBank database to excised bands of DGGE gel (denaturing gradient 55-75%) representative of day 14 from the 14 Day Moderately Elevated Copper and Glucose Microcosms. Bands correlate to the red arrows in **Figure 12.** Closest relatives were found using sequences from both forward and reverse primer, unless otherwise noted.

Band	Closest Relative in GenBank	Identities
1	*Arthrobacter* sp.	409/412 (99%)
2	*Arthrobacter* sp.	396/398 (99%)
3	*Arthrobacter* sp.	433/438 (98%)
4	*Streptomyces paucisporeus*	408/413 (98%)
	Kitasatospora sp.	405/413 (98%)
5	*Streptomyces* sp.	462/473 (97%)
	Kitasatospora sp.	461/474 (97%)
6	*Streptomyces* sp.	401/406 (98%)
	Kitasatospora sp.	400/407 (98%)
7	*Streptomyces paucisporeus*	426/434 (98%)
	Kitasatospora sp.	426/438 (97%)
8	*Arthrobacter defluvii* (1)	353/367 (96%)
	Arthrobacter sp. (2)	361/374 (96%)
9	*Arthrobacter defluvii* (1)	338/348 (97%)
	Arthrobacter defluvii (2)	330/337 (97%)
10	Not Sequenced Successfully	
11	*Streptomyces* sp.	419/426 (98%)
	Kitasatospora sp.	418/427 (97%)
12	*Streptomyces* sp.	464/473 (98%)
13	*Streptomyces* sp.	446/456 (97%)
	Kitasatospora sp.	445/457 (97%)
14	*Streptomyces paucisporeus*	425/433 (98%)
	Kitasatospora sp.	425/437 (97%)
15	*Streptomyces* sp.	418/426 (98%)
	Kitasatospora sp.	417/427 (97%)
16	*Streptomyces* sp.	59/468 (98%)
	Kitasatospora sp.	458/469 (97%)
17	*Streptomyces* sp.	462/471 (98%)
	Kitasatospora sp.	461/472 (97%)
18(1)	*Arthrobacter nitroguajacolicus*	365/378 (96%)
19	*Streptomyces* sp.	462/472 (97%)
	Kitasatospora sp.	461/473 (97%)

(1) Only the forward primer amplified successfully

(2) Only the reverse primer amplified successfully

Table 10: Summary of closest relatives in the GenBank database to excised bands of two DGGE gel representative of day 14 samples from the 14 Day Moderately Elevated Copper and Glucose Microcosms, tabulated by presence in treatments.

Treatment(s)	Closest Relative in GenBank	Denaturing Gradient*
$Cu_0dx_{1,2}$	***Streptomyces* sp.** ***Kitasatospora* sp.**	**both**
Cu_0dx_2	***Streptomyces* sp.** ***Kitasatospora* sp.**	**55-75%**
$Cu_{500}dx_{0,1}$	**u. Rubrobacteracea**	**60-85%**
$Cu_{500}dx_2$	***Arthrobacter nitrogujacolicu***	**55-75%**
$Cu_{500}dx_{1,2}$	***Arthrobacter* sp.**	**both**

* Denaturing gradient in the Day 14 DGGE gels from which the bands were excised

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