Interactive Effects of Copper on Alfalfa Growth, Soil Copper, and Soil Bacteria

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Abstract

Copper sulfate ($CuSO_4$) foot baths are a management practice used by dairy farms in an effort to control hoof infections. As an unintended consequence, agricultural soils experience Cu accumulation when spent foot baths are disposed of in wastewater lagoons utilized for irrigation purposes. We investigated the effect of Cu applications (up to 1000 mg kg^{-1}) to a Xeric Haplocalcid (Declo series) and a Typic Calciaquoll (Logan series) on alfalfa (*Medicago sativa*) growth and Cu concentration, soil total and diethylenetriaminepentaacetic acid (DTPA)-extractable Cu, and the soil bacterial community diversity using ribosomal intergenic spacer analysis (RISA). Copper application up to 250 mg kg^{-1} did not affect alfalfa growth; above 500 mg Cu kg^{-1} alfalfa did not grow. Increasing Cu application rates increased alfalfa Cu content grown in both soils. Regardless of initial application rate, 48 to 80% of the added Cu was still plant-available at the end of the study. Comparing DTPA-extractable Cu to alfalfa Cu concentrations, 63 or 95 mg kg^{-1} of DTPA-extractable soil Cu for the Declo and Logan soils, respectively, would be detrimental in terms of cattle dietary Cu intake. For Declo soils, bacterial diversity remained relatively stable across all Cu application rates; Logan soils saw a peak in bacterial diversity at the 50 mg kg^{-1} Cu application rate. Cluster analysis revealed differences in the bacterial RISA profiles between the lower and higher Cu application rates. To prevent excessive alfalfa Cu accumulation and negative impacts on the soil bacterial community, it is suggested available soil Cu not exceed 63 mg kg^{-1} in agroecosystems associated with these soil series.

Keywords: Alfalfa, Cattle wastes, Soil copper, Soil bacteria

1. Introduction

Agricultural soil Cu accumulation can be a concern to producers receiving manure sources with elevated Cu concentrations. In particular, dairy operations utilize between 5 to 10% (w:v) $CuSO_4$ concentrations (equivalent to 12,500 to 25,000 mg Cu L^{-1}) in foot baths to control hoof infections, with spent foot baths typically washed out of dairy barns and into liquid waste lagoons. Once $CuSO_4$ enters the waste lagoon the soluble Cu concentration decreases due to dilution and Cu binding to organic phases, which binds approximately 90 to 95% of the Cu (Stehouwer and Roth 2009). However, 625 to 2500 mg Cu L^{-1}, depending on initial foot bath Cu content, remains in a soluble form. Because excessive soluble soil Cu can be toxic to plants (Paschke and

Redente 2002; White and Brown 2010), Cu-enriched liquid dairy waste applied as irrigation water to agricultural crops raises concerns regarding how plants and soils are impacted.

Soil Cu occurs in several forms: in the soil solution; on soil exchange sites; specifically sorbed; occluded in soil oxides; in the lattice structure of primary and secondary minerals; and in organic residues and living organisms (Adriano 1986). In soils with pH values greater than 7.0, such as those typical of arid regions, soluble Cu can react to form CuO, $CuCO_3$, or mixed hydroxy-carbonate mineral species (McBride and Bouldin 1984; Ponizovsky et al. 2007). However, in basic soils the majority of Cu is strongly adsorbed to soil organic matter (OM) due to greater OM solubility and increasing pH dependent charge associated with increasing soil pH (McBride and Blasiak 1979). Copper complexation in calcareous soils has been shown to increase with increasing OM content, thus decreasing Cu availability to plants (Ghasemi-Fasaei et al. 2006).

Detrimental impact to plants due to Cu application above plant Cu requirements, regardless of potential soil precipitation or complexation reactions, has been observed. Copper mainly impairs root growth through inhibition of lateral root development and new seedling root growth initiation (Pahlsson 1989). Subsequently, plants tend to be stunted, chlorotic, or necrotic (Pierzynski et al. 2000). Brun et al. (2001) studied corn (*Zea mays* cv. Gaucho) root and shoot growth when grown in Cu-contaminated vineyard soils (up to 251 mg total Cu kg^{-1}). Root Cu concentrations (23 to 584 mg kg^{-1}) were greater than shoot concentrations (7 to 17 mg kg^{-1}), with total Cu and cation exchange capacity, and total Cu, soil pH, and OM explaining 81 and 85% of the variability in root and shoot Cu content, respectively. Sonmez et al. (2006) studied the effect of increasing concentrations (0, 1000, 2000 mg Cu kg^{-1}) to tomato (*Lycopersicon esculentum* (L.) Mill. Cv. F144), observing a decrease in plant height, total yield, number of fruit, and dry root weight with increasing Cu application.

Not only are excessive Cu concentrations detrimental to plants, they may also toxic to microorganisms. Ranjard et al. (2006) investigated the effects of Cu (16 and 48 kg ha^{-1}) on indigenous soil microorganisms in a calcareous silty clay soil, showing that two months following Cu application the control soil bacterial and fungal populations were significantly different from Cu-treated soils regardless of application rate. Ranjard et al. (2006) carried the experiment out to four and twelve months following application, observing no difference between treatments and the control at both time steps. The authors suggested that a transitory effect of the Cu stress might be partly due to the progressive reduction of soil Cu bioavailability over time. Yet time does not always reduce bioavailability, as shown by Sauve (2006) who noted that soil OM degradation by microorganisms was inhibited by 10, 20, and 50% with 154, 193, and 285 mg Cu kg^{-1} soil at a site exposed to decades of Cu contamination.

The Cu concern in Idaho is increasing because of rising dairy $CuSO_4$ foot bath usage; Idaho is currently the third largest dairy producing state in the US (~ 550,000 head of dairy cows; USDA NASS 2009) with most of the dairy industry is located in south-central Idaho. Land application of Cu, from spent foot baths, may detrimentally affect alfalfa raised for dairy cow feed. Thus, the main objectives of this pilot study were to quantify the effects of Cu addition on: 1) alfalfa growth; 2) soil total and subsequently extractable Cu characteristics; and 3) soil bacterial community structure and diversity. Soil extractable Cu concentrations were also used to determine an estimate of extractable soil Cu associated with alfalfa Cu content and the maximum tolerable Cu level in cattle feed based on National Research Council (2005) guidelines.

2. Materials and Methods

2.1 Soils

The Declo (loam; coarse-loamy, mixed, superactive, mesic Xeric Haplocalcid) and Logan (silty clay loam; fine-silty, mixed, superactive, mesic Typic Calciaquoll) soil series were utilized for this study as they are extensive in south-central Idaho (63,860 and 6,270 ha, respectively; USDA NRCS 2009). Surface soils (0-30 cm) were collected, air dried and passed through a 6.35-mm sieve. A part of this material passed through a 2-mm sieve and analyzed for certain properties (Table 1) and the rest material was used for the pot experiment. Soil texture was determined by the hydrometer method (Gee and Or, 2002). Soil pH (Thomas 1996) and electrical conductivity (EC; Rhoades 1996) were determined on a saturated paste extract, and cation exchange capacity via the method outlined by Sumner and Miller (1996) for soils containing carbonates. Total C was determined using the dry combustion method outlined by Nelson and Sommers (1996), while $CaCO_3$ and inorganic C content were determined by a modified pressure-calcimeter method (Sherrod et al. 2002); organic C was determined via difference between total C and inorganic C, and OM was calculated by multiplying organic C content by 1.724. Nitrate-N and NH_4-N were determined following methods outlined by Mulvaney (1996), Olsen-extractable P was determined as outlined by Kuo (1996), and total Ca, Mg, Na, K, Fe, P, Mn, Zn, Mo, and Cu using a 4 *M* HNO_3 digest (Bradford et al. 1975).

2.2 Experimental Setup

1.5 kg of soil was placed into individual 23 cm tall x 10 cm diameter pots with no drain holes. All pots received P (KH_2PO_4 in liquid form) at a rate equivalent to 108 kg P ha^{-1} based on Olsen-extractable P and the University of Idaho fertilizer recommendations for alfalfa (Stark et al. 2002). Pots were brought to 70% of field capacity using tap water three times per week during the study period. Six alfalfa seeds were planted into each pot and allowed to establish over a 5 week period, after which plants were thinned to three per pot. Pots then received Cu solutions, as $CuSO_4$, at concentrations of 0 (control), 50, 100, 250, 500, or 1000 mg Cu kg^{-1}, and plants were allowed to grow for approximately three weeks. The experimental design was completely randomized with four replicates.

2.3 Plant and Soil Chemical Analyses

Pot cumulative evapotranspiration (ET) losses were calculated by summing daily gravimetric ET losses for the first 14 days following copper application. Live plants were harvested ~ 2.54 cm above the soil surface 23 days after Cu was applied, placed in paper bags, oven dried at 60°C for 72 hours, biomass determined, and then ground to pass a 20-mesh sieve. A 0.50-g subsample was placed in a 100-mL beaker and ashed at 500°C for 5 hours. The samples were allowed to cool, weighed, and then 10 mL of 1 *M* HNO_3 was added. The samples were then heated on a hot plate until condensation no longer occurred on the inside of the beaker. Then, all samples were brought to a 50 mL final volume by weight with de-ionized H_2O, stirred, filtered through Whatman #50 filter paper, and analyzed for total Cu using inductively coupled plasma optical emission spectroscopy (ICP-OES).

Following harvest, soils were removed from pots, air-dried, and ground to pass a 2-mm sieve. Soils were extracted for plant-available Cu using diethylenetriaminepentaacetic acid (DPTA; Lindsay and Norvell 1978) and for total Cu with 4 *M* HNO_3 (Bradford et al. 1975). All extracts were filtered through Whatman #50 and analyzed using ICP-OES. The quantity of Cu accumulated by alfalfa and the DTPA-extractable soil Cu were compared using linear regression analysis to determine an upper level soil DTPA Cu concentration associated with the National Research Council (2005) maximum tolerable Cu level in cattle feed.

If necessary, data were $\log_{10}$ transformed to improve normality and reduce heteroscedascity before analysis (Steel and Torrie, 1980). Then, all soil and plant statistical tests were performed using the Proc GLM model in SAS software version 9.1 (SAS Institute 2002). Differences were examined using analysis of variance at a significance level (p) of 0.05 with mean separation determined using Fisher's Protected LSD test.

2.4 Soil Bacterial Community Analysis

In addition to collecting a pre-study soil sample, soil subsamples were collected immediately following plant harvest and placed in a -80°C freezer for bacterial community analysis. The pre-study soils consisted of soil devoid of plant and copper additions and all soil subsamples for bacterial analysis were collected from the alfalfa root zone. One replicate of each subsample was selected at random, and deoxyribonucleic acid (DNA) was extracted using a SoilMaster DNA Extraction Kit (Epicentre, Madison, WI), and with an additional DNA purification step using an UltraClean GelSpin DNA Extraction Kit (MO BIO Laboratories, Inc., Carlsbad, CA). The DNA quantity was determined via Biophotometer (Eppendorf AG, Hamburg, Germany), while quality was assessed by electrophoresis on a 1% agarose gel stained with SYBR Safe (Invitrogen, Carlsbad, CA). Only DNA with a 260/280 ratio between 1.7 - 1.9, with a majority of the fragments greater than 5 kb in size, was used in this study.

Extracted DNA was analyzed by ribosomal intergenic spacer analysis (RISA) using primers ITSF (5' – GTCGTAACAAGGTAGCCGTA – 3') and ITSReub (5' – GCCAAGGCATCCACC – 3') to amplify the variable length region between the 16S and 23S rDNA genes of the soil bacterial populations (Cardinale et al. 2004). Final reaction concentration of reagents were 10 ng of DNA, 1x PCR buffer, 1.5 U of *Taq* DNA polymerase (New England Biolabs, Ipswich, MA), 0.2 mM of each deoxynucleoside triphosphate, and 0.25 µM of each primer in a final volume of 25 µl. Amplifications were performed at 94°C for 3 min, followed by 30 cycles of 94°C for 45 s, 55°C for 1 min, 72°C for 2 min, with a final extension at 72°C for 7 min. A total of 1 µl of each amplification reaction was loaded, and RISA fragments were resolved on 3.7% KB^{Plus} polyacrylamide gels (LI-COR Inc., Lincoln, NE) of 66 cm length and 0.2 mm thickness. Profiles were run under denaturing conditions for 15 h at 3000 V / 60 A on a LiCor 4300 DNA Analyzer (LI-COR Inc., Lincoln, NE). Gel images were analyzed using Phoretix 1D software (Nonlinear Dynamics, Newcastle upon Tyne, United Kingdom). Bands (hereafter referred to as phylotypes) were determined, and matched between soil RISA profiles based on their electrophoretic mobility.

Soil bacterial diversity was determined from the RISA profiles using the Shannon-Wiener diversity index [*H*; (Margalef 1958)]. For *H*, p_i was calculated as $p_i = n_i/N$ with n_i representing the peak intensity of individual phylotypes to the *i*th phylotype in a profile, and *N* representing the total peak intensity of the profile. Evenness [(*J*; (Pielou 1966)] of the soil RISA profiles was calculated using sample richness (*S*) which was calculated as the total number of phylotypes for a given profile.

Cluster analysis of soil RISA profiles was performed by the unweighted pair-group method with arithmetic averages (UPGMA), using a Pearson correlation coefficient, via the Phoretix 1D software (Nonlinear Dynamics, Newcastle upon Tyne, United Kingdom).

3. Results and Discussion

3.1 Alfalfa ET, Growth, and Alfalfa Cu Concentration

Cumulative ET was not affected by increasing Cu applications up to 100 mg kg^{-1} for the Declo and Logan soils (Figure 1). However, ET decreased significantly above these Cu rates, likely because the alfalfa was either stressed or dead. Menon et al. (2005) observed similar results while investigating the effect of topsoil heavy metal contamination on newly established vegetation. The authors placed contaminated topsoil (2700 mg kg^{-1} Zn, 385 mg kg^{-1} Cu, 63 mg kg^{-1} Pb, 10 mg kg^{-1} Cu) over non-contaminated subsoil seeded with various plants and monitored soil water potential and plant transpiration over several years. Heavy metal contaminated topsoils maintained significantly greater soil water potentials than controls, indicating that less water was lost from the contaminated soil (Menon et al., 2005). The authors also noted a reduction in poplar (*Populus tremula*) transpiration in metal contaminated soil as compared to controls. Decreases in transpiration due to heavy metal exposure may be explained by decreases in root growth (Pahlsson, 1989), blockages of xylem tissue (Lamoreaux and Chaney, 1977; Robb et al., 1980), or decreased stomatal closure induced by water stress (Schlegel et al., 1987) which could result in reduced growth.

Alfalfa growth was unaffected by Cu application up to 250 mg kg^{-1} for the Logan soil while a quadratic growth response was observed for the Declo soil (Table 2). No alfalfa plants survived the 500 and 1000 mg kg^{-1} Cu concentrations in both soils, and thus plant Cu content for these rates are not shown. Similar plant responses have been observed in other studies. Sonmez et al. (2006) applied up to 2000 mg Cu kg $soil^{-1}$ to tomato (*Lycopersicon esculentum* (L.) Mill. Cv. F144), noting total yield, fruit number, dry root weight, and plant height decreased with increasing application rate. Ginocchio et al. (2006) showed that above ~ 300 mg Cu kg^{-1} lettuce (*Lactuca sativa* var. capitata) yield began to decrease. Strandberg et al. (2006) studied Cu-spiked soil effects on growth of Black Bindweed (*Fallopia convolvulus*), showing that shoot growth was reduced to 50% at a Cu application of 280 mg kg^{-1}, and was absent above 400 mg kg^{-1}.

Alfalfa Cu concentrations increased with increasing Cu application rate for both soils (Table 2). The 250 mg kg^{-1} Cu application rate likely stressed alfalfa more so than the other Cu rates, thus causing a reduction in ET and/or biomass. Because the pH of the two soils were similar (pH 7.9), differences in plant Cu concentrations between the two soils were likely due to textural differences associated with clay content, and to quantities of $CaCO_3$ and OM present which helped form insoluble Cu precipitates and organic Cu complexes. The Declo soil is classified as a loam (i.e. less clay) while the Logan soil is a silty clay loam (i.e. more clay). It has been shown that increasing soil clay content causes an increase in Cu sorption (Maftoun et al., 2002; Ghasemi-Fasaei et al., 2006). The Declo and Logan soils also contained 11 and 49% $CaCO_3$, and 1.2 and 2.5% OM, respectively (Table 1). Greater quantities of clay, $CaCO_3$, and OM in the Logan soil, as compared to the Declo soil, likely reacted with more soluble Cu and made Cu less available for alfalfa uptake. And, as Cu rate increased, Cu likely overwhelmed soil sorption sites which led to greater Cu availability and detrimental plant effects.

Soil clays, $CaCO_3$, and OM influence Cu sorption, however, in soils with pH values greater than 7.0 soluble Cu has been shown to react and form CuO, $CuCO_3$, $Cu_2(OH)_2CO_3$ (malachite), and $Cu(OH)_2$ (McBride and Bouldin, 1984; Ponizovsky et al., 2007; Ma et al., 2006). Rodriguez-Rubio et al. (2003) showed that Cu sorption on calcareous soils was enhanced by addition of calcite and OM, but Cu sorption decreased with the removal of the soil carbonate fraction. The authors suggested CuO, $Cu(OH)_2$, and malachite were controlling Cu availability. McBride and Bouldin (1984) found evidence of malachite precipitation in a Cu contaminated calcareous soil, yet the soil contained 5.3% OM and greater than 95.5% of the Cu in soil solution was complexed with organic species. Maftoun et al. (2002) concluded that Cu retention in calcareous soils was related to $CaCO_3$ and OM, yet McBride and Blasiak (1979) suggested that the majority of Cu is strongly adsorbed to OM in calcareous soils due to both greater OM solubility and greater pH dependent charge present. When present in calcareous soils, it has been shown that increasing OM content (0.4 to 4.8%; within the range of our study) decreases the quantity of plant-available Cu (Ghasemi-Fasaei et al., 2006) due to Cu complexation with organic species. Between 80 and

100% of Cu added to neutral or calcareous soils has been shown to be organically complexed (Cavallaro and McBride, 1978; McBride and Blasiak, 1979).

3.2 Soil Total and DTPA-Extractable Cu, and Associated Alfalfa Cu Content

Soil total and DTPA-extractable Cu content increased with increasing Cu application rate (Table 3), as was expected. Between 48 to 67% of the total Cu measured in the Declo soil was plant-available as determined by the DTPA extraction, while 70 to 80% was plant-available in the Logan soil. The remainder of Cu was likely sorbed to OM or precipitated as inorganic phases, as previously outlined. Other research has shown lower DTPA-extractable Cu concentrations after short incubation times. Ghasemi-Fasaei et al. (2006) demonstrated that after 20 days of incubation, DTPA extracted only 20% of the total soluble Cu (5 mg kg^{-1}) added to calcareous soils. Greater Cu rates in the current study likely saturated soil sorption sites, and thus more Cu remained available as compared to that found by Ghasemi-Fasaei et al. (2006).

Soil DTPA-extractable Cu was used to determine an estimate of extractable soil Cu associated with alfalfa copper content and the maximum tolerable Cu level in cattle feed (40 mg kg^{-1}) based on National Research Council (2005) guidelines (Fig. 2). A first order polynomial equation fit the observed Declo and Logan well (both R^2 = 0.977; dashed lines Fig. 2). Based on the predicted fit for the Declo soil, 63 mg kg^{-1} of DTPA-extractable soil Cu would produce a 40 mg Cu kg^{-1} alfalfa response and be detrimental to cattle. Following the same approach for the Logan soil, 95 mg kg^{-1} of DTPA-extractable soil Cu would be detrimental to cattle health. Greater soil DTPA extractable Cu can be tolerated in the Logan soil due to the aforementioned influence of increased clay, $CaCO_3$, and OM content. These values can be used as an estimate for determining soil DTPA Cu concentrations associated with alfalfa Cu concentration cattle feeding risk.

Stehouwer and Roth (2009) suggested that if copper was added gradually to soil (< 11 kg ha^{-1} yr^{-1}; which is not uncommon (Rankin, 2008)) then approximately 170 kg ha^{-1} could be added to light textured, low OM soils (< 1%; Stehouwer, personal communication) without causing crop toxicity. Heavier textured soils with moderate to high OM contents could receive three to five times as much Cu without experiencing crop toxicity symptoms (Stehouwer and Roth, 2009). However, Klingberg (2009) recommended that continued use of high copper content manures on the same field over many years should be avoided because of the potential for crop toxicity.

The Declo soil series is light textured (loam) with low OM content (1.2%; Table 1). Alfalfa grown on these soils requires on average 40 ha cm of irrigation water (US Department of Interior, 2009). Total Cu from south central Idaho dairy lagoon effluent was recently measured at 35.4 mg L^{-1} (D. Tarkalson, personal communication). Assuming 5% Cu availability (Stehouwer and Roth, 2009) in the effluent and if effluent water were solely used to irrigate alfalfa, a total of 17.5 kg Cu ha^{-1} yr^{-1} would be applied to Declo soils. Assuming 170 kg of plant-available Cu ha^{-1} could be added before crop toxicity was observed, approximately 10 years would be required to reach this Cu level. One should keep in mind that this calculation does not account for crop removal or potential changes in soil Cu availability which would lengthen the time until crop toxicity is observed. In the case of the Logan soil, it is heavier textured (silty clay loam) and contains twice as much OM as the Declo soil, and thus could possibly receive greater Cu quantities before experiencing crop toxicity symptoms.

3.3 Effect of copper sulfate on soil bacterial community diversity

To assess the impact of copper sulfate concentrations on the soil bacterial communities, we examined the bacterial RISA profiles of two different soil series growing alfalfa under varying copper treatment regimens. The UPGMA clustering analysis of the bacterial RISA profiles of both the Declo (Figure 3) and Logan (Figure 4) soil series divided the Cu treated soils into two clusters. For the Declo soils, the first and second clusters consisted of the 0 and 50 mg kg^{-1} (73% similarity) and 100, 250, 500, and 1000 mg kg^{-1} (69% similarity) Cu application treatments, respectively. Together, the two clusters had a 65% similarity, with 59% similarity to the pre-study sample. For the Logan soils, the first cluster included the pre-study sample along with the 0, 50, and 100 mg kg^{-1} (66% similarity) Cu application treatments; the second cluster consisted of the 250, 500, and 1000 mg kg^{-1} (65% similarity) Cu application treatments and had 61% similarity to the first cluster. The differences in similarity between the soils receiving Cu application could not be attributed solely to loss of bands in profiles receiving larger dosages of Cu, as the richness (S) of the profiles remained consistent between the treatments (Table 4); on average, Declo soils had 62.8 ± 2.1 bands per profile and Logan soils had 76.5 ± 3.1 bands per profile.

Based on the bacterial RISA profiles, Shannon-Weiner diversity indices and the corresponding evenness values were calculated (Table 4). For the Declo soil series, *H* remained stable with a decrease in overall diversity between soils with no copper application (*H* = 3.88) and 1000 mg Cu kg^{-1} (*H* = 3.81). For the Logan soil series, *H* demonstrated an increase at 50 mg kg^{-1} before trending downward until 1000 mg kg^{-1}. Evenness (*J*) remained high (*J* > 0.875) for all treatments, indicating no individual species dominated any of the Cu treatments. The

pattern of bacterial diversity in the Logan soils was similar to a study by Zhang et al. (2009) who researched bacterial diversity in response to Cd, another heavy metal known to stress bacterial populations; both the Logan soils and the soils studied by Zhang et al. (2009) revealed a peak in bacterial diversity at the lower concentrations of heavy metal contamination, a response also previously detailed by Giller et al. (1998). For the Logan soils, this increase in diversity could have been in response to either increased levels of Cu bioavailability (Ranjard et al., 2006), or environmental stress (Huysman et al., 1994). In comparison to the Logan soils, the Declo soils demonstrated a different response to Cu concentrations. Rather than peak at low levels of contamination, the diversity remained relatively stable regardless of the Cu concentration; however, there was an increase in diversity over the pre-study soils (H = 3.74) upon the planting of alfalfa (H = 3.88). This result runs counter to studies which have reported decreasing microbial diversity under high concentrations of heavy metals (Giller et al., 1998). However, our results are supported by Zhang et al. (2009) who also observed little effect on the structure and diversity of soil bacterial communities under long-term Cd stress. The researchers attributed this to the heterogeneity of soils, and how impacts upon bacterial community structure and diversity by heavy metals could be countered by other soil characteristics such as total organic carbon, total nitrogen, and nutrient status. Indeed, a report by Speir et al. (1999) indicated that soil acidification played a significant role in reducing microbial enzymatic activity; this inhibition could be directly attributed to acidification rather than the introduction of heavy metals into the soil profile. In the current study, pH remained stable for both the Declo (pH 8.10 ± 0.2) and Logan (pH 7.9 ± 0.2) soils with neither representing an acidic environment, likely attributed to the high $CaCO_3$ content in both soil series.

4. Conclusions

The objectives of this investigation were to identify Cu application effects on alfalfa growth and Cu concentration, total and DTPA-extractable soil Cu content, and the soil bacterial community. In addition, soil DTPA-extractable Cu was used to determine an estimate of extractable soil Cu associated with alfalfa copper content and the maximum tolerable Cu level in cattle feed based on National Research Council (2005) guidelines. Our results demonstrate that alfalfa growth was generally unaffected by Cu applications up to 250 mg kg^{-1}; 500 and 1000 mg kg^{-1} Cu application rates killed alfalfa. Although increasing Cu application rates caused an increase in alfalfa Cu content, only Cu application rates greater than 250 mg kg^{-1} decreased ET in both soils. The DTPA-extractable Cu content increased with increasing Cu application, and following 23 days since Cu application 48 to 80% of the added Cu was still plant-available. Above 63 and 95 mg kg^{-1} of DTPA-extractable soil Cu was associated with the maximum dietary alfalfa Cu limit (40 mg kg^{-1}) for cattle in Declo and Logan soil, respectively. Bacterial community analysis revealed that soils clustered according to low and high Cu application rates. For the Logan soils, bacterial diversity peaked at 50 mg kg^{-1} while Declo soil bacterial diversity remained relatively stable throughout the entire course of Cu applications. These differences could potentially be explained by differences in the characteristics of the individual soil series.

Based on the predicted DTPA-extractable soil Cu to detrimental animal dietary consumption values, it is suggested that plant-available soil Cu concentrations not exceed 63 mg kg^{-1} in these agroecosystems. In regards to bacterial community structure and diversity, our analysis indicates that the effects of Cu application rates are most likely soil series dependent, but that generally the bacterial communities are able to withstand higher rates of Cu in their environment. Most states do not have environmental regulations in place regarding Cu handling and land application. However, as with any land application of waste program it is suggested that soil testing is conducted on a routine basis to ensure optimum crop yields along with environmental protection.

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Table 1. Certain properties of the Declo and Logan soil series prior to Cu application

Property	Units	Declo Soil Series	Logan Soil Series
Texture		loam	silty clay loam
pH		7.9	7.9
EC	dS m^{-1}	1.02	0.73
CEC	$cmol_c$ kg^{-1}	14	14
Total C	g kg^{-1}	20	73
$CaCO_3$	g kg^{-1}	110	490
Inorganic C	g kg^{-1}	13	59
Organic C	g kg^{-1}	7.0	14
Organic Matter	g kg^{-1}	12	25
NO_3-N	mg kg^{-1}	11.6	9.82
NH_4-N	mg kg^{-1}	5.20	4.75
Olsen P	mg kg^{-1}	13	19
Total Ca	mg kg^{-1}	30560	85600
Total Mg	mg kg^{-1}	6180	29800
Total Na	mg kg^{-1}	398	414
Total K	mg kg^{-1}	1430	866
Total Fe	mg kg^{-1}	3290	1150
Total P	mg kg^{-1}	706	405
Total Mn	mg kg^{-1}	252	291
Total Zn	mg kg^{-1}	27.2	27.0
Total Mo	mg kg^{-1}	ND	ND
Total Cu	mg kg^{-1}	6.52	5.04

Table 2. Influence of copper application on alfalfa (*Medicago sativa*) growth and copper concentration in the Declo and Logan soil series

	Declo Soil Series		Logan Soil Series	
Cu Application	Alfalfa Growth	Alfalfa Cu Concentration	Alfalfa Growth	Alfalfa Cu Concentration
(mg kg^{-1})†	(g pot^{-1})	(mg kg^{-1})	(g pot^{-1})	(mg kg^{-1})
0	2.82 (0.57) a‡	12.7 (1.2) a	4.28 (0.65) a	11.2 (2.0) a
50	3.22 (0.45) ab	28.4 (11.4) b	4.02 (0.33) a	17.0 (1.6) b
100	3.05 (0.52) a	47.9 (13.2) c	3.80 (0.58) a	31.9 (9.9) c
250	2.10 (0.78) b	79.0 (11.0) d	3.45 (0.95) a	74.7 (27.1) d

†The 500 and 1000 mg kg^{-1} Cu applications were not included in the statistical analysis because no plants survived.

‡ Values within parentheses represent one standard error of the mean. Different lower case letters within a column indicate significant difference between copper application rates (Fisher's LSD, p=0.05, n=4).

Table 3. Influence of copper application on total and DTPA-extractable copper in the Declo and Logan soil series

	Declo Soil Series		Logan Soil Series	
Cu Application	Total Cu	DTPA Cu	Total Cu	DTPA Cu
(mg kg^{-1})	---------- mg kg^{-1} ----------			
0	7.74 (0.10) a‡	0.97 (0.05) a	3.81 (0.11) a	0.67 (0.04) a
50	62.8 (7.83) b	30.2 (4.27) b	46.6 (4.76) b	32.5 (2.26) b
100	115 (22.5) c	67.6 (13.5) c	110 (7.87) c	87.2 (6.71) c
250	272 (6.60) d	175 (22.3) d	232 (27.6) d	186 (26.2) d
500	468 (63.5) e	289 (39.1) e	410 (164) e	286 (101) e
1000	957 (17.4) f	640 (18.6) f	845 (79.8) f	640 (50.6) f

‡ Values within parentheses represent one standard error of the mean. Different lower case letters within a column indicate significant difference between copper application rates (Fisher's LSD, p=0.05, n=4).

Table 4. Bacterial diversity indices as affected by alfalfa (*Medicago sativa*) and copper application to the Declo and Logan soil series

Soil Series	Plant	Cu Application (mg kg^{-1})	*S*	*H*	*J*
Declo	None	0	74	3.74	0.8763
Declo	*M. sativa*	0	65	3.88	0.8737
Declo	*M. sativa*	50	61	3.87	0.8808
Declo	*M. sativa*	100	62	3.83	0.8818
Declo	*M. sativa*	250	65	3.85	0.8918
Declo	*M. sativa*	500	64	3.82	0.9120
Declo	*M. sativa*	1000	60	3.81	0.9099
Logan	None	0	78	4.04	0.9275
Logan	*M. sativa*	0	81	4.02	0.9040
Logan	*M. sativa*	50	79	4.22	0.9609
Logan	*M. sativa*	100	77	4.04	0.9299
Logan	*M. sativa*	250	75	3.91	0.9061
Logan	*M. sativa*	500	73	3.92	0.9387
Logan	*M. sativa*	1000	74	4.01	0.9319

S = Sample Richness; H = Shannon-Wiener Diversity Index; J = Evenness.

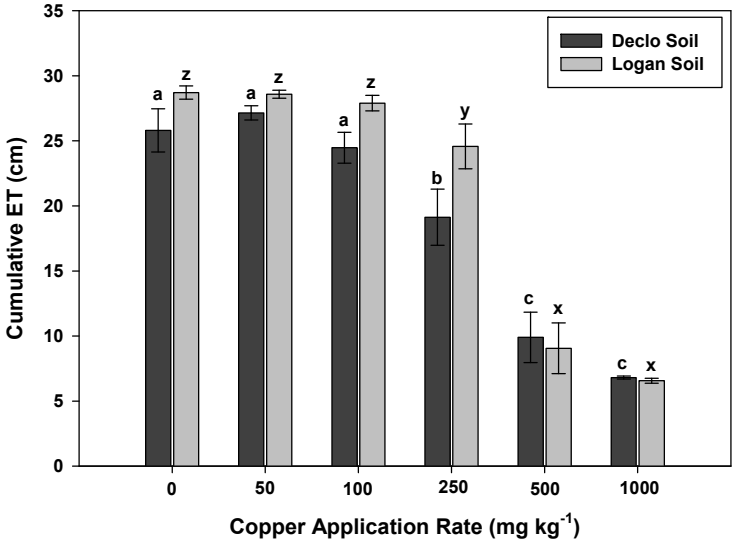

Figure 1. Cumulative alfalfa evapotranspirational (ET) losses for the Declo and Logan soils for the 14 days following copper application (mean +/- SE). Different letters above the bars for either the Declo or Logan soils indicate a significant difference in ET between copper application rates (Fisher's LSD, p=0.05, n=4)

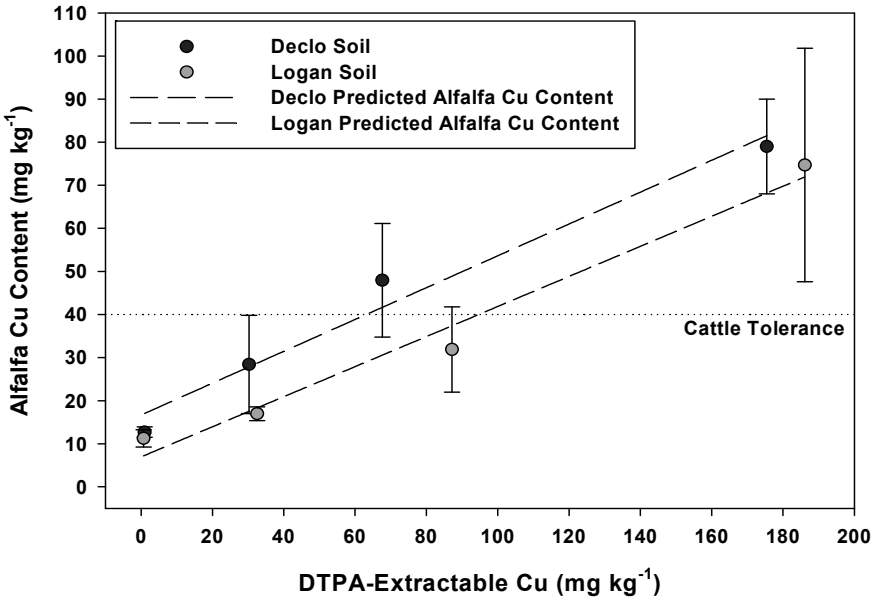

Figure 2. Alfalfa copper concentration versus DTPA-extractable soil copper concentration (mean +/- SE). Dashed lines represent the predicted alfalfa copper concentration as compared to either Declo or Logan DTPA-extractable Cu content. The solid horizontal line represents cattle (40 mg kg^{-1}) maximum tolerable dietary Cu content based on National Research Council (2005) recommendations

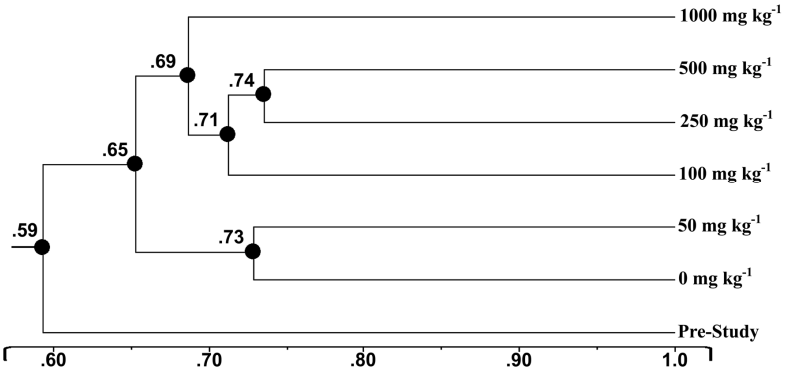

Figure 3. UPGMA dendrogram representing the similarity of bacterial RISA profiles from Declo soil with increasing Cu application rates. The percentages of similarity amongst the RISA profiles were calculated using the Pearson coefficient. Each profile represents one subsample of each treatment chosen at random

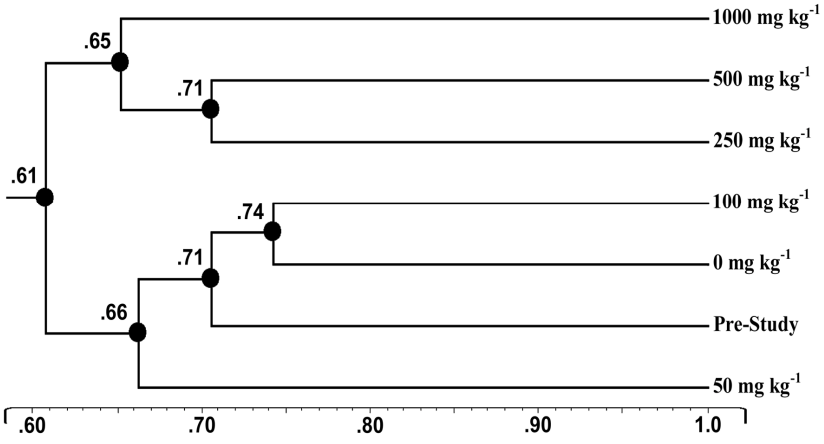

Figure 4. UPGMA dendrogram representing the similarity of bacterial RISA profiles from Logan soil with increasing Cu application rates. The percentages of similarity amongst the RISA profiles were calculated using the Pearson coefficient. Each profile represents one subsample of each treatment chosen at random

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