Long-Term Use of Copper-Containing Fungicide Affects Microbial Properties of Citrus Grove Soils

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Long-term use of Cu-containing fungicides in citrus groves has resulted in Cu buildup in the soil, but the effects of Cu contamination on microbial properties of citrus grove soils remain poorly understood. The objective of this study was to investigate the influence of long-term application of Cu-containing fungicides on microbial biomass, and bacterial community structure and diversity in five representative commercial citrus groves (5, 21, 27, 36, and 43 yr of planting history, respectively) soils, with one adjacent pasture soil as a reference. With increasing planting time of citrus, Cu concentrations in the soils increased, while microbiological properties including microbial biomass carbon (Cmic), microbial quotient (MQ), and the diversity of operational taxonomic units based on denaturing gradient gel electrophoresis (DGGE) community fingerprinting decreased. Stepwise multiple regression analysis showed that Cmic, microbial quotient, and bacterial community diversity were affected by Cu concentration and other soil properties such as total N and available P, but the effect of Cu was dominant. These results indicate that long-term application of Cu-containing fungicides has adverse effects on microbial biomass and bacterial community diversity in citrus grove soils. Sequencing of partial 16S rRNA gene fragments revealed that a shift of total bacterial community composition occurred as a result of Cu contamination, and the soils of more severely Cu-polluted citrus groves were dominated by bacteria g-Proteobacterium, Acidobacteria, Firmicutes, and b-Proteobacterium, whereas certain strains of s-Proteobacterium, g-Proteobacterium, Firmicutes, and Cyanobacteria were the dominant bacteria in the young citrus grove soils.

Abbreviations: AP, available P; C_{mic}, microbial biomass carbon; C_{org}, total organic carbon; DGGE, denaturing gradient gel electrophoresis; LSD, least significant difference; MQ, soil microbial quotient; PCR, polymer chain reaction; SI, Shannon index; TCu, total recoverable Cu; TN, total N; UPGMA, unweighted pair-group method average.

Microorganisms in soil ecosystem are responsible for many fundamental ecological processes, such as the biogeochemical cycling of chemical elements and the decomposition of plant and animal residues (Kent and Triplett, 2002). Thus, functional diversity and community structure of microorganisms are important for soil ecosystem health. The changes of microbial community structure and activities, as a result of natural processes or human activities, may have lasting negative effects on soil ecosystem dynamics if critical microbial community functions are compromised (Deng et al., 2009; Viti et al., 2008).

Deleterious effects of heavy metals on soil microorganisms have been of concern for decades. There is strong evidence that microorganisms are generally more sensitive to heavy metals than other organisms in soil biocoenosis (Giller et al., 1998). Previous reports have shown that short-term or long-term exposure of soils to toxic metals resulted in reduced microbial diversity and activity, as a result of selection of heavy metal resistant or tolerant organisms that are less metabolically efficient (Nakatsu et al., 2005; Viti et al., 2008). Copper is an essential micronutrient, but at excessive levels, it can adversely affect microbial activity. Kandeler et al. (1996) observed that microbial biomass, enzyme activity, and functional diversity of soil microbial communities decreased with increasing Cu pollution at 100 mg kg^{-1} Cu in three soils (Calcaric Phaeozem, Eutric Cambisol, and Dystric

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Lithosol) from Austria. Microbial biomass and metabolic quotient were also reduced when the Cu level was raised from 112 to 182 mg Cu kg^{-1} soil (Khan and Scullion, 2000). There were negative correlations between soil microbial biomass, phosphatase activity, and NH_4NO_3 extractable Cu in soil (Wang et al., 2007). Analysis of the phospholipid fatty acids profile showed that microbial community structure shifted with the [Cu^{2+}] gradient in the Cu-enriched soils (Deng et al., 2009).

Pollution of Cu to soils is on the rise worldwide from both point and nonpoint sources, and in comparison, nonpoint-source pollution of Cu poses a greater threat to the environment and ecosystem functions due to its widespread nature (He et al., 2006, 2009). Florida is well known for its citrus production with a history of over 150 yr and nearly three quarters of the total U.S. citrus production is grown in Florida (United States Department of Agriculture–National Agricultural Statistics Service [USDA-NASS], 2009). However, in the last two decades, an increased amount of Cu-containing fungicides have been used to cure/prevent canker diseases, particularly for grapefruit trees (Driscoll, 2004). Repeated use of Cu-containing fungicides has increased the total Cu concentration in some citrus grove soils up to 250 mg kg^{-1} as compared with background levels of approximately 10 mg kg^{-1} (Yang et al., 2009). Laboratory simulation and field monitoring studies had been conducted to examine the biogeochemical aspects of Cu in citrus groves soils and subsequent impacts on soil quality (He et al., 2009). Environmental concerns about the side effects of copper-based fungicides have addressed their potential for accumulation in soils and the effects on soil biota such as earthworms (Paoletti et al., 1994), nematodes (Jaworska and Gorczyca, 2002), and Florida apple snail (Rogevich et al., 2008). However, to date, minimal information is available regarding the changes in microbial properties in citrus grove soils due to the accumulation of excess Cu in soils from repeated use of Cu-containing fungicides. The aim of this study was to investigate the impact of Cu contamination on soil microbial biomass and bacterial community structure and diversity, and the results will provide information for evaluation of the potential environmental risks of long-term application of Cu-containing fungicides in the citrus production systems.

MATERIALS AND METHODS

Site Description and Sample Collection

Five commercial groves of grapefruit (Citrus *paradise* McFad.) in the Indian River area south Florida, with a planting history of 5, 21, 27, 36, and 43 yr, respectively, were selected for this study (Table 1). The selection of field sites was based on similar texture (sandy with sand fraction being approximately 900 g kg^{-1}) of the same soil type (fine loamy siliceous hyperthermic Typic Glossaqualfs) according to soil survey of the Indian River County Area, Florida (United States Department of Agriculture–Soil Conservation Service [USDA-SCS], 1980), and these fields were all pastureland before converted into citrus groves. The groves received a similar amount of Cu-containing fungicide each year, applied four to five times per year right from the beginning of the citrus trees being planted. Both copper sulfate ($CuSO_4$) and copper hydroxides [$Cu(OH)_2$] were used as fungicides to prevent or cure citrus diseases; however, in the last decade a major citrus disease, canker, occurred in this area, and increased amounts of the fungicides with higher proportion of $Cu(OH)_2$ were applied as grapefruit trees are very susceptible to this disease.

Eighteen cores of random samples were collected from each grove to form three composite samples for each site. Soil samples were collected from the middle of a citrus bed and between two neighboring trees (approximately 1 m from citrus tree trunk) at a depth of 0 to 20 cm (after removing the litter layer). An adjacent pasture field which had not received any artificial Cu input was chosen for collecting reference soil samples and these soils were collected from marginal transects with no grass cover. The selection of this field as control is based on its similarity in basic soil properties (of the same soil type with similar texture), hydrological and climate conditions (within the same area and of similar landscape), and cropping history (pastureland) to those citrus groves before citrus cultivation, so that the effects of anthropogenic inputs of Cu from citrus planting can be singled out. A portion of each soil sample was air-dried at ambient temperature, crushed, and sieved to pass through a 2-mm sieve for the analysis of physicochemical properties. The remaining moist soil samples were stored in the dark at 4°C before microbial property analysis. Soil C_{mic} was assayed within a week after collection, and for soil bacterial community property analysis, soil DNA was extracted shortly after soil collection, and the DNA samples were kept at −20°C before further analysis in <1 mo.

Determination of Soil Physicochemical Properties

Soil pH was measured using a pH/conductivity meter (Model 220, Denver Instrument, Denver, CO) following USEPA Method 150.1.

Table 1. Sampling location and relevant physicochemical characteristics of the studied soils†.

Soil/planting history	Location of sampling (GPS coordinates)	pH (H_2O)	C_{org}	Available P	Available K	Total N	Total Cu	$CaCl_2$–Cu
			mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	g kg^{-1}	mg kg^{-1}	mg kg^{-1}
Reference soil	27° 35.784N 080° 37.654W	5.98 ± 0.46ab	5.73 ± 0.18a	26.27 ± 3.04a	83.85 ± 6.56a	0.55 ± 0.04a	10.29 ± 0.13a	0.00 ± 0.00a
5 yr	27° 35.781N 080° 37.634W	5.25 ± 0.30a	5.23 ± 0.28a	142.8 ± 46.22b	28.59 ± 4.46b	0.31 ± 0.02b	19.86 ± 3.49a	0.07 ± 0.02b
21 yr	27° 29.544N 080° 37.969W	5.46 ± 0.54ab	5.47 ± 0.21ab	110.2 ± 4.23b	24.75 ± 2.46b	0.73 ± 0.34abcd	164.1 ± 2.90b	0.14 ± 0.04b
27 yr	27° 26.310N 080° 39.796W	6.08 ± 0.26b	12.68 ± 1.10bcd	214.6 ± 19.40c	50.05 ± 8.11c	1.22 ± 0.11c	195. 8 ± 37.04b	0.11 ± 0.01b
36 yr	27° 26.304N 080° 40.337W	6.25 ± 0.16b	12.18 ± 0.24c	179.4 ± 32.45c	32.39 ± 3.20d	1.00 ± 0.12cd	210.5 ± 17.71b	0.13 ± 0.02b
43 yr	27°26.310N 080° 39.785W	6.08 ± 0.29b	9.58 ± 0.81d	206.2 ± 3.37c	20.32 ± 2.84e	0.74 ± 0.16d	256.5 ± 47.40b	0.31 ± 0.08c

† Data shown are expressed as means ± SD obtained from three replicates. Corg, total soil organic C (mg kg^{-1}); mean values with different letters indicate significantly different ($p < 0.05$) according to the least significant difference (LSD) test.

Total organic carbon (C_{org}) in soil was determined using C/N analyzer (Vario Max CN Macro Elemental Analyzer, Elemental Analysen system GmbH, Hanau, Germany). Available P and K concentrations were determined according to Bray and Kurtz (1945) and Jones (1973), respectively. Total recoverable Cu in the soils was determined by digesting the sample following USEPA Method 3050B, which uses repeated additions of nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) to extract elements and metals including Ba from soil. Soil available Cu was measured by 0.01 mol L^{-1} $CaCl_2$ extraction procedure (He et al., 2009). After extraction, the suspension was centrifuged at 7500 × *g* for 30 min and then the supernatant was passed through a Whatman #42 filter paper to remove any suspended materials. The concentrations of Cu in the digested solution or soil extracts were determined using inductively coupled plasma optical emission spectrometry (ICP–OES, Ultima 2, JY Horiba Inc., Edison, NJ) following USEPA Method 200.7. All the analyses were conducted following a quality assurance and quality control plan (NELAC certificate #: E76888) of the University of Florida Soil and Water Science Laboratory at the Indian River Research and Education Center in Fort Pierce, Florida. The detection limit for Cu was 0.3 μg L^{-1}, with a recovery of 90 to 110%.

Microbial Biomass Carbon

Soil C_{mic} was determined by the fumigation extraction method according to Vance et al. (1987). Total organic carbon in the extracts was determined immediately after extraction using a liquid TOC analyzer (liquid TOC Trace, Elemental Analysis system GmbH, Hanau, Germany). Micobial biomass C was calculated according to the equation: $C_{mic} = E_C/K_{EC}$ (Vance et al., 1987), where E_C was the difference in extractable organic C between the fumigated and unfumigated samples, and $K_{EC} = 0.45$ (Ocio et al. 1991). Soil MQ (ratio of C_{mic}/C_{org}) was calculated for all soil samples and expressed as mg C_{mic} $g^{-1}C_{org}$.

DNA Extraction and PCR Amplification of 16S rRNA Fragments

Approximately 0.25 g of soil was extracted to obtain DNAs using a PowerSoil DNA Isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. The resolution of extracts in a 1.0% agarose gel containing ethidium bromide (0.3 μg mL^{-1}) was used to estimate the DNA quantity and quality. Changes in bacterial community of different soil samples were detected by DGGE of polymerase chain reaction (PCR) products from 16S rRNA genes as previously described (Muyzer et al., 1996). Briefly, the V3 region of the 16S rRNA gene was amplified by using primer 341F with a GC clamp attached to the 5′ end (40-nucleotide GC-rich sequence, 5′-CCTACGGGAGGCAGCAG-3′) and primer 907R (5′-CCGTCAATTCMTTTGAGTTT-3′) (Casamayor et al., 2002; Integrated DNA Technologies, Coralville, IA). The PCR mixture contained 1× PCR buffer (Promega, Madison, WI), 3.5 mM $MgCl_2$ (Promega, Madison, WI), 0.8 μmol of each deoxynucleoside triphosphate (Promega, Madison, WI), 0.1% bovine serum albumin (Promega, Madison, WI), 0.5 μM (each) forward and reverse primers, 1 U/25μL of Taq polymerase (Promega, Madison, WI), and 1 to 50 ng of template DNA. Amplification was performed by 5 min of denaturation at 94°C and then 30 cycles of 30 s each at 92, 55, and 72°C, followed by a final extension at 72°C for 15 min. Polymerase chain reactions were run in Eppendorf Mastercycler gradient thermal cycler (Model 5321, Eppendorf, Germany).

Denatured Gradient Gel Electrophoresis

A DCode Universal Mutation Detection System (GE Healthcare bio-sciences Co, Piscataway, NJ) was used to perform DGGE analysis. The gels were made with a denaturing gradient ranging from 45 to 65% (100% denaturant is defined as a mixture of 7 mol L^{-1} urea and 40% deionized formamide). Between 0.5 and 1 μg of the PCR products were loaded per well and run at 80 V for 16 h. The gels were stained with ethidium bromide solution and the images were captured using a Gel DOC XR Imaging system (Bio-Rad, Cambridge, MA). Three replicates of each sample were loaded on one gel to compare the DGGE patterns of all different soils.

Analysis of Denatured Gradient Gel Electrophoresis Patterns

Denaturing gradient gel electrophoresis patterns were analyzed with GelCompar 4.0 program (Applied Maths, Ghent, Belgium) as described by Smalla et al. (2001). Similarity matrix and dendrogram of the DGGE profiles were generated based on Pearson correlation coefficient and unweighted pair-group method average (UPGMA), respectively. The lower limit for band detection was set by the band peak height threshold of 0.1% of the total detected optical density in the lane. Microbial diversity was examined by the Shannon diversity indices (H′) (Shannon and Weaver, 1963). The equation for the Shannon index (SI) is: $H' = -\sum(p_i)\log(p_i)$, where p_i represents % of the integrated density of a band, relative to the sum of all the bands in a lane. Species evenness of the bacterial community was indicated by the intensity of three most intense bands in each community expressed as percentages of total DNA intensity in a community.

Isolation and Sequencing of Dominant Denatured Gradient Gel Electrophoresis Bands

Isolation and sequencing of dominant DGGE bands were done according to Sun et al. (2004). The PCR products were checked for purity and expected size and quantified as described above. The re-amplified products were purified with the UltraClean PCR cleanup DNA purification kit (Mo Bio Laboratories, Inc., Carlsbad, CA). The DNA sequencing was performed with the BigDye Terminator Cycle Sequencing Kit v 3.1 on the AB 3730xl DNA analyzer (Applied Biosystems, Foster City, CA). The sequence was read from both directions with primers 341F and 907R, respectively. Sequence data from this study are listed within the Genbank database under accession numbers GU589556-GU589569. Sequences were compared with the GenBank database by using Nucleotide Blast (Altschul et al., 1990). The ClustalX v2.0 program (Larkin et al., 2007) was used to assemble and align all of 16S rRNA gene sequences including the reference sequences obtained from GenBank database. The sequence distance matrix for all pair wise sequence combinations was analyzed by the use of MEGA4.1 (Tamura et al., 2007) with the neighbor-joining method of phylogenetic tree construction with 1000 bootstrap replicates.

Data Analysis

Data were analyzed using SPSS 11.0 software. One-way ANOVA was used to compare soil physicochemical and microbial properties, using the least significant difference (LSD) test to evaluate whether the means were significantly different at a *p*-value of <0.05. Bivariate correlation analyses were performed to determine the association between soil parameters, and the stepwise multiple regression analysis was used to determine the contribution of major soil properties to the changes of soil microbial biomass and bacterial community structures.

RESULTS

Total Recoverable and Available Copper in Soils

The concentrations of total recoverable Cu and available Cu measured by $CaCl_2$ extraction significantly varied among the studied soils and were related to land use history (Table 1). Total recoverable Cu in the reference soil was only 10.29 mg kg^{-1}, which was close to the background Cu level of representative soils in Florida (Ma et al., 1997), while in soils under citrus production, the concentration ranged from 19.86 mg kg^{-1} for the 5-yr grove to 256.5 mg kg^{-1} for the 43-yr grove. On average, total recoverable Cu increased by approximately 6 mg kg^{-1} per year. Similar to total recoverable Cu, $CaCl_2$–extractable Cu in the soils was also related to planting time.

Soil Microbial Biomass

Both soil C_{mic} contents and MQ decreased with the grove age or planting history (Fig. 1). Planting of citrus for 5, 21, 27, 36, and 43 yr caused 10.9, 37.8, 53.8, 53.7, and 57.8% of reduction in the C_{mic}, respectively, as compared with the reference soil. Multiple variable regression analysis indicated that total recoverable Cu can explain 92% of the variance in C_{mic} and 86% of the variance in MQ, with organic C and nutrient condition (N and P) accounting for the rest (Table 2).

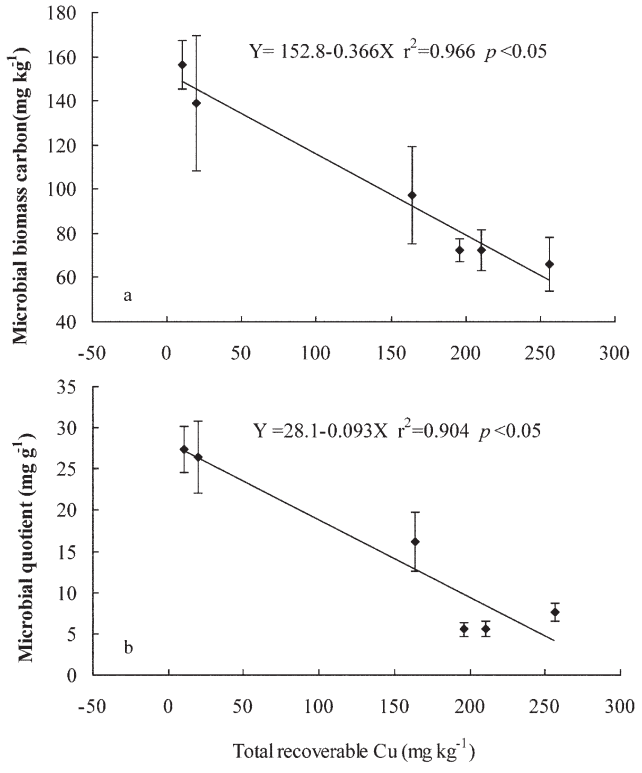

Fig. 1. Changes of microbial biomass C (a) and soil microbial quotient(MQ) (b) in relation to total recoverable Cu in soils. Error bars indicate standard errors.

Denaturing Gradient Gel Electrophoresis Analysis of Bacterial Community

Bacterial DGGE profiles generated from the 16S rRNA fragments revealed the richness and eveness of the bacterial community in soil samples (Fig. 2). A significantly higher number of visible bands were detected in the reference soil and the soil from the 5-yr grove, indicating the presence of high number of bacterial taxa. Soil samples from the 43-yr grove showed the simplest DGGE pattern (Table 3). Clustering of the profiles revealed that bacterial community structures in the groves aged 36 and 43 yr showed the highest similarity, with an average band sharing coefficient of 0.95, followed by groves aged 27 and 36 yr, with an sharing coefficient of 0.94 (Fig. 3).

The Shannon diversity index of the bacterial community based on DGGE fingerprinting data had a tendency to decrease with the citrus grove age (Table 3). Significant differences were found between the reference soil and the soils from the citrus groves aged 27, 36, and 43 yr, and between the soils from groves aged 5 and 43 yr, and 21 and 43 yr ($p < 0.05$). The distribution evenness of the bacterial community was analyzed based on the intensity of the three most intense bands. As it was seen in Table 3, DGGE bands from the control soil were more evenly distributed than corresponding bands from Cu contaminated soils, with the top three ribotypes comprising about 20% of the total bacterial community detected. While the least evenly distribution of bacterial ribotypes occurred in the grove aged 36 yr, in which the top three ribotypes comprised of 30.7% of the total bacterial community detected. Multiple variable regression analysis indicates that the SI value was weakly related to pH, organic C, and available nutrients (N, P, K), but very significantly negatively correlated with total recoverable Cu in the soil ($r = -0.976, p < 0.001$).

Sequencing of Denaturing Gradient Gel Electrophoresis Bands

The prominent bands in each sample from the DGGE gel were excised and sequenced to obtain further information about the shift of dominant bacteria populations in the citrus grove soils of different planting ages (Fig. 2). A total of 42 bands were sequenced and related to 14 kinds of bacteria according to sequence analysis. A neighbor-joining phylogenetic tree was constructed to visualize the relationships between the sequences from the citrus grove soils and related organisms from the GenBank database (Fig. 4). According to Fig. 4, Bands P7 and P10 have close relationships with b-Proteobacteria and Firmicutes, respectively.

A comparison of the DGGE patterns revealed several bands which were detected in the reference soil but not in the citrus grove soils, while a few dominant bands were characteristic of

Cu contaminated soil samples only. Bands P1 and P14, which showed 95% similarity to *Bacillus megaterium* and 98% similarity to *Nitrospira* sp., respectively, existed in all soil samples. This may indicate that these two bacteria strains are general population in the soils and can survive the gradually accumulated Cu. Bands P3 and P11were the apparently dominant population in the citrus grove aged 5 yr, but not detectable in the other soil samples. Band P9, which could be assigned to *Bacillus pocheonensis* strain G3 with a 99% similarity, existed only in the soils from the citrus groves aged 5 and 21 yr. Similar information was observed for Band P7, which presented in the citrus groves aged 5, 21, and 27 yr, but was not found in the citrus groves aged 36 and 43 yr. Of all the soil samples, the strongest bacteria shift occurred in the 21-yr grove, which was characterized by the appearance of some bacteria which became dominant in the soils from the groves aged 27, 36, and 43 yr, including some strains of g-Proteobacterium, Firmicutes, and Acidobacteria bacteria.

Table 2. Multiple variable regression of microbial biomass C (C_{mic}) or microbial quotient (MQ) with total recoverable Cu (TCu), available P (AP), total N (TN), and organic carbon (C_{org}).

Variables	Partial R^2	Model R^2	F value	Pr > F
	C_{mic}			
Total recoverable Cu	0.9234	0.9234	48.23	0.0023
Total N	0.0416	0.9650	3.57	0.1552
Available P	0.0266	0.9917	6.40	0.1271
Organic carbon	0.0081	0.9998	33.72	0.1086
Regression equation	C_{mic} = 171.37- 0.1415TCu -0.2197AP-46.492TN+2.7786C_{org}			
	MQ			
Total recoverable Cu	0.8572	0.8572	24.02	0.0080
Total N	0.1221	0.9794	17.76	0.0244
Available P	0.0189	0.9983	21.97	0.0426
Organic carbon	0.0014	0.9997	4.28	0.2868
Regression equation	MQ = 35.98–0.0298TCu-11.39TN-0.0244AP-0.3044C_{org}			

DISCUSSION

Accumulation of Copper in Citrus Groves Soils

Total recoverable Cu concentration in soils was linearly related to the age of citrus grove ($Y = 11.33 + 5.98\ X$, $r^2 = 0.96$, $p < 0.05$). Crop variety and soil type may also affect Cu accumulation in the soil to a certain extent (He et al., 2009). Since all the five citrus groves were planted with grapefruit and belonged to the same soil type with similar management practices and therefore, their significant difference in total Cu concentration can be attributed to the time under citrus production or the external Cu input from repeated application of Cu-containing fungicides (He et al., 2009).

The toxicity of Cu to plants depends more on its available rather than total concentration (Giller et al., 2009). In the present study, available Cu estimated by the $CaCl_2$ extraction procedure in the soils was significantly correlated with total recoverable Cu ($r = 0.82$, $p < 0.05$), this is in accordance with Wang et al. (2009), who observed that 0.01 mol L^{-1} $CaCl_2$–extractable Cu has a high correlation with total Cu in the soils.

Effect of Copper and Other Soil Properties on Soil Microbial Biomass Carbon

Soil microbial biomass has been proposed as a sensitive endpoint for defining the impact of contaminants such as heavy metals on soil biological functioning (Giller et al., 1998), and a number of soil properties may affect soil microbial biomass (He et al., 2003). In the present study, both bivariate correlation and multiple variable regression analysis showed that soil C_{mic} and MQ values in the citrus grove soils were mainly affected by soil Cu accumulation, and the influence of other soil variables such as C_{org} and nutrients (N, P, K) were limited. This agrees with several previous studies: for example, V⟨squez-Murrieta et al. (2006) found that long-term contamination of soil with heavy metals had an adverse effect on microorganisms as evidenced by a marked decrease in C_{mic}. A laboratory incubation experiment also showed that addition of 50 to 600 mg kg^{-1} Cu decreased soil C_{mic} and microbial biomass N sharply (El-Ghamry et al., 2000). The results from this study and the literature indicate that Cu contamination causes soil quality

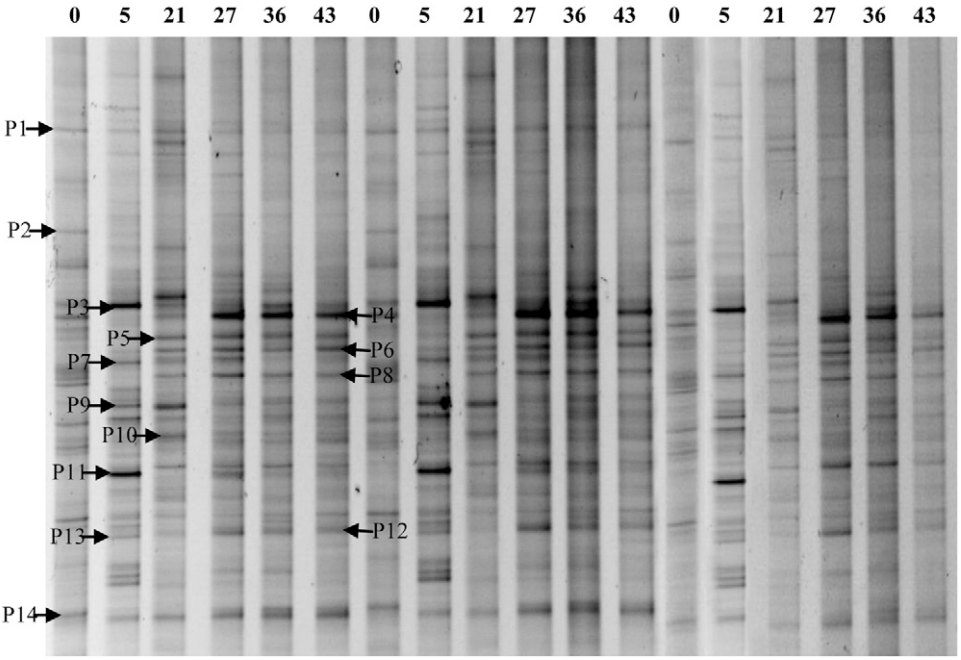

Fig. 2. Denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA fragments of microbial communities from citrus grove soils. Lanes 1–18 are PCR products amplified from the reference soil (labeled as 0), soils from citrus groves aged 5, 21, 27, and 34 yr (labeled as 5, 21, 27, 36, and 43), respectively (three replicates for each sample). DNA bands that were cut, purified and sequenced were labeled as P1-P14, which were listed within the Genbank database under accession numbers GU589556-GU589569, respectively.

Table 3. Shannon diversity index and the evenness of total bands of studied soils.

Soils/planting history	Shannon index (H′)†	Evenness of soil bacterial community‡
Reference soil	1.45 ± 0.08 a	20.52% ± 1.22% a
5 yr	1.43 ± 0.06 ab	22.74% ± 2.85% ac
21 yr	1.34 ± 0.04 ab	22.94 ± 1.72% ac
27 yr	1.29 ± 0.05 bcd	26.03 ± 3.49% ac
36 yr	1.21 ± 0.03 cd	30.15 ± 1.24% bc
43 yr	1.20 ± 0.04 d	26.73 ± 2.08% c

† Data shown are means ± SD obtained from three replicates.

H′ = −Σ(pi)log(pi), where pi represents % of the integrated density of a band, relative to the sum of all the bands in a lane.

‡The evenness of soil bacterial community was expressed as the intensity of three most intense bands in each community expressed as percentages of total DNA intensity in the lane. Different letters indicate significantly different means ($p < 0.05$) according to the least significant difference test.

degradation by a significant reduction in microbial biomass. It was reported that $CaCl_2$–extratable Cu lead to a better understanding of Cu toxicity than total recoverable Cu (Wang et al., 2009). However, the results from this study showed a slightly better correlation between total recoverable Cu and C_{mic} ($r = 0.89$, $p < 0.05$) than that between $CaCl_2$–extratable Cu and C_{mic} ($r = 0.76$, $p < 0.05$). This is likely due to the coarse texture of the soils, with a significant portion (48.7%) of the total recoverable Cu being extractable to Mehlich 3 reagent as observed in our previous study (He et al., 2009), implying Cu in the sandy soils exhibits high bioavailability. In addition, $CaCl_2$ is a very weak extractant which measures only a portion of available Cu and thus this method may not represent Cu bioavailability in soils.

Soil MQ reflects the linkage and interactions between the two parameters and the quantity of C immobilized into the microorganisms (Insam and Domsch, 1988). Brookes (1995) suggested that MQ might be a better indicator of soil pollution than either microbial activity or biomass measurement alone. Soil MQ in the citrus grove soils had a tendency to decrease with increasing grove age. This observation is consistent with previous studies (Merrington et al., 2002; Wang et al., 2009). However, previous laboratory incubation studies showed that MQ tended to increase at low external Cu input (<200 mg kg^{-1}) but decreased at higher Cu loadings (>200 mg kg^{-1}; Sun et al., 2007). This discrepancy may be due to the difference in exposure time to Cu

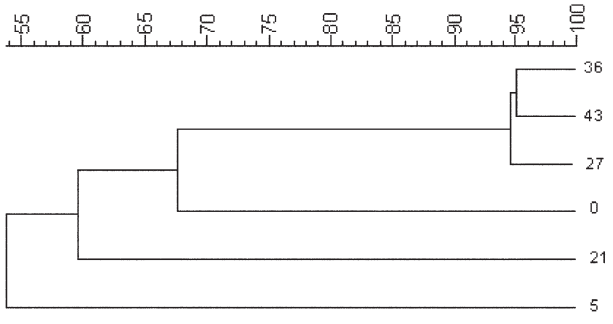

Fig. 3. Cluster analysis of DGGE profile. Similarity matrix and dendrogram of the DGGE profiles were generated based on Pearson correlation coefficient and UPGMA, respectively.

as the laboratory simulation studies measured the responses of soil microorganisms to immediate, acute toxicity (disturbance), whereas the measurements of field samples provided information on the long-term chronic toxicity which accumulates gradually (Giller et al., 1998). These results suggest that the microbial responses measured in the short-term assays may not be able to indicate the long-term effects of Cu contamination on microbial properties under the field conditions.

Effect of Copper Accumulation on Soil Bacterial Community Structure

Long-term exposure to heavy metals has been reported to result in adaptation of some microbial communities which could survive in the metal-polluted soils and subsequent changes of soil microbial community structure (Kunito et al., 1999). In the majority of studies, a decrease in bacterial diversity and a change in community structure were observed in the soils polluted by Cu (Smit et al., 1997; Tom-Petersen et al., 2003; Brandt et al., 2010), although comparable bacterial diversity or no change in microbial diversity were also reported (Brandt et al., 2010; Ellis et al., 2003; Linton et al., 2007).

In the present study, the bacterial diversity and evenness in each community calculated by DGGE profiles evidenced clear differences between the reference soil and the soils from the citrus groves aged 36 and 43 yr. The bacterial diversity tended to decrease with the grove age or total recoverable Cu concentration in the soils, with the most uniform soil community being detected in a more severely polluted soil (from the 36-yr grove). This may indicate that Cu contamination affected soil bacterial diversity, or at least, altered the community species composition sufficiently to establish a different pattern, as previously reported (Dell'Amico et al., 2008). Moreover, according to the cluster analysis of the DGGE profile, the soils from the citrus groves aged 27, 36, and 43 yr showed high similarity, and it was interesting to note that the DGGE profile of the reference soil was much closer to that of the above three soils than the soils from the 5-yr and 21-yr groves. This may be due to the fact that the response of bacterial community structure to Cu contamination in soils is complex, influenced by the toxicity of Cu to microorganisms, and the change in other soil properties caused by Cu accumulation or other agricultural practices (Jiang, 2010). Stepwise regression analysis also showed that although the Shannon diversity index of bacterial communities was predominantly negatively affected by total recoverable Cu, it was also affected by other soil properties such as available P. This may be related to the low solubility of Cu phosphates (He and Zhou, 1998) and available P in soil acts as a retention agent for Cu.

A shift of dominant bacteria in soil encountered with Cu-containing fungicides was noticed in the present study. Two out of the five dominant bacteria in the citrus groves with higher level of potential Cu contamination were related to Acidobacteria, a recently recognized phylum of bacteria, which are acidophilic as implied by the name (Barns et al., 2007). Since they were recently discovered and majority of them are not culturable, and

the ecology and metabolism of these bacteria are still not well understood (Eichorst et al., 2007). The complete genome sequence analysis of *Acidobacteria* showed that this kind of bacteria are supposed to be long-lived, divide slowly, exhibit slow metabolic rates under low-nutrient conditions, and are well equipped to tolerate fluctuations in soil moisture (Ward et al., 2009). Linton et al. (2007) also reported that high number of bacteria similar to acidophiles were detected in long-term heavy metal contaminated Ombrotrophic Peats, and suggested that this kind of acid-tolerant bacteria may render heavy metals more bioavailable and thus more toxic to other organisms. The presence of *Acidobacteria* in long-term Cu contaminated soil suggested that this group of bacteria may play an important role in heavy metal contaminated soils.

Proteobacteria and Firmicutes bacteria were detectable in all soil samples, though the strains of each phylum are different between the more severely polluted citrus groves and the young citrus grove soils. The Proteobacteria are a major group of bacteria, many of which are responsible for N fixation. Many Firmicutes produce endospores which enable them to survive under extreme conditions, and consequently they are found in a variety of environments. Autotrophic nitrifiers were reported to be the most sensitive group to toxicants, such as heavy metals, within the bacterial community and they have been proposed as toxicity biosensors (Dell'Amico et al., 2008). However, the bacterium P14, which shows a 98% similarity with unculturable *Nitrospira* sp., was detected in both the reference soil and Cu-contaminated soils, indicating that this Nitrospira strain may be Cu-tolerant.

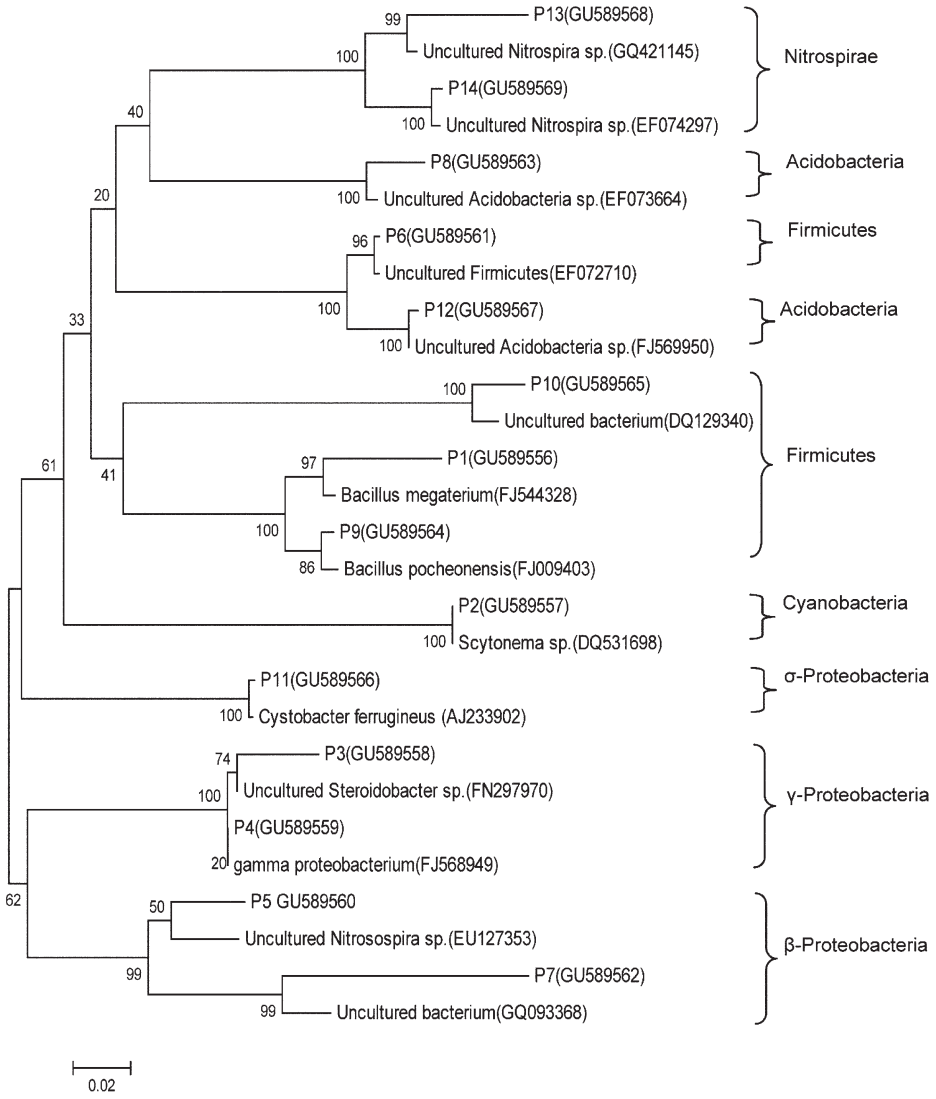

Fig. 4. Phylogenetic relationship between the 16S rRNA gene sequences from excised bands from DGGE gels (P1-P14) and their most closely related sequences from the GenBank database. Bacterial divisions are indicated. The scale bar indicates a change of 2%. The information of bands P1-P14 was shown in Fig. 2.

A shift of bacterial community in Cu contaminated soils was also reported in several studies; however, in the case of dominant bacteria, different results have been recorded (Tom-Petersen et al., 2003; Dell'Amico et al., 2008). We hypothesized that the bacteria survived in the soils contaminated with a high level of Cu may be tolerant to Cu stress through phylogenetic and functional adaption. It was reported that long-term exposure to Cu had probably selected a Cu-tolerant community composed of two types of bacteria: the one originally resistant to Cu and the other with an acquired resistance (Bamborough and Cummings, 2009; Dell'Amico et al., 2008). However, it is difficult to discriminate between the two types. In this respect, further analyses of the impact of Cu contamination on soil microorganisms would be valuable.

CONCLUSIONS

Long-term application of Cu-containing fungicides resulted in Cu buildup in the soil and significantly decreased soil C_{mic} and MQ. Diversity indices based on DGGE fingerprinting data generally decreased with increasing total recoverable Cu, suggesting that soil bacterial diversity was adversely affected by Cu. The dominant microbes in the polluted soils are most likely those with greater tolerance to environmental stresses, including certain strains of γ-Proteobacterium, Acidobacteria, Firmicutes and β-Proteobacterium. However, other soil physicochemical characteristics, which showed variation among the sampling citrus groves, might also contribute to the changes in soil microbial biomass and bacterial community diversity.

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REFERENCES

Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403–410.

Bamborough, L., and S.P. Cummings. 2009. The impact of increasing heavy metal stress on the diversity and structure of the bacterial and actinobacterial communities of metallophytic grassland soil. Biol. Fertil. Soils 45:273–280. doi:10.1007/s00374-008-0323-1

Barns, S.M., E.C. Cain, L. Sommerville, and C.R. Kuske. 2007. Acidobacteria phylum sequences in uranium-contaminated subsurface sediments greatly expand the known diversity within the phylum. Appl. Environ. Microbiol. 73:3113–3116. doi:10.1128/AEM.02012-06

Brandt, K.K., R.J.N. Frandsen, P.E. Holm, and O. Nybroe. 2010. Development of pollution-induced community tolerance is linked to structural and functional resilience of a soil bacterial community following a five-year field exposure to copper. Soil Biol. Biochem. 42:748–757. doi:10.1016/j.soilbio.2010.01.008

Bray, R.H., and L.T. Kurtz. 1945. Determination of total, organic and available forms of P in soils. Soil Sci. 59:39–45. doi:10.1097/00010694-194501000-00006

Brookes, P.C. 1995. The use of microbial parameters in monitoring soil pollution by heavy metals. Biol. Fertil. Soils 19:269–279. doi:10.1007/BF00336094

Casamayor, E.O., C. Pedrós-Alió, G. Muyzer, and R. Amann. 2002. Microheterogeneity in 16S ribosomal DNA-defined bacterial populations from a stratified planktonic environment is related to temporal changes and to ecological adaptations. Appl. Environ. Microbiol. 68:1706–1714. doi:10.1128/AEM.68.4.1706-1714.2002

Dell'Amico, E., M. Mazzocchi, L. Cavalca, L. Allievi, and V. Andreoni. 2008. Assessment of bacterial community structure in a long-term copper-polluted ex-vineyard soil. Microbiol. Res. 163:671–683. doi:10.1016/j.micres.2006.09.003

Deng, H., X.F. Li, W.D. Cheng, and Y.G. Zhu. 2009. Resistance and resilience of Cu-polluted soil after Cu perturbation, tested by a wide range of soil microbial parameters. FEMS Microbiol. Ecol. 70:137–148.

Driscoll, P.J. 2004. Copper toxicity on Florida citrus—Why did it happen? Proc. Florida State Hort. Soc. 117:124–127.

Eichorst, S.A., J.A. Breznak, and T.M. Schmidt. 2007. Isolation and characterization of soil bacteria that define Terriglobus gen. nov., in the phylum Acidobacteria. Appl. Environ. Microbiol. 73:2708–2717. doi:10.1128/AEM.02140-06

El-Ghamry, A.M., C. Huang, and J. Xu. 2000. Dynamic response of chlorsulfuron herbicide to nitrogen mineralization and the ratio of microbial biomass nitrogen to nitrogen mineralization in the soil. J. Environ. Sci. (China) 12:252–256.

Ellis, R.J., P. Morgan, A.J. Weightman, and J.C. Fry. 2003. Cultivation-dependent and-independent approaches for determining bacterial diversity in heavy-metal-contaminated soil. Appl. Environ. Microbiol. 69:3223–3230. doi:10.1128/AEM.69.6.3223-3230.2003

Giller, K.E., E. Witter, and S.P. McGrath. 1998. Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: A review. Soil Biol. Biochem. 30:1389–1414. doi:10.1016/S0038-0717(97)00270-8

Giller, K.E., E. Witter, and S.P. McGrath. 2009. Heavy metals and soil microbes. Soil Biol. Biochem. 41:2031–2037. doi:10.1016/j.soilbio.2009.04.026

He, Z.L., and Q.X. Zhou (Ed.). 1998. Equilibrium of beneficial and pollution elements in soils. (In Chinese.) Environ. Sci. Press, Beijing.

He, Z.L., J. Fan, X. Yang, and P.J. Stoffella. 2009. Effects of copper contamination on soil quality and transport to the environment. p.1892–1898. *In* Progress in environmental science and technology. Proc. of 2009 Int. Symp. on Environ. Sci. and Technol.Vol.2, Part B. Science Press, Beijing, China.

He, Z.L., X.E. Yang, V.C. Baligar, and D.V. Calvert. 2003. Microbiological and biochemical indexing systems for assessing acid soil quality. Adv. Agron. 78:89–138. doi:10.1016/S0065-2113(02)78003-6

He, Z.L., M. Zhang, X.E. Yang, and P.J. Stoffella. 2006. Release behavior of copper and zinc from sandy soils. Soil Sci. Soc. Am. J. 70:1699–1707. doi:10.2136/sssaj2005.0255

Insam, H., and K.H. Domsch. 1988. Relationship between soil organic carbon and microbial biomass on chronosequences of reclamation sites. Microb. Ecol. 15:177–188. doi:10.1007/BF02011711

Jaworska, M., and A. Gorczyca. 2002. The effect of metal ions on mortality, pathogenicity and reproduction of entomopathogenic nematodes *Steinernema feltiae* Filipjev (*Rhabditida, Steinernematidae*). Pol. J. Environ. Stud. 11:517–519.

Jiang, X.J. 2010. Long-term tillage effects on bacterial biomass and community structure distribution within water stable aggregates. p. 90–93. *In* 2010 19th World Congress of Soil Science, Soil Solutions for a Changing World, Brisbane, Australia.

Jones, J.B. 1973. Soil testing in the United States. Commun. Soil Sci. Plant Anal. 4:307–342. doi:10.1080/00103627309366451

Kandeler, E., C. Kampichler, and O. Horak. 1996. Influence of heavy metals on the functional diversity of soil communities. Biol. Fertil. Soils 23:299–306. doi:10.1007/BF00335958

Kent, A.D., and E.W. Triplett. 2002. Microbial communities and their interactions in soil and rhizosphere ecosystems. Annu. Rev. Microbiol. 56:211–236. doi:10.1146/annurev.micro.56.012302.161120

Khan, M., and J. Scullion. 2000. The effect of soil type on microbial responses to metals in sludge. Environ. Pollut. 110:115–125. doi:10.1016/S0269-7491(99)00288-2

Kunito, T., K. Senoo, K. Saeki, H. Oyaizu, and S. Matsumoto. 1999. Usefulness of the sensitivity-resistance index to estimate the toxicity of copper on bacteria in copper-contaminated soils. Ecotoxicol. Environ. Saf. 44:182–189. doi:10.1006/eesa.1999.1821

Larkin, M.A., G. Blackshields, N.P. Brown, R. Chenna, P.A. McGettigan, H. McWilliam, F. Valentin, I.M. Wallace, A. Wilm, R. Lopez, J.D. Thompson, T.J. Gibson, and D.G. Higgins. 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–2948. doi:10.1093/bioinformatics/btm404

Linton, P.E., L. Shotbolt, and A.D. Thomas. 2007. Microbial communities in long-term heavy metal contaminated Ombrotrophic Peats. Water Air Soil Pollut. 186:97–113. doi:10.1007/s11270-007-9468-z

Ma, L.Q., F. Tan, and G.H. Willie. 1997. Concentrations and distributions of eleven metals in Florida soils. J. Environ. Qual. 26:769–775. doi:10.2134/jeq1997.00472425002600030025x

Merrington, G., S.L. Rogers, and L. Van Zwieten. 2002. The potential impact of long-term copper fungicide usage on soil microbial biomass and microbial activity in an avocado orchard. Aust. J. Soil Res. 40:749–759. doi:10.1071/SR01084

Muyzer, G., S. Hottenträger, A. Teske, and C. Wawer. 1996. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA- A new molecular approach to analyse the genetic diversity of mixed microbial communities. p. 1–23. *In* A.D.L. Akkermans et al. (ed.) Molecular microbial ecology manual 3.4.4. Kluwer Academic Publishers, Dordrecht.

Nakatsu, C.H., N. Carmosini, B. Baldwin, F. Beasley, P. Kourtev, and A. Konopka. 2005. Soil microbial community responses to additions of organic carbon substrates and heavy metals (Pb and Cr). Appl. Environ. Microbiol. 71:7679–7689. doi:10.1128/AEM.71.12.7679-7689.2005

Ocio, J.A., P.C. Brookes, and D.S. Jenkinson. 1991. Field incorporation of straw and its effects on soil microbial biomass and soil inorganic N. Soil Biol. Biochem. 23:171–176. doi:10.1016/0038-0717(91)90131-3

Paoletti, M.G., D. Sommaggio, G. Petruzzelli, B. Pezzarossa, M. Barbafieri, and M. Sterzynska. 1994. 4th Seminar on Apterygota, Bialowieza, Poland. Pol. Pismo Entomol. 64:113–122.

Rogevich, E.C., T.C. Hoang, and G.M. Rand. 2008. The Effects of water quality and age on the acute toxicity of copper to the Florida apple snail, *Pomacea paludosa*. Arch. Environ. Contam. Toxicol. 54:690–696. doi:10.1007/s00244-007-9106-1

Shannon, A.E., and W. Weaver. 1963. The Mathematical theory of communication. Univ. Illinois Press, Urbana, IL.

Smalla, K., G. Wieland, A. Buchner, A. Zock, J. Parzy, S. Kaiser, N. Roskot, H. Heuer, and G. Berg. 2001. Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: Plant-dependent enrichment and seasonal shifts revealed. Appl. Environ. Microbiol. 67:4742–4751. doi:10.1128/AEM.67.10.4742-4751.2001

Smit, E., P. Leeflang, and K. Wernars. 1997. Detection of shifts in microbial community structure and diversity in soil caused by copper contamination using amplified ribosomal DNA restriction analysis. FEMS Microbiol. Ecol. 23:249–261. doi:10.1111/j.1574-6941.1997.tb00407.x

Sun, H.Y., S.P. Deng, and W.R. Raun. 2004. Bacterial community structure and diversity in a century-old manure-treated agroecosystem. Appl. Environ. Microbiol. 70:5868–5874. doi:10.1128/AEM.70.10.5868-5874.2004

Sun, Q., Z.L. He, X. Yang, and J. Shentu. 2007. Microbiological response to copper contamination of a paddy soil. Bull. Environ. Contam. Toxicol. 78:469–473. doi:10.1007/s00128-007-9213-8

Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software. Version 4.0. Mol. Biol. Evol. 24:1596–1599. doi:10.1093/molbev/msm092

Tom-Petersen, A., T.D. Leser, T.L. Marsh, and O. Nybroe. 2003. Effects of copper amendment on the bacterial community in agricultural soil analyzed by the T-RFLP technique. FEMS Microbiol. Ecol. 46:42–53.

USDA-NASS. 2009. 2008–09 Citrus summary: Production, price and value. Available online at http://www.nass.usda.gov/Statistics_by_State/Florida/Publications/Citrus/cspre/cit92409.pdf (verified 23 Mar. 2011).

USDA-SCS. 1980. Soil survey of Indian River County Area, Florida. USDA-NRCS Office, Vero Beach, FL.

Vance, E.D., P.C. Brookes, and D.S. Jenkinson. 1987. Microbial biomass measurements in forest soil: The use of the chloroform fumigation-incubation method in strongly acid soils. Soil Biol. Biochem. 19:697–702. doi:10.1016/0038-0717(87)90051-4

Vásquez-Murrieta, M.S., I. Migueles-Garduño, O. Franco-Hernández, B. Govaerts, and L. Dendooven. 2006. C and N mineralization and microbial biomass in heavy-metal contaminated soil. Eur. J. Soil Biol. 42:89–98. doi:10.1016/j.ejsobi.2005.10.002

Viti, C., D. Quaranta, R. De Philippis, G. Corti, A. Agnelli, R. Cuniglio, and L. Giovannetti. 2008. Characterizing cultivable soil microbial communities from copper fungicide-amended olive orchard and vineyard soils. World J. Microbiol. Biot. 24:309–318. doi:10.1007/s11274-007-9472-x

Wang, Q.Y., D.M. Zhou, and L. Cang. 2009. Microbial and enzyme properties of apple orchard soil as affected by long-term application of copper fungicide. Soil Biol. Biochem. 41:1504–1509. doi:10.1016/j.soilbio.2009.04.010

Wang, Y., J. Shi, H. Wang, Q. Lin, X. Chen, and Y. Chen. 2007. The influence of soil heavy metals pollution on soil microbial biomass, enzyme activity, and community composition near a copper smelter. Ecotoxicol. Environ. Saf. 67:75–81. doi:10.1016/j.ecoenv.2006.03.007

Ward, N.L., J.F. Challacombe, P.H. Janssen, B. Henrissat, P.M. Coutinho, M. Wu, et al. 2009. Three genomes from the phylum Acidobacteria provide insight into the lifestyles of these microorganisms in soils. Appl. Environ. Microbiol. 75:2046–2056. doi:10.1128/AEM.02294-08

Yang, Y., Z.L. He, Y. Lin, E.J. Phlips, P.J. Stoffella, and C.A. Powell. 2009. Temporal and spatial variations of copper, cadmium, lead, and zinc in Ten Mile Creek in South Florida, USA. Water Environ. Res. 81:40–50. doi:10.2175/106143008X296479