Microb Ecol (2011) 61:844–852
DOI 10.1007/s00248-010-9795-2

ENVIRONMENTAL MICROBIOLOGY

Low Concentration of Copper Inhibits Colonization of Soil by the Arbuscular Mycorrhizal Fungus *Glomus intraradices* and Changes the Microbial Community Structure

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Received: 17 August 2010 / Accepted: 14 December 2010 / Published online: 20 January 2011

Abstract Common agricultural practices result in accumulation of copper in agricultural soils worldwide. The effect of bioavailable copper ($[Cu]_{bio}$) on colonization of soil by the AM fungus *Glomus intraradices* and other soil microorganisms was investigated in microcosms containing copper-amended soil. To avoid indirect effects through the plant, copper was only added to root-free microcosm compartments. $[Cu]_{bio}$ was measured using a *Pseudomonas fluorescens* biosensor strain. In the range of 0–1.5 μg g^{-1} $[Cu]_{bio}$, a log–log linear relationship between added copper and $[Cu]_{bio}$ was found. Microbial colonization of the root-free compartment was evaluated by whole-cell fatty acid analysis (WCFA) and amplified rDNA restriction analysis (ARDRA). The WCFA analysis showed that the AM fungus soil colonization was severely inhibited by Cu with a 50% reduction of mycorrhizal growth at 0.26 μg g^{-1} $[Cu]_{bio}$. The growth of other main microbial groups was not significantly affected by copper. However, ARDRA analysis showed a very strong effect of copper on the bacterial community composition probably caused by an increased proportion of Cu-resistant bacteria. Our results suggest that problems with plant yield may arise when converting slightly copper-contaminated soils to land uses such as low-input and sustainable agriculture that are dependent on AM fungal symbiosis.

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Introduction

Copper (Cu) is currently introduced to agricultural soil ecosystems through manure because Cu is often used as a growth promoter in pig production [1]. The use of sewage sludge as a fertilizer and the application of Cu-containing pesticides may also represent significant Cu input to these systems [2, 3]. As a consequence of the above practices, Cu is currently accumulating in many agricultural soils [1]. At lower levels, Cu is an essential mineral nutrient to most organisms, but, at higher levels, Cu is toxic and has negative effects on soil biota. Among soil-dwelling organisms, bacteria are generally considered more sensitive to metal toxicity than other microorganisms, invertebrates, or plants [4, 5].

Low-input systems, such as organic farming, rely heavily on exploitation of beneficial microorganisms developing and interacting at soil–plant interfaces. Thus, high Cu levels in soil may comprise a problem when a farmer switches from high-input systems with ample supply of chemical fertilizers to low-input systems. Plant-beneficial soil microorganisms play a major role for plant growth and health in low-input systems due to their effects on, e.g., cycling of nutrients and protection of the plant against pathogens or abiotic stress. Hence, it is of concern that pseudomonads, which often protect plants against plant fungi [6], and nitrogen-fixing rhizobia are sensitive indicators for metal stress [4, 7–9].

Arbuscular mycorrhizal (AM) fungi represent another group of beneficial microorganisms that have important functions in promoting plant nutrition and health. AM fungi colonize most crop plants [10], and it has been suggested that the fungi may protect plants against metal toxicity (e.g., from Cu, Cd, Pb and Zn) in metal-contaminated soils [11]. Previous investigations of indigenous AM fungi in polluted soil as compared with unaffected soil have, however, been complicated by variations in several soil characteristics in addition to metal content [12]. The AM fungus *Glomus intraradices* is frequently used as an inoculant in agriculture [13], and it could be speculated that the practice of using AM inoculants might be compromised by the increasing amounts of Cu in soil. The Cu sensitivity of this potential inoculant has, to our knowledge, not been compared with the sensitivity of other soil microorganisms. Furthermore, there are relatively few reports addressing Cu impacts on AM colonization and plant growth in simple and well-controlled experimental systems [14].

The impact of Cu on soil microorganisms is caused by the toxicity of bioavailable pools of the metal. Cu bioavailability is strongly dependent on chemical speciation [15] and can be measured by bacterial reporter strains that respond with a changed reporter gene expression in relation to the amount of bioavailable Cu. We have previously used the Cu-specific *Pseudomonas fluorescens* reporter strain DF57-Cu15 [16] in combination with chemical methods to relate amounts of bioavailable Cu to impacts on soil microorganisms in several different model systems and soil types, and to identify factors affecting bioavailability [7, 15, 17–19]. Collectively, these studies have revealed that particle-associated Cu pools do not contribute significantly to bioavailable, and thus toxic, Cu and that the bioavailable fraction of total dissolved Cu species may vary from just 5% up to 100% depending on soil conditions.

The objectives of the current study were to examine the effects of Cu on hyphal growth of *G. intraradices* in a well-defined root-free soil system and to compare the Cu impact on the AM fungus to that exerted on other soil fungi, bacteria, and protozoa. To provide a link to previous impact studies, we monitored the amounts of bioavailable Cu and thereby the Cu exposure level, by the *P. fluorescens* DF57-Cu15 reporter strain.

Methods

Model Organism

The AM fungus *G. intraradices* Schenck and Smith (isolate BEG 87) with maize (*Zea mays* L. cultivar Nescio) as host plant was used as a model organism. The *G. intraradices* inoculum was obtained from a dried 12-week-old maize pot culture consisting of soil/sand mixture (1:1 volume ratio), AM fungal propagules, and 5–10 mm maize root segments.

Experimental Soil

The soil was collected from Højbakkegaard experimental farm (Denmark, 55° 40′ N, 12° 18′ E), University of Copenhagen. The soil was a sandy clay loam (clay, 15%; silt, 18%; and sand, 65%) from a field treatment with no history of P and Cu fertilization [20]. One part of the soil (*w*/*w*) was mixed with one part of quartz sand (average grain size, 0.5 mm; size range, 0.2–1.4 mm). The soil/sand mix was autoclaved at 120°C for 45 min three times with a 1-day interval between treatments to remove the indigenous species of AM fungi. This soil was termed “autoclaved soil”. A soil suspension was obtained by mixing one part of natural soil with five parts of de-ionized Milli-Q water (Millipore; *w*/*w*) in a Warren blender for 10 min. The suspension was filtered through a nylon mesh with a pore size of 50 μm to remove the propagules of indigenous AM fungi. A portion (1 kg) of autoclaved soil was mixed with 100 ml soil suspension and incubated in darkness at 20°C for 2 weeks to restore the indigenous microbial community. This soil was termed “reinoculated soil”. The applied protocol for autoclaving and reinoculation was initially tested in a pilot experiment that demonstrated that it selectively removed indigenous AM and created a microbial community similar to the corresponding community in natural soil (data not shown).

Microcosms

A three-compartment growth system [21] was used as microcosms, where the plants were grown in a central root compartment (Fig. 1). The side compartments were separated from the central compartment by a 38-μm nylon mesh, allowing in-growth by fungal hyphae and other microorganisms but not by plant roots. The root compartments were filled with 800 g of a mixture of 15 parts

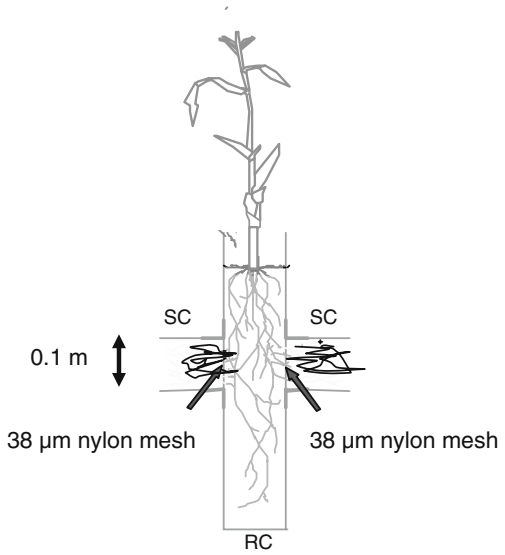

Figure 1 The three-compartment growth system used in the study. Plant roots growing in the central root compartment are excluded from the side compartments by a 38-µm nylon mesh, whereas the hyphae of the AM fungi may grow through the mesh. *RC* root compartment, *SC* side compartment

reinoculated soil and one part of *G. intraradices* inoculum (*w*/*w*) [21]. Microcosms were planted with two seeds of maize or left unplanted to serve as negative controls.

Four stock solutions of $CuSO_4$: 0 mM, 1.6 mM, 6.3 mM, and 23.6 mM in double-distilled water were prepared. The stock solutions were added to and thoroughly mixed into one batch of autoclaved soil respectively, in a ratio of 1:10 (*v*/*w*) giving four soils with Cu levels ($[Cu]_{add}$) of 0, 10, 40, and 150 µg g^{-1} fresh weight (fw). The side compartments of the microcosms were filled with 121 g of Cu-amended soil using five replicate microcosm per Cu level, giving in total 40 (2×4×5) microcosms. No Cu-amended soil was added to the root compartment, since we did not want the Cu to affect the plant roots.

The experiment was performed in a greenhouse at Research Centre Flakkebjerg (University of Aarhus, Denmark) from April to June 2007. Temperature settings were arranged so minimum day and night temperatures were 20°C and 16°C, respectively. Minimum day length was 16 h, and when needed, natural daylight was supplemented with 150 mole m^{-2} s^{-1} at foliage level by daylight lamps (Radium RNP-T high pressure sodium lamps). The microcosms were set up at soil field capacity and watered by weight at least every second day to maintain the water content. From 2 weeks after sowing, 30 µg N g^{-1} soil fw was added every second week to the root compartment as a NH_4NO_3 solution. During the 8 weeks of growth period, each growth unit received in total 90 mg nitrogen.

After incubation, the shoots were cut off, the roots were washed out of the root compartment, and the shoot and root fresh weights were estimated. The water content of the side compartments was estimated as the weight loss when drying soil samples at 105°C overnight. The pH was determined using paper pH indicator (4.0–7.0) on supernatants obtained by suspending 1 g soil in 10 ml Milli-Q water, shaking for 1 min and allowing the soil to sediment for 2 h. Other soil samples from the side compartments were stored at −20°C for further analyses.

Whole-Cell Bacterial Biosensor Assay for Determination of Bioavailable Cu

Bioavailable Cu in soil–water extracts were estimated using the Cu-specific biosensor strain *P. fluorescens* DF57-Cu15 [16]. Bioavailable Cu was operationally defined as Cu species that were able to induce biosensor expression of Cu-regulated *lux*AB genes (bioluminescence) within a 90-min incubation period. Bioavailable Cu was quantified by comparing biosensor bioluminescence determined in soil-derived samples with an external standard curve of corresponding bioluminescence values determined in $CuSO_4$ standard solutions as described previously [17]. Hence, $[Cu]_{bio}$ was determined with reference to the freely dissolved Cu^{2+} concentration in the external standard solutions and expressed as micrograms Cu per gram fresh weight soil.

Biosensor cell suspensions were prepared in a minimal medium consisting of 100 mM KCl, 20 mM 4-(2-hydroxyethyl)piperazine-1-ethylsulfonic acid (HEPES) (pH 7.2), 7.6 mM $(NH_4)_2SO_4$, 4 mM glycerol-2-phosphate disodium salt, and 0.8% *w*/*v* glucose. Biosensor cell suspensions were mixed with Cu standard solutions or environmental samples and incubated for 90 min. Then, bacterial bioluminescence (peak emission at 490–500 nm) was determined using a Fluostar Optima multi-well plate luminometer (BMG Labtech GmbH, Offenburg, Germany). All bioluminescence values were determined as the mean of two analytical replicates.

Extraction of Fatty Acids

Whole-cell fatty acids (WCFA) were extracted from root-free soil samples (1 g) by a four-step fatty acid extraction procedure according to a method by Sasser [22]: (1) saponification, (2) methylation, (3) extraction, and (4) base wash. To enable quantification of the extracted fatty acid methyl esters an internal standard, nonadeconoate fatty acid methyl ester was added to each sample. Analysis of fatty acid methyl esters were performed according to Parsley [23] using the software package Sherlock Version 6.0 (MIDI Inc., Newark, DE, USA) with HP Chemstation (Hewlett Packard, Pablo Alto, CA, USA) and HP6890 GC fitted with a 25 m fused silica capillary column (HP part no. 19091B-102) and hydrogen as carrier gas. The injector temperature was 250°C, and the detector temperature was

300°C. The temperature program was as follows: initial temperature 170°C increasing to 270°C at 5°C min^{-1}. Aliquots of 2 μl per sample were injected. The MIDI software automatically controlled all gas chromatography operations including calibration, subsequent sample sequencing, peak integration, and naming. Calibration standards contained a mixture of straight-chain, saturated, and hydroxy fatty acid methyl esters with a length of ten to 20 carbons (MIDI Part No. 1200A).

Several different whole-cell fatty acids were used as biomarkers for different groups of microorganisms. Fatty acid 16:1ω5c was used for AM fungi [21], 18:2ω6,9 for saprotrophic fungi [24, 25], branched chain localized on positions iso and anteiso (i14:0, i15:0, a15:0, i16:0, i17:0, and a17:0) for Gram-positive bacteria, cyclopropyl (cy17:0, cy19:0), and hydroxylic fatty acid (10:03OH, 12:02OH, 12:0 3OH, 16:0 2 OH, 16:0 3OH, 18:0 3OH) for Gram-negative bacteria and 10 Me 16:0, 10 Me 17:0, and 10 Me 18:0 for actinomycetes [26]. The fatty acid 20:4 was used as a biomarker for protozoans [27].

Amplified Ribosomal DNA Restriction Analysis (ARDRA) of Soil Bacterial Communities

DNA was extracted from 0.5 g freshly thawed soil using a FastDNA SPIN Kit for soil (Cat. No. 6560–200, Quantum Biotechnologies/Bio101) according to the instructions provided by the manufacturer. Amplified ribosomal DNA restriction analysis (ARDRA) was carried out as described previously [7]. Polymerase chain reaction (PCR) was performed using primers amplifying a 1.4 kb region of the 16S rRNA gene [7]. PCR products of the correct size were pooled from eight replicate PCR reactions and purified using the QIAquick Gel Extraction Kit (Cat. No. 28706, Qiagen) before restriction digestion for 20 h at 37°C with *Rsa*I (New England Biolabs, Beverly, MA). Restriction fragments were finally separated on 2% agarose gels, stained with ethidium-bromide and visualized using a Model 2000 GelDoc imager (Bio-Rad, Hercules, CA).

Digital Image Analysis of ARDRA Band Patterns

The ARDRA band patterns generated were analyzed using the software Quantity One version 4.6.3 (Bio-Rad, Hercules, CA). Briefly, each restriction DNA fragment in each gel was matched to a standard DNA marker (2log DNA ladder) for size determination. Band fluorescence intensities were corrected for background fluorescence by lane-based background subtraction and subjected to Gauss modeling according to the manufacturer's recommendations. Equally sized DNA restriction fragments in different samples (i.e., different lanes) were matched by setting a tolerance value of 3%. Finally, Gauss peak intensities were recorded for all ARDRA bands. Data matrices of Gauss peak intensities for individual samples were normalized relative to the sample with the lowest total Gauss peak intensity.

Statistical Analyses

Effects of the treatments on shoot and root weight and water content were evaluated with ANOVA using SPSS 11.0 (SPSS Inc.) selecting $p<0.05$ as the level of significance. To determine highest observed no-effect concentration (HNOEC) and lowest observed effect concentration (LOEC) of $[Cu]_{bio}$ on WCFA 16:1ω5c, significant difference between control and treatments were evaluated with a single-sided Dunnetts test using $p<0.05$ as the level of significance. Regression analysis of WCFAs for the microbial groups against $[Cu]_{bio}$ and multivariate effect of Cu treatments on ARDRA patterns were evaluated with the free source statistical package PAST1.84 [28]. The ARDRA data matrix was subjected to principal coordinate analysis (PCoA) and NPMANOVA using Bray–Curtis similarity index.

Results

Cu Amendment and Plant Growth

Bioavailable copper $[Cu]_{bio}$ increased with added Cu and reached a maximum of 1.57 μg g^{-1} fw in soil from side compartments of unplanted microcosms receiving the high Cu amendment of 150 μg g^{-1} fw of soil (Table 1). The presence of a plant in the microcosm significantly reduced $[Cu]_{bio}$, and there was a linear relationship between the natural logarithms (ln–ln) of added and bioavailable Cu: $\ln([Cu]_{bio}) = -3.33 \pm 0.138(\text{standard error, SE}) + 1.58 \pm 0.083(\text{SE}) \times \ln([Cu]_{add})$. The growth of the plants as determined by shoot and root weights were not affected by the addition of Cu to the side compartments (Table 2). The pH of the side compartments ranged from 5.0 to 5.1 in all side compartments, and the presence of plants did not affect the pH. However, the presence of plants significantly lowered the water content in the side compartments of the microcosms (Table 2), while no effect of Cu amendment on the water content of the side compartments was noted.

Effect of Cu on Biomass of *G. intraradices* and Other Microbial Groups as Estimated by WCFA

The biomass of AM fungi in side compartments of microcosm where no plants were grown ranged between 2 and 4 μg 16:1ω5c g^{-1} dry weight (dw) and was unaffected by Cu addition. In microcosms where plants were grown, the biomass of *G. intraradices* that established in the side

Table 1 Levels of bioavailable Cu $[Cu]_{bio}$ in soil from the plant root-free side compartments as affected by Cu amendment and presence or absence of a growing plant in the neighboring root compartment

Cu treatment[a], μg g^{-1} fw	No plant, μg g^{-1} fw	Plant, μg g^{-1} fw
0	b.d.	b.d.
10	0.048±0.011	0.019±0.005
40	0.391±0.028	0.176±0.016
150	1.57±0.31	1.21±0.09
Statistics[b]		
Cu	<0.001	
Plant	<0.001.	
Cu×plant	n.s.	

$[Cu]_{bio}$ was determined by the Cu-specific *P. fluorescens* DF57-Cu15 biosensor after 8 weeks of incubation. Data are presented as means±standard error (n=5)

b.d. below detection limit (<0.005 μg g^{-1}); *n.s.* not significant

[a] Only the plant-free side compartments were amended with Cu in order to protect the plant growing in the main compartment from Cu toxicity

[b] Statistical analysis of significant differences were evaluated by two-way ANOVA using $p<0.05$ as the level of significance. The analysis was made on treatments of Cu addition (Cu), presence/absence of plant grown in the microcosm root compartment (plant), and interaction of treatments (Cu×plant)

compartments was reduced by the added Cu and was found to be negatively correlated with the observed $[Cu]_{bio}$ (Fig. 2, p_{uncorr}=0.003). The relationship between $[Cu]_{bio}$ (μg Cu g^{-1} fw) and the AM biomass (μg 16:1ω5c g^{-1}) fitted an exponential decay function where: $AM_{[Cu]bio} = AM_{[Cu]bio=0} \times e^{-D[Cu]bio}$ and D is the decay constant [μg ($[Cu]_{bio}$) g^{-1}(soil)]$^{-1}$. This type of relationship means that the percentage decrease in $AM_{[Cu]bio}$ for each unit increase in $[Cu]_{bio}$ is constant. This equation can be written in its linear form: $\ln(AM_{[Cu]bio}) = \ln(AM_{[Cu]bio=0}) - D[Cu]_{bio}$. We found that our data best fitted an equation where $\ln(AM_{[Cu]bio=0}) = 4.33 \pm 0.74(SE)$ $\ln(\mu g(16{:}1\omega 5c)g^{-1}(soil))$ and $D = 2.68 \pm 0.47(SE)(\mu g([Cu]_{bio})g^{-1}(soil))^{-1}$. The increase in $[Cu]_{bio}$ that halves the AM biomass (EC_{50}) in soil was found to be 0.26 μg g^{-1} fw (95% confidence interval, 0.16–0.34). If calculated from the found relationship between total added Cu and $[Cu]_{bio}$, a $[Cu]_{bio}$ of 0.26 μg g^{-1} fw corresponds to 55 μg g^{-1} fw added Cu (95% confidence interval, 41–65). HNOEC was 10 μg g^{-1} fw $[Cu]_{add}$ corresponding to 0.019 μg g^{-1} fw $[Cu]_{bio}$, and LOEC was 40 μg g^{-1} fw $[Cu]_{add}$ corresponding to 0.17 μg g^{-1} fw $[Cu]_{bio}$ (Table 1).

We found no significant correlation between $[Cu]_{bio}$ and the amount of WCFAs representing total fungi, Gram-positive bacteria, Gram-negative bacteria, actinomycetes, or protozoa, as p_{uncorr} was greater than 0.2 for all relationships (data not shown). Hence, in contrast to the observations for the AM fungus, biomasses of all the other large microbial groups were not affected by the levels of $[Cu]_{bio}$ measured

Table 2 Growth of maize (shoot and root fresh weight) in the main compartment of microcosms as affected by Cu amendment to plant root-free side compartments and impact of plant growth on the water content and pH in the plant root-free side compartments after 8 weeks of incubation

Treatment[a]	Shoot weight, g fw	Root weight, g fw	Water content, % dw	pH
0 μg Cu g^{-1} fw				
Plant	21.5±2.6	14.5±0.7	7.7±0.7	5.0
No plant	n.a.	n.a.	11.3±0.3	5.1
10 μg Cu g^{-1} fw				
Plant	17.8±1.4	18.8±2.6	8.6±1.1	5.0
No plant	n.a.	n.a.	12.8±1.3	5.1
40 μg Cu g^{-1} fw				
Plant	17.0±1.5	18.1±0.5	7.3±1.2	5.0
No plant	n.a.	n.a.	12.2±0.6	5.1
150 μg Cu g^{-1} fw				
Plant	17.6±0.3	16.5±1.8	6.6 ±0.4	5.0
No plant	n.a.	n.a.	12.4±0.5	5.0
Statistics[b]				
Cu	n.s.	n.s.	n.s.	
Plant			<0.001	
Cu×Plant			n.s.	

The standard errors of the means are given (n=5)

n.a. not applicable, *n.s.* not significant

[a] Only the plant-free side compartments were amended with Cu in order to protect the plant growing in the main compartment from Cu toxicity

[b] Statistical analysis of significant differences were evaluated by two-way ANOVA using $p<0.05$ as the level of significance. The analysis was made on treatment of Cu addition (Cu). Also, concerning water content, the treatment of plant grown in microcosm (plant) and interaction of treatments (Cu×Plant) were analyzed

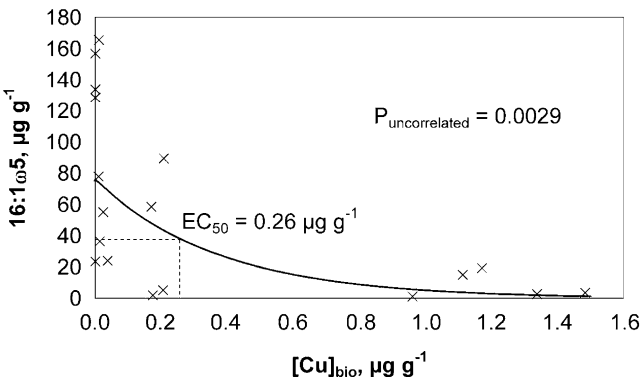

Figure 2 The growth of the AM fungus *G. intraradices* as affected by different levels of bioavailable Cu in microcosms with maize as host plant. Fungal biomass was determined in plant-free, Cu-amended side compartments after 8 weeks of incubation, whereas Cu was not added to the main root compartment. The *line* describes an exponential decay function fitted to the data. EC_{50} value is indicated in the diagram

in this study. Consequently, there was also no correlation found between AM biomass and any of the other microbial groups.

Effect of $[Cu]_{bio}$ on the Bacterial Community Composition as Estimated by ARDRA

To evaluate if Cu addition changed the bacterial community in the soil systems, the ARDRA data for the bacterial communities were subjected to multivariate analysis. The principal coordinates 1, 2, and 3 explained 38%, 21%, and 13% of the variation, respectively. A separation of the bacterial communities was found along coordinates 1 and 2 as shown by the PCoA (Fig. 3). The bacterial communities of the side compartments where Cu was added at 150 μg g^{-1} fw of soil were clearly separated from the communities in the control soil, p_{same}=0.016 (NPMANOVA). The communities from the soils with Cu amendments of 10 and 40 μg g^{-1} fw were not significantly different from each other (p_{same}=0.17). They were, on the other hand, different from the communities from both the control soils and 150 μg g^{-1} fw Cu soil although more related to the control soil (p_{same}=0.016 and 0.017, respectively) than to the soil amended with Cu at 150 μg g^{-1} fw (p_{same}=0.0083 and 0.0077, respectively). Hence, the composition of the bacterial communities was a very sensitive indicator for Cu impact in this investigated model system as it responded to $[Cu]_{bio}$ as low as 0.019 μg g^{-1} fw as found in the microcosms containing $[Cu]_{add}$ at 10 μg g^{-1} fw (Table 1).

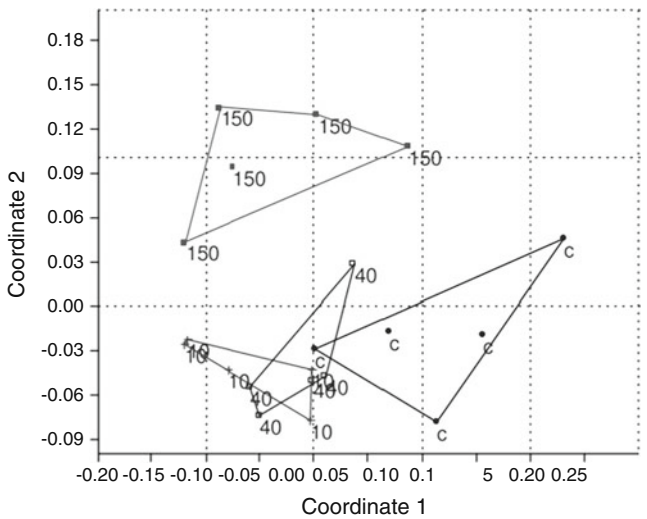

Figure 3 A principal coordinates analysis of the bacterial populations after an 8-week incubation obtained by ARDRA analyses. The axes explain 38% and 21% of data variation for the coordinates 1 and 2, respectively. The *numbers* describes the amount of Cu added to the soils and the *c* indicates the control soils where no Cu was added

Discussion

The majority of studies addressing impacts of Cu in soil microorganisms have relied on total soil Cu as the sole descriptor of Cu exposure. However, it is well established that total pools of soil metals comprise poor descriptors of metal bioavailability and thus toxicity in soil [29, 30]. In the current study, we have used a robust Cu-specific biosensor for determination of $[Cu]_{bio}$ allowing us to expand the current data for Cu impacts with a compilation of Cu toxicity data where Cu exposure was determined with the same biosensor (Table 3). This approach provides a direct comparison of Cu sensitivity across a range of microbial toxicity endpoints in soil.

Combined with the data shown in Table 3, our data indicate that *G. intraradices* constitutes a sensitive indicator for adverse Cu impacts on soil microbial communities as hyphal growth responded already at low levels of $[Cu]_{bio}$ (LOEC 0.17 μg g^{-1} fw), whereas the total biomass of other microbial groups was not affected by levels of $[Cu]_{bio}$ up to 1.4 μg g^{-1} fw as estimated by WCFA. Community analysis based on phospholipid and ester-linked fatty acids have previously been useful to determine impacts of metal pollution in soil, see, e.g., Hinojosa et al. [31].

A more detailed comparison of the response to Cu of *G. intraradices* BEG 87 used in current study with that of other AM fungi needs to rely on experiments, for which only total Cu concentrations were determined. Relatively few studies have investigated specific impacts of Cu in simple model systems. Hence, our observation that *G. intraradices* is sensitive to Cu (EC_{50}=55 μg g^{-1} fw of soil) corresponds well with the observations made by Graham et al. [32] as these authors found a EC_{50} of ca. 40 μg g^{-1} for growth of *G. intraradices* in field soils.

An analysis of Cu toxicity in other AM fungi reveals a more complex Cu tolerance pattern. For example, Gildon and Tinker [33] found that soil amendments with Cu at 5 μg g^{-1} of soil reduced the proportion of infected root length by *Glomus mossae* by 10% while an amendment with 30 μg g^{-1} lead to an 80% reduction. On the other hand, Chen et al. [34] demonstrated that *G. mossae* colonized several plant species grown in Cu mine tailings, which contained Cu at about 232 μg g^{-1} of soil. These results suggest that Cu sensitivity may vary among AM fungi and that they may acquire resistance to Cu. This is underlined by the studies of Liao et al. [14] who demonstrated differences in the sensitivity of different AM fungi to Cu when tested in sand culture experiments with maize as the plant component. Hence, *Glomus caledonium* proved more tolerant to Cu than *Acaulospora laevis* while Wang et al. [35] demonstrated that colonization of maize roots in pot cultures by the AM fungus *Acaulospora mellea* was significantly lowered only at Cu amounts as high as 400–800 μg g^{-1} of soil.

Table 3 Calculated highest no-observed effect concentrations (HNOECs) and lowest observed effect concentrations (LOECs) for various microbial toxicity endpoints based on data from previous studies, where Cu exposure was quantified by the *P. fluorescens* DF57-Cu15 biosensor assay

Microbial toxicity endpoint	Reference	Cu exposure	HNOEC (μg g^{-1})		LOEC (μg g^{-1})	
		(months)	$[Cu]_{total}$	$[Cu]_{bio}$	$[Cu]_{total}$	$[Cu]_{bio}$
Field experiments						
Community size						
Viable count (bacteria)	[39]	21	117	0.20	>117	>0.20
	[39]	21	117	0.20	>117	>0.20
Total cell count (bacteria)	[29]	60	82	0.16	>82	>0.16
Community function						
Basal soil respiration (bacteria and fungi)	[Brandt, unpublished]	55	97	0.18	>97	>0.18
Substrate-induced respiration (bacteria and fungi)	[Brandt, unpublished]	55	97	0.18	>97	>0.18
Potential nitrification (bacteria and Archaea)	[Brandt, unpublished]	55	97	0.18	>97	>0.18
Community tolerance						
Cu resistance (bacterial isolates)	[39]	21	9	b.d.	117	0.20
[^{3}H]leucine incorporation (bacteria)	[29]	60	10	b.d.	82	0.16
Community structure						
Bacterial community structure (T-RFLP)	[16]	2–21	9	b.d.	54	0.10
	[29]	60	82	0.16	>82	>0.16
Laboratory experiments						
Community size						
Viable count (bacteria)	[7]	4–6	600	0.20	>600	>0.20
Pseudomonas spp. viable count	[7]	4–6	12	b.d.	184	0.09
Total cell count (bacteria)	[29]	0.25–3	82	0.16	>82	> 0.16
Microbial biomass (bacteria and fungi)	[41]	1	60	n.a.	250	0.13
Community function						
[^{3}H]leucine incorporation (bacteria)	[29]	0.25–3	49	0.18	159	0.44
Basal soil respiration (bacteria and fungi)	[29]	0.25–3	49	0.18	159	0.44
Substrate-induced respiration (bacteria and fungi)	[29]	0.25–3	49	0.18	159	0.44
Respiratory quotient (bacteria and fungi)	[29]	0.25–3	159	0.44	509	22
Community tolerance						
Cu resistance (*Pseudomonas* spp. isolates)	[7]	4–6	12	b.d.	184	0.09
[^{3}H]leucine incorporation (bacteria)	[29]	0.25–3	9	b.d.	49	0.18
Community structure						
T-RFLP (bacteria)	[29]	0.25–3	49	0.18	159	0.44
ARISA (bacteria)	[41]	1	250	0.14	>250	>0.14
ARISA (fungi)	[41]	1	250	0.14	>250	>0.14
CLPP (bacteria)	[29]	0.25–3	159	0.44	509	22
UP-PCR (*Pseudomonas* spp.)	[7]	0.25–3	184	0.09	600	0.20
Indicator organisms						
P. fluorescens toxicity biosensor	[15]	0.002	53	n.d.	184	0.84

T-RFLP terminal restriction fragment length polymorphism, *ARISA* automated ribosomal intergenic spacer analysis, *CLPP* community-level physiological profiling, *UP-PCR* universally primed polymerase chain reaction, *b.d.* below detection limit, *n.a.* data not available

Even studies performed for complex natural populations of AM fungi and typically addressing complex metal contaminations (including contamination with Cu) show that AM fungi can colonize plants growing in metal-contaminated soil [11, 36–38]. In such a study, Ortega-Larrocea et al. [37] found that AM spore formation and diversity was reduced by increasing total metal contents (Zn increased from ca. 50 to ca. 300 μg g^{-1}, Pb from ca. 10 to ca. 60 μg g^{-1}, Cu from ca. 10 to ca. 50 μg g^{-1}, and Cd from ca. 0.2 to ca. 2 μg g^{-1}) of soil irrigated with metal-

containing waste water. Nevertheless, *Glomus* populations not including *G. intraradices* were found and dominated these soils. Furthermore, da Silva et al. [36] showed that different *Glomus* species, again not including *G. intraradices*, were able to grow in soils polluted by copper mine wastes containing up to 600 mg dm^{-3} of total Cu and 434 mg dm^{-3} of Fe. The AM fungal isolates obtained from metal-contaminated sites might be useful for bioremediation of contaminated sites as AM fungi appear to be able to lower the amount of heavy metals available to the plants by partly unknown mechanisms [11, 38].

The current analysis of ARDRA profiles demonstrated that the bacterial community structure served as the most sensitive microbial toxicity endpoint for Cu with significant differences in ARDRA profiles being detected at a $[Cu]_{bio}$ level of just 0.019 μg g^{-1}. This is much lower than the LOECs obtained from other studies (Table 3), where LOECs for microbial Cu toxicity data based on related DNA fingerprinting methods ranged from 0.1 to >0.44 μg g^{-1}. The mycelium of *G. intraradices* colonizing the side compartments could have facilitated the transport of AM fungus-associated bacteria [21] along the growing hyphae. Hence, the observed impact of Cu amendments on bacterial community structure may reflect an indirect impact through Cu toxicity affecting AM fungal growth and metabolism, but also direct toxic effects of Cu towards bacteria colonizing the soil. Since pollution-induced bacterial community tolerance is known to be a highly sensitive indicator of Cu toxicity affecting soil microbial communities [29], the observed change in the bacterial community was most likely caused by a direct effect of Cu on the bacteria, resulting in an increased proportion of Cu-resistant bacteria as already observed in several studies [7, 39, 40].

Commercial AM fungal inoculants used to promote plant growth typically contain a single fungal isolate that most commonly belong to *G. intraradices* [13]. The current study suggests that even a moderate increase in Cu levels by 10 μg g^{-1} in agricultural soil may constitute a threat to their future use in low-input agriculture, where the dependence on these plant growth-promoting microorganisms is high. Due to the very long soil Cu residence time [3], contaminated soil thus may be unavailable for low-input agriculture for extended periods. Whether it is feasible to develop metal-tolerant *G. intraradices* isolates into inoculants for improvement of plant growth even in moderately Cu-contaminated agricultural systems remains to be explored.

Acknowledgments This work was funded by the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS), and by the Center for Environmental and Agricultural Microbiology (CREAM) funded by the Villum Kann Rasmussen Foundation. We thank Professor L.S. Jensen for access to nutrient depleted soil.

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