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Response of the microbial community to copper oxychloride in acidic sandy loam soil

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ABSTRACT

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Aims: Determining the response of different microbial parameters to copper oxychloride in acidic sandy loam soil samples using cultivation-dependent and direct microscopic techniques.

Methods and Results: Culturable microbial populations were monitored for 245 days in a series of soil microcosms spiked with different copper oxychloride concentrations. Microbial populations responded differently to additional Cu. Protistan numbers and soil metabolic potential decreased. Experiments with more soil samples revealed that metabolic potential was not significantly affected by ≤100 mg kg^{-1} additional Cu. However, a negative impact on protista was noted in soil containing only 15 mg kg^{-1} EDTA-extractable Cu. The negative impact on protistan numbers was less severe in soils with a higher phosphorous and zinc content.

Conclusions: Bacterial populations responded differently, and protista were most sensitive to elevated Cu levels. Protistan numbers in soil from uncultivated land were higher and seemed to be more sensitive to additional Cu than the numbers of these organisms in soil originating from cultivated land.

Significance and Impact of the Study: Protistan sensitivity to small increases in Cu levels demonstrates the vulnerability of the soil ecosystem to Cu perturbations, especially when the importance of protista as link in the flow of energy between trophic levels is considered.

Keywords: copper oxychloride, metabolic potential, microbial community, protista, soils.

INTRODUCTION

Copper (Cu)-containing compounds have widely been used in agricultural practices as fertilizers (Baker and Senft 1995) and fungicides (Flores-Vélez *et al.* 1996). While Cu concentrations are usually low in virgin soils from viticultural areas, copper oxychloride is annually applied on vineyards as a fungicide to control a significant number of plant diseases (Nel *et al.* 1999). Inevitably this Cu ends up in the soil of the vineyard and the adjacent pristine natural vegetation. In addition, in many cases these soils are acidic, making Cu more mobile and bio-available than it is in alkaline soils (McBride 1994; Kunito *et al.* 1999).

While soil fungi are more tolerant to heavy metal contamination than bacteria (Doelman 1985; Hiroki 1992), the sensitivity of other eucaryotes, such as protista, was utilized in the development of bioassays for the bio-availability of heavy metals in soils (Forge *et al.* 1993). For example, it was found that growth of *Colpoda steinii* was reduced by 50% in aqueous solutions containing Cu concentrations as low as 0·25 mg kg^{-1}.

Recently, bacterial and protistan activity, diversity and abundance in the presence of $CuCl_2$ amendments to soil were studied in a series of microcosms (Ekelund *et al.* 2003). These experiments were conducted with acidic sandy agricultural soil supplemented with *ca* 1·1 g kg^{-1} ground barley straw. As virgin soils in pristine areas are not

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supplemented with agricultural wastes, our interest, however, lay in the microbial response to Cu perturbations in soils that originate from viticulture areas that have not been supplemented with organic material. The aim of this study was therefore to determine the response of different microbial parameters to copper oxychloride in uncultured acidic sandy loam soil using cultivation-dependent and direct microscopic techniques in soil that has a low natural Cu content. To achieve this, it was necessary to also include soils from cultivated land in our investigation. The experiments entailed routine monitoring of the numbers of actinomycetes, fluorescent bacteria, heterotrophic microorganisms and *Pseudomonas* strains over a period of 245 days in a series of soil microcosms. At the end of this period measurement of the metabolic potential of the microbial community, as well as direct protozoan counts, were used to determine the impact of copper oxychloride on the soil microbial community. The negative impact of additional Cu on the metabolic potential and protistan numbers in additional samples taken from acidic sandy loam soil, which differed in physical and chemical composition, was subsequently investigated.

MATERIALS AND METHODS

Soil microcosms

Soil was collected from five different sampling sites, covered with vines, grasses or indigenous fynbos vegetation (Kruger and Van Wilgen 1992) in the wine producing region of the Western Cape Province, South Africa (Table 1). After the organic matter at the surface of each sampling site was removed, the top 30 cm of soil was collected. Approximately 600 kg of soil was allowed to dry for 2 weeks at 30°C, whereafter the soil was sieved (pore size 2 mm).

The physical and chemical properties of the sieved soil were determined using standard protocols. The soil was classified according to the Soil Classification Working Group (1991), while soil texture was determined using the hydrometer method (Van der Watt 1966). Determination of organic

Table 1 Characteristics of the soil samples used in the experimentation

Characteristics of soil	Sample I	Sample II	Sample III	Sample IV	Sample V
Classification	Sandy loam	Sandy	Sandy loam	Sandy loam	Sandy loam
Physical characteristics texture					
Stone (%)	0·54	0·00	0·00	0·00	0·00
Sand (%)	68·50	93·00	75·00	79·50	60·30
Silt (%)	22·80	3·20	16·10	15·40	17·90
Clay (%)	5·70	3·80	8·90	5·10	21·80
Chemical characteristics					
Organic carbon (%)	3·54	0·75	1·61	1·71	1·50
Total nitrogen (%)	0·20	0·05	0·16	0·13	0·13
Ammonium (mg kg^{-1})	5·40	1·53	0·35	4·57	3·54
Nitrate and nitrite (mg kg^{-1})	3·20	5·51	27·74	45·88	11·75
Phosphorous (mg kg^{-1})	29·00	22·00	43·00	10·00	117·10
Copper (mg kg^{-1})	1·37	0·83	3·14	1·63	1·10
Zinc (mg kg^{-1})	6·80	6·60	2·63	1·87	4·43
Manganese (mg kg^{-1})	80·0	17·50	47·37	34·93	26·60
Boron (mg kg^{-1})	0·86	0·07	0·64	0·14	0·31
Exchangeable cations					
Calcium (cmol kg^{-1})	4·70	1·40	2·24	2·50	8·63
Potassium (cmol kg^{-1})	0·63	0·10	0·33	0·35	0·32
Sodium (cmol kg^{-1})	0·11	0·03	0·16	0·18	0·11
Magnesium (cmol kg^{-1})	3·25	0·33	1·06	1·17	1·04
CEC (cmol kg^{-1})*	8·69	2·26	5·37	5·46	10·09
pH (KCl)	6·10	5·10	4·30	4·70	5·90

Sample I, uncultivated soil obtained from Nietvoorbij experimental farm (Stellenbosch) on which the natural fynbos vegetation was removed 20 years earlier resulting in the growth of various indigenous grasses; sample II, potting soil prepared from the same soil as sample I by adding sterile sand; sample III, soil obtained from a 2-year-old vineyard on a commercial wine farm near Stellenbosch; sample IV, virgin soil originating from the same farm as sample III, but collected beneath typical indigenous fynbos vegetation; sample V, soil originating from a 17-year-old vineyard on a commercial wine farm near Somerset West.

*Cation exchange capacity.

carbon was carried out with the Walkey-Black method (Nelson and Sommers 1982), and the total nitrogen content by digestion in a LECO FP-528 nitrogen analyser, while the ammonium, as well as the nitrate and nitrite content was determined in a 1-M KCl extract (Bremner 1965). Phosphorous was determined in a Bray-2 extract (Thomas and Peaslee 1973), Cu, zinc and manganese in a di-ammonium EDTA extract (Beyers and Coetzer 1971), and boron in a hot water extract according to the methods of the Fertilizer Society of South Africa (1974). Exchangeable cations were determined in a 1-M ammonium acetate extract (Doll and Lucas 1973), and the pH using the method of McClean (1982). The main physical and chemical properties of the sieved soil from each sampling site are listed in Table 1.

The first series of soil microcosms was prepared from sample I by adding a different concentration of copper oxychloride ($CuCl_2{\cdot}2CuO{\cdot}4H_2O_4$) to each of nine aliquots of soil. Each of these aliquots was subdivided into triplicate microcosms in polythene bags, each containing *ca* 2 kg of soil resulting in a soil column 10 cm in diameter and *ca* 20 cm in height. The concentration of bio-available copper in the top 10 cm of each triplicate, as determined after 70 days of incubation in a di-ammonium EDTA extract of the soil (Beyers and Coetzer 1971), was 1·9 ± 0·1 (control without additional Cu); 12·2 ± 0·1; 22·6 ± 0·7; 33·8 ± 4·5; 42·1 ± 3·4; 59·5 ± 3·4; 125·7 ± 2·9; 516·1 ± 6·7 and 1112·4 ± 52·2 mg kg^{-1}.

Microcosms prepared from samples II–V were the same size as the first series of microcosms and were exposed to the same range of copper oxychloride concentrations, but received fewer treatments. The concentration of EDTA-extractable copper in each microcosm triplicate after 70 days of incubation, for each of these samples, is listed in Table 2.

Analyses conducted on microcosms prepared from soil sample I

Periodic analyses of microbial numbers. Sterile distilled water was added to each microcosm to result in a soil moisture content of 15% (v/w). Thereafter 200 ml water was added every 2 weeks to each microcosm. None of this water leached to the bottom of the microcosms, which were incubated at 22°C for 245 days. To enumerate culturable microbial populations soil dilution plates from the top 5 cm of soil in each microcosm were prepared on days 1, 3, 7, 14, 70 and 245. At each of these time intervals the top 5 cm of soil in individual microcosms was mixed using a spatula before samples were taken for microbial analyses. In each case the mixing procedure was repeated immediately after the samples were taken, but this time by mixing the top 10 cm of soil.

Different microbial growth media designed to be selective for heterotrophic microbes, actinomycetes, fluorescent bacteria and pseudomonads were used in the microbial analyses. The delimitation of these microbial populations was subjected to the physiological ability of microbes to grow on each of the selective media. To obtain general heterotrophic counts, tryptone soya agar (TSA, Biolab, Midrand, South Africa) was used as isolation medium. Actinomycetes were enumerated using sodium caseinate agar. This medium (pH 6·7) consisted of 0·2 g l^{-1} sodium caseinate, 0·5 g l^{-1} K_2HPO_4, 0·2 g l^{-1} $MgSO_4$, 0·01 g l^{-1} $FeCl_3$ and 15 g l^{-1} agar. Fluorescent bacterial counts were performed on King's medium B (KMB, Biolab^{TM}) and *Pseudomonas* strains were enumerated using *Pseudomonas* CFC medium (Oxoid^{TM}). Plates were incubated at 30°C, except for the actinomycete enumeration plates, which were incubated at 22°C for 5–7 days.

For the microbial numbers obtained on each of the media mentioned above, Statistica Software (StatSoft, Tulsa, OK, USA) Version 6 was used to conduct a repeated measures analysis over the six sampling dates with nine levels of administered Cu as treatment. If significant interaction existed between the incubation period and the Cu treatments, these interaction plots were either, interpreted, or separate one-way ANOVA was performed at each sampling date to determine the nature of the differences among the Cu treatments for that day. In all other cases the main effect least square (LS) means plots were interpreted. When there were indications of

Table 2 Concentration of copper in the soil microcosms

	Cu concentration in EDTA extract of soil†			
Cu added*	Sample II	Sample III	Sample IV	Sample V
0	0·83 ± 0·23	3·14 ± 0·31	1·63 ± 0·23	1·05 ± 0·06
30	29·47 ± 1·96	19·06 ± 1·04	15·34 ± 1·01	25·31 ± 0·65
100	101·89 ± 9·40	60·14 ± 4·74	56·75 ± 1·02	89·57 ± 1·01
1000	1008·15 ± 41·81	608·17 ± 26·02	652·49 ± 25·74	973·3 ± 14·76

*Concentration of copper (in mg kg^{-1}) administered to each soil microcosm.
†Concentration of copper (in mg kg^{-1}) determined in a di-ammonium EDTA extract according to the methods of Beyers and Coetzer (1971). Values represent the mean and standard deviation of three repetitions. Copper oxychloride was used in the preparation of all the series of Cu concentrations.

nonnormality in the residuals, the interaction plots of the LS means were performed using bootstrap methods. These plots are better for interpretation in nonnormal residual cases. The closer the bootstrap intervals are to the usual LS means intervals, the closer the residuals are to normality.

Whole community metabolic profiles

Whole community metabolic analyses (Garland 2000) were performed on four of the five samples (samples II–V) on day 70. The remaining series (sample I) was allowed to incubate for 245 days before analyses. Soil suspensions were prepared from microcosms that received 0, *ca* 30, *ca* 100 and *ca* 1000 mg kg^{-1} Cu, by transferring 10 g of soil to 90 ml sterile distilled water. Each suspension was shaken for 10 min and allowed to settle for 2 h. The supernatant was then used to inoculate duplicate sets (150 μl per well) of Biolog^{TM} Eco microplate wells (Biolog, Hayward, CA, USA), and incubated at 22°C for 24–48 h. Utilization of the carbon source in each well, indicated by a reduction of the tetrazolium dye, was recorded as either positive or negative.

The data from the series of microcosms prepared from soil samples II–V, obtained after 70 days of incubation (four random soil samples, each subsampled and treated in triplicate with increasing levels of Cu), were analysed using ANOVA (Statistica Version 6, Statsoft, Tulsa, OK, USA). Univariate tests of significance were performed on observations made in triplicate for the soil samples at four different concentrations of administered Cu (Table 2). Where application of the tests revealed that the factors had a significant effect on the numbers of carbon sources utilized, the differences between treatments were further separated at $P \leq 0{\cdot}05$, using Bonferroni (Dunn) *t*-tests. The results obtained on the first series of microcosms after 245 days of incubation were analysed using cluster analyses (Statistica Version 6).

Protistan numbers following exposure to Cu

Protistan counts were performed after 70 days of incubation on all series of microcosms (samples I–V). Series one (sample I) was also sampled and protistan counts determined after 245 days of incubation. The procedure of Griffiths and Ritz (1988) was followed, whereby 5·0 g of soil from each microcosm that received 0, 30, 100 and 1000 mg kg^{-1} Cu, was added to 50 ml Tris-HCl buffer (pH 8·0) and shaken for 10 min on a wrist shaker. After shaking, the soil was allowed to settle for 1 min, after which a 1-ml sample was removed 5 cm below the meniscus in each tube. This was then added to 0·1 ml of 0·4% (w/v) iodonitrotetrazolium and incubated at 25°C for 4 h. After fixing the cells in 2·5% glutaraldehyde, each aliquot was loaded onto a 5-ml Percoll phosphate column in sterile 15 ml polypropionate centrifuge tubes. The column was allowed to settle for 30 min after which it was centrifuged at 3000 *g* for 2 h. The supernatant was decanted and stained with 1 ml of 5 μg ml^{-1} diamidinophenyl indole. This suspension was subsequently filtered with low vacuum through black filters (47 mm diameter, 0·8 μm pore size, Osmonics, Minnetonka, MN, USA), and the protista enumerated using epifluorescence microscopy at 400× magnification.

The data obtained after 70 days of incubation (five random soil samples, each subsampled and treated in triplicate with increasing levels of Cu) were analysed using ANOVA (Statistica Version 6). Univariate tests of significance were performed on observations made in triplicate for the soil samples at four different concentrations of administered Cu (0, 30, 100 and 1000 mg kg^{-1} Cu). Where application of ANOVA revealed that the factors had a significant effect on protistan numbers, the differences between treatments were further separated at $P \leq 0{\cdot}05$, using Bonferroni (Dunn) *t*-tests. For the first series of microcosms, mean and standard deviation were also calculated for protistan numbers obtained in triplicate microcosms after 70 and 245 days of incubation.

Effect of soil characteristics on impact of copper on protistan numbers

Our objective was to identify those characteristics listed in Table 1 that correlated with the percentage reduction in protistan numbers, compared with the control, after 70 days of incubation in the presence of an additional 1000 mg kg^{-1} Cu (Table 3). Statistica Version 6 was used to compute the correlation of each variable versus percentage reduction in protistan numbers, and to identify three variables which correlated best with percentage reduction in protistan numbers. In addition, an all subsets regression procedure, as well as a stepwise regression procedure were carried out, and the two variables that yielded the strongest regression relationship with the percentage reduction in protistan numbers were identified.

RESULTS

Impact of Cu on microbial numbers in soil sample I

In all soil microcosms their was an increase in microbes enumerated as heterotrophic plate counts during the initial 14 days of incubation, after which they remained at *ca*

Table 3 Percentage reduction in protistan numbers, compared to the control, after 70 days of incubation in the presence of an additional 1000 mg kg^{-1} Cu

Sample I	Sample II	Sample III	Sample IV	Sample V
55·6	59·1	68·9	78·7	48·8

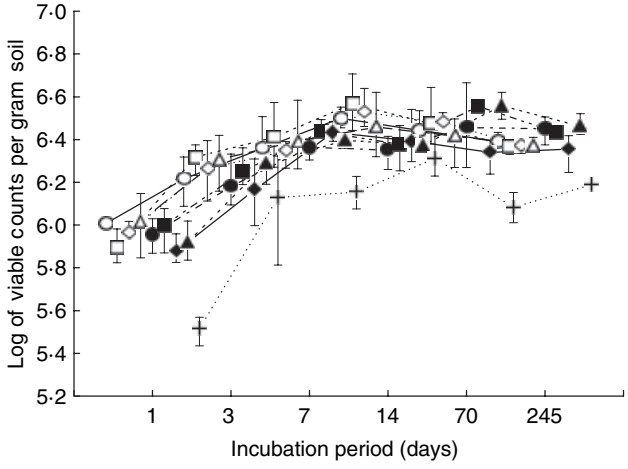

Fig. 1 General heterotrophic counts in soil microcosms prepared from soil sample I (see Table 1). Each value represents the mean of three repetitions; vertical bars denote 0·95 bootstrap confidence intervals. Concentration of Cu (mg kg^{-1}) added to each microcosm: (○) 0, (□) 10, (◇) 20, (△) 30, (●) 40, (■) 50, (◆) 100, (▲) 500 and (+) 1000

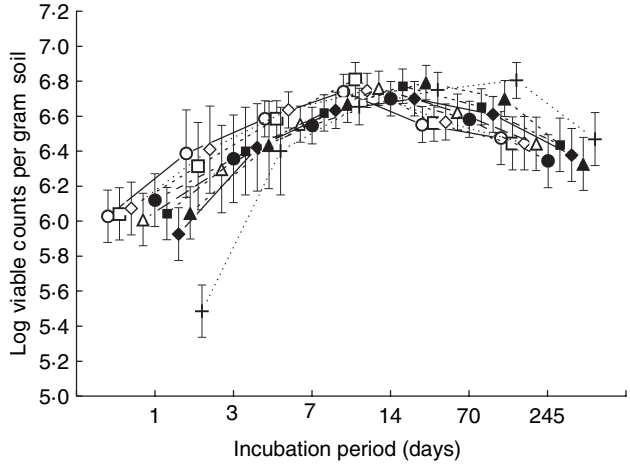

Fig. 2 Fluorescent bacterial counts, obtained on King's medium B, in soil microcosms prepared from soil sample I (see Table 1). Each value represents the mean of three repetitions; vertical bars denote 0·95 confidence intervals. Concentration of Cu (mg kg^{-1}) added to each microcosm: (○) 0, (□) 10, (◇) 20, (△) 30, (●) 40, (■) 50, (◆) 100, (▲) 500 and (+) 1000

$3{\cdot}2 \times 10^6$ cells g^{-1} soil (Fig. 1). A bootstrap repeated measures analysis (over 245 days) of the heterotrophic plate counts, showed no difference among the treatments, except those treated with *ca* 1000 mg kg^{-1} Cu, which was generally significantly lower, except at days 3 and 14. Heterotrophic plate counts in microcosms treated with *ca* 1000 mg kg^{-1} generally only reached *ca* 1×10^6 cells g^{-1} soil during the 245 days incubation period.

Despite the initial negative impact of Cu on the fluorescent bacterial numbers in microcosms that received 1000 mg kg^{-1} Cu, these bacteria increased significantly in number when compared with similar numbers recorded for microcosms that received lesser amounts of Cu (*ca* $6{\cdot}3 \times 10^6$ bacteria g^{-1} soil, Fig. 2).

The numbers of bacteria that grew on the medium designed to be selective for pseudomonads ranged between $3{\cdot}2 \times 10^4$ and $6{\cdot}3 \times 10^6$ cells g^{-1} soil during the incubation period. The initial response of these bacterial populations differed depending on the concentration of the administered Cu (Fig. 3). Pseudomonad numbers in the control microcosms remained the same during the initial 7 days of incubation. However, during the same period a significant increase in the numbers of these bacteria was noted in microcosms that received 30 mg kg^{-1} Cu. The numbers of pseudomonads in microcosms treated with 100 mg kg^{-1} Cu also increased (but not significantly) during the initial 7 days of incubation. During this period, a significant decrease in pseudomonads was noted in those microcosms that received 1000 mg kg^{-1} Cu. However, at the end of the incubation period, the pseudomonad numbers in all the microcosms had decreased to similar levels in all microcosms. The numbers of actinomycetes, culturable on sodium caseinate agar, in all the microcosms that received copper oxychloride remained similar to that in the control microcosms at *ca* $3{\cdot}2 \times 10^6$ actinomycetes g^{-1} soil (results not shown).

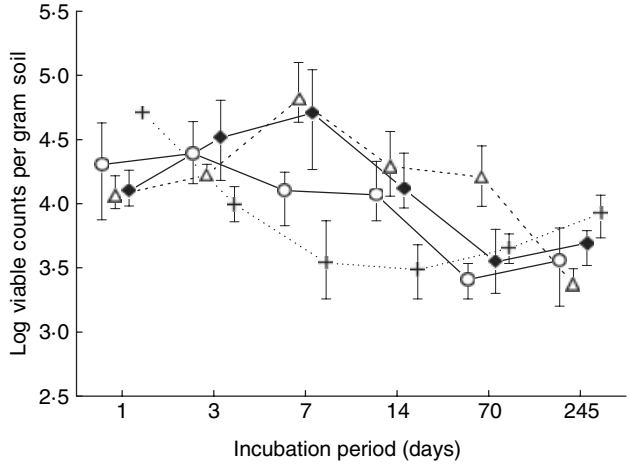

Fig. 3 Pseudomonad counts, obtained on *Pseudomonas* CFC medium, in soil microcosms prepared from soil sample I (see Table 1). Each value represents the mean of three repetitions; vertical bars denote 0·95 bootstrap confidence intervals. Concentration of Cu (mg kg^{-1}) added to each microcosm: (○) 0, (△) 30, (◆) 100 and (+) 1000

Impact of Cu on whole community metabolic profiles

The data obtained on the utilization of the series of carbon compounds in the Biolog™ Eco microplates by the soil

microbes of samples II–V, after an incubation period of 70 days in the presence of different concentrations of additional Cu, were analysed using ANOVA, followed by univariate tests of significance and Bonferroni (Dunn) *t*-tests. The numbers of carbon sources that were subsequently found to be utilized by soil microbes in the presence of 0, 30, 100 and 1000 mg kg^{-1} additional Cu, calculated as the LS means of four repetitions representing soil samples II–V, were respectively 27·0, 23·8, 23·0 and 6·5, which are presented as the midpoints of four 95% confidence intervals. The confidence interval for 1000 mg kg^{-1} did not overlap the other intervals, indicating a significant difference with the other Cu treatments. These significant differences between 1000 Cu mg kg^{-1} and the other treatments were also reflected in the cluster analysis on the metabolic profiles of the micro-organisms present in the microcosms prepared from the different soil samples (Fig. 4). Only two well-defined clusters were observed, one represented microcosms containing 100 mg kg^{-1} Cu and less, while the other represented microcosms containing 1000 mg kg^{-1} Cu.

The cluster analysis on the reduction of metabolic potential in the presence of 1000 mg kg^{-1} additional Cu was similar to the results obtained with soil sample I after 245 days of incubation in the presence of the same series of Cu concentrations (Fig. 5).

Impact of Cu on protistan numbers

The protistan numbers in soil microcosms prepared from soil samples I after 245 days of incubation are depicted in Fig. 6. Compared with the control, a significant reduction in protistan populations was observed in soil that received ≥30 mg kg^{-1} Cu. Similarly, after 70 days of incubation a significant reduction in protistan numbers was observed in microcosms prepared from samples I–IV in the presence of ≥30 mg kg^{-1} additional Cu and in microcosms prepared from sample V in presence of 1000 mg kg^{-1} additional Cu (Fig. 7).

Interestingly, after 70 days of incubation, a significant difference in protistan numbers was also observed between some of the untreated microcosms (controls) originating from different soil samples (Fig. 7). The soil samples with higher protistan numbers in these microcosms also showed a greater percentage reduction in protistan numbers (compared with the control, in the presence of an additional 1000 mg kg^{-1} Cu). This percentage reduction in protistan numbers for the different soil samples is depicted in

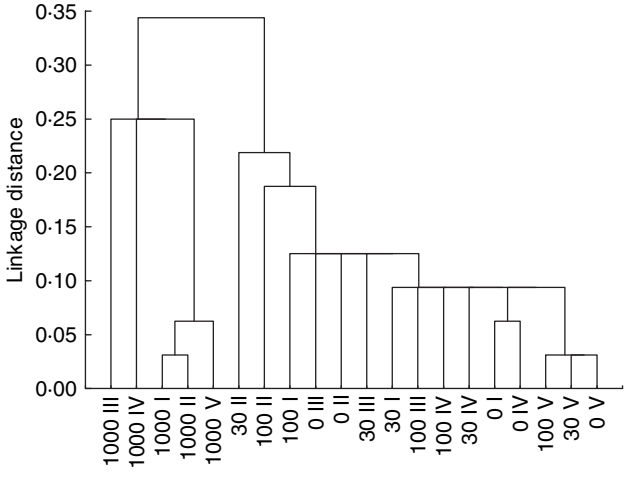

Fig. 4 Dendrogram illustrating shifts in metabolic profiles after incubating microcosms prepared from soil samples II–V, that were challenged with different copper oxychloride concentrations, for a period of 70 days. The *y*-axis represents the linkage distance, while the *x*-axis represents different microcosms with different copper concentrations (mg kg^{-1}) added to it (Arabic numerals) and prepared from different soil samples (Roman numerals)

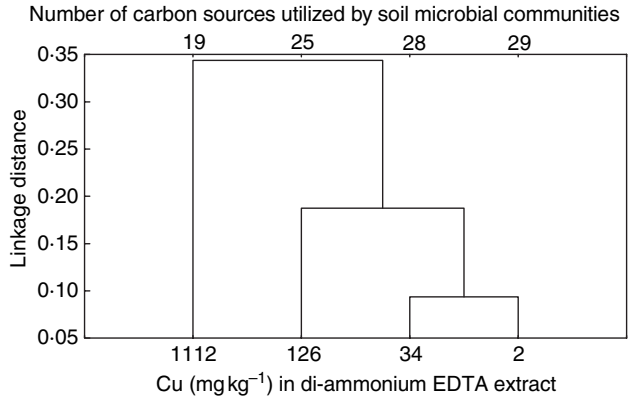

Fig. 5 Dendrogram, illustrating shifts in metabolic profiles after incubating the microcosms prepared from soil sample I, containing different copper oxychloride concentrations, for a period of 245 days

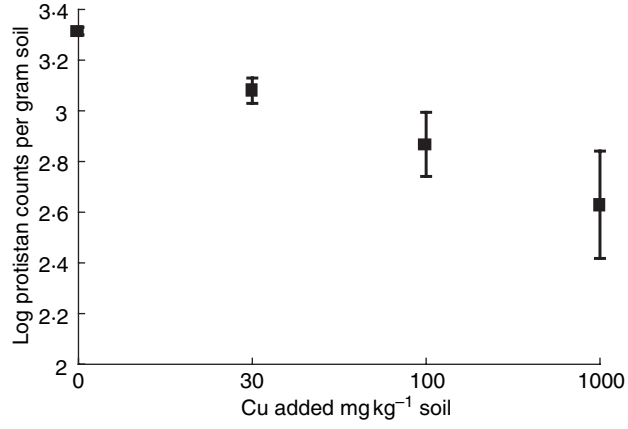

Fig. 6 Protistan counts in soil microcosms prepared from soil sample I after 245 days of incubation. Each value represents the mean of three repetitions; vertical bars denote standard deviations

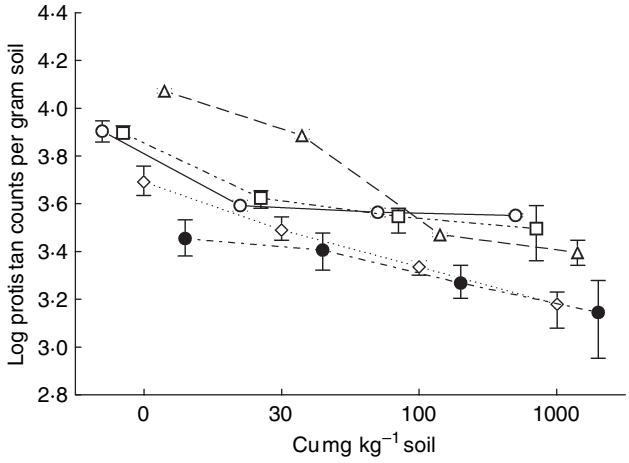

Fig. 7 Protistan counts in soil microcosms after 70 days of incubation. Each value represents the mean of three repetitions; vertical bars denote 0·95 bootstrap confidence intervals. The different soil samples analysed: (○) sample I, (□) sample II, (◇) sample III, (△) sample IV and (●) sample V

Table 3. The characteristic (variable) listed in Table 1 that showed the strongest correlation with this percentage reduction was the nitrate and nitrite content of the soil ($P = 0{\cdot}045$). To a lesser extent soil zinc ($P = 0{\cdot}162$) and phosphorous ($P = 0{\cdot}194$) content also correlated with the percentage reduction in protistan numbers. When an all subsets regression procedure was conducted, the two variables that yielded the strongest regression relationship with the percentage reduction in protistan numbers were however found to be the zinc and phosphorous content of the soil, explaining 99·9% of the variation in the model. A decrease in both these variables resulted in a greater percentage reduction in protistan numbers as a result of the additional Cu. Nitrate and nitrite content of the soil was negatively correlated with the soil zinc content to such an extent that nitrate and nitrite was excluded as variable upon inclusion of soil zinc content in the regression equation.

DISCUSSION

Culturable populations of micro-organisms, either total populations or specific bacterial groups, have been used to monitor changes in soil biota in response to land management (Maltby 1975; Kale and Raghu 1989). Although plate counts correlated poorly with microbial biomass and enzymatic measurements of microbial growth (Frankenberg and Dick 1983), evidence suggested that plate count techniques are useful in comparative studies of specific microbial populations (Harris and Birch 1992). A study conducted by Smit *et al.* (1997) showed no differences using direct counting, but a lower diversity of isolates was found in soils contaminated with 750 kg ha^{-1} Cu, compared with uncontaminated soil. It has been demonstrated that 150 mg kg^{-1} Cu in soil may inhibit carbon mineralization in acidic sandy loam soil when added as a component of sewage sludge (Chander and Brookes 1991). It was also found that amendment of soil with heavy metal-containing sewage sludge, resulting in concentrations of 0·6 mg kg^{-1} Cd, 11 mg kg^{-1} Ni, 102 mg kg^{-1} Zn and 21 mg kg^{-1} Cu, changes the bacterial community structure, as determined by molecular techniques such as DNA reassociation analysis (Sandaa *et al.* 1999). Similarly, substrate utilization analyses were used to reveal shifts in the community structure of physiologically active soil bacteria when challenged with elevated levels of heavy metals originating from sewage sludge (Bååth *et al.* 1998).

In our study, it was found that different physiological groups reacted differently to the addition of Cu to the soil. In addition, increased Cu concentrations in the soil did not necessarily result in a decrease in the numbers of a particular microbial population. This was evident for the numbers of pseudomonads in the soil (Fig. 3), indicating that other factors in addition to the intrinsic abilities to resist high Cu concentrations play a role in the regulation of pseudomonad populations under these conditions. For example, an increase in pseudomonad numbers may be a consequence of a negative impact of these higher Cu concentrations on the competitors and/or protistan predators of the pseudomonads. It is known that, because of their delicate external membranes, protists are highly sensitive to elevated concentrations of heavy metals (Foissner 1994). It is therefore not unlikely that the decrease that was observed in protistan numbers at higher soil Cu concentrations (Figs 6 and 7) could have resulted in an increase in bacterial prey that is not as sensitive to the heavy metal as these eucaryotes. The abundance or observed absence of physiologically related microbial groups in soil therefore may be a result of both the direct and indirect effects of Cu.

During the first 14 days of incubation of soil sample I, significant increases occurred in the general microbial and fluorescent bacterial numbers in the soil microcosms (Figs 1 and 2). This phenomenon is not uncommon and was also demonstrated by Franzleubbers *et al.* (2000). It may be attributed to the fact that the soil was re-wetted after it was dried for 2 weeks prior to initiation of the experiment. However, after 70 days of incubation, the numbers of bacteria generally seemed to have reached equilibrium. This period of time was also found to be sufficient for a much larger soil mesocosm to reach steady state (Lawrence *et al.* 1993). Likewise, it was found that when the microbial populations in soil microcosms prepared from sandy loam soil were monitored, the soil phospholipid fatty acid profiles generally took 70 days to return to a profile that is close to the original soil (Ekelund *et al.* 2003). Consequently, we decided to compare the metabolic profiles and protistan

numbers in four more series of microcosms, after 70 days of incubation, using soils with similar physical and chemical composition, but with dissimilar histories regarding periods under agricultural management and chemical and physical composition (soil samples II–V, Table 1).

As was found in soil sample I (Figs 5 and 6), the addition of copper oxychloride to soil samples II–V had a negative impact on both protistan numbers (Fig. 7) and the metabolic potential of the soil microbial community (Fig. 4). However, the metabolic potential, which is an indication of the abundance of physiological groups able to utilize a specific carbon source, was not as severely affected by the addition of Cu to the soil as the protists. A significant negative impact was only observed in the metabolic potential of the soil that received *ca* 1000 mg kg^{-1} Cu (Figs 4 and 5). This may indicate, as Ekelund *et al.* (2003) also concluded after similar experiments, that functional substitution has occurred within the microbial populations challenged with the lower Cu concentrations.

Although the protistan numbers, and the impact of the additional Cu on these numbers, differed between the different soils, the protists were found to be the most sensitive to additional Cu. In some soils, perturbations resulting in as little as 15 mg kg^{-1} Cu, determined in a di-ammonium EDTA extract, had a significant negative impact on protist numbers (Fig. 7, Table 2). These findings are in contrast to the results of Ekelund *et al.* (2003), who found in similar experiments that after 70 days increased Cu levels impacted negatively on protistan diversity, but not on protistan abundance. We are of the opinion that this discrepancy may be as a result of the fact that, in contrast to the work of Ekelund *et al.* (2003), we did not amend the soil with potentially Cu absorptive organic material. The latter would enhance Cu retention by the soil (Rodriguez-Rubio *et al.* 2003) thus alleviating the toxic effects of this heavy metal. The chemical and physical composition of the soils may also play a role in the interactions of Cu in soil; for example in our study the impact of an additional 1000 mg kg^{-1} Cu on the percentage reduction in protistan numbers was less severe in soils containing a higher zinc and phosphorous content than in soils containing lower concentrations of these two elements. Furthermore, it was found that a direct correlation existed between the reduction in protistan numbers as a result of 1000 mg kg^{-1} additional Cu, and the nitrate and nitrite content of the soil. These findings are in accordance with literature, as it is well known that nitrate decreases the adsorption maximum of Cu to some soils (Yu *et al.* 2002). In contrast, phosphate increases the adsorption of copper ions to certain soil components (McBride 1994), thus reducing bioavailability. Cu and Zn are furthermore known to be involved in similar physico-chemical interactions with soil components (McBride 1994; Agbenin and Olojo 2004).

Interestingly, after 70 days of incubation, untreated microcosms from uncultivated soils (samples I, II and IV) contained more protists than Cu treated microcosms from cultivated soils (samples III and V). Also, the percentage reduction in protistan numbers as a result of the additional Cu was higher in virgin uncultivated soil when compared with the cultivated soils (Table 3). These findings are important as they demonstrate the sensitivity of protista in acidic sandy loam soil, originating from pristine or near-pristine areas, to perturbations with Cu that is often liberally applied to adjacent vineyards. The pivotal role of protists in the mineralization process (Griffiths 1994), and the negative impact of relatively low Cu concentrations on the populations of these microbes in soil, suggest that soil processes in these soils may be notably effected at much lower levels of heavy metal than is commonly believed.

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