Changes in the succession and diversity of protozoan and microbial populations in soil spiked with a range of copper concentrations

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Abstract

We studied microbial and protozoan activity, diversity and abundance as affected by Cu$^{2+}$ amendments ranging from 0 to 1000 μg g$^{-1}$ over a 70-day period. At the end of the experiment the microbial population size, as indicated by substrate-induced respiration, had normalized for all Cu$^{2+}$ concentrations, but 1000 μg g$^{-1}$. Protozoan abundance was negatively affected by Cu$^{2+}$, although, only in the first few weeks. A more detailed analysis of the individual components that make up the microbial and micro-faunal populations (phospholipid fatty acid (PLFA) profile and protozoan morphotypes), however, yielded a somewhat more complex picture. For the three highest Cu$^{2+}$ amendments (160, 400 and 1000 μg g$^{-1}$), there still was a significant reduction in number of differentiable protozoan morphotypes. The bacterial PLFA pattern suggested a shift from Gram-negative towards Gram-positive bacteria for the high amendments, a process where protozoan grazing most likely played a significant role. The ratio of the trans/cis isomers of the 16:1ω7 fatty acid indicated that Cu$^{2+}$, even at low and medium concentrations, induced physiological changes in the microbial population. The relatively slight changes in total microbial and micro-faunal abundance and activity, also at the highest Cu$^{2+}$ concentrations, probably reflected the ability of the community to compensate for loss of taxa by functional substitution.

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1. Introduction

Heavy metals including copper are a potential problem in agricultural soil because they are supplied to the soil along with pig slurry (Christie and Beattie, 1989), sludge from wastewater treatment plants (Tabatabai, 1976) or pesticides (Vasseur et al., 1988). Industrial wastes may locally cause extremely high concentrations in soil (Giller et al., 1998; Tabatabai, 1976). There is an increasing body of evidence suggesting that microorganisms are far more sensitive to heavy metal stress than plants and animals inhabiting the polluted soils (Giller et al., 1998). Heavy metal impact on microorganisms may include decreases in microbial biomass and changes in community structure, reduced symbiotic N$_2$-fixation in legumes (McGrath, 1994) and a reduced carbon mineralization, which may ultimately lead to accumulation of deep layers of organic matter (Giller et al., 1998).

Considering the severe effects heavy metals may have on soil ecosystems and the important role protozoa play in soil ecosystems as regulators of the bacterial and fungal populations (Ekelund, 1998; Ekelund and Ronn, 1994), the literature on heavy metal effects on soil protozoa is surprisingly sparse. Kandeler et al. (1992) observed effects on testate amoebae both in terms of number of species and abundance in soil influenced by copper emitted from a smelter, whereas Boon et al. (1998) saw no effect on protozoan numbers in a soil heavily polluted with copper. Most studies on heavy metals and soil protozoa have been performed on ciliates, often involving only a few species (Campbell et al., 1997; Forge et al., 1993; Nicolau et al., 1999; Pratt et al., 1997); see Foissner (1999) for more details on this subject.

Many soil microorganisms cannot be cultivated, which impedes our knowledge on microbial diversity in soil (Torsvik et al., 1990). Similarly, a large fraction of the soil protozoa remains undescribed (Foissner, 1999), although, most can be assigned to genus level. Still, neither for
bacteria, fungi nor for protozoa, are we able to link the large taxonomic diversity in soil to the functioning of the microbial ecosystems (Griffiths et al., 2000). It is, e.g. not established if a certain level of protozoan diversity is required to ensure maximal performance, and if all groups have to be present in order to sustain it. Neither is it known which role protozoa play when microbial communities recover after soil perturbation (Coûteaux and Darbyshire, 1998). A more thorough understanding of these mechanisms would be helpful when trying to evaluate the vulnerability of microbial ecosystems upon contamination with toxic compounds (e.g. heavy metals).

In the present study, we used a microcosm approach to study interactions between the protozoan grazers and their potential food items, with major focus on the bacteria, along a broad range of Cu²⁺ concentration in soil. Samples were taken 2, 7, 14 and 70 days after the Cu²⁺ amendments to ensure that the population dynamics of main microbial groups could be monitored over a relatively large time span to reveal their growth dynamics when the amount of bioavailable Cu²⁺ supposedly had reached a stable level. We measured protozoan abundance and diversity by conventional microscopy. The phospholipid fatty acid (PLFA) technique was used to estimate the in situ structure and size of the entire microbial population. The accumulated and substrate-induced respiration (SIR) were used to estimate microbial activity and population size, respectively. Using this broad array of techniques we were particularly interested in answering the following questions: (1) At which levels in the soil will Cu²⁺ perturb the microbial population? (2) How are size and diversity of the protozoan population related to an increasing Cu²⁺ concentration and can changes in the other microbial populations be assigned hereto? (3) To which extent will microbial populations return towards their original structure (resilience) after a Cu²⁺ perturbation event?

Table 1
Important characteristics of the clay loam soil (Schroeder, 1984; British system) used in the experiments

<table>
<thead>
<tr>
<th>Component</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coarse sand (≥ 200 μm)</td>
<td>69%</td>
</tr>
<tr>
<td>Fine sand (20–200 μm)</td>
<td>21.7%</td>
</tr>
<tr>
<td>Silt (2–20 μm)</td>
<td>2.8%</td>
</tr>
<tr>
<td>Clay (&lt; 2 μm)</td>
<td>4.1%</td>
</tr>
<tr>
<td>Organic carbon</td>
<td>1.2%</td>
</tr>
<tr>
<td>pH</td>
<td>6.5</td>
</tr>
<tr>
<td>Water holding capacity</td>
<td>15%</td>
</tr>
</tbody>
</table>

conditions. Important soil characteristics are presented in Table 1.

2.2. Microcosm setup and sampling

The soil was mixed thoroughly with finely ground barley straw (1.125 mg straw g⁻¹ soil dw). Soil microcosms were then prepared in 116-ml serum flasks with 10.0 g soil and 3 ml distilled water (corresponding to a soil dry matter content of about 77%), which contained CuCl₂ to give each of 10 final Cu²⁺ concentrations: 0, 0.7, 1.6, 4.1, 10, 25, 64, 160, 400 and 1000 mg Cu²⁺ kg⁻¹ dry soil. After inoculation, the microcosms were sealed with airtight rubber stoppers and incubated in darkness at 10 °C. On day 2, 7, 14 and 70 after start of incubation three replicate microcosms from each Cu²⁺ level were destructively sampled. Accumulated CO₂ in each flask was determined by injecting 1 ml from its headspace air into a gas chromatograph with a thermal conductivity detector (Mikrolab, Århus, Denmark). Results from our laboratory have shown that variation in atmospheric contents of CO₂ and O₂, within the limits observed in this experiment, have no significant effects on microbial growth (Christensen and Ekelund unpublished results). Wet soil samples, each of 7.0 g, was taken out for subsequent analysis of SIR and PLFA profiles (see below).

2.3. Measurements of protozoa

We added 100 ml ‘Modified Neff’s amoeba saline’ (Page, 1988) to the remaining soil in each flask and agitated the soil suspensions for half an hour on a ‘Heidolph Promax 2020’ end to end shaker (Struurs Kebo Lab ag., Denmark) at 390 rev min⁻¹. The soil suspensions were used to prepare microtiter plates for enumeration of bacterivorous protozoa (Ekelund, 1999). An 0.3 g l⁻¹ autoclaved solution of Tryptic Soy Broth (Difco Bacto®, Detroit, Michigan, USA) in Modified Neff’s amoeba saline was used as growth medium in the microtiter plates. Data from microtiter plates were converted to protozoan numbers by a computer programme as detailed in Rønn et al., (1995). Protozoan diversity (number of different discernable morphotypes) for each treatment was determined as described by Ekelund (1999). Heterotrophic flagellates were assigned to the lowest taxonomic level possible, by an examination in the microscope. The presence of ciliates, naked and testate
amoebae, and forms with heliozoan morphology were also recorded.

2.4. Substrate-induced respiration

The SIR assay (Anderson and Domsch, 1978) was performed on 300-mg portions of soil that were placed in 3-ml Venoject tubes and supplied with 4 mg of a glucose and talcum mixture (1/4, w/w) (Anderson and Domsch, 1985), corresponding to about 3 mg glucose g\(^{-1}\) of dry soil. After thorough mixing the Venojects were capped, incubated in the dark at 20 °C for 30 min and flushed for 1 min with a technical gas (79% N\(_2\) and 21% O\(_2\)) to remove any CO\(_2\). Headspace gas was sampled 1.5 and 3 h after the initial flushing and analysed for its concentration of respired CO\(_2\) on a Hewlett Packard 6890 GC equipped with a Porapack-Q column and a thermal conductivity detector at an oven temperature of 30 °C. The concentration of CO\(_2\) in the headspace was determined by comparing the obtained areas to a CO\(_2\) standard curve.

2.5. Phospholipid fatty acid analysis

Microbial biomass and community structure were assessed using PLFA analysis. The procedure for extraction of PLFAs was modified after Frostegård et al. (1993). Soil samples were extracted with a mixture of chloroform, methanol and 0.15 M citrate buffer (pH 4.0) in the proportion 1/2/0.8 (v/v/v). The pooled supernatants from two repeated extractions were split in two phases by addition of chloroform and citrate buffer, after which the lipid-containing phase was transferred to burned glass tubes and evaporated under streaming N\(_2\). Classes of lipids were separated on a silicic acid (100–200 mesh size, Sigma) column, and the PLFAs were retained and dried under N\(_2\). Methyl nonadecanoate was added as an internal standard and the PLFAs were subsequently derivatised by mild alkaline methanolysis (Dowling et al., 1986).

Samples were analysed for their content of fatty-acid methyl esters on a Hewlett Packard 6890 GC equipped with an auto injector, a flame ionisation detector and a 60 m HP5 (5% phenyl methyl siloxane) capillary column. Hydrogen was used as the carrier gas (2 ml min\(^{-1}\)) and injections were made in split-less mode. The initial oven temperature was held at 80 °C for 5 min, increased at 20 °C min\(^{-1}\) to 160 °C and increased at 5 °C min\(^{-1}\) to 270 °C, at which level it was maintained in 5 min. Inlet and detector temperatures were 230 and 270 °C, respectively. Identification of fatty acids was performed as in Frostegård et al. (1993) and by comparison to the retention time of known fatty acids. Nomenclature of fatty acid follows Tunlid and White (1992). Attempts were made to estimate proportions of the following main groupings of the microbial population in samples by using PLFAs (nmol per unit dry soil) indicative of these groups: Gram-positive bacteria (PLFAs i15:0, a15:0, i16:0, i17:0, a17:0) (O’Leary and Wilkinson, 1988), Gram-negative bacteria (18:1ω7, cy17:0, cy19:0) (Wilkinson, 1988), fungi (PLFA 18:2ω6,9) (Federle, 1986).

3. Results

3.1. Respiration

Cu\(^{2+}\) had a significant effect on accumulation of CO\(_2\) in the examined soil (Fig. 1, P < 0.001, two way ANOVA on log transformed data). During the whole experiment, treatments with the three highest Cu\(^{2+}\) concentrations differed significantly from the unamended control, whereas the respiration in general was unaffected at the lowest Cu\(^{2+}\) concentrations (P < 0.05, Tukey two way comparison).

Cu\(^{2+}\) as well as time had a significant effect on the microbial biomass calculated from the SIR (Fig. 2, P < 0.001, two way ANOVA). Likewise, we observed a significant interaction between the two factors (P < 0.05). At day 7, the 400 and 1000 µg g\(^{-1}\) treatments showed a significantly lower microbial biomass than the control (P < 0.05, Tukey two way comparison); although not consistently significant, this tendency was also observed at the highest Cu\(^{2+}\) concentrations at the two later harvests.

3.2. PLFAs

Cu\(^{2+}\) concentration and time, as well as the interaction between the factors, influenced the soil content of total microbial PLFA significantly (P < 0.01, two way ANOVA). The soil content of total microbial PLFA (Fig. 3a) was similar regardless of the Cu\(^{2+}\) concentrations at day 2, 7 and 70; on day 14, however, the unamended control soil contained significantly more total microbial PLFA than in any of the other treatments (P < 0.01, Tukey pairwise comparison). This pattern was also observed for PLFAs typically found in Gram-negative bacteria (Fig. 3b).

PLFAs typically found in Gram-positive bacteria (Fig. 3c) were significantly affected by the concentration of Cu\(^{2+}\) (P < 0.05, two way ANOVA), and a significant interaction between day and concentration (P < 0.001) was likewise observed, whereas no significant change with time was observed. Gram-positive PLFAs were measured in similar amounts for all Cu\(^{2+}\) treatments at day 2, while at day 7 this value was increased ca. 100% in the controls and decreased by 30–40% in the 10 and 160 µg g\(^{-1}\) treatments. This pattern was even more pronounced at day 70.
the concentration of Gram-positive PLFA in the unamended controls again dropped to the level observed at the start of the experiment, while in the treatments with 10–1000 μg g⁻¹ Cu²⁺ it increased with increasing Cu²⁺ concentrations compared to the day-2 values.

As for the bacterial PLFAs, there was a significant variation between fungal PLFAs depending on both day and Cu²⁺ concentration (P < 0.05, two way ANOVA). Still, the fungal PLFA pattern (Fig. 3d) differed somewhat from the pattern observed for the main bacterial groups. The concentrations were not significantly different at day 2, although a tendency for a decrease was seen in the treatments with the two highest Cu²⁺ concentrations. Between day 2 and 7 the fungal PLFA content increased slightly for all treatments, though significantly only for the 1000 μg g⁻¹ amendment (P < 0.005). The fungal PLFAs in the unamended control continued to increase until day 14. On day 70 all treatments contained significantly more fungal PLFA than on day 2 (P < 0.001) with the most pronounced increase at the highest Cu²⁺ concentration.

Subjecting all PLFAs to a PCA showed that at day 2 the 1000 μg g⁻¹ treatment was located apart from the rest of the treatments that grouped together close to the origin (Fig. 4). At day 7 and 14, the 0 and 1.6 μg g⁻¹ treatments were located in the upper part of the diagram and visa versa for the 160 and 1000 μg g⁻¹. After 70 days the system returned towards the original status, as observed at day 2, except for the 1000 μg g⁻¹ treatment, indicating
that the microbial community structure was permanently altered only at the highest Cu\(^{2+}\) concentration. The overall pattern throughout the experiment showed a continuous distribution (from 0 to 1000 \(\mu\)g g\(^{-1}\)) of the values along the ordinate (from top to bottom), indicating that PC2 represented the variation arising directly from the Cu\(^{2+}\) treatments. Although PC1 contributed to a large part of the variation (78%), a direct connection to the Cu\(^{2+}\) concentrations was not observed. In the loading plot (data not shown) typical PLFAs from Gram-positive (i15:0, a15:0) and Gram-negative (18:1\(\omega_7\)) bacteria contributed considerably to the variation in each direction along the PC1-axis.

### 3.3. Trans/cis ratio of PLFA 16:1\(\omega_7\)

The trans/cis ratio of PLFA 16:1\(\omega_7\) (Fig. 5) was affected by time and Cu\(^{2+}\) concentrations (both \(P < 0.0001\), two way ANOVA), which also resulted in a significant interaction (\(P < 0.001\)). Through the 70 days the trans/cis ratio in the 0, 160 and 1000 \(\mu\)g g\(^{-1}\) treatments was stable, although decreased by about 35% at the two highest concentrations. Contrasting, in the 1.6 and 10 \(\mu\)g g\(^{-1}\) treatments the trans/cis ratio decreased dynamically through the first 14 days followed by an increase towards the end of the experiment, this development being strongest at 10 \(\mu\)g g\(^{-1}\).
3.4. Protozoa

Total protozoan abundance (Fig. 6) was affected significantly \( (P < 0.01, \text{two way ANOVA on log transformed data}) \), but to a much smaller extent than the other properties. No significant differences in protozoan abundance between concentrations were seen after day 7.

The number of discernible morphotypes was significantly affected by addition of \( \text{Cu}^{2+} \) on all sampling dates (Fig. 7, \( P < 0.001, \text{one way ANOVA} \)). Throughout the experiment, the protozoan diversity for the three highest \( \text{Cu}^{2+} \) concentrations remained lower than in the other treatments and significantly \( (P < 0.05, \text{Tukey two way comparison}) \) different from the unamended control. Only \textit{Heteromita globosa}, \textit{Cercomonas minimus}, and naked amoebae were able to withstand 1000 \( \mu \text{g} \text{g}^{-1} \text{Cu}^{2+} \), and there was a clear tendency that some forms only occurred in samples with a low \( \text{Cu}^{2+} \) addition (data not shown).

4. Discussion

4.1. Microbial activity

We amended the soil with barley straw in order to mimic a post-harvest situation, where plant residues induce a high microbial activity (Christensen et al., 1992; Rønn et al., 1996). The large amount of \( \text{CO}_2 \) released in the microcosms during the experimental period (Fig. 1) demonstrates that the straw material did stimulate the microbial activity. In the treatments with no or low \( \text{Cu}^{2+} \) amendment the total amount of carbon mineralised during the 70 days made up about 60% of the original amount of C applied as straw, whereas only about 30% was mineralised in the 1000 \( \mu \text{g} \text{g}^{-1} \)
treatment. Thus, at high concentrations, Cu\(^{2+}\) had a strong negative effect on the microbial mineralization performance. Likewise, the microbial biomass, determined as SIR, was reduced at the highest Cu\(^{2+}\) concentrations (Fig. 2). This estimate of biomass did not correlate with the content of total PLFAs in the soil. However, where the SIR probably measures the active, and to a lesser degree the inactive part of the microbial population (Anderson and Domsch, 1978), the PLFA method includes the entire microbial population (Tunlid and White, 1992). Choosing a total PLFA concentration of 30 nmol g\(^{-1}\) dry soil and a carbon content of 17 C (calculated weighed mean) atoms per fatty acid, this corresponds to approximately 6 \(\mu\)g C g\(^{-1}\) dry soil in microbial membrane fatty acids—or 2% of the biomass C if using a SIR value of 300 \(\mu\)g g\(^{-1}\) dry soil. Similar amounts of PLFA C relative to total cellular C have been observed in bacteria (Balkwill et al., 1988) and fungi (Klamer and Bååth, pers. comm.). Although these calculations should be taken with precaution, they indicate that the present SIR and PLFA estimates are within comparable ranges.

4.2. Protozoa

Both abundance and diversity of the protozoa in the soil microcosms were affected by the Cu\(^{2+}\) amendments (Figs. 6 and 7). Total protozoan abundance, though, was only affected significantly at day 2 and 7. The pattern on day 2 probably reflects a dose-related protozoan death, whereas the day-7 pattern reflected that growth was inhibited also for relatively small concentrations, supporting previous findings that protozoa are most susceptible to toxic compounds during growth (Ekelund et al., 1994). Further ahead in the succession, the more Cu\(^{2+}\) tolerant strains were probably favoured, blurring the toxic effect on the system. We attribute the apparent tendency to an inverse dose-response relation on day 70 to growth of small opportunistic forms, a suggestion, which is also supported by the actual observed composition of morphotypes (data not shown). The effect on the protozoan diversity was much more pronounced and the number of morphotypes was lowered at the three highest Cu\(^{2+}\) concentrations. A stronger effect on diversity than on abundance may be expected, since some protozoa are more susceptible to certain toxic xenobiotics than others (Ekelund, 1999; Ekelund et al., 2000; Griffiths et al., 2000). Implementation of other techniques for the study of protozoan diversity would probably have allowed discovery of additional protozoan types like the autochthonous forms (Petz et al., 1985, 1986) and might have revealed even larger differences between Cu\(^{2+}\)-amended and unamended systems.
4.3. PLFA patterns

The changes in population biomass levels as indicated by the PLFA plots (Fig. 3a–d) probably reflect dynamic steady-state situations governed by input from microbial growth and removal by micro-faunal grazing in combination with toxic effects of the applied Cu$^{2+}$. In the unamended systems, total microbial, Gram-negative and Gram-positive PLFA (Fig. 3a–c) increased to a certain level, after which the grazing exceeded the growth and the populations declined again. Increasing the Cu$^{2+}$ level to 1.6 and 10 $\mu$g g$^{-1}$ led to inhibition of the microbial growth, while the protozoa were left unaffected, which resulted in a reduced population level at day 14, where the unamended systems peaked. For the 160 and 1000 $\mu$g g$^{-1}$ treatments, effects on the protozoan community structure (Fig. 7) caused a less efficient grazing performance, thus allowing growth of microorganisms with a high Cu$^{2+}$ tolerance experiencing a diminished grazing pressure. This is supported by the fact, that many protozoa have relative specific food preferences (Ekelund and Rønn, 1994; Rønn et al., 2001). Comparison of the PLFA patterns of the Gram-negative and Gram-positive bacteria (Fig. 3b and c), strongly suggests that the protozoan types surviving the high Cu$^{2+}$ concentrations for more than 14 days preferred to prey on Gram-negative bacteria. Their PLFA value was nearly halved from day 14 and on, while the opposite pattern was seen for Gram-positive bacteria.

The fungal PLFA graphs (Fig. 3d) indicate that grazing pressure on fungi was insignificant in this study, which is probably related to the generally rather small populations of fungivorous protozoa in soil (Ekelund, 1998; Hekman et al., 1992). Some nematodes likewise graze on fungi, but they probably played an insignificant role here, as their ability to colonise a substrate depends on migration from a large surrounding soil volume (Griffiths and Caul, 1993) not present in this study. Chander et al. (2001) and Huysman et al. (1994) found indications that heavy metal contamination enhanced the proportion of fungal biomass relative to the bacterial, as in our experiment. The enhanced fungal population late in the experiment were probably also able to exploit the recalcitrant straw residues better than the bacteria.

The PCA plots show the most important trends in the variation in the entire fatty acid data set and it is clear that the two highest Cu$^{2+}$ concentrations affected the microbial community differently than the 0 and 1.6 $\mu$g g$^{-1}$ treatments. The effect of the 1000 $\mu$g g$^{-1}$ treatment was evident already after 2 days, indicating that this load of Cu$^{2+}$ had a direct toxic effect on the microorganisms. Moreover, the community structure seemed permanently changed only at 1000 $\mu$g g$^{-1}$ as its data point never grouped up with the other treatments, which seemed to return to the original population structure.

Pure-culture studies have shown that microorganisms change the proportion of membrane trans fatty acids relative to the respective cis isomers as a way to cope with various changes in their life conditions (Guckert et al., 1986; Heipieper et al., 1994, 1996). The microorganisms use the trans/cis ratio to adjust the fluidity of the membrane. We observed that Cu$^{2+}$ amendment led to a significant decrease in the trans/cis ratio (Fig. 5), for the 1.6 and 10 $\mu$g g$^{-1}$ Cu$^{2+}$ treatments. It appears that at low concentrations, Cu$^{2+}$ interacts with the cell membrane phospholipids and thereby decreases its fluidity (Suwalsky et al., 1998). Under such conditions, the living cells may decrease their trans fatty acid content in order to keep the fluidity at a proper level.

4.4. Conclusion

Cu$^{2+}$ added to soil affected both bacteria and protozoa regarding activity, physiology and diversity. Except for the highest concentrations, this effect was transient although Gram-positive bacteria seemed to be more directly affected by the Cu$^{2+}$ than Gram-negative bacteria. The relative slight changes in total microbial and micro-faunal abundance and activity, even at high levels of Cu$^{2+}$, probably reflect an ability of the microbial community to quickly compensate for loss of taxa by functional substitution. The return of the community to a PLFA composition close to the origin after 70 days (except for the highest Cu$^{2+}$ concentrations) could be indicative of such a substitution. The apparent permanent changes in the number of protozoan taxa groups and microbial PLFA profiles, suggest that absorption of Cu$^{2+}$ to soil particles did not happen to an extent that could alleviate the toxic effects of Cu$^{2+}$ even within this relatively long period of time.

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References


