

In Situ Bioremediation through Mulching of Soil Polluted by a Copper–Nickel Smelter

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ABSTRACT

Bioremediation of a heavy metal-polluted soil was investigated in a 3-yr field experiment by adding mulch to a polluted forest floor. The mulch consisted of a mixture of compost and woodchips. The remediation treatment decreased the toxicity of the soil solution to bacteria as determined by the [³H]-thymidine incorporation technique, that is, by measuring the growth rate of soil bacteria extracted from unpolluted humus after exposing them to soil solution containing heavy metals from the experimental plots. Canonical correlation analysis was performed in order to identify the chemical and microbiological changes in the soil. The pH of the mulched organic layer increased by one unit. The concentration of complexed Cu increased and that of free Cu²⁺ decreased in the soil solution from the mulch treatment. According to basal respiration and litter decomposition, microbial activity increased during the 3 yr following the remediation treatment. The [³H]-thymidine incorporation technique was also used to study the growth rate and tolerance of bacteria to Cu. The bacterial growth rate increased and the Cu tolerance decreased on the treated plots. The structure of the microbial community, as determined by phospholipid fatty acid (PLFA) analysis, remained unchanged. The results indicate that remediation of the polluted soil had occurred, and that adding a mulch to the forest floor is a suitable method for remediating heavy metal-polluted soil.

UNLIKE organic pollutants, metals cannot be degraded into a harmless form such as carbon dioxide, but persist indefinitely in the environment. The only approaches available for remediating heavy metal-polluted soils are to remove the metals or to convert the metals into less bioavailable forms. Both approaches are employed in phytoremediation, that is, the use of pollutant-accumulating plants to remove metals from soil, or the use of plants to reduce the bioavailability of heavy metals (Salt et al., 1998). The immobilization of heavy metals by a wide range of binding agents has been tested in laboratory conditions. Cation exchange resins, limestone, clays, ferrous sulfate (Czupyrna et al., 1989), minerals (Chen et al., 1997; García-Sánchez et al., 1999), synthetic zeolites (Czupyrna et al., 1989; Gworek, 1992), hydrous manganese oxide, steel shots and beringite (a modified aluminosilicate) (Mench et al., 1994), and biomass residues (Fisher et al., 1998) have been suggested for the in situ remediation of heavy metal-polluted soil. In field experiments, limestone

(Mälkönen et al., 1999), gravel sludge (Krebs et al., 1999), sewage sludge and fly ash (Kelly and Tate, 1998), beringite and compost (Vangronsveld et al., 1995a,b; Vangronsveld et al., 1996), and Fe-rich limed compost (Li et al., 2000) have been found to immobilize metals. Because one chemical extraction method is often inadequate to measure the degree of immobilization of all the components (Vangronsveld and Clijsters, 1992), we also fractionated Cu in the soil solution into Cu²⁺ and complexed Cu.

In order to assess the success of remediation, some bioassays are also needed. Immobilization has resulted in an increase in tree growth (Mälkönen et al., 1999), a decrease in heavy metal concentrations in plants (Krebs et al., 1999), a reduction in soil phytotoxicity, and successful revegetation (Li et al., 2000; Vangronsveld et al., 1995b). The recovery of nutrient cycling mediated by soil microbes is often of major importance in heavy metal-polluted sites. An increase in microbial activity (Mälkönen et al., 1999; Kelly and Tate, 1998) and establishment of a mycorrhizal network (Vangronsveld et al., 1996) have been reported. More information is needed concerning the effects of remediation on soil microbiota. To our knowledge this has not been studied using methods describing microbial activities and biomass, bacterial Cu tolerance, and structure of the microbial community.

Compost is considered to be a good amendment agent for bioremediating heavy metal-polluted soil (Vangronsveld and Clijsters, 1992; Li et al., 2000). Unfortunately, very little information has been published about the effects of compost amendments on the stabilization of heavy metal-contaminated soils. Sewage sludge has been used successfully in remediating mine spoils, and a good review of these studies is presented in Sabey et al. (1990). A mixture of compost and lime (Li et al., 2000) or modified aluminosilicate (Vangronsveld et al., 1996) was mixed with soil that was heavily polluted with Zn and Cd especially. Copper is the main pollutant at the study site where we mulched the forest floor with a mixture of compost and woodchips, an easily available and inexpensive waste material. Addition of mature compost to soil is known to enhance soil fertility by

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Abbreviations: AO, number of bacterial cells; BIOL, canonical biological variable; CCA, canonical correlation analysis; CHEM, canonical chemical variable; Cu_{comp}, complexed copper in soil solution; Cu_{ex}, exchangeable copper concentration in soil; DM, dry matter weight; IC₅₀, inhibition concentration (i.e., bacterial copper tolerance); MDS, multidimensional scaling; Ni_{ex}, exchangeable nickel concentration in soil; OM, organic matter weight; PLFA, phospholipid fatty acid; PLFA_{bact}, an indicator of bacterial biomass; PLFA_{fung}, an indicator of fungal biomass; T, treated; TdR, [³H]-thymidine incorporation rate (i.e., bacterial growth rate); TdR/AO, specific bacterial growth rate, U, untreated.

modifying the chemical, physical, and biological properties of the soil. A comprehensive review of this subject is presented in Dick and McCoy (1993). Compost increases the water-holding capacity of the soil, the soil pH, and microbial activity. The organic matter in compost also complexes metals into less bioavailable forms (Vangronsveld and Clijsters, 1992). Copper especially is known to form stable complexes with organic matter (Baker and Senft, 1995). Compost also introduces new active microbiota and provides a nutrient source for the microorganisms. We added woodchips to the compost in order to increase the amount of slow-release carbon. Mulching a polluted forest floor with a layer of organic material has several advantages in the remediation of heavy metal-polluted soil, since it prevents drying and erosion of the soil and thus promotes revegetation.

We aimed to decrease the bioavailable fractions of heavy metals in the soil by mulching the forest floor with a mixture of compost and woodchips. The subsequent increase in soil pH would precipitate heavy metals, and the organic matter addition would increase heavy metal complexation. Our hypothesis was that bioremediation of the soil would result in (i) an increase in microbial activities, (ii) a decrease in bacterial heavy metal tolerance, and (iii) a change in the structure of the microbial community. This study on soil bioremediation is the microbial part of the "Recovery of a Boreal Forest Ecosystem from Long-Term Heavy-Metal Pollution" research project.

MATERIAL AND METHODS

Study Site

The bioremediation experiment was established at a distance of 0.5 km from a Cu-Ni smelter in southwestern Finland (61°19'N, 22°9'E). The study site was of the relatively infertile *Calluna* forest site type (Cajander, 1949), with a tree cover consisting of Scots pine (*Pinus sylvestris* L.). The ground vegetation on the study sites has almost completely disappeared (Salemaa et al., 2001), and the growth of trees is extremely poor (Mälkönen et al., 1999).

The experiment was established on an esker, the soil consisting of sorted fine or fine/coarse sand with no stones. The soil was classified as an orthic Podzol (Anonymous, 1988). The original humus layer over the podzolized mineral soil was relatively thin (0–3 cm) mor (A_{O1}/A_{O2} ; F/H) with a clearly pronounced, dry, undecomposed litter layer. The structure of the humus layer (A_{O1}/A_{O2} ; F/H) has changed, and has an extremely low amount of fine-root biomass (Helmisaari et al., 1999), high heavy metal concentrations (Derome and Lindroos, 1998), and an average organic matter content of 400 g kg^{-1} . In this and earlier publications it is therefore called the organic layer. Derome and Lindroos (1998) reported a clear increasing gradient in many heavy metal concentrations in the organic layer with decreasing distance to the smelter. Total concentrations of Cu, Ni, Fe, Zn, Cd, Pb, and Cr were 6000, 460, 18 600, 520, 5.0, 310, and 31 mg kg^{-1} , respectively. According to Derome and Nieminen (1998), there is also a severe shortage of Ca, Mg, and K in a plant-available form, the exchangeable concentrations being 580, 40, and 132 mg kg^{-1} , with reduced concentrations of these nutrients in the Scots pine needles. However, the accumulation of heavy metals and sulfur has not had any effects on soil acidity, that is, on pH

or on exchangeable acidity in the organic layer and uppermost mineral soil layer (Derome and Lindroos, 1998).

More than 50 yr of heavy metal accumulation in the soil has had direct toxic effects on the soil microbiota. The overall microbiological activity has decreased drastically (Fritze et al., 1989), the structure of the microbial community has changed, and the bacterial community is highly resistant to heavy metals (Pennanen et al., 1996; Fritze et al., 1997). This is reflected in a decreased rate of litter decomposition.

Experimental Design

In summer 1996 we marked out 36 sample plots at the site. Each sample plot was 5 × 5 m, including a 1-m-wide buffer zone. Eighteen of the plots were covered with a 5-cm-thick layer of mulch (treated = T), and the other 18 plots were left untreated (untreated = U). The mulch was spread directly over the layer of undecomposed plant litter on the forest floor. The mulch consisted of a mixture of compost and woodchips (1:1, volume). The compost was 14 mo old and had been produced in outdoor windrows from a mixture of organic household waste and coarse woodchips (diam. ca. 5 cm) at the Ämmässuo Waste Handling Centre (Espoo, Finland). According to Mäkelä-Kurtto and Sippola (1995, 1996), the average nutrient concentrations (per dry matter) of compost that is sold as a garden soil amendment and produced at the same waste handling centre are: NH_4-N , 370 mg; NO_3-N , 760 mg; Ca, 28 g; K, 16 g; Mg, 3 g; P, 2 g; Fe, 7 g; Al, 800 mg; Mn, 300 mg; Cu, 60 mg; Zn, 250 mg; Ni, 3 mg; Cd, 0.6 mg; Pb, 51 mg; and Cr, 2.6 mg kg^{-1} . According to our own measurements, the pH of the compost was 7.7, total organic C content 280 g kg^{-1} , and total N content 26 g kg^{-1} , giving a C to N ratio of 11. The C to N ratio is used as a measure of compost maturity; in mature compost the ratio is between 10 and 12 (Chefetz et al., 1996). The mulch was prepared 1 wk before spreading by mixing the compost with woodchips (diam. <20 mm) of Scots pine and Norway spruce [*Picea abies* (L.) Karst.] stemwood. The carbon content of stemwood is ca. 500 g kg^{-1} C, but the contents of N and other nutrients are insignificant in this context. The mulch contained 320 g C and 20 g N kg^{-1} dry matter, giving a C to N ratio of 16, and pH 6.3. Compost (excluding the woodchips) was added to the plots at a dose of 5.4 kg m^{-2} (dry matter weight).

Soil samples were taken from the 0- to 3-cm organic layer below the polluted litter layer on each plot. One composite sample (five replicates) was taken from each plot using a spoon (area 10 cm^2) after the litter layer (U plots) or the mulch and the litter layer (T plots) had been removed. Care was taken to ensure that the T samples did not contain any mulch. The samples were taken to the laboratory within 1 or 2 d after sampling. The fresh samples were sieved (mesh size 28 mm) and stored for 1 d at room temperature in order to stabilize the microbiota, and then stored at 4°C until analysis. The samples for phospholipid fatty acid (PLFA) analysis were frozen immediately, and those for exchangeable metal analyses were air-dried. Soil samples were collected in autumn 1996, spring 1997, autumn 1997, spring 1998, and autumn 1998.

Chemical Analyses

Total C and N in the mulch were determined by dry combustion (LECO [St. Joseph, MI] CHN-600). Dry matter weight (DM) was determined by drying overnight in an oven at 105°C, and the organic matter content (OM) as loss in weight on ignition (550°C). The pH was measured from a fresh soil-water suspension (1:3, volume). Exchangeable copper (Cu_{exc}) and nickel (Ni_{exc}) were determined by extracting 2 g of air-dried

soil with 100 mL of 0.1 M BaCl₂ on a shaker for 1 h. The extract was filtered and the Cu and Ni concentrations determined by atomic absorption spectrometry (AAS).

Copper fractionation into free Cu²⁺ ions and complexed Cu (Cu_{comp}) was carried out on the soil solution extracted from samples taken at the last sampling round. Soil solution was obtained from the fresh soil samples by centrifugation for 40 min at 30 100 × *g* on a Beckman (Fullerton, CA) centrifuge (J2-21M/E) with a fixed angle rotor (JA-14) at 5°C. The samples were placed in special two-part, nylon centrifuge tubes (Geisler, 1996). After centrifugation, the soil solution was removed from the bottom section of the centrifuge tube, and then diluted to 50 mL for Cu fractionation. Fractionation into Cu²⁺ and Cu_{comp} was performed by passing the diluted soil solution sample through a cation exchange column (Amberlite 120 Plus, Na⁺ form; ICN Biomedicals, Costa Mesa, CA) (Berggren, 1989). The Cu concentration in the sample was determined before and after passage through the column by atomic absorption spectrometry. The Cu concentration in the effluent was considered to be Cu_{comp} (neutral or negatively charged species), and the difference between the Cu concentration before and after passage through the column was considered to be the concentration of Cu²⁺ ions.

Toxicity Test

The toxicity of the soil solution (prepared as described above) to bacteria was studied on samples taken at the last sampling round by the [³H]-thymidine incorporation technique in order to measure the bacterial growth rates. The [³H]-thymidine incorporation rate was determined as described by Bååth (1992a,b) and modified by Kiikkilä et al. (2000). We used bacteria extracted from an unpolluted forest site (Fritze et al., 2000). Soil solution (0.2 mL) from the plots was added to 1.8 mL of bacterial suspension, and the [³H]-thymidine incorporation procedure was then performed as described below. It was expected that the more toxic the soil solution, the less [³H]-thymidine would be incorporated.

Microbial Activities

Microbial activity was measured as basal respiration (BR). The CO₂ evolved in 24 h was determined by gas chromatography as described by Pietikäinen and Fritze (1995). Litter decomposition was studied with litter bags (nylon net bag, 7 × 7 cm, of 1-mm pore size), containing green Scots pine needles (1 g DM) collected from an unpolluted area. The litterbags (20 on each sample plot) were inserted immediately under the polluted litter layer. The bags on the T plots were covered by the litter layer and the mulch. The bags were removed after three growing seasons, dried, washed, and weighed. The litter weight lost was calculated.

Bacterial growth rate and copper tolerance were determined by the [³H]-thymidine incorporation technique. Soil equivalent to 1.3 g of organic matter was shaken in 100 mL of distilled water for 1 h at 250 rpm at 4°C. The soil suspension was centrifuged for 10 min (750 × *g*). The supernatant (i.e., the bacterial suspension) was then incubated at 22°C for 2 h with [³H]-labelled thymidine. The growing bacteria incorporate [³H]-thymidine, and the incorporation rate (TdR) was measured by counting the radioactivity in a Wallac (Turku, Finland) 1411 liquid scintillation counter using the fine-tuned external standard method (Anonymous, 1991). In the copper tolerance assay the bacterial suspension was mixed with a solution containing 0, 0.0001, 0.001, 0.01, and 0.1 M Cu. Growth inhibition (IC₅₀) was calculated as the log Cu concentration (*M*) giving a 50% reduction in [³H]-thymidine incorpo-

ration. The lower the absolute IC₅₀ value, the greater is the tolerance of the bacterial community. The isotope dilution procedure, as described by Pollard and Moriarty (1984), was performed on four replicate samples of both treatments taken at the last sampling round. Since the degree of participation was ca. 33% for both treatments (U and T), dilution of the isotope was not taken into account in the calculations. The supernatant used for determining the bacterial growth rate was also used to determine the number of bacterial cells in the sample. The diluted soil suspension was filtered through a black 0.2-μm Poretics polycarbonate membrane (Osmonics, Minnetonka, MN) and the cells were stained with acridine orange. The number of cells (AO) was counted under a Leitz Laborlux S epifluorescence microscope (Ernst Leitz, Wetzlar, Germany). The specific [³H]-thymidine incorporation depicting the growth rate of the bacterial cells (TdR/AO) was calculated.

Structure of the Microbial Community

The microbial community structure was analyzed as described by Frostegård et al. (1993a) and Pennanen et al. (1999) by extracting the microbial-derived phospholipid fatty acids (PLFAs) from the organic soil sample. Different subsets of the microbial community have different PLFA patterns in their cell membrane, and a treatment-induced change in the PLFA pattern is an indication of a changed microbial community. To briefly summarize this procedure, 0.5 g fresh weight of organic soil was extracted with chloroform–methanol–citrate buffer mixture (1:2:0.8) and the lipids separated into neutral lipids, glycolipids, and phospholipids on a silicic acid column. The phospholipids were subjected to a mild alkaline methanolysis, and the fatty acid methyl esters were analyzed by gas chromatography (flame ionization detector) using a 50-m HP-5 (phenylmethyl silicone) capillary column (Hewlett–Packard, Palo Alto, CA). Helium was used as a carrier gas. The temperatures of the injector and detector were 230 and 270°C, respectively. The initial temperature of the oven was 50°C and it was raised at the rate of 30°C min⁻¹ to 160°C, then at the rate of 2°C min⁻¹ to 270°C, after which the oven was kept for 5 min at the final temperature of 270°C. Peak areas were quantified by adding methyl nonadecanoate fatty acid (19:0) as an internal standard.

Fatty acids are designated in terms of the ratio between the total number of carbon atoms and the number of double bonds, followed by the position of the double bond with respect to the methyl end of the molecule. The prefixes *i* and *a* indicate iso- and anteiso branching, *br* indicates unknown branching, and *cy* indicates a cyclopropane fatty acid. *Me* refers to the position of the methyl group with respect to the carboxyl end of the chain. The prefix *C* (C15:1) indicates that the PLFA has 15 carbon atoms and one double bond, but the arrangement of the carbon atoms (e.g., branching position) was not confirmed. The individual PLFAs were expressed as percentage of the total amount of PLFAs detected in a soil sample (mol%). The total sum of PLFAs was used as an indicator of microbial biomass (PLFA_{tot}). The sum of PLFAs considered to be predominantly of bacterial origin (i15:0, a15:0, 15:0, i16:0, 16:1ω9, 16:1ω7t, i17:0, a17:0, 17:0, cy17:0, 18:1ω7, and cy19:0) was chosen as an index of the bacterial biomass (PLFA_{bact}) (Frostegård and Bååth, 1996). The amount of PLFA 18:2ω6,9 was used as an indicator of the fungal biomass (PLFA_{fung}) because it is suggested to be mainly of fungal origin in the soil (Federle, 1986) and it is known to correlate with the amount of ergosterol (Frostegård and Bååth, 1996), a sterol found only in fungi. The ratio between

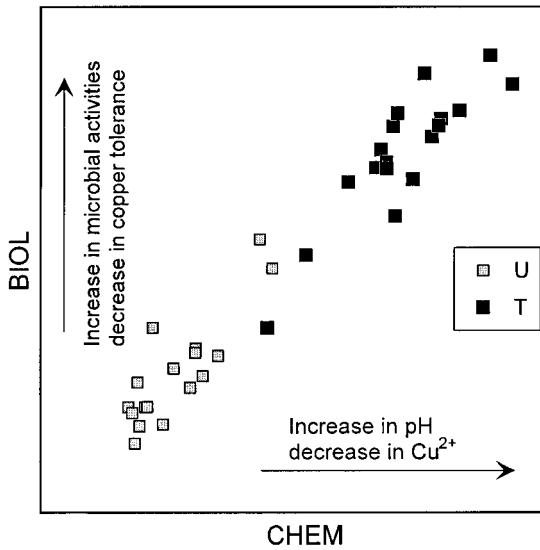


Fig. 1. Plot of the untreated (U) and the treated (T) sample plots of the last sampling date on the first canonical variables, CHEM (the chemical data set) and BIOL (the biological data set) from canonical correlation analysis. The arrows indicate the influence of the most important variables on formation of the canonical variables.

fungal and bacterial PLFAs was used as an index of the fungal/bacterial ($PLFA_{fung}/PLFA_{bact}$) biomass in the soil.

Statistical Analyses

The results are calculated per organic matter content (OM). Canonical correlation analysis (CCA), performed for each sampling date separately, was used to investigate the relationships between chemical and biological variables. It generates pairs of linear combinations from two sets of original variables such that the correlation is maximal between the pairs of the new canonical variables (Gittins, 1985). A canonical variable is a linear summary of the set of input variables (Gittins, 1985). The chemical dataset consisted of pH, Cu_{exc} , Cu^{2+} , Ni_{exc} , DM, OM, and the biological dataset of basal respiration (BR), TdR, mass loss of litter (ML), AO, IC_{50} , TdR/AO, $PLFA_{tot}$, $PLFA_{bact}$, $PLFA_{fung}$, and $PLFA_{bact}/PLFA_{fung}$, depending on which variables were determined on the sampling in question. The new canonical variables are called CHEM and BIOL. Graphical presentations of CCA are scatter plot diagrams of the sample plots on CHEM (x axis) and BIOL (y axis) (Fig. 1). Canonical structure (i.e., correlations between the original variables and canonical variables) was applied to the figure with the arrows of the original variables indicating the influence of the most important original variables on formation of the new canonical variable. Redundancy analysis, which can be seen as a part of the CCA, was used to determine the proportion of the variation that the canonical variables explain either in their own or the alternate data set (Van den Wollenberg, 1977).

Prior to the CCA the relationships between individual variables were examined by plotting the variables against each other. Log transformations were made for Cu^{2+} and Cu_{exc} variables in order to make the relationships between the variables linear. Canonical correlation analyses were performed on SAS using the CANCELL procedure (SAS Institute, 1996).

To examine the statistical difference between the treatments, one-way analysis of variance (ANOVA) was performed for

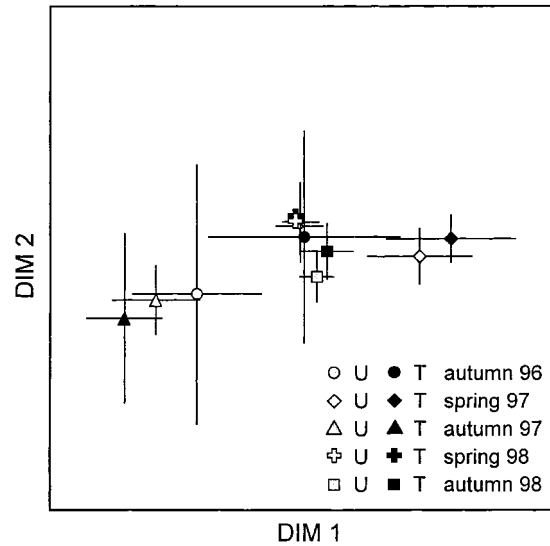


Fig. 2. Nonmetric multidimensional scaling (MDS) ordination of the sample plots with respect to sampling date and treatments. Symbols indicate the mean of 18 replicate untreated (U) and treated (T) plots, and bars indicate the standard deviation of the replicates.

the canonical variables CHEM and BIOL (Fig. 1) and for the variables measured in the soil solution (Table 2).

The PLFA pattern was explored with global nonmetric multidimensional scaling (MDS), using the program package PC-ORD (McCune and Mefford, 1999), which considers the rank order of distances (Minchin, 1987). Five repeated samplings resulted in 180 sample units and 31 PLFAs. The principle on which MDS operates is to find a representation of the data in a few dimensions, the distances in the ordination of the sample plots reflecting the (dis)similarities between the respective PLFA patterns as closely as possible. The pairwise dissimilarities were computed using a Bray–Curtis coefficient, which is widely used in community-level studies (Clarke, 1999), and has been found to be a robust measure of quantitative dissimilarity (Faith et al., 1987). Graphical presentation of MDS is in the form of scatter plot diagrams (Fig. 2 and 3) about the sample plots. The final ordination diagram is

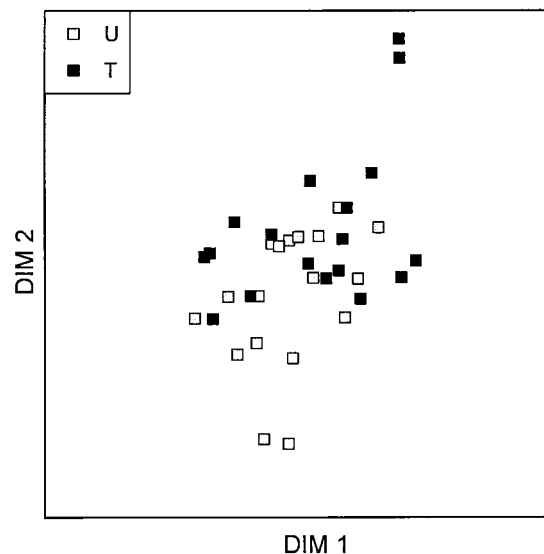


Fig. 3. Plot of the untreated (U) and the treated (T) sample plots of the last sampling date in autumn 1998 from multidimensional scaling (MDS) ordination in Fig. 2.

Table 1. Chemical and biological variables of the heavy metal-polluted organic soil on untreated (U) and treated (T) plots. Variables were analyzed during three growing seasons after the remediation treatment (the addition of mulch).

Variable†	Autumn 1996		Spring 1997		Autumn 1997		Spring 1998		Autumn 1998	
	U	T	U	T	U	T	U	T	U	T
DM (g kg ⁻¹)	670 (20)‡	620 (20)	530 (20)	510 (20)	570 (20)	460 (10)	480 (40)	530 (30)	460 (10)	450 (20)
OM (g kg ⁻¹)	270 (20)	260 (30)	350 (30)	380 (30)	480 (20)	520 (20)	440 (40)	460 (30)	540 (20)	530 (30)
pH	4.1 (0.0)	4.8 (0.1)	4.4 (0.1)	5.4 (0.1)	4.0 (0.0)	5.4 (0.1)	4.3 (0.4)	4.8 (0.2)	4.2 (0.0)	5.3 (0.1)
Cu _{exc} (μg g ⁻¹)§	4480 (190)	3850 (180)	4140 (110)	3030 (130)	3950 (80)	3200 (150)	3880 (120)	2610 (180)	3800 (100)	2860 (120)
Ni _{exc} (μg g ⁻¹)	515 (26)	464 (24)	412 (28)	317 (16)	378 (25)	380 (20)	390 (40)	432 (32)	391 (26)	372 (20)
BR (μg CO ₂ g h ⁻¹)	7.3 (0.3)	7.8 (0.5)	10.1 (1.1)	11.3 (1.5)	6.2 (0.2)	10.3 (0.6)	9.7 (0.5)	17.5 (3.0)	6.7 (0.3)	10.5 (0.4)
TdR (mol × 10 ⁻¹¹ g ⁻¹ h ⁻¹)	nd¶	nd	-1.2 (0.1)	nd	2.3 (0.2)	3.0 (0.1)	-2.2 (0.3)	2.6 (0.2)	-1.7 (0.1)	-2.6 (0.1)
IC ₅₀	nd	nd	nd	nd	1.6 (0.1)	4.6 (0.4)	9.9 (1.0)	10.7 (0.7)	4.1 (0.5)	6.0 (0.4)
AO (cell × 10 ¹⁰ g ⁻¹)	nd	nd	nd	nd	2.6 (0.2)	3.6 (0.6)	2.4 (0.3)	2.6 (0.3)	4.7 (0.4)	5.5 (0.5)
TdR/AO (mol × 10 ⁻²²)	1790 (80)	1590 (80)	1950 (80)	1810 (60)	1260 (70)	1300 (60)	1580 (120)	1510 (120)	1610 (70)	1540 (80)
PLFA _{tot} (nmol g ⁻¹)	684 (25)	594 (26)	708 (30)	647 (22)	541 (33)	553 (29)	579 (45)	544 (42)	634 (29)	597 (31)
PLFA _{bact} (nmol g ⁻¹)	54.1 (2.5)	49.8 (4.7)	35.5 (2.1)	31.5 (1.7)	29.9 (1.7)	30.6 (2.1)	45.1 (5.0)	47.2 (4.4)	36.2 (4.2)	32.6 (1.9)
PLFA _{fung} /PLFA _{bact}	0.08 (0.00)	0.08 (0.01)	0.05 (0.00)	0.05 (0.00)	0.06 (0.00)	0.06 (0.00)	0.08 (0.01)	0.09 (0.01)	0.06 (0.01)	0.05 (0.00)
ML (%)	nd	nd	nd	nd	nd	nd	nd	nd	47 (0.3)	51 (0.3)

† DM, soil dry weight; OM, organic matter content; Cu_{exc}, Ni_{exc}, exchangeable Cu and Ni, respectively; BR, microbial respiration activity; TdR, bacterial growth rate; IC₅₀, bacterial copper tolerance; AO, number of bacterial cells; TdR/AO, specific bacterial growth rate; PLFA_{tot}, total microbial biomass; PLFA_{bact}, bacterial biomass; PLFA_{fung}/PLFA_{bact}, ratio of fungal to bacterial biomass; ML, litter decomposition (i.e., percent of litter weight loss).

‡ The standard error of the mean (n = 18) is given in parentheses.

§ Results are calculated per mass of organic matter (OM).

¶ nd, not determined.

interpreted as follows: the closer the two sample plots are on the ordination, the more similar is their PLFA pattern. The vector fitting procedure was performed with the PC-ORD program in order to study the correlation between the ordination and the environmental variables that could possibly separate the treatments or the sampling dates (pH, Cu_{exc}, DM, OM).

Prior to MDS, the mole percentages of the PLFA values were double-square root transformed (y^{0.25}) to down-weight the influence of very abundant PLFAs. The single missing PLFA values (45) were replaced by the mean of each sampling date. A total of 18 outlier samples, of which 16 were from the third sampling, were excluded. PC-ORD autopilot mode with medium thoroughness was used to compute the ordination.

RESULTS

Chemical Analyses

During the first summer, application of the mulch increased the pH of the organic layer in 17 wk from 4.1 to 4.8 (Table 1). In the following spring the pH was 4.4 on the untreated (U) and 5.4 on the treated (T) plots. The Ni_{exc} concentration in the organic layer did not vary systematically between the treatments. The Cu_{exc} concentration was lower on the T plots from the first sampling onward, the Cu_{exc} concentration on the last sampling being 2860 ± 120 and 3800 ± 100 μg g⁻¹ for the T and U plots, respectively. The Cu²⁺ concentration in the soil solution was clearly lower (p < 0.001) on the T plots (1.6 ± 0.2 mg L⁻¹) than on the U plots (9.1 ± 0.9 mg L⁻¹) on the last sampling (Table 2). At the same time, the proportion of Cu_{comp} out of total Cu and the concentration of DOC in the soil solution were higher (p < 0.001) on the T plots than on the U plots. The values for the T and U plots for Cu_{comp}/Cu_{tot} were 0.41 ± 0.04 and 0.04 ± 0.01, and for DOC 134 ± 8 and 76 ± 4 mg L⁻¹, respectively.

Toxicity Test

The toxicity of the soil solution to bacteria on the T plots 3 yr after application of the mulch was lower (p < 0.001) than that on the U plots. When the soil solution from the T plots was added to a bacterial suspension extracted from unpolluted humus the bacterial growth rate was higher than that with the soil solution from the U plots. The [³H]-thymidine incorporation for the T and U plots was 2.3 and 1.4 ± 0.1 × 10⁻¹¹ mol [³H]-thymidine g⁻¹ h⁻¹, respectively (Table 2). There was strong correlation between [³H]-thymidine incorporation and the Cu²⁺ concentration in the soil solution (Pearson; r = -0.72).

Table 2. Results of the soil solution of organic soil from the experimental plots in autumn 1998.

Variable†	Untreated	Treated
DOC (mg L ⁻¹)	76 (4)‡a***	134 (8)b
Cu ²⁺ (mg L ⁻¹)	9.1 (0.9)a	1.7 (0.2)b
Cu _{comp} /C _{tot}	0.04 (0.01)a	0.41 (0.04)b
TdR (mol × 10 ⁻¹¹ h ⁻¹)	1.4 (0.1)a	2.3 (0.1)b

*** Means followed by a different letter, for each row, are significantly different (p < 0.001).

† DOC, dissolved organic carbon; Cu_{comp}/Cu_{tot}, proportion of complexed Cu out of total Cu; TdR, toxicity of soil solution (i.e., the growth rate of bacterial suspension (TdR) extracted from unpolluted humus after exposing them to soil solution containing heavy metals).

‡ The standard error of the mean (n = 18) is given in parentheses.

Microbial Activities

The microbial activity measured as basal respiration was slightly higher on the T plots than on the U plots one summer after addition of the mulch (Table 1). The difference between the treatments subsequently increased, the values at the last sampling being 10.5 ± 0.4 and $6.7 \pm 0.3 \mu\text{g CO}_2 \text{ g h}^{-1}$, respectively. Litter decomposition was faster on the T plots after three growing seasons, the loss in weight of the needles on the T plots being $50.9 \pm 0.3\%$ and on the U plots $46.5 \pm 0.3\%$. The bacterial growth rate, the number of cells, and the specific growth rate per bacterial cell were higher on the T plots on each sampling occasion that they were measured. There was a slight difference between the treatments with respect to bacterial copper tolerance after 1 yr. The tolerance on the T plots decreased subsequently, the IC_{50} values at the last sampling being -2.6 ± 0.1 and $-1.7 \pm 0.1 \log M \text{ Cu}$ for the T and U plots, respectively. The indicators of bacterial and fungal biomass determined on the basis of the PLFA analysis were not affected by the treatments.

Canonical Correlation Analysis

The canonical correlation analysis (CCA) of the chemical and biological variables for the last sampling is presented in Fig. 1. The exchangeable Ni concentration had no effect on the CCA, and was excluded from the CCA for the last sampling. Copper in ionic form (Cu^{2+}) was selected to represent the pollution in CCA.

The canonical structure, providing the correlations of the original variables with their first canonical variables, and the proportion of explained variances, are presented in Table 3 for the last sampling date. The first canonical variable (CHEM), formed from the chemical data set, explained 48% of the total variance in the chemical data set, suggesting that the first canonical variable provided a fairly effective summary of the original chemical variables. The first biological canonical variable (BIOL)

Table 3. Summary of the canonical correlation analysis in autumn 1998.

Chemical variable†	CHEM	Biological variable†	BIOL
pH	0.97‡	BR	0.91
Cu^{2+}	-0.91	TdR	0.89
DM	-0.17	ML	0.78
OM	-0.01	AO	0.58
		IC_{50}	-0.85
		TdR/AO	0.26
		PLFA_{tot}	-0.14
		$\text{PLFA}_{\text{bact}}$	-0.17
		$\text{PLFA}_{\text{fung}}$	-0.23
		$\text{PLFA}_{\text{bact}}/\text{PLFA}_{\text{fung}}$	-0.15
%C§	48	%B	33
%B	45		

† DM, soil dry weight; OM, organic matter content; BR, microbial respiration activity; TdR, bacterial growth rate; IC_{50} , bacterial copper tolerance; AO, number of bacterial cells; TdR/AO, specific bacterial growth rate; PLFA_{tot} , total microbial biomass; $\text{PLFA}_{\text{bact}}$, bacterial biomass; $\text{PLFA}_{\text{fung}}$, fungal biomass; $\text{PLFA}_{\text{fung}}/\text{PLFA}_{\text{bact}}$, ratio of fungal to bacterial biomass; ML, litter decomposition (i.e., percent of litter weight loss).

‡ The correlations between the original and the first canonical variables CHEM (chemical data set) and BIOL (biological data set).

§ %C, standardized variance of the chemical variables explained by CHEM; %B, standardized variance of the biological variables explained by CHEM and BIOL.

explained less (33%) of the total variation in the biological data set. The correlation between the first canonical variables CHEM and BIOL (canonical correlation) was 0.97 ($p < 0.0001$).

The interpretation of CCA with respect to the treatments is presented in Fig. 1, where the sample plots are plotted along the first canonical axes CHEM and BIOL, the arrows describing the canonical structure (Table 3). The T sample plots are mostly situated in the right upper corner and, according to the canonical structure, are characterized by a high pH and low Cu^{2+} concentration and high microbial activity, high bacterial growth rate, and low bacterial copper tolerance. A lower pH, higher Cu^{2+} concentration and lower microbial activity and higher bacterial copper tolerance characterize the U plots.

As all the biological variables were not determined on the other sampling dates, the CCA results (not shown) are not comparable and the time trend cannot be precisely interpreted. However, the main results of the CCAs were rather similar from the second sampling onward. The separation of the treatments was clearly visible from the second sampling onward. From the second to the fifth sampling time the canonical variables CHEM and BIOL differed significantly ($p < 0.001$) between the treatments in ANOVA. The r^2 values for CHEM were 0.53, 0.82, 0.43, and 0.82 for the second, third, fourth, and fifth sampling time, respectively. The respective r^2 values for BIOL were 0.58, 0.82, 0.35, and 0.78.

Structure of the Microbial Community

The PLFA pattern was subjected to the multidimensional scaling procedure. A two-dimensional solution was selected for MDS ordination (autopilot mode in program PC-ORD), the minimum stress value obtained being 0.14. The PLFA pattern differed between the sampling dates, but not between the treatments (Fig. 2). This was further confirmed by the results of the vector fitting procedure. None of the environmental variables had significant correlation with the ordination (vectors not shown), indicating that chemical variables, which separated the treatments in CCA (i.e., the Cu_{exc} concentration and pH) did not correlate with the sample plot ordination. The variables that varied between the sampling dates (i.e., DM and OM) did not show any correlation with the ordination. However, when the T and U plots of the last sampling date were plotted (Fig. 3), the treatments were slightly separated. Overall, however, the differences in the PLFA patterns were very small (Table 4).

DISCUSSION

The aim of this bioremediation experiment was to diminish the toxicity of heavy metal-polluted soil to the microbiota. According to the results of the [^3H]-thymidine incorporation analysis, the toxicity of the soil solution to bacteria decreased on the treated plots. The decrease in toxicity is due to the conversion of toxic metal ions into less bioavailable forms (e.g., by precipita-

Table 4. Amounts of phospholipid fatty acids (PLFAs) in organic soil three growing seasons after the remediation treatment.

PLFA	Untreated	Treated
mol%		
i14:0	0.33 (0.01)†	0.38 (0.01)
14:0	2.52 (0.06)	2.49 (0.07)
i15:0	12.18 (0.19)	11.50 (0.18)
a15:0	3.22 (0.07)	3.46 (0.07)
C15:1	1.01 (0.07)	0.95 (0.07)
15:0	0.87 (0.01)	0.91 (0.02)
i16:1	1.00 (0.06)	1.04 (0.07)
i16:0	5.00 (0.11)	5.05 (0.10)
16:1 ω 9	0.68 (0.02)	0.74 (0.02)
16:1 ω 7c	5.11 (0.07)	5.04 (0.08)
16:1 ω 7t	0.84 (0.02)	0.79 (0.02)
16:1 ω 5	2.08 (0.05)	2.27 (0.05)
16:0	16.80 (0.36)	17.19 (0.29)
br17:0	0.36 (0.01)	0.37 (0.01)
10Me16:0	8.16 (0.26)	8.23 (0.19)
i17:0	2.10 (0.05)	2.06 (0.06)
a17:0	1.43 (0.03)	1.50 (0.02)
17:1 ω 8	0.57 (0.01)	0.55 (0.01)
cy17:0	2.18 (0.04)	2.19 (0.03)
17:0	0.72 (0.01)	0.74 (0.01)
br18:0	1.58 (0.06)	1.56 (0.08)
10Me17:0	1.07 (0.05)	1.11 (0.04)
18:2 ω 6,9	1.98 (0.12)	2.12 (0.09)
18:1 ω 9	5.70 (0.10)	5.83 (0.11)
18:1 ω 7	3.12 (0.07)	3.38 (0.05)
18:1	1.01 (0.10)	1.02 (0.08)
18:0	4.09 (0.11)	4.31 (0.11)
19:1a	0.34 (0.01)	0.41 (0.01)
10Me18:0	0.98 (0.06)	0.91 (0.05)
19:1b	2.08 (0.10)	2.06 (0.09)
cy19:0	6.95 (0.11)	6.43 (0.11)
20:5	0.88 (0.04)	0.84 (0.04)
20:4	0.12 (0.01)	0.18 (0.01)
20:0	2.32 (0.08)	2.37 (0.10)

† The standard error of the mean ($n = 18$) is given in parentheses.

tion as the soil pH increases) (Alloway, 1995). The addition of mulch resulted in a one pH-unit increase in the organic layer in this experiment. The concentration of exchangeable Ni remained the same, while that of exchangeable Cu decreased by 30%. This result is in accordance with the findings of earlier studies. The concentration of exchangeable Cu was found to decrease more than that of exchangeable Ni after organic substances were added to soil (Ross, 1996). At the same polluted site as that used in this study, Derome and Nieminen (1998) reported Ni to be readily leached and Cu to be strongly retained in the organic layer.

Another important process affecting the bioavailability of metals in soils, in addition to a change in soil pH, is complexation between metals and organic substances (Alloway, 1995). Compost addition has resulted in a reduction in the phytotoxicity of soil (Vangronsveld et al., 1995b; Li et al., 2000) but, to our knowledge, metal speciation has not been investigated in field remediation studies. However, speciation has been studied after sewage sludge application. The Ni and Zn applied in sludge remained in chemical forms that were available for plant uptake, but the major portion of the Cu was partitioned into the relatively resistant organic fraction, which probably exhibits low bioavailability (Sloan et al., 1997). In compost most of the Cu is found in the organic fraction. The complexing capacity of compost was demonstrated by Giusquiani et al. (1992), who found that Cu and to a lesser extent Ni complexed with dissolved organic matter. Most of the Cu (77%) and one third of the Ni

in compost occurred in the organic fraction, while the leachable fraction of Cu was less than 10% (Tisdell and Breslin, 1995). We found that the mulch application resulted in an increase in DOC in the soil solution, as well as in Cu complexation, and a corresponding decrease in exchangeable Cu in the soil. Thus we conclude that soluble and particulate organic matter in the compost had complexed Cu into less bioavailable forms.

The decreasing Cu concentration in our field remediation experiment had reduced bacterial tolerance to Cu after two growing seasons. In laboratory conditions, bacterial tolerance to copper developed rapidly after the Cu concentration in the soil had increased (Díaz-Raviña and Bååth, 1996). However, the effect of a decreasing Cu concentration on the heavy metal tolerance of bacteria has not been studied very much. Kelly and Tate (1998), who studied bacterial tolerance to Zn by the plate count method, found that Zn-tolerant bacteria were not affected by decreasing soluble Zn concentrations.

Tolerance and adaptation of microorganisms to heavy metals are common phenomena. The increased abundance of tolerant organisms in a polluted environment can be due to genetic changes, to physiological adaptations involving no alterations to the genotype, or to the replacement of metal-sensitive species with species that are already tolerant to heavy metals (Bååth, 1989). A change in species composition has been proposed as the main reason for the change in metal tolerance of microbial populations in laboratory studies (Díaz-Raviña et al., 1994; Frostegård et al., 1993b), and in a field study carried out by Pennanen et al. (1996). In these studies, the heavy metal tolerance of the bacterial community, determined by [³H]-thymidine incorporation, was accompanied by a change in the microbial community structure, as determined by the PLFA technique. In the present study the microbial community structure showed no changes after 2 yr, and only slight changes after 3 yr. Despite this, the copper tolerance of the bacterial community decreased after 2 yr of exposure to the mulch. The PLFA pattern would have changed if the microbes from the mulch had become dominant in the polluted organic layer. Therefore, the results support the alternative hypotheses of genetic change or physiological adaptation of the Cu tolerant bacteria to diminishing toxic concentrations of heavy metals.

Recovery of the microbiota would occur if the structure of the microbial community gradually became similar to that on unpolluted sites. The PLFA patterns for the less-polluted areas at Harjavalta, studied by Pennanen et al. (1996), and for the mulched plots at our study site, showed some rather similar trends. The relative quantities of the eucaryotic (Amano et al., 1992) PLFAs, 18:2 ω 6,9 and 20:4, increased, while the PLFAs i16:0, br17:0, and br18:0, common in gram-positive bacteria (O'Leary and Wilkinson, 1988), decreased with decreasing Cu concentrations along the heavy-metal pollution gradient at Harjavalta. On the treated plots in our remediation study, the PLFAs 18:2 ω 6,9 and 20:4 also increased when the Cu concentration decreased. However, most of the PLFAs did not change as a result of the remediation treatment. Fritze et al. (1997) also

studied the impact of liming at Harjavalta on polluted soil. The PLFAs 16:1 ω 5 and 20:4 increased and i15:0, 16:1 ω 7, br18:0, and cy19:0 decreased on the limed plots, as was the case on the plots covered with mulch in this study. However, the differences in the relative abundance of PLFAs in our remediation study were very small, and therefore these signs of the recovery of the microbial community are only tentative.

A change in the microbial community was found in the remediation study of Kelly and Tate (1998), who investigated the microbial community using the BIOLOG procedure. The metabolic profiles of the sites treated with sewage sludge were clustered close to the least-contaminated sites, indicating a change in the microbial community toward unpolluted sites. However, they found no change in bacterial metal tolerance using the plate count method. In our study, the microbial community did not appear to change rapidly toward the structure of an unpolluted community, but instead the community that was adapted to heavy metals seemed to lose its heavy metal tolerance when the metal concentrations decreased.

Some similar features were found between the effects of the remediation experiment, in which the pH increased by about one unit, and forest liming. The total microbial biomass remained unchanged after liming (Frostegård et al., 1993a) and after the remediation treatment in this study. Liming has frequently been reported to increase microbial activity (Zelles et al., 1987; Persson et al., 1989; Illmer and Schinner, 1991) and the bacterial growth rate (Bååth and Arnebrandt, 1994), as was the case in this study. Forest liming (Frostegård et al., 1993a) and ash application (Bååth et al., 1995) alter the microbial community structure toward one more dominated by gram-negative bacteria. These authors found an increase in the PLFAs i14:0, 16:1 ω 5, 16:1 ω 9, 18:1 ω 7, and 19:1a, and a decrease in, for example, the PLFAs i15:0 and cy:19, as a result of limestone or ash application. Similar slight changes were also found on the mulched plots in our remediation study. However, nine PLFAs, which Frostegård et al. (1993a) and Bååth et al. (1995) reported to correlate with pH, showed an opposite change or none at all in this remediation study. Bååth et al. (1995) discussed the reason for the altered PLFA pattern after the increase in pH. They concluded that it was not pH as such that was the reason for the altered PLFA pattern, but rather the change in substrate quality and quantity (i.e., in the available soil organic matter). The amount of dissolved organic carbon increased on the treated plots in our study, indicating an increase in such substrates.

In conclusion, application of mulch to a heavy metal-polluted soil decreased the toxicity of the soil solution to bacteria. The decreased toxicity was reflected in the organic layer as increased microbial activity and bacterial growth rate, and as decreased tolerance of the bacteria to heavy metals. The variables that did not change or changed only slightly were the exchangeable Ni concentration in the soil, and the microbial biomass and the structure of the microbial community. The positive changes indicate remediation of the polluted soil. How-

ever, the mulch, which consisted of compost and woodchips, has not yet decomposed completely, and final conclusions about remediation cannot be drawn until a number of years have passed. Further research could focus on the addition of chemical agents to the mulch in order to further increase Cu immobilization, as well as that of Ni and other metals.

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Ecological Risk Assessment

Factors Affecting the Ratio of Cation Exchange Capacity to Clay Content in Lignite Overburden

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ABSTRACT

Unusually high cation exchange capacity (CEC) values relative to clay content are frequently reported for lignite overburden and minesoils. The CEC to percent clay ratio is commonly greater than one and would require that the average charge of the clay fraction be greater than 100 cmol_c kg⁻¹. A comparison of methods for particle-size distribution suggests that the major reason lignite overburden samples have CEC to percent clay ratios greater than one is incomplete dispersion of aggregates of clay minerals or shale fragments. Preliminary investigations revealed the presence of shale fragments, smectite, and partially weathered mica in the silt fraction. Methods commonly used in soil textural analysis underestimated clay content by approximately 24%. The silt fraction may, therefore, provide a “hidden” source of CEC. Another important factor influencing the CEC to percent clay ratio was the presence of organic materials (lignite) in the samples. Lignite may make a significant contribution to CEC in overburden materials. In a study designed to estimate the pH-dependent charge of both the mineral and organic fractions, the CEC of overburden organic constituents was determined to be approximately 158 cmol_c kg⁻¹ at pH 8.2. The high CEC to percent clay ratio in lignite overburden and minesoils may be resolved by adjusting methods for clay determination to optimize dispersion and by accounting for CEC due to organic materials. An alternative approach is to use existing methodology and use correction factors to account for incomplete dispersion of clay minerals and the charge contributions of organic materials.

THE Railroad Commission of Texas uses the CEC to percent clay ratio of minesoils and overburden samples as one measure of the adequacy of laboratory data submitted by the mining companies. Cation exchange capacity to percent clay ratios greater than one are frequently reported for overburden and minesoils in the Gulf Coast lignite region. This suggests a clay fraction dominated by minerals with CEC greater than 100 cmol_c kg⁻¹ (vermiculite and/or high charge smectite), excessive levels of organic materials (lignite), or errors in the method of CEC, percent clay, or organic carbon determination.

The mineralogy of overburden and native soils overlying Texas lignite has been investigated and described

by several researchers (Askenasy, 1977; Dixon et al., 1980; Senkayi et al., 1983; Arora et al., 1984). The most commonly occurring clay minerals in fluvial overburden sediments are smectite, kaolinite, mica, vermiculite, and chlorite (Dixon et al., 1980; Senkayi et al., 1983; Arora et al., 1984). Distribution of these minerals varies widely with depth and location.

Smectite is an important mineral in Wilcox Group overburden. Arora et al. (1984) found smectite to be the predominant clay mineral in the fine clay fraction of most east Texas lignite overburden samples with amounts ranging from 21 to 46%. High charge smectite has been identified in these overburden materials (Egashira et al., 1982; Senkayi et al., 1985). These researchers suggest that this mineral may be the result of mica weathering. High charge smectite has charge properties intermediate between those of smectite and vermiculite with a layer charge near that of vermiculite. Kaolinite is the most abundant mineral in the coarse clay (2–0.2 μm) size fraction and the second most abundant, after smectite, in the fine clay fraction of Wilcox Group overburden (Arora et al., 1984). Mica is common in the clay fraction of these overburden materials. Mica is generally present in lower quantities than either smectite or kaolinite (Dixon et al., 1982). Chlorite has been identified in the <2.0-μm clay fraction of Texas lignite overburden (Askenasy, 1977; McAllister, 1981; Senkayi et al., 1981, 1983; Arora et al., 1984) and occurs primarily in the reduced overburden zone (Senkayi et al., 1983). The sand and silt fractions of these materials consist primarily of quartz and feldspars with lesser amounts of mica, kaolinite, and smectite (Arora et al., 1984). Other minerals may include gypsum, jarosite, barite, calcite, siderite, magnesite, dolomite, rhodochroite, and pyritic minerals (Dixon et al., 1982). Also siderite may weather to form goethite, hematite, and todorokite (Senkayi et al., 1986).

Reasonable hypotheses for the rather high CEC to percent clay ratios include incomplete dispersion of aggregates of clay minerals in the determination of clay content, the presence of lignite or other organic material not readily oxidized by the conventional method of or-

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