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Academic dissertation in Environmental Protection Science
Faculty of Agriculture and Forestry
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Front cover: A mulched plot at Harjavalta. Photograph by M. Salemaa (2000).

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Original publications

This thesis is based on the following articles, referred to in the text by their Roman numerals:

I Kiikkilä, O. Pennanen, T., Pietikäinen, J., Hurme K-R. and Fritze, H. 2000. Some observations on the copper tolerance of bacterial communities determined by the (^{3}H)-thymidine incorporation method in heavy metal polluted humus. Soil Biology & Biochemistry 32: 883–885.

II Kiikkilä, O., Pennanen, T., Perkiömäki, J., Derome, J. and Fritze, H. 2002. Organic material as a copper immobilising agent: a microcosm study on remediation. Basic and Applied Ecology (in press).

III Kiikkilä, O., Perkiömäki, J., Barnette, M., Derome, J., Pennanen, J., Tulisalo, E. and Fritze, H. 2001. In situ bioremediation through mulching of soil polluted by a copper-nickel smelter. Journal of Environmental Quality 30: 1134–1143.

IV Kiikkilä, O., Derome, J., Brügger, T., Uhlig, C. and Fritze, H. 2002. Copper mobility and toxicity of soil percolation water to bacteria in a metal polluted forest soil. Plant and Soil (in press).

Studies were carried out mainly by Oili Kiikkilä. Taina Pennanen was responsible for the phospholipid fatty acid analyses and John Derome for the fractionation of copper.

List of abbreviations

d.m.	dry matter weight
o.m.	organic matter weight
Cu_{exc}	exchangeable copper
Cu_{comp}	complexed copper
Cu_{tot}	total copper
BR	microbial respiration activity
m.l.	mass loss of litter, litter decomposition
TdR	[^{3}H]-thymidine incorporation rate describing bacterial growth rate
IC_{50}	inhibition concentration describing bacterial copper tolerance
AO	the number of bacterial cells
PLFA	phospholipid fatty acid
mol%	the relative abundance of the PLFA in question
$PLFA_{tot}$	an indicator of microbial biomass
$PLFA_{bact}$	an indicator of bacterial biomass
$PLFA_{fung}$	an indicator of fungal biomass

Abstract

Remediation through mulching with organic matter of soil polluted by a copper-nickel smelter

Remediation of soil polluted by a Cu-Ni smelter was studied by adding organic matter onto polluted soil. The study sites were at 2 km (laboratory experiment) and 0.5 km (field experiment) distance from the Outokumpu Harjavalta smelter which has had a severe effect on the forest ecosystem. The total copper concentrations in the organic soil were 1 600 mg kg^{-1} dry matter (2 km) or 5 800 mg kg^{-1} dry matter (0.5 km). A laboratory microcosm study with nine different organic mulch materials and a field study with one, a mixture of compost and woodchips, were carried out. The success of remediation treatments was assessed by measuring exchangeable copper concentration in the soil, the speciation of copper in soil water, and the toxicity of soil water to bacteria. An important objective in remediation is the recovery of microbial activities, since the nutrient cycling in heavy metal polluted soil is in general disturbed. The microbial activity, the number of bacterial cells, the bacterial growth rate, the bacterial copper tolerance and the structure of the microbial community in the organic soil were studied during a 16 months period in the laboratory and a 27 months period in the field. The toxicity of the soil water to bacteria, the bacterial growth rate and copper tolerance was measured using the [^{3}H]-thymidine incorporation method. The procedure to prepare the bacterial suspension for the [^{3}H]-thymidine incorporation into the macromolecules of bacteria was modified for the soils of low microbial activity.

During the 16 months of laboratory incubation the exchangeable copper concentration in the polluted soil remained on the same level in the control and in the treatments involving barkchips, humus, or peat. The exchangeable copper concentration in the soil decreased during the incubation when the soil was mulched with compost, mixture of compost and woodchips, mixture of compost and barkchips, sewage sludge, or garden soil. However, the Cu^{2+} concentration in soil water or the toxicity of soil water to bacteria was on the same level in all the treatments after 16 months of incubation. No microbial response direct attributable to remediation was detected in the laboratory.

In the field, mulching the polluted soil with the mixture of compost and woodchips converted copper into less toxic forms, which was detected as a lower exchangeable Cu concentration in the soil, a lower Cu^{2+} concentration in the soil water, and as decreased toxicity of soil water to bacteria after

mulching. This was mainly a consequence of increased complexation with particulate and dissolved organic matter, and increased soil pH. However, also leaching of copper down the soil profile increased slightly after mulching the forest floor. The microbial response to remediation was clear. The microbial activities increased and the tolerance of the bacteria to Cu decreased in the organic layer. Since the structure of the microbial community did not differ between the treatments it was the original microbiota that became measured.

Mulching the exposed mineral soil after the removal of the polluted organic layer had a similar but greater influence on Cu speciation and the toxicity of soil water than mulching the forest floor. However, also the leaching of Cu down the soil profile was the highest when the exposed mineral soil was mulched. Thus mulching the polluted forest floor was a more successful remediation treatment.

1. Harjavalta as a case study of environmental pollution

In Finland, the largest heavy metal polluted areas are situated around Harjavalta and Tornio (Kubin *et al.* 2000) where the area of forest death is less than 1 km^2. The largest heavy metal polluted area in the northern hemisphere is situated in NW Russia in the Kola Peninsula where the area of forest death is 600–1 000 km^2 and the area of visible damage is 39 000 km^2 (Oleksyn and Innes 2000). Another large area is found in Sudbury, Canada, where mining and smelting activities have created 170 km^2 of barren land and 700 km^2 of stunted open woodland (Winterhalder 2000).

Many studies on the effects of heavy metals on the forest ecosystem have been performed at Harjavalta, in SW Finland, which is part of the southern boreal coniferous zone. Harjavalta is situated on an esker that runs to the SE of the Cu-Ni smelter. Along the forested esker runs the pollution gradient, from 0.5 to 8 km in length, which has often been examined. The forest on the esker consists mainly of Scots pine, *Pinus sylvestris* L., and is situated on dryish, relatively nutrient-poor sites (Mälkönen *et al.* 1999). According to the Finnish forest site type classification (Cajander 1949), the forest along the esker varies from *Vaccinium* to *Calluna* type (Derome and Lindroos 1998b). According to Mälkönen *et al.* (1999) the soil comprises of sorted glaciofluvial sediments and the texture of the mineral soil is classed as sorted fine or fine/coarse sand with no stones. The soil type is ferric podzol, with organic mor layer (F+H) ranging between 1 to 3 cm, an E horizon 6 to 15 cm, and a B_s horizon 26 to 39 cm, in thickness.

This section reviews the work on heavy metal deposition and the effects of pollution on the forest ecosystem that has been published in scientific journals since 1975. The aim is to outline the extent the metal pollution affects the ecosystem.

1.1 Emissions, deposition and contamination

A copper smelter started to operate at Harjavalta in 1945 and a nickel smelter in 1960. In addition to copper and nickel, the emissions contain also zinc, lead, cadmium, arsenic, mercury, and sulphur (Table 1).

Elevated Cu concentration in forest mosses was detected as far as 30–40 km distance from the smelter between 1985 and 1995 (Kubin *et al.* 2000). With moss bags, a method where moss (*Spaghnum sp.*) bags hang in trees to collect deposition, was observed high airborne pollution of copper, nickel,

Table 1. Annual emissions from Harjavalta smelter. Emissions before 1985 are estimated. (Data from Outokumpu Harjavalta Metals Ltd)

Year	Dust	Cu	Ni	Zn	Pb	As
	t year^{-1}					
1945-84	1100	98	47	216	55	
1985	1100	98	47	216	55	
1986	1200	126	46	232	60	
1987	1800	140	96	162	94	
1988	1000	104	45	103	48	
1989	1000	80	33	190	70	
1990	960	80	31	160	80	
1991	640	80	14	90	45	
1992	280	60	10	12	9	
1993	250	50	7	13	6	11
1994	190	40	6	6	3	5
1995	70	17	1.4	1.7	0.5	0.2
1996	195	49	1.2	5.3	1.9	4.2
1997	360	70	3	14	4	10
1998	132	23	1.7	6.1	2.4	10
1999	48	6	0.8	4.2	1.0	1.8

zinc, lead, cadmium (Hynninen 1986) and mercury (Hynninen and Lodenius 1986) between 1981 and 1982 up to the distance of 9 km. Up to 4 km during 1992–1996 bulk deposition in open areas and stand throughfall, i.e. deposition inside the stand, was contaminated with sulphate and heavy metals (Derome and Nieminen 1998). The deposition of Cu was high close to the smelter the bulk deposition being 160 mg m^{-2} y^{-1}, and stand throughfall 360 mg m^{-2} y^{-1} (Derome and Nieminen 1998). At 8 km both the bulk deposition and stand throughfall were ca. 3 mg m^{-2} y^{-1}. The respective values for Ni close to the smelter were 70 and 140 mg m^{-2} y^{-1} and for Zn 20 and 40 mg m^{-2} y^{-1}, whilst at 8 km the values were ca. one tenth of these. In addition to emissions the dust from the degraded forest floor and slagheaps located near-by increased the deposition of metals (Nieminen *et al.* 1999).

A clear, logarithmically decreasing gradient, studied at the distances of 0.5, 2, 4 and 8 km, was found in the forest soil for Cu, Ni, Zn, Cd, Fe, Pb, Cr and S (Derome and Lindroos 1998b). Elevated concentrations of heavy metals were also found in peatlands near the smelter (Veijalainen 1998; Nieminen *et al.* 2001) (Table 2). The total Cu concentration in the organic layer of the forest soil was 5 800 mg kg^{-1} d.m. (Derome and Lindroos 1998b) and the exchangeable ($BaCl_2$) Cu concentration was 4 700 mg kg^{-1} d.m. at 0.5 km from the smelter (Derome and Nieminen 1998). The respective values for Ni were 460 and 420 mg kg^{-1} d.m. and for Zn 520 and 130 mg kg^{-1} d.m.

Table 2. Total concentrations of Cu, Ni, Zn and Cd in soil near the smelter (0.5–1 km). The concentration in the reference area (at 8 km or further) is in parentheses.

	Cu	Ni	Zn	Cd	Reference
	mg kg^{-1} d.m.				
Peat					Veijalainen 1998
Surface	3600 (170)	470 (50)	460 (86)	3.7 (0.9)	
0–10	1200 (75)	240 (16)	240 (16)	3.7 (0.9)	
10–20	180 (10)	5 (0)	110 (29)	1.5 (0.1)	
Peat					Nieminen *et al.* 2001
2 cm	4400 (6)	870 (3)	560 (53)		
14 cm	45 (5)	260 (7)	570 (60)		
Soil					
Organic layer	5800 (150)	460 (40)	520 (60)	4.6 (0.7)	Derome and Lindroos 1998b
Organic layer[a]	4700 (120)	420 (37)	130 (47)		Derome and Lindroos 1998
Mineral Soil					Derome and
0–5 cm	270 (1.5)	25 (0)	10 (1)		Lindroos 1998
5–10 cm	27 (0.4)	5.4 (0)	2.9 (0.4)		
10–20 cm	16 (0)	3.2 (0)	1.8 (0)		
20–30 cm	12 (0.2)	2.1 (0)	1.4 (0)		
Percolation water					Derome and
5 cm	0.6 (0.02)	0.5 (0.01)			Lindroos 1998a
20 cm	1.1 (0.01)	0.9 (0)			

[a] Exchangeable ($BaCl_2$) metals

(Table 2). Total Fe concentration in organic soil was 18 600 mg kg^{-1} d.m. (Derome and Nieminen 1998). Uhlig *et al.* (2001) found extremely high total Cu concentration in organic soil under *Empetrum nigrum* patches, 49 000 mg kg^{-1} d.m. at 0.5 km and 12 000 mg kg^{-1} d.m. at 4.0 km. At the vertical scale, leaching of Cu, Ni, Zn and SO_4-S down to 40 cm depth in the soil profile was observed, Zn being the most mobile element and Cu being strongly bound to organic layer (Derome and Nieminen 1998).

Cu, Ni, Zn and Cd concentrations in different trophic levels have been reported to be high near the smelter. Cu has usually been found in higher concentrations than Ni or Zn although Zn has been emitted more than Cu until 1992 (Table 1). Elevated heavy metal concentration have been reported in understorey vegetation (Helmisaari *et al.* 1995), pine (Derome and Nieminen 1998), spruce (Heliövaara and Väisänen 1991), birch (Koricheva and Haukioja 1995), insects (Heliövaara *et al.* 1990), spiders (Koponen and Niemelä 1995), and birds (Eeva and Lehikoinen 1995).

1.2. Vegetation

The Scots pine forest stand close to the smelter has clearly suffered from pollution. The tree growth has been extremely poor (Mälkönen *et al.* 1999) and the understorey vegetation has drastically changed (Salemaa *et al.* 2001). The total coverage and the number of species decreased towards the smelter and vegetation was almost absent up to a distance of 0.5 km from the smelter (Salemaa *et al.* 2001) (Table 3). On the most polluted sites, *Empetrum nigrum*, *Arctosthaphylus uva-ursi*, and *Vaccinium uliginosum*, clonal dwarf shrubs, have survived in small patches. Vigorous regrowth and phenotypic plasticity have improved the survival of *A. uva-ursi* and *V. uliginosum* (Salemaa *et al.* 1999). *E. nigrum* possesses an internal heavy metal tolerance (Monni *et al.* 2000a). However, decreased chlorophyll and organic acid, and an increased abscisic acid concentration in stems and leaves indicated a reduction in the physiological activity of *E. nigrum* near the smelter (Monni *et al.* 2000c). The tolerance mechanisms of *E. nigrum* may include accumulation of heavy metals in older tissues, the restriction of the metal transport to the green leaves (Uhlig *et al.* 2001), localisation of metals in certain cell compartments (vacuoles, cell walls, cytoplasm), possible detoxification of metals by phenolics (Monni *et al.* 2001), and accumulation and immobilisation of metals in the litter beneath *E. nigrum* patches (Uhlig *et al.* 2001). Of the dwarf shrubs, *Calluna vulgaris*, growing first at 1.2 km to the NW of the smelter, proved to be least resistant to Cu (Monni *et al.* 2000b). Although germinable seeds of *C. vulgaris, Betula pubescens*, *Pinus sylvestris* and *V. uliginosum* were found in the most contaminated soil, seedlings of trees and dwarf shrubs were absent close to the smelter (Salemaa and Uotila 2001).

With regards to moss, *Pohlia nutans* and *Ceratodon purpureus* were the only moss species surviving in small patches on the most contaminated site (Salemaa *et al.* 2001) (Table 3). At 8 km distance the frequency of *Pleurozium schereberi* and *Dicranum spp.* began to increase (Salemaa *et al.* 2001). However, the Cu concentration in their tissues were still considerably higher at 8 km than those in background areas (Helmisaari *et al.* 1995). The reindeer lichens (*Cladina spp.*) appeared to be more tolerant than forest mosses, they increased in frequency at 4 km (Salemaa *et al.* 2001). Epiphytic lichens were absent up to 2 km, on an area of 8.8 km^2, in 1970 (Laaksovirta and Silvola 1975) and up to 4 km in 1987 (Fritze *et al.* 1989).

Table 3. The change in species abundance close to the smelter, - damaged, + benefit. The number in the parentheses refers to the distance where the point frequency % is more than 0.02. + in parentheses refers to the benefit in the moderately polluted area. The year in the parentheses refers to the study year. [a]rare, except at the distance in the parentheses

Species		**Reference**
Vascular plants		
Arctostaphylus uva-ursi (L.) Sprengel	- (2)	Salemaa *et al.* 2001 (1993)
Calluna vulgaris (L.) Hull	- (4)	
Carex clobularis L.	(1)[a]	
Empetrum nigrum L.	- (1)	
Pinus sylvestris	- (0.5)	
Vaccinium uliginosum L.	- (1)	
Vaccinium vitis idaea L.	- (1)	
Mosses		
Dicranum polysetum Sw.	- (8)	Salemaa *et al.* 2001 (1993)
Dicranum scoparium Hedw.	- (8)	
Cerantodon purpureus (Hedw.) Brid.	- (1)	
Polytrichum juniperum Hedw.	(1)[a]	
Pleurozium schreberi (Brid.) Mitt.	- (8)	
Pohlia nutans (Hedw.) Lindb.	- (0.5)	
Ground lichens		
Cetraria islandica (L.) Ach.	- (2)	Salemaa *et al.* 2001 (1993)
Cladina rangiferina (L.) Nyl.	- (3)	
Cladina arbuscula (Wallr.) Hale&W.L.Club	- (3)	
Cladina stellaris (Opiz) Brodo	- (4)	
Cladonia spp.	- (2-3)	
Epiphytic lichens		
Hypogymnia physodes L.	- (4)	Fritze *et al.*1989 (1987)
Pseudevernia furfuracea (L.) Zopf	- (4)	
Usnea hirta (L.) Wigg.	- (7)	
Bryoria fuscescens (Gyelnik) Brodo & Hawksw	- (7)	
Platismatia glauca (L.) Culb&Culb	- (7)	
Epiphytic algae		
Scoliciosporum chlorococcum	+	Fritze *et al.*1989 (1987)
Endophytic fungi		
Cenangium ferrucinosum Fr:Fr	-	Helander 1995 (1992)
endophtic fungi total	-	Lappalainen *et al.* 1999 (1993-94)
Hormonema sp.	+	
Fusicaldium sp.	-	
Gnomonia setacea (Pers.) Ces. and de Not	-	
Soil animals		
Enchytraeids (Oligochaeta, Enchytraeidae)	-	Haimi and Siira-Pietikäinen 1996
Microarthropods	-	(1993-94)
Collembolans (Collembola)	-	
Nematodes (Nematoda)	-	

Table 3. (continued)

Bark bug (Heteroptera, Aradidae)		
Aradus cinnamomeus Panzer	- (+)	Heliövaara and Väisänen 1990a (1987-89)
Tortricid moths (Lepidoptera, Tortricidae)		
Retinia resinella L.	- (+)	
Rhyacionia pinicolana Doubleday	- (+)	
Blastesthia turionella L.	- (+)	
Blastesthia posticana Zetterstedt	- (+)	
Leaf- miners (Lepidoptera, Eriocraniidae)		
Eriocrania, solitary species	-	Koricheva 1994 (1992-93)
Eriocrania cicatricella Zetterstedt	+	
Geometrid moth (Lepidoptera, Geometridae)		
Epirrita autumnata Bkh.	-	Ruohomäki *et al*, 1996 (1990)
Aphids (Homoptera)		
Cinaria pini L.	+	Heliövaara and Väisänen, 1990a
Pineus pini Gmelin	+	Heliövaara and Väisänen, 1989b (1987)
Schizolachnus pineti Fabricius	+	
Diprionid (Hymenoptera, Diprionidae)		
Diprion pini L.	- (+)	Heliövaara *et al.* 1990
Ants (Hymenoptera, Formicidae)	(+)	Koponen and Niemelä 1995 (1992)
Formica fusca L. or *F. lemani* L.	+	Koricheva *et al.* 1995 (1993)
Beetles (Coleoptera, Scolytidae)		
Xylechinus pilosus Ratzb.	+	Heliövaara and Väisänen 1991
Tomicus piniperda L.	+	
Pityogenes chalcographus L.	-	
ground living beetles (Coleoptera)	-	Koponen and Niemelä 1995 (1992)
Coccinella septempunctata L.	+	
Mites (Acarina, Eriophyidae)		
Aceria leionotus Nalepa	-	Koricheva *et al.* 1996 (1993)
Aceria longisetosus Nalepa	-	
Acalitus rudis Canestrini	-	
Aceria varia Nalepa		
Eriophyes diversipunctatus Nalepa		
Phyllocoptes populi Nalepa		
Spiders (Araneae)		
Xerolycosa nemoralis Westring	+	Koponen and Niemelä 1993
Alopecosa aculeata Clerck	-	
Oedothorax apicatus Blackwall	+	
Erigone atra Blackwall	+	
Agyneta rurestris C.L. Koch	+	
Zelotes petrensis C.L. Koch	-	
Tapinocyba pallens O.P.-Cambridge	-	
Silometopus elegans O.P.-Cambridge	-	
Walckenaeria antica Wider	-	
Walckenaeria atrotibialis O.P.-Cambridge	-	
Birds		
Parus major L.	-	Eeva and Lehikoinen 1996 (1993)
Ficedula hypoleuca Pallas	-	

1.3. Nutrient cycling and soil organisms

Inhibition of nutrient cycling and the displacement of base cations from cation exchange sites by Cu and Ni cations has resulted in a decrease of base cation (Ca, Mg, K) concentrations in the organic layer (Derome and Lindroos 1998b). Trees have not been able to utilise the nutrient pools in the mineral soil presumably due to the toxic effects of Cu and Ni in the plant fine roots, including ectomycorrhizal root tips (Helmisaari *et al.* 1999) since Mg, Ca, and Mn concentrations in Scots pine needles were low (Derome and Nieminen 1998). In contrast, trees obtained sufficient K from the soil, since despite K leached from the needle tissues close to the smelter, the needle K concentrations were relatively high (Nieminen *et al.* 1999). Autumnal nutrient retranslocation, i.e. transport of nutrients from the senescing needles to the remaining organs for overwinter storage, of P and K in Scots pine was less efficient close to the smelter than at 8 km (Nieminen and Helmisaari 1996). The retranslocation of nutrients was suggested to be inhibited by non-pathogenic endophytic fungi (Wilson 1993). However, endophytes seemed not to be a reason for the decreased nutrient retranslocation since the number of endophyte infected needles was lower close to the smelter than further away (Helander *et al.* 1995).

The number of soil animals has clearly decreased (Table 3) and their community structure strongly altered close to the smelter (Haimi and Siira-Pietikäinen 1996). Since at 2 km the number of soil animals has only slightly decreased, soil animals appeared to be quite resistant to heavy metals. An indication of increased Cu resistance of the enchytraeid worm, *Cognettia sphagnetorum*, Vejdovsky, usually the only abundant enchytraeid species found in northern coniferous forest soils, has been found near the smelter (Salminen and Haimi 2001). It seems that the presence of patches of lower metal concentrations was mitigating the effects of the metals on enchytraeid populations (Salminen and Haimi 1999).

The overall microbiological activity in the soil has decreased drastically near the smelter. Microbial respiration activity, the amount of fungal hyphae, starch, pectin, xylan and cellulose hydrolysers, i.e. physiological groups of bacteria (Fritze *et al.* 1989), microbial biomass, measured as fumigation-extraction and substrate induced respiration, ATP content, and fungal biomass, measured as ergosterol concentration (Fritze *et al.* 1996) decreased towards the smelter. The toxicity of a soil extract, measured with a standard *Photobacterium phosphoreum* test, increased towards the smelter (Vanhala and Ahtiainen 1994). The structure of the microbial community, measured with phospholipid fatty acid analysis, had changed and the bacterial community was highly resistant, measured using thymidine incorporation

method, to Cu but not to Cd, Ni, or Zn (Pennanen *et al.* 1996; Fritze *et al.* 1997). The fungal part of the microbial biomass was more sensitive to heavy metals than bacterial part (Pennanen *et al.* 1996). The decreased microbial activities have been reflected in a decreased rate of litter decomposition which could be seen as a changed structure of the humus layer (F+H) and as a 6–8 cm thick layer of accumulated brown needle litter on the top of the forest floor near the smelter (Fritze *et al.* 1989). The rate of litter decomposition has been influenced by the accumulation of Cu, Ni and Zn in brown needle litter and root litter, collected at the site (McEnroe and Helmisaari 2001). The accumulation of metals to unpolluted green needle litter was also observed (Ohtonen *et al.* 1990).

1.4. Herbivores and pathogens on trees

The adverse effects caused by forest pests increased with pollutant load as bark bugs, diprionids, tortricids, aphids, and bark beetles were abundant in the moderately polluted pine stands (Heliövaara and Väisänen 1990a), and near the smelter the Scots pines were heavily infested by aphids and bark beetles – *Xylechinus pilosus* being the most abundant bark beetle species in spruce and *Tomicus piniperda* in pine (Heliövaara and Väisänen 1991) (Table 3). Close to the smelter the cocoons of the defoliator species were smaller than further away (Heliövaara and Väisänen 1989a) but the smaller females produced more viable eggs (Heliövaara and Väisänen 1990a). Many insect species, however, suffered from severe pollution. *Pityogenes chalcographus*, which is one of the most common bark beetle species associated with spruce in Finland, was almost absent near the smelter (Heliövaara and Väisänen 1991). Also bark bugs, diprionids and tortricids were scarce in the immediate vicinity of the smelter (Heliövaara and Väisänen 1990a) (Table 3). Insects such as a moth *Epirrita autumnata* (Ruohomäki *et al.* 1996) and gall mite species on birch (*Betula pubescens* and *B. pendula*) (Koricheva *et al.* 1996) were also scarce near the smelter. In contrast, densities of mites on European aspen (*Populus tremula* L.) were not affected by the pollution (Koricheva *et al.* 1996) (Table 3).

Great differences in metal concentrations between the insect species feeding on Scots pine were observed near the smelter (Table 4). The highest concentration was measured in a sap-feeding aradid bug (*Aradus cinnamomeus*, the Cu concentration being 800 mg kg^{-1}. The lowest Cu concentration was measured in a gall-forming tortricid moth (*Retinia resinella*), 40 mg kg^{-1} (Heliövaara *et al.* 1987). Metal levels were higher in the needles (500 mg kg^{-1}) than in the insects *Neodiprion sertifer* (50 mg kg^{-1}), except in the case of Cd. Cd accumulated in the insects, the

Table 4. The concentrations of Cu, Ni, Zn and Cd in different plant species, cocoons of the insects, ants, spiders and faeces of birds near the smelter. The concentration in the reference area (8 km or further) is in parentheses.

Species	Cu	Ni	Zn	Cd	Reference
	mg kg^{-1} d.m.				
Pohlia nutans	1390 (270)				Helmisaari *et al.* 1995
Empetrum nigrum					
Last annual shoot	180 (20)				Helmisaari *et al.* 1995
	86 (22)[a]	30 (13)	50 (16)	0.5 (0.1)	Uhlig *et al.* 2001
Older living parts	1500 (30)				Helmisaari *et al.* 1995
	340 (90)[a]	120 (40)	220 (40)	1.1 (0.5)	Uhlig *et al.* 2001
Cladina arbuscula	160[a] (60)				Helmisaari *et al.* 1995
Picea abies (L.) Karsten					
Bark	600 (40)	100 (15)	300 (180)	1.2 (1.1)	Heliövaara and Väisänen
Phloem	75 (6)	80 (6)	340 (170)	1.1 (1.2)	1991
Wood	6 (1)	8 (1)	40 (10)	0.2 (0.1)	
Pinus sylvestris					
Bark	1500 (30)	390 (9)	190 (21)	5.6 (0.5)	Heliövaara and Väisänen
Phloem	66 (6)	35 (5)	120 (56)	5.1 (1.5)	1991
Wood	11 (3)	6.9 (1.2)	25 (7.8)	0.7 (0.2)	
Trunk wood	0.9[a] (1.1)	0.4 (0.2)	7.9 (5.2)	0.3 (0.3)	Harju *et al.* 1997
Needles	500 (10)	140 (10)		1.3 (0.2)	Heliövaara and Väisänen 1990b
Needles	210 (9)	44 (5)	83 (33)		Derome and Nieminen 1998
Stems (1-22 years)	2 (0.4)				Helmisaari *et al.* 1995
Fine roots	480 (75)				
Fine roots	590 (21)	110 (15)	70 (90)	2.1 (0.6)	Helmisaari *et al.* 1999
Betula pubescens Ehrh.		51 (10)	250 (210)		Koricheva and Haukioja
Foliage	96 (10)				1995
Betula pendula Roth.		40 (10)	220 (180)		
Foliage	64 (10)				
Aradus cinnamomeus[b]	800 (40)	110 (10)		13 (7)	Heliövaara *et al.* 1987
Retinia resinella	40 (5)	7 (2)		1.6 (0.2)	
Panolis flammea (Denis and Schiffermüller)	70 (10)	10 (1)		2 (0.1)	Heliövaara and Väisänen
Bupalus piniarius L.	90 (10)	1.6 (0)		0.6 (0.1)	1990c
Diprion pini L.	70 (10)	8 (1)			Heliövaara *et al.* 1990
Gilpinia socia Klug	60 (20)	10 (2)			
Neodiprion sertifer (Geoffroy)	80 (20)	7 (1)		2 (0.5)	Heliövaara and Väisänen 1989c
Gilpinia virens Klug	60 (10)	5 (0)		1 (0)	
G. frutetorum Fabricius	90 (20)	8 (2)		2 (0)	
Microdiprion pallipes Fallén	130 (20)	20 (2)		4 (1)	
ground living ants[b]	300 (30)			6 (4)	Koponen and Niemelä 1995
	180 (20)	30 (5)			Eeva and Lehikoinen 1996
ground living spiders	2000 (800)			20 (20)	Koponen and Niemelä 1995
Parus major	320 (50)	45 (5)	550 (350)		Eeva and Lehikoinen 1996
Ficedula hypoleuca	420 (70)	55 (5)	700 (250)		

[a] 4-6 km distance, [b] adults

concentration in the adults was 2.6 mg kg^{-1} which is higher than that in their food (1.3 mg kg^{-1}) or in their faeces (0.7 mg kg^{-1}) (Heliövaara and Väisänen 1990b). The low nutritional quality and high metal contents of pine needles increased the mortality of diprionids (Heliövaara and Väisänen 1990d) although the outbreaks of diprionids were also common (Heliövaara *et al.*

1991). The susceptibility of *N. sertifer* to virus and other diseases increased near the smelter but the mortality of *N. sertifer* caused by parasitoids decreased.

Means of defence against herbivores for trees include the production of resin and the phenolics in the bark, phloem, and foliage (Herms and Mattson 1992). Phenolics can also act as antidesiccation agents (Loponen *et al.* 1997). The resin flow decreased towards the smelter, indicating a decreased defence level, but the phenolic concentration increased, as a response to pollution, in Scots pine (Kytö *et al.* 1998) and in birch (Loponen *et al.* 1997). Compensatory growth, as a response to simulated herbivore, of two willow species, *Salix borealis* (Fries.) Nasar. and *S. caprea* L., was reduced near the smelter (Zvereva and Kozlov 2001). The endophytic fungal flora may affect their host plants positively by enhancing the resistance of the plant to pathogens (Butin 1992). Suppression of these non-pathogenic endophytes by air pollution did not promote the development of pathogenous *Gremniella abietina* (Lagerb.) Morelet, causing Scleroderris canker disease (Ranta *et al.* 1994).

Increased densities of leaf-miner species, which as pathogens are of minor importance, have been recorded around pollution source (Kozlov and Haukioja 1993). The solitary *Eriocrania* species (Koricheva and Haukioja 1992) were found to be scarce whilst the gregarious *Eriocrania cicatricella* (former *E. haworthi*) was abundant near the smelter (Koricheva and Haukioja 1994). The authors suggested that *E. cicatricella* possesses higher tolerance for pollutants than solitary species. Only host plant quality was found to be related to the population density of the solitary *Eriocrania* species (Koricheva and Haukioja 1992; 1995) when also several other aspects, such as: larval parasitism (Koricheva 1994), ant predation of miners (Koricheva *et al.* 1995), and the densities of endophytic fungi (Lappalainen *et al.* 1999) were studied.

Some changes in the ground-living arthropod fauna have also been reported. Beetles, except *Coccinella septempunctata*, were scarce near the smelter (Koponen and Niemelä 1995). Differences in diversity and species composition of spiders (Table 3), ants and bugs were observed along the pollution gradient although there were little differences in the total numbers (Koponen and Niemelä 1993 and 1995).

1.5. Birds

During 1991–1997, the survival (Eeva and Lehikoinen 1998) and behaviour (Eeva *et al.* 2000b) of two hole-nesting passerines, Pied Flycatcher (*Ficedula hypoleuca*) and Great Tit (*Parus major*) were studied around Harjavalta.

F. hypoleuca was more susceptible to pollutants than *P. major*, the response of which was weaker in many aspects. The breeding success of *P. major* was below background levels up to 3–4 km from the smelter (Eeva and Lehikoinen 1996) whilst *F. hypoleuca* was affected severely only next to the smelter (ca 1 km) (Eeva and Lehikoinen 1995; 1996). No clear differences in the female condition (Eeva *et al.* 1997a), or in the density of ectoparasites in the nestlings (Eeva *et al.* 1994) of these two bird species in relation to the pollution were found. The different responses of these two bird species were probably due to their different diet (Eeva and Lehikoinen 1996).

The poor breeding success of *P. major* was suggested to be related to habitat changes that have taken place around the smelter, e.g. a scarcity of suitable insect food for nestlings (Eeva and Lehikoinen 1996). The proportion of green larvae in the diet of the nestlings was smaller (Eeva *et al.* 1997b) and the nestlings were lighter (Eeva *et al.* 1998) in the vicinity of the smelter than further away. Air pollution was found to fade the yellow colour in plumage of the *P. major*. Pale plumage might affect mate choice, and predict reduced winter survival (Eeva *et al.* 1998). However, better wintering conditions next to human habitation may in general compensate for the possible detrimental effects of pollutants on the *P. major* population (Eeva and Lehikoinen 1998).

The low local survival rate of *F. hypoleuca* adult females near the smelter was suggested to be caused by higher emigration from the low quality habitat (Eeva and Lehikoinen 1998). However, *F. hypoleuca* nestlings were directly affected by increased amount of heavy metals and the low availability of calcium-rich food items in their diet near the smelter (Eeva *et al.* 2000a). The pollution related stress of *F. hypoleuca* was detected in biomarkers from blood and liver (Eeva and Lehikoinen 1998) and as growth abnormalities of legs and wings and changes in egg shell quality near the smelter (Eeva and Lehikoinen 1995; 1996). The authors suggested that heavy metals might accumulate more in ground living, mobile, often adult, prey items of *F. hypoleuca* than in foliage living, less mobile, often larval, prey items of *P. major*. The concentrations of Cu, Ni and Pb in ants were higher close to the smelter than further away (Table 4) and correlated positively with *F. hypoleuca* nestling faecal concentrations (Eeva and Lehikoinen 1996). Close to the smelter the heavy metal concentrations in ground-living ants and spiders (Koponen and Niemelä 1995) were higher than the concentrations of defoliator species (e.g. Heliövaara and Väisänen 1990c) (Table 4).

The breeding success of *P. major* and *F.hypoleuca* has markedly improved in the vicinity of the smelter between the years 1991 and 1997, and the lead concentrations of the nestlings have decreased by about 90% during this

time (Eeva and Lehikoinen 2000). The birds have probably benefited from the decrease in emissions and the practical remediation actions, revegetation after replacing the excavated polluted soil, in parks and along roadsides at Harjavalta. The vegetation recovery has probably promoted the recovery of herbivorous insect populations.

In conclusion, the effects and mechanisms of heavy metal deposition on forest ecosystem are diverse. Cu concentration is high in the forest soil and in different trophic levels of the forest ecosystem. Clear effects on organisms were observed up to ca. 4 km distance from the smelter. However, slight changes in organisms were observed as far as 8 km and in deposition 30 km distance from the smelter.

2. Remediation through *in situ* stabilisation of heavy metal polluted soil

Remediation of heavy metal polluted soils aims to improve the soil properties by removing or immobilising the pollutants. In the immobilisation process metals are converted into forms that are less mobile and less available for plants and microbiota.

Remediation actions for the recovery of plants and animals are needed on soils polluted by atmospheric emissions from burning of fossil fuels or mining and smelting activities, industrial wastes, and on abandoned mine workings. On agricultural soils remediation may also be needed following the spreading of polluted industrial or municipal sewage sludge, fertilisers or pesticides. Vangronsveld and Cunningham (1998) and Knox *et al.* (2000a, b) have recently reviewed remediation techniques of heavy metal polluted soils. Remediation has primarily been based on techniques where the polluted soil is excavated and transported to special landfills. Less commonly soils have been remediated by soil washing e.g. by different extractants. New perspectives on remediation include phytoextraction, i.e. removing pollutants using specific metal accumulating plants, microbial-based techniques, and electroreclamation. Many techniques are expensive and environmentally invasive. Environmentally gentle and less expensive techniques in the remediation are often based on *in situ* stabilisation through revegetation.

Revegetation of industrial barren land was studied around the Severonikel smelter, NW Russia, where the seedling performance was improved by wind sheltering and watering during two growing seasons (Kozlov and Haukioja 1999). Revegetation is often combined with immobilisation of the metals by different soil additives (i.e. immobilisation agents). In this overview the emphasis is placed on copper and the remediation through *in situ* stabilisation of the natural landscape that has been degraded mainly by atmospheric metal pollution. *In situ* stabilisation has also been used to vegetate tailings and mine spoils by adding sewage sludge into soil (e.g. Sabey *et al.* 1990; Kramer *et al.* 2000) and on agricultural soils after the use of polluted sewage sludge. Remediation for agricultural use has been studied by adding gravel sludge (Krebs *et al.* 1999), zeolites (Edwards *et al.* 1999), steel shots, or beringite (Boisson *et al.* 1998; Vangronsveld *et al.* 2000a) into soil.

2.1. Immobilisation agents

In laboratory conditions immobilisation of Zn, Cd, Ni, Pb or Cu in soil has been found with several inorganic and organic agents (Table 5).

In laboratory conditions Cu_{exc} concentration in soil decreased by 28% and Cu concentration in *Phaseolus vulgaris* by 77% after the addition of beringite. The respective values after compost addition were 24% and 51% (Vangronsveld and Clijsters 1992). Synthetic zeolites have decreased Cu_{exc} in soil by 60% (Edwards *et al.* 1999) and Cu in *Lolium perenne* L. by 82% (Rebedea and Lepp 1995). The respective values after a polyacrylate polymer addition were 72% and 77% (Torres and De Varennes 1998). Hydroxyapatite has decreased Cu_{exc} concentration in soil by 97% and Cu in *Zea mays* L. by 72% (Boisson *et al.* 1999a).

Zeolites, gravel sludge, lime, beringite, compost, and sewage sludge have been used as immobilisation agents in the field. These *in situ* stabilisation experiments were often made on soils severely polluted by Zn, Cd, and Pb. Only few reports were found on soils polluted severely by Cu. The results were reported mainly 1–6 years after the start of the experiment.

Table 5. Remediation agents studied in laboratory.

Inorganic agents	
Zeolites	Gworek 1992a and b, Rebedea and Lepp 1995, Chlopecka and Adriano 1996 and1997, Garcia-Sanchez *et al.* 1999, Edwards *et al.* 999
Beringite	Vangronsveld and Cljisters 1992, Mench *et al.* 1994b, Boisson *et al.* 1999b, Oste *et al.* 2001
Fe-oxides	Chlopecka and Adriano 1996 and 1997
Mn-oxides	Mench *et al.* 1994a and b, Boularbah *et al.* 1996, Sappin-Didier *et al.* 1997, Hettiarachchi *et al.* 2000
Apatite	Ma *et al.* 1993, Chlopecka and Adriano 1996, Laperche *et al.* 1997, Chen *et al.* 1997, Boisson *et al.* 1999a and b
Phosphate rocks	Ma *et al.* 1995
Lime	Chlopecka and Adriano 1996, Shuman and Li 1997
Modified montmorillonite compounds	Lothenbach *et al.* 1997
Steel shots	Mench *et al.* 1994a and b, Sappin-Didier *et al.* 1997
Polyacrylate polymer	Torres and De Varennes 1998
Organic agents	
Biomass residues	Fisher *et al.* 1998
Sewage sludge	Sabey *et al.* 1990
Compost	Vangronsveld and Clisters 1992, Shuman and Li 1997

2.1.1. Zeolites

Zeolites are crystalline, hydrated aluminosilicate of alkali and alkaline earth cations that possess infinite three dimensional crystal structure (Mench *et al.* 1998). Nearly 50 natural chemical forms have been recognised and more than 100 forms have been synthesised. The use of zeolites for pollution control primarily depends on their ion exchange and ion-trapping capabilities (Mench *et al.* 1998).

Copper emissions from a copper rod rolling factory at Prescot, Merseyside, UK, since 1975, has resulted in significant changes in the surrounding grassland species composition the copper tolerant *Agrostis capillaris* L. being the dominant plant species (Lepp *et al.* 1997). At this site Vangronsveld *et al.* (2000a) performed a study in which large quantities of dead undecomposed plant material was removed before synthetic zeolites were added, at a rate of 1 l m^{-2}, as a slurry. The authors found that 18 months later, the water extractable Cu concentration in soil had decreased (Table 6) and the root growth of *A. capillaris* had increased. They also performed another experiment on arable soil contaminated with Cd that had been emitted by a Cd pigment manufacturer in 1970s at Staffordshire, UK. After zeolites was added a clear reduction in the Cd concentration of sown plants were recorded (Table 6) (Vangronsveld *et al.* 2000a).

2.1.2. Gravel sludge

Gravel sludge consisted of 42% clay minerals, 31% $CaCO_3$, and to a lesser extent quartz, plagioclase, dolomite and organic matter (Krebs *et al.* 1999). Gravel sludge in a powder form was tilled into 20 cm depth (8–15 g kg^{-1} d.m.) into a soil contaminated with Zn and Cd emissions from a metal-smelter at Giornico, Switzerland, and into a soil contaminated with Zn, Cu and Cd polluted sludge at Dotticon, Switzerland (Krebs *et al.* 1999). Gravel sludge application increased soil pH by 0.5 unit and decreased metal concentrations in soil and in sown plants (Table 6). The metal immobilisation effect of gravel sludge was at least partially a pH effect mostly due to calcium carbonate.

2.1.3. Lime

The first large scale attempt to remediate a heavy metal polluted landscape was in Sudbury, Ontario, Canada. More than 30 km^2 of barren land polluted by mining and smelting activities has been treated since 1978 with surface application of ground dolomite limestone (1 kg m^{-2}), with or without an accompanying fertiliser, and grass-legume seed application (Winterhalder

Table 6. Metal concentrations in soil or in plants before the remediation treatment. The percentage of decrease after the remediation treatment is in the parentheses.

Immobilisation agent	Cu	Zn	Cd	Ni
		mg kg^{-1} d.m.		
Synthetic zeolites				
Vangronsveld *et al.* 2000a				
grassland soil	15[a] (74)	2.8[a] (50)	0.8[a] (44)	
Lactuca sativa L. (arable soil)			11 (54)	
Spinacia oleracea L. (arable soil)			8.1 (40)	
Gravel sludge				
Krebs *et al.* 1999				
arable soil	1.0[b] (28)	1.9[b] (99)		
Lactuca sativa L.	12 (0)	290 (24)	1.1 (36)	
Lolium perenne	60 (36)	600 (77)	0.4 (50)	
Lime				
Winterhalder 1996				
forest soil	3[a] (50)			5[a] (66)
Deschampsia caespitosa (L.) Trin.	35 (29)			60 (50)
Pinus banksiana Lamb.	42 (81)	65 (58)		32 (34)
Li *et al.* 2000				
town park soil		195[c] (20)	2.0[c] (17)	
Festuca rubra L.	12 (20)	620 (40)	3.9 (20)	
Mälkönen *et al.* 1999				
forest soil, sandy	1700[d] (36)			300[d] (2)
Pinus sylvestris, needles	260 (8)	160 (0)		
Helmisaari *et al.* 1999				
Pinus sylvestris, roots	590 (49)	70 (0)		110 (44)
Beringite and compost				
Vangronsveld *et al.* 1996				
sandy soil		10[a] (79)		
Phaseolus vulgaris L.		900 (88)	8.2 (86)	
Sewage sludge and fly ash				
Kelly and Tate 1998				
sandy loam soil		1100[e] (85)	63[e] (83)	
Sewage sludge				
Li *et al.* 2000				
town park soil		195[c] (98)	2.0[c] (98)	
Festuca rubra	12 (22)	620 (70)	3.9 (20)	

Extracts used [a] H_2O, [b] $NaNO_3$, [c] $Sr(NO_3)_2$, [d] $BaCl_2$, [e] $CaCl_2$

1996). Nine years later the treatment had lead to an increase in pH by 2 units to pH 6, a decrease in soil and plant metal concentrations (Table 6) and revegetation by grasses and woody plants.

Li *et al.* (2000) reported on a remediation experiment in a town park in Palmerton, USA, where the soil, with sparse vegetation of Zn-resistant grasses, was badly disturbed by erosion. Limestone treatment, which

consisted of applying 0.9 kg m^{-2} $CaCO_3$, the equivalent using dolomite agricultural limestone, increased soil pH from 6 by 0.6 unit and reduced the availability of metals to plants (Table 6).

In the forest soil polluted by the Cu-Ni smelter at Harjavalta, a soil remediation experiment was started in 1992 (Mälkönen *et al.* 1999). The treatments consisted of liming (0.2 kg m^{-2}, granulated Mg rich limestone), applying a powdered slow release mineral mixture, and stand-specific fertilisation determined on the basis of needle and soil analyses (Mälkönen *et al.* 1999). Liming had positive effects on soil chemistry during the 5 study years. It increased exchangeable Ca and Mg concentrations (Derome 2000) and reduced exchangeable Cu and Ni in the soil (Mälkönen *et al.* 1999) (Table 6) with the decreased leaching of metals down the soil profile (Derome and Saarsalmi 1999). Positive effects on tree growth and survival were also detected, liming being the most successful treatment. All the fertiliser treatments increased volume growth of Scots pine (Mälkönen *et al.* 1999) and liming increased the growth and survival of fine roots (Helmisaari *et al.* 1999) and reduced the detrimental effects of heavy metals on experimental seedling survival (Salemaa and Uotila 2001). Liming also increased the phenolic concentration of the phloem, indicating an improvement in the defence level against pathogens of Scots pine (Kytö *et al.* 1998) but did not affect soil decomposer animal community (Haimi and Mätäsniemi 2001). The microbiological response to remediation is reviewed in details in the Results and discussion section.

2.1.4. Beringite and compost mixture

Beringite, a modified aluminosilicate, also called cyclone ashes, originated from the burning of coal refuse from the former coal mine in Beringen, Belgium, (Mench *et al.* 1998). The product contains 52% SiO_2 and 30% Al_2O_3. The pH of the product was strongly alkaline (ca. 11), because of the presence of MgO and CaO. The high metal immobilising capacity of beringite was supposed to be based on the combination of chemical precipitation, ion exchange, and crystal growth (Mench *et al.* 1998).

In Maatheide, Belgium, a zinc smelter operated from 1904 until 1974 and created a desert like area of about 1.4 km^2 where the highest observed total Zn concentration in soil was 16 000 mg kg^{-1} d.m. (Vangronsveld *et al.* 1995). An experimental area of 3 ha was treated with a mixture of beringite and compost. Beringite (12 kg m^{-2}), and compost from municipal waste (10 kg m^{-2} wet weight) was mixed in the upper 35 cm topsoil layer. A seed mixture of metal- and drought-tolerant grasses was sown (Vangronsveld *et al.* 1995). After 5 years the soil pH had increased by ca. 2 units to 7.5, the

organic matter content and cation exchange capacity in the soil increased and the exchangeable Zn concentration in the soil decreased (Table 6) (Vangronsveld *et al.* 1996; 2000a). The papers report successful revegetation with clearly decreased phytotoxicity symptoms. Vegetation was healthy and regenerating by vegetative means and seeds. Also the total number of nematodes in soil was increased.

2.1.5. Sewage sludge

At Palmerton, Pennsylvania, the smelting of zinc ore in a narrow valley between 1898–1980 has contaminated the naturally acid soil with Zn, Cd, Cu, and Pb giving rise to 8 km^2 of barren to sparsely vegetated land (Sopper 1989). An experimental area was successfully revegetated when a mixture of sewage sludge (0.5 kg m^{-2} d.m.), power plant fly ash (1–2.5 kg m^{-2}), and lime (0.2 kg m^{-2}) was applied on the soil surface and metal-tolerant ecotypes of grasses were sown (Oyler 1988; Sopper 1989). An increase in pH from 4.5–6.0 to 6.2–6.9 and a decrease in soluble metal concentrations in soil was detected (Table 6) (Kelly and Tate 1998). Li *et al.* (2000) mixed composted sewage sludge, 22 kg m^{-2} d.m., containing 4% Fe and 30% calcium carbonate equivalent, and NPK fertilisation, into soil 15 cm deep in a town park at Palmerton and sowed grasses. The treatment increased the soil pH from 6 by 1.3 units and reduced of the shoot metal concentrations (Table 6) – the effects remaining stable for over 6 years. The treatment resulted in successful revegetation and a disappearance of visual symptoms of metal phytotoxicity.

3. The aim of the study

The aim of the study was to investigate the remediation of forest soil polluted by a Cu-Ni smelter. The *in situ* stabilisation through mulching with organic matter was studied. Since the nutrient cycle in heavy metal polluted soils is, in general, disturbed an important objective in remediation is the recovery of microbial activities. Low microbial activities are accompanied by the increased tolerance to the heavy metals. If the metal stress is removed it is possible that microbial population might lose the tolerance previously acquired thus leaving more energy for metabolism and thereafter also the microbial activities could increase.

The addition of new organic material onto the polluted soil aims to decrease the fractions of heavy metals that are toxic to microbiota by increasing heavy metal complexation. Applying an organic mulch has also other advantages. It can increase the soil pH and thus precipitation of heavy metals, and it can prevent drying and erosion of soil thus promoting revegetation. Organic matter introduces new microbiota into the polluted soil and provides a nutrient source for them.

The Harjavalta area is suitable for studying the remediation of heavy metal polluted forest ecosystem. Knowledge about the forest ecosystem around Harjavalta provides a good possibility to follow the effect of remediation actions on the whole ecosystem. My focus in the thesis is in Cu because it is the dominant pollutant at Harjavalta, it is very toxic to microbiota (Bååth 1989), and it forms complexes with organic matter (Baker and Senft 1995) that are less toxic to bacteria (Hughes and Poole 1991).

I hypothesised that the soil copper fractions that are toxic to bacteria would decrease after mulching. I further hypothesised that if remediation occurred the bacterial tolerance to copper would decrease, the microbial activities would increase, and the microbial community would change.

4. Material and methods

4.1. The modification of the [^{3}H]-thymidine incorporation method (I)

The bacterial growth rate, the bacterial copper tolerance and the toxicity of the soil water to bacteria was studied by the [^{3}H]-thymidine incorporation method. For this the preparation of the bacterial suspension was modified. Two homogenisation techniques (crushing and rotary shaking) and three filtering techniques (glass wool, acid washed glass wool and polyester net) for preparing the bacterial suspension were tested.

The polluted soil was sampled at a distance of 2 km from the Harjavalta Cu-Ni smelter. Undecomposed litter, dwarf shrubs, and lichens were removed, and the organic layer (F+H) (1 to 3 cm) was sampled.

4.2. Experimental design of the microcosm study (II)

Remediation of heavy metal polluted forest soil was studied using nine different organic immobilisation agents in the laboratory microcosm study. The organic soil was sampled at a distance of 2 km from the Harjavalta Cu-Ni smelter. The microcosms were established in 2 l plastic pots. A 50 mm layer (0.8 l) of mulch (organic remediation agent) was spread over the surface of the polluted organic soil (1 l). The treatments are presented in Table 7. The microcosms were kept at 20–28°C in the dark, and moistened with distilled water to 50% of water holding capacity once a week. After 1, 4, 10 and 16 months of incubation three microcosms of each treatment were destructively sampled.

4.3. Experimental design in the field (III and IV)

Remediation through mulching with organic matter was studied at 0.5 km distance from the Harjavalta smelter. The mulch in the field consisted of a mixture of compost and woodchips, which was also used in the microcosm study (II). Woodchips was added to the mulch in order to increase the amount of slow-release carbon source for microbiota and especially for fungi since the biomass of fungi is decreased on heavy metal polluted soils (Pennanen *et al.* 1996). The input of C through mulching was 2 kg m^{-2}. Compost (excluding the woodchips) was added on plots at the rate of 5.4 kg m^{-2} d.m.

Table 7. The treatments, the origin, pH and C:N ratio of the mulch materials in the beginning of the microcosm experiment.

Mulch material		pH	C:N
Composted sewage sludge	Helsinki Metropolitan area sewage plant	7.7	16:1
Compost	Mature compost from Ämmässuo Waste handling Centre, Helsinki Metropolitan area	7.7	11:1
A mixture of compost and woodchips	The woodchips (diameter < 20 mm) from Scots pine and Norway Spruce stemwood (1:1, volume)	6.4	17:1
A mixture of compost and barkchips	The barkchips from Scots pine and Norway Spruce (Kekkilä Ltd) (diameter < 50 mm) (1:1, volume)	6.1	23:1
Garden soil	Commercial soil (Kekkilä Ltd)	6.7	29:1
Green leaves	Downy birch (*Betula pubescens*)	5.8	11:1
Barkchips		4.9	160:1
Humus	F+H layers, mixed Scots pine and Norway Spruce stand	4.1	33:1
Peat	Low humufied *Sphagnum* peat (Kekkilä Ltd)	4.5	46:1
Control	Polluted F+H layers	3.9	25:1

Each of the 36 sample plots 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 (mulch treatment), and the other 18 plots were left untreated (control). The mulch was spread directly over the layer of undecomposed plant litter on the forest floor in summer 1996.

In 24 plots seedlings of four plant species were planted in the pockets (2 l) filled with mulch. In each plot were 49 pockets. For each plant species three plots were then mulched and three was not mulched. Plots with empty mulch pockets and plots with no pockets were established, respectively. One additional treatment without mulch pockets was established in summer 1997. The polluted litter layer (from 0 to 15 cm) and organic soil layer were removed before mulching (the exposed mineral soil mulched). The treatments are shown in Fig. 1.

Soil samples were taken from the organic layer (F+H) below the polluted litter layer on each plot (III). Five samples (each area 10 cm^2) to form a composite sample was taken from each plot using a spoon after the litter layer or the mulch and the litter layer had been removed. Soil samples were collected 4, 11, 16, 23 and 27 months after mulching between October 1996 and September 1998. In 1998 the roots of the planted seedlings were still totally in the mulch pockets and thus the effects of plants on remediation were not included in the experimental layout of this thesis.

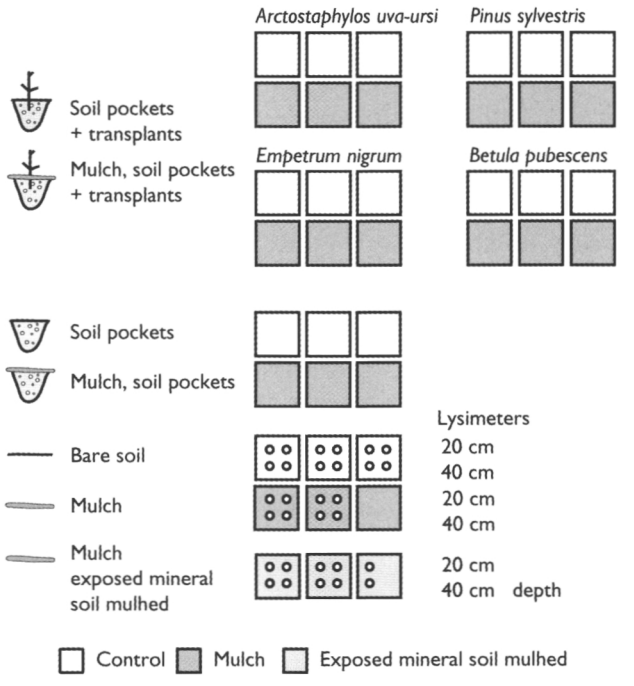

Fig. 1. The experimental design in the field.

Soil percolation water was collected by zero tension lysimeters from control, mulch treatment, and the exposed mineral soil mulched treatment (IV). The plate lysimeters (40 × 25 cm, stainless steel) were installed at the depths of 20 and 40 cm in the plots without mulch pockets. The replicate number of lysimeters at both 20 cm and 40 cm depth was 6 in the control, 4 in the mulch treatment and 5 in the exposed mineral soil mulched treatment (Fig. 1). The lysimeters were sampled once a month between April and October 1999. A total of 36 samples at 20 cm and 38 at 40 cm depth were collected.

4.4. Heavy metal pollution of the soils studied

The total heavy metal concentrations in the organic layer at 2 km distance from the smelter (I, II) were as follows: Cu 1 600 (Derome and Lindroos 1998b) or 3 000 (Pennanen *et al.* 1996), Ni 220, Fe 6 100, Zn 160, Pb 130, Cr 19, and Cd 2.1 mg kg^{-1} d.m. (Derome and Lindroos 1998b). The organic matter content, determined as loss in weight on ignition, was 33%.

The organic layer at a distance of 0.5 km from the smelter (III, IV) contained total Cu 5 800, Ni 460, Fe 20 000, Zn 520, Pb 300, Cr 31, and Cd 5 mg kg^{-1} d.m. (Derome and Lindroos 1998b) and the average organic matter content varied from 26 to 54%.

4.5. Physico-chemical and microbiological analyses

A brief summary of the methods is given here. The detailed descriptions of the methods are in the publications I–IV. The references and analyzed samples are presented in Table 8. The samples consisted of mulches, organic soil, extracts from mulches and soil (bacterial suspension), and soil water fractions. The soil water fractions are referred to as the soil solution and percolation water throughout the text. The soil solution of the organic layer was obtained from the fresh organic soil samples by centrifugation and percolation water was obtained with lysimeters.

From soil samples, dry matter weight, organic matter content, pH, and the concentrations of exchangeable Cu, Cu^{2+} and complexed Cu were measured (Table 8). The concentrations of total Cu, Cu^{2+}, complexed Cu and dissolved organic carbon were measured from soil water samples.

The bacterial growth rate and copper tolerance were determined using the [^{3}H]-thymidine incorporation method. The method was modified for soils with low microbial activity by using shaking and filtering through a polyester net to prepare the bacterial suspension (I). Then the [^{3}H]-thymidine incorporation procedure was performed as Bååth *et al.* (1992b). In the bacterial copper tolerance assay, the bacterial suspension was mixed with a range of $CuSO_4$ concentrations. The growth inhibition was calculated as the Cu concentration (M) giving a 50% reduction in [^{3}H]-thymidine incorporation. The higher the inhibition concentration, the greater is the tolerance of the bacterial community. The toxicity of the soil solution and percolation water to bacteria is expressed as the degree of inhibition of soil water on the growth rate of bacteria extracted from humus of an unpolluted forest site. After staining with acridine orange, the bacterial cells in the suspension were counted.

Microbial respiration activity was measured as the CO_2 evolved. Litter decomposition was studied with litterbags containing green Scots pine needles collected from an unpolluted area. The litterbags were inserted immediately under the polluted litter layer. The bags were removed 27 months after mulching. The litter weight lost was calculated. The growth rate of bacteria was also used as a variable describing microbial activities.

Table 8. The parameters and methods that were used in the articles. Articles are referred I, II, III, and IV, the numbers in the parentheses refer to the sampling occasions (months after mulching) when the parameter was not measured each sampling.

Abbreviation	Parameter	Method	Publication for method reference	Sample	Used in articles
C N	Total carbon and nitrogen	dry combustion	Nelson and Sommers 1996	mulch	II, III
d.m.	Dry matter	105°C		soil	I, II, III
o.m.	Organic matter	550°C	Howard and Howard 1990	soil	I, II, III
pH	pH	water suspension		mulch soil soil water[a]	II, III I, II, III II(16), III(27), IV
DOC	Dissolved organic carbon	TOC analyser	Greenberg *et al.* 1981	soil water	II(16), III(27), IV
Cu_{exc}	Exchangeable copper	$BaCl_2$ extraction	Tamminen and Starr 1990	soil	II, III
	Total Cu	Ion exchange column	Berggren 1989	soil extract[b]	I
Cu_{comp}	Complexed Cu			soil water	II(16), III(27), IV
Cu^{2+}	Free Cu				
BR	Basal respiration	CO_2 evolved	Pietikäinen and Fritze 1995	soil	I, II, III
m.l.	Mass loss of litter	litter bags	Fritze 1989	soil	III(27)
TdR	[^{3}H]-Thymidine incorporation rate	[^{3}H]-thymidine incorporation	Bååth 1992a	soil	I, II(10-16), III(11-27)
IC_{50}	Inhibition concentration	[^{3}H]-thymidine incorporation	Bååth 1992b	soil	I, II(10-16), III(11-27)
DI	Degree of inhibition	[^{3}H]-thymidine incorporation	–	soil water	II(16), III(27), IV
AO	Number of bacterial cells	Acridine orange staining	Bååth and Arnebrant 1994	soil	II(10-16), III(16-27)
PLFA	Phospholipid fatty acid pattern	Phospholipid fatty acid	Frostegård *et al.* 1996; Pennanen *et al.* 1999	soil extract soil mulch extract[b]	I II, III II(1,16)
$PLFA_{tot}$ $PLFA_{bact}$ $PLFA_{fung}$	Indicator of total microbial bacterial fungal biomass	Phospholipid fatty acid	Frostegård *et al* 1996	soil	II, III

[a] Soil water was obtained by centrifugation i.e. the soil solution, or collected by lysimeters i.e. percolation water, [b] Soil extract and mulch extract were obtained after homogenisation by shaking and centrifugation.

The structure of the microbial community was analysed by extracting the microbial-derived phospholipid fatty acids from the soil samples. 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.

The individual PLFAs were expressed as a mole percentage of the total amount of PLFAs detected in a soil sample. The total amount of PLFAs was used as an indicator of soil microbial biomass. The sum of PLFAs 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, and the amount of PLFA 18:2ω6,9 was used as an indicator of the fungal biomass (Frostegård and Bååth 1996).

4.6. Data analyses

The results of the soil analysis were calculated per organic matter content. The effect of the different types of mulch on the chemical and biological variables was detected using the a priori Dunnett's test to compare the treatments to the control. Canonical correlation analysis (Gittins 1985) was used to investigate the relationships between chemical and biological variables. The PLFA pattern was explored with global non-metric multidimensional scaling (Clarke 1999). Percolation water results were subjected to analysis of variance, with depth and treatment as the main effects, and followed by Tukey's test. Linear regressions of DOC and the toxicity of the soil water to bacteria on metal fractions in soil water were performed. Prior to the analyses, the necessary logarithmic, square root, or exponential transformations were made to normalise the distribution of the variables.

5. Results and discussion

5.1. The [^{3}H]-thymidine incorporation method (I)

The [^{3}H]-thymidine incorporation method, used to measure the bacterial growth rate and Cu tolerance in soil, was modified for the purposes of this study (I). The polluted soil, which had a very low microbial respiration activity (5 μg CO_2 g^{-1} d.m. h^{-1}), gave low mean incorporation values with high standard deviations. This made it difficult to assess the change in the inhibition concentration (IC_{50}) values of Cu after remediation treatments. Therefore the preparation of the bacterial suspension was investigated in order to raise the thymidine incorporation rate (TdR) of soil.

The method is based on the incorporation of radioactive [^{3}H]-thymidine into the macromolecules of bacteria extracted from soil after homogenisation and centrifugation (Bååth 1992a). The technique was previously modified by Bååth (1992b) to determine the heavy metal tolerance of soil bacterial communities and applied to field samples of heavy metal polluted soils by Pennanen *et al.* (1996).

In the original method of Bååth (1992a), bacteria were extracted from soil by intensive crushing and the bacterial suspension was filtered with glass wool to remove humus particles. However, the glass wool increased the pH of the suspension from ca 5 to 7, and gave a lower TdR (8 pmol thymidine g^{-1} d.m.) than the unfiltered suspension (61 pmol thymidine g^{-1} d.m.). In addition, the IC_{50} value was clearly lower after glass wool filtration (0.03 mM Cu) than in the unfiltered suspension (100 mM Cu). Therefore acid washed glass wool and polyester net was tested to filtrate the suspension. When acid washed glass wool or polyester net filtration was used TdR increased compared to glass wool filtration. Therefore the former was preferred as better filtering methods. Also the alternative homogenisation method, rotary shaking, which however had only a minor effect on TdR and the IC_{50} value was tested. In the analysis of articles II, III and IV, the rotary shaking and the filtration with polyester net was used to process the bacterial suspension.

5.2. Remediation studies (II, III and IV)

To study the bioremediation through mulching of heavy metal polluted soil nine mulch materials were tested in the microcosms (II). In the field (III, IV) one of them, the mixture of compost and woodchips, was tested. I focus on this treatment when I compare the laboratory and field experiments.

5.2.1 Copper

During the 16 months of laboratory incubation (II) the Cu_{exc} concentration in the underlying polluted soil was 25–40% lower in the treatments mulched with garden soil, compost, the mixture of compost and woodchips, the mixture of compost and barkchips, and sewage sludge than in the control. The mixture of compost and woodchips, the mulch used also in the field experiment, decreased Cu_{exc} in soil by 33% from 300 mg kg^{-1} d.m. compared to the control. However, in the laboratory incubation the decrease in Cu_{exc} concentration was not reflected in the Cu^{2+} concentration of the soil solution (II).

In the field, the Cu_{exc} concentration in soil was 26% lower in the mulch treatment than in the control (2100 mg kg^{-1} d.m.) 27 months after mulching (III). Cu^{2+} in the soil solution was 81% lower (III) and in percolation water at 20 cm depth 61% (IV) lower in the mulch treatment than in the control. In percolation water at 20 cm depth the total Cu concentration was 31% lower in the mulch treatment than in the control. Liming reduced Cu_{exc} concentration in the organic soil by 36% in 4 years at Harjavalta from 1 700 mg kg^{-1} d.m. (Table 6) (Mälkönen *et al.* 1999), and by ca 50% in soil percolation water at 20 cm depth (Derome and Saarsalmi 1999). Thus the decrease in Cu concentration was on the same level after mulching and after liming. Other remediation experiments shown in Table 6 were performed on soils with a relatively low Cu concentration. Cu_{exc} concentration had decreased by 28%, from 60 mg kg^{-1} organic C, after the addition of gravel sludge (Krebs *et al.* 1999) and 74%, from 15 mg kg^{-1} d.m. in grassland, after the addition of zeolites (Vangronsveld *et al.* 2000a).

The lowest obtained Cu_{exc} concentration in organic soil in the mulch treatment was 1 200 mg kg^{-1} d.m. (III). At 2 km, the Cu_{exc} concentration has been 300 mg kg^{-1} d.m. (II) or 1 000 mg kg^{-1} d.m. (Derome and Lindroos 1998b). Thus, compared to the pollution gradient at Harjavalta, the Cu_{exc} concentration remained at higher level after mulching than it was at 2 km distance from the smelter. The total Cu concentration in the soil solution was 2.7 mg l^{-1} in the mulch treatment and 0.06 mg l^{-1} at 4 km distance (Derome and Lindroos 1998a). In the percolation water at 20 cm depth the total Cu was 1.1 mg l^{-1} in the mulch treatment (IV) and 0.03 mg l^{-1} at 4 km (Derome and Lindroos 1998b). Thus, the total Cu concentrations in soil water after mulching remained clearly higher than at 4 km.

One sink for the observed decrease in Cu_{exc} concentration after mulching was probably the precipitation of Cu due to the increased soil pH (Alloway 1995). In the field (III), the addition of the mulch resulted in ca. one pH-unit increase in the organic layer varying in the mulch treatment between 4.8

and 5.4 during the study. In the laboratory the soil pH was 4.4 in the control and 4.1 in the mixture of compost and woodchips treatment after 16 months of incubation (II). Thus, the pH of the underlying polluted soil did not markedly change in the microcosms. Another possible sink for the observed decrease in Cu_{exc} concentration was the formation of complexes with particulate organic matter (Ross 1996) carried down into the underlying polluted soil from the mulch. It was assumed that relatively large amounts of particulate material were released especially by the compost and the sewage sludge. However, no increase in the organic matter content, measured by the weight loss on ignition (a rough method), was detected in the microcosms (II) or in the field (III). An additional sink for the decreased Cu^{2+} concentration in soil water in the field was probably the formation of complexes with dissolved organic carbon after mulching. DOC concentration was 76 mg l^{-1} in the control and 130 mg l^{-1} in the mulch treatment (III). The respective values for Cu_{comp} concentration were 0.25 and 1.1 mg l^{-1}. In percolation water, the concentrations of both DOC and Cu_{comp} were also higher in the mulch treatment than in the control (IV). In the microcosms, the complexation with DOC was not detected since DOC and Cu_{comp} were not correlated, and in the compost, the mixtures of compost, and sewage sludge microcosms, the DOC concentration was lower than in the control microcosms after 16 months of incubation. DOC concentration was 130 mg l^{-1} in the compost and woodchips mixture and 220 mg l^{-1} in the control (II).

The formation of soluble organo-metal complexes can increase the mobility of heavy metals down the soil profile (Li and Shuman 1997a, b). This has been shown in soils amended with organic wastes, e.g. poultry litter (Jackson *et al.* 1999) or sewage sludge (Brown *et al.* 1997). Mulching the forest floor slightly increased the mobility of Cu compared to the control but leaching was higher after the polluted organic soil and litter was removed before mulching (exposed mineral soil mulched treatment) (IV). Increased leaching of heavy metals is obviously an undesirable phenomena in remediation, although it has been rarely studied. In a semi-field experiment with *ex situ* lysimeters, Vangronsveld *et al.* (2000a) found a strong increase in Cu percolating from polluted soils mixed with compost. When a combination of compost and beringite was mixed with polluted soil the authors reported a slight reduction in percolated Cu compared to untreated soil. There was little information available about whether organo-metal complexes are leached into groundwater. One study detected increased DOC concentration in groundwater after 15 years of heavy application of spent mushroom substrate, rich in DOC, on an agricultural field (Kaplan *et al.* 1995). Albeit, the concentration of DOC in the groundwater was only 2–7%

of that at a depth of 1 m. A laboratory study by Giusquiani *et al.* (1992) also showed that only 5–10% of the DOC extracted from composted sewage sludge leached down to a depth of 50 cm, and 70–80% retained in the upper 10 cm layer. The soil therefore has a high absorption capacity for water-soluble organic matter and mulching the forest floor with compost is not likely to markedly increase the leaching of heavy metals into the groundwater.

5.2.2. The toxicity of soil to bacteria

The change in the toxic fractions of Cu can be assessed by measuring the toxicity of soil to bacteria. Boularbah *et al.* (1996) used a ß-galactosidase-producing strain of *Escheria coli*, which responds to low levels of heavy metals (Bitton *et al.* 1996), and found that the toxicity of soil decreased following the addition of hydrous manganese oxides into polluted soil. Vangronsveld *et al.* (2000b) found the toxicity of soil to bacteria to be decreased after remediation treatment with beringite, when they applied the bioluminescence method with heavy metal-specific (Zn, Cd) biosensor strain *Alcaligenes eutrophus*. Bacteria extracted from unpolluted coniferous humus were used as biosensors in the present study. The inhibition (DI) that the soil water exhibited to bacterial growth rate was measured by TdR method.

The regression of DI on Cu^{2+} concentration in the bacterial suspension was calculated using the samples of II, III and IV articles. The regression was found to be approximately linear between 20 and 80% DI and between 0.05 and 1.5 mg l^{-1} Cu^{2+} ($DI = 77+17\times \ln Cu^{2+}$, $p < 0.001$, $r=0.73$, $n=88$) (Fig. 2). The regression equation was used to calculate the comparable DI values for the soil solution of the field experiment (III), which was diluted 2-fold compared to the other solutions (II, IV) in the analysis procedure in order to reach the optimum Cu^{2+} concentration to measure the bacterial growth rate.

DI varied very little in the microcosms, between 69–75% (Fig. 2). DI in the control (69%) was lower than in the compost and woodchips mixture (74%). In the field, however, the toxicity of the soil solution to bacteria was clearly lower in the mulch treatment than in the control (Fig. 2). DI in the soil solution was 56% in the mulch treatment and 85% in the control. In percolation water, DI was 29 and 36% at 20 cm and 23 and 25% at 40 cm in the mulch treatment and in the control, respectively.

Cu^{2+} seemed to be the best variable to explain the toxicity of Cu in soil because the correlation between DI and Cu_{tot} was lower than between DI and Cu^{2+} calculated from the data of II, III, and IV articles. The Cu^{2+} in the soil solution also explained more of the biological variation than Cu_{exc} in soil when the canonical correlation analysis was performed (III). Cu^{2+} is generally assumed to be the most toxic form to bacteria, but according to

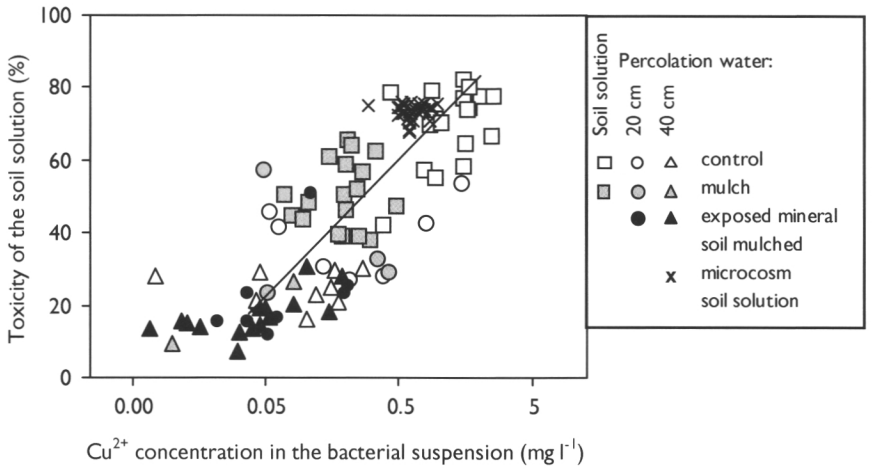

Fig 2. The relationship between the toxicity of solution (DI), measured by TdR method, and Cu^{2+} concentration in the bacterial suspension. Samples are the soil solution and the percolation water at 20 and 40 cm depth in the field, and the soil solution of the microcosm samples.

the review of Giller *et al.* (1998) this has rarely been demonstrated. The most toxic forms of Cu to microbiota were dilute HCl and $CaCl_2$ extractable Cu when the microbial response was measured as the microbial biomass (C_{mic}/ Organic C), dehydrogenase activity (Aoyoma and Nagumo 1997), or the bacterial Cu tolerance (Kunito *et al.* 1999).

5.2.3. Microbial activities and the number of bacterial cells

Microbial activities in the microcosms (II) were in general slightly lower in the compost, mixtures of compost, sewage sludge and garden soil treatments than in the peat, humus or control treatments after 16 months of incubation. Microbial respiration activity was 4.8 μg CO_2 g^{-1} h^{-1} in the control and 4.1 μg CO_2 g^{-1} h^{-1} in the compost and woodchips mixture treatment. The respective values for bacterial growth rate were 26 and 20 pmol thymidine g^{-1} h^{-1} and for the number of bacterial cells 72 and 59 × 10^9 cells g^{-1}. In the field, 27 months after mulching (III), BR was 6.7 μg CO_2 g^{-1} h^{-1} in the control and 10.5 μg CO_2 g^{-1} h^{-1} in the mulch treatment. TdR was respectively 17 and 30 pmol thymidine g^{-1} h^{-1} and AO 41 and 60 × 10^9 cells g^{-1}. BR was 7–80%, TdR 8–180% and AO 8–100% higher in the mulch treatment than in the control during the study. The litter was decomposed 9% more in the mulch treatment than in the control. Thus, the microbial activities in the field were remarkably increased after mulching with compost and woodchips mixture. After sewage sludge and fly ash addition on Zn and Cd polluted soil,

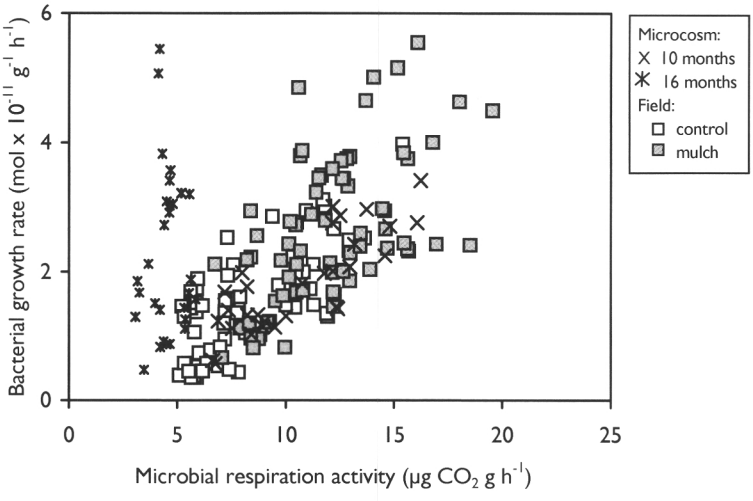

Fig. 3. Bacterial growth rate and microbial respiration activity in the organic soil from the microcosm experiment after 10 and 16 months of incubation and in the control and mulch treatment in the field.

dehydrogenase activity and total viable plate counts increased (Kelly and Tate 1998). The number of bacterial cells increased (Vangronsveld *et al.* 2000a) and mycorrhizal network established in the roots of the sown grasses (Vangronsveld *et al.* 1996) after the addition of compost and beringite. After liming microbial respiration activity increased ca 50% in the heavily Cu polluted soil at Harjavalta (Fritze *et al.* 1996).

The correlation between bacterial growth rate and microbial respiration activity was studied. In figure 3, TdR and BR of all 195 samples from microcosms (II) and field experiment (III) are plotted. When the samples taken after 16 months (II), were excluded the correlation was fairly strong ($r=0.72$, $n=165$).

5.2.4. The microbial community

The structure of the microbial community varied during the study, however no clear change attributable to remediation was detected in the laboratory (II) or in the field (III). Liming of polluted soil at Harjavalta changed the PLFA pattern (Fritze *et al.* 1997). The relative quantities of PLFAs 20:4 and 16:1ω5 increased and i15:0, 16:1ω7t, br18:0 and cy19:0 decreased on the limed plots, as was the case in general for the mulch treatment. The relative quantity of 20:4 increased and br18:0 decreased with decreasing Cu concentration in soil along the heavy-metal pollution gradient at Harjavalta

(Pennanen *et al.* 1996). After mulching most of the PLFAs did not change as could be predicted from Pennanen *et al.* (1996). Thus the microbial community seemed not to change towards the unpolluted sites but rather the slight change might be a consequence of the increase in pH or some other factors related to that. However, the differences in the relative abundance of PLFAs after mulching were very small, and therefore these signs of the changes in the microbial community are only tentative. Changes in microbial community have been found in other remediation studies, such as after liming as mentioned above. Liming changed the PLFA pattern at 4 km from the smelter where Cu_{tot} concentration in soil was 1100 mg kg^{-1} d.m. (Fritze *et al.* 1997). On these sites, also the metabolic profiles of the microbial community, measured using BIOLOG method, changed. The BIOLOG method also indicated change after the application of sewage sludge and fly ash on Zn and Cd polluted soils (Kelly and Tate 1998).

The tolerance of the bacterial community to Cu did not reflect the decreased Cu_{exc} concentration in the microcosms. However, tolerance to Cu decreased in the field after mulching. In the laboratory, 50% inhibition concentration (IC_{50}) was 8 mM Cu in both the control and the mixture of compost and woodchips treatment after 16 months of incubation (II). In the field IC_{50} in the control was 60, 25, 6, and 20 mM Cu 11, 16, 23, and 27 months after the treatment, respectively (III). In the mulch treatment the respective values were 30, 1, 3, and 3 mM Cu. Thus 16 months after mulching, the tolerance was clearly decreased in the mulch treatment to about the same level as at 2 km, where the microcosms soil samples were collected. Pennanen *et al.* (1996) detected about the same IC_{50} values, 2 mM Cu at 2 km and 20 mM Cu at 0.5 km.

When the metal stress was removed by reinoculating the metal tolerant bacteria in an unpolluted soil sample the bacterial tolerance to copper, measured by the TdR method, was shown to decrease within the first week (Díaz-Raviña and Bååth 2001). I found the tolerance to Cu to decrease 16 months after mulching in the field. Preliminary results of Vangronsveld *et al.* (2000a) suggested a loss of bacterial tolerance to several metals 5 years after the addition of compost and beringite into polluted soil. A contrary result was obtained by Kelly and Tate (1998), who found no change in bacterial tolerance to Zn, measured using the plate count method, although the soluble Zn concentration decreased and microbial activity increased 3 years after the application of sewage sludge and fly ash.

Tolerance and adaptation of microorganisms to heavy metals are common phenomenon. The increased or decreased abundance of tolerant organisms can be due to genetic changes, to physiological adaptations involving no

alterations to the genotype, or to the changes in species composition (Bååth 1989). A change in species composition has been proposed as the main reason for both the increase (Frostegård *et al.* 1993; Díaz-Raviña *et al.* 1994; Pennanen *et al.* 1996) and decrease (Díaz-Raviña and Bååth 2001) in metal tolerance of microbial populations. In those studies, the change in heavy metal tolerance of the bacterial community, determined by TdR method, was accompanied by a change in the microbial community structure, determined by the PLFA technique. In the present study the microbial community structure showed no changes after 23 months, and only slight changes after 27 months. Despite this, the copper tolerance of the bacterial community decreased 16 months after the exposure to the mulch. Therefore, the results support the alternative hypotheses of genetic change or physiological adaptation of Cu tolerant bacteria to diminishing toxic concentrations of heavy metals. The microbiota that were carried from the mulch down into the underlying polluted soil were presumably not able to maintain their activity in polluted soil either in the laboratory or in the field. This was confirmed with the result that the PLFA pattern of the underlying polluted organic soil in the microcosms did not reflect the PLFA patterns of the mulch extracts (II).

One can hypothesise that the decreased IC_{50} value, measured by the TdR method, may reflect the increased bacterial growth rate instead of decreased tolerance, since rapid growing bacteria are in general more sensitive to external disturbances. Fritze *et al.* (2000) used the TdR method and found that increased microbial activity was accompanied by the increased sensitivity to Cd, after wood ash application. Therefore one possible interpretation could be that in the mulch treatment the faster growing bacteria were more sensitive to external disturbances and thus also to Cu. However, this hypothesis is not supported by the fact that TdR did not correlate with IC_{50}, when the data from articles II and III were included (n=200) (Fig. 4).

The data suggests that the bacterial community had lost its Cu tolerance as a result of decreased toxic Cu fractions in the soil. An alternative hypothesis is that instead of the decrease in the toxic Cu in soil, the main factor for decreasing IC_{50} values in the mulch treatment was the increase in soil pH by one unit (III). This hypotheses is supported by the result of the laboratory experiment where neither the soil pH nor IC_{50} value changed although the Cu_{exc} decreased. If the bacteria had not been adapted to higher pH after mulching, this might have also caused the sensitivity to Cu, since the tolerance of an organism to toxic compounds may change depending on the growth conditions e.g. pH (Bååth 1989). The effect of elevated pH on Cu tolerance was detected in article I, where bacteria growing in pH that was

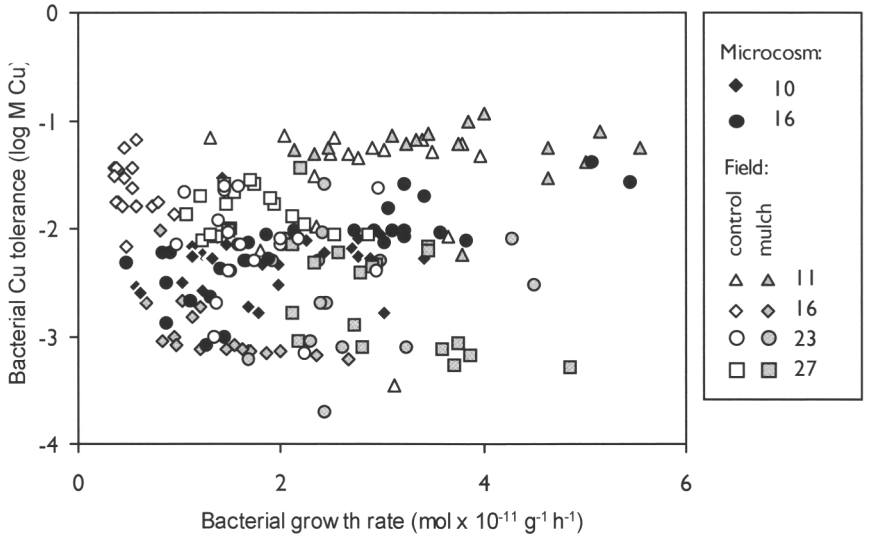

Fig. 4. Bacterial Cu tolerance and growth rate of the samples.

higher than the actual soil pH were more sensitive to Cu than the bacteria growing near the actual soil pH. However, the results from the field (III) showed that pH had increased 4 months after mulching but a clear decrease in IC_{50} was detected only 16 months after mulching. Bååth (1996) found that TdR decreased if the pH of the growth medium was changed from the actual soil pH. The same was detected in article I when the TdR decreased if the pH in the growth medium was higher than in soil. The pH adaptation developed rapidly, in one year, after wood-ash fertilisation Bååth *et al.* 1995; Bååth 1996). Thus it seems probable that following mulching, the bacteria was adapted to the increased pH and that the increased pH was not the main factor explaining the decreasing Cu tolerance in the mulch treatment. The reason was rather the decreased toxic Cu concentration in the soil. In the microcosms there were other microbial successional factors (discussed in Section 5.2.5.) which may have affected the microbial variables rather than the soil Cu or pH.

Soil pH, however, has an indirect effect on the bacterial tolerance to Cu because the availability of metals to microbiota is pH dependent. Metal tolerance increased in soil with relatively low total Cu concentration (94 mg kg^{-1} d.m.) when the soil pH had decreased from 7 to 5 after the addition of polluted sewage sludge (Witter at al. 2000). On the other hand, Pennanen *et al.* (1998) found no increased bacterial Cu tolerance after a low metal and acid load, when the soil pH had decreased from 4.1 to 3.95.

The soil pH affects also microbial activities. The higher pH itself, or some factors related to that, like the change in the organic matter quality, is beneficial to bacteria. I suppose that the quality of organic matter changed after mulching since DOC concentration increased (III, IV). It is well known that microbial activities, including bacterial growth rate, increase with increasing pH in forest soil (Bååth *et al.* 1992; Bååth and Arnebrant 1994). Also in polluted soil samples, the microbial respiration activity increased after liming at Harjavalta (Fritze *et al.* 1996; 1997). Therefore, the increased soil pH, or related factors, after mulching had probably increased microbial activities.

To summarise the changes in polluted organic soil after mulching; the microbial activities increased as a consequence of the increased pH. The increase in pH and the immobilisation effect of the mulch decreased the toxic Cu concentration. Because the metal stress decreased also the Cu tolerance decreased and that further increased the activities of the original microbiota.

5.2.5. Assessment of the microcosm experiment (II)

Several contrary results were obtained in the laboratory and in the field experiment. In the microcosms the decrease in the Cu_{exc} concentration was not reflected in the Cu^{2+} or Cu_{comp} concentrations in the soil solution, toxicity of the soil solution, or microbial activities as was hypothesised. Cu_{exc} in the soil did not correlate with Cu^{2+} but did correlate positively with Cu_{comp} in the soil solution. These results were contrary to my hypotheses and the results obtained in the field study.

One explanation could be that the $BaCl_2$ extractable Cu_{exc} in the microcosms did not reflect the available amount of Cu to microbiota. At the end of the experiments the mean and SD of Cu_{exc} concentration calculated from all microcosms (n=30) was 720±150 mg kg^{-1} o.m. (II), in the field in the mulch treatment (n=18) 2 900±500 mg kg^{-1} o.m., and in the control (n=18) 3 800±400 mg kg^{-1} o.m (III). In the microcosms, where the Cu_{exc} was lower than in the field, the Cu^{2+} concentration and DI of the soil solution remained at relatively high levels being 2.2±0.5 mg l^{-1} and 73±2%, respectively. In the field the Cu^{2+} concentration in the mulch treatment was 1.7±0.9, and DI 56±7%. In the control the respective values were 9.1±3.8 mg l^{-1} and 85±8%.

The soil pH did not increase in any of the laboratory treatments contrary to the one unit increase after mulching in the field. Thus, in the laboratory experiment the pH did not affect the chemical speciation of Cu or the microbial activities. Microbial activities were very low at the end of the

incubation. Cells in the underlying polluted soil probably died gradually owing to the toxicity of the organic soil, the stressful conditions, or the loss of available carbon sources in the microcosms. As an indication of the loss of available carbon sources Frostegård *et al.* (1996) suggested the decrease in the abundance of the PLFA 16:1ω5. The amount of 16:1ω5 was higher in the extract of sewage sludge, compost, the compost mixtures or garden soil than bark, peat or humus at the end of the experiment. Therefore the carbon sources seemed not to be depleted in the microcosms mulched with sewage sludge, compost, the compost mixtures or the garden soil. This was, however, not supported by the result that DOC was lower under these mulches than under the control. Microbial cells dye throughout the course of the incubation. The differences in microbial activities may therefore also be due to the successive breakdown of dead cells, and a concomitant increase in the microbiota feeding on them. This could similarly be the reason for the succession in the PLFA patterns of the soil during the incubation (Frostegård *et al.* 1996). The microbial activities were among the highest in the control between 4 and 16 months, indicating stressful and maybe partly anaerobic conditions in the mulched microcosms. The conditions in the microcosm environment for microbiota are different from those in the field. The lack of living roots, the destroyed physical structure of the soil and the limited amount of available carbon set limits when the microbiological properties of soil are studied in a laboratory experiment.

6. Conclusion

In the microcosm study no microbial response direct attributable to remediation was detected. In the field, mulching the polluted soil with the mixture of compost and woodchips converted copper into less toxic forms. The tolerance of the bacteria to Cu decreased and the activities of the original microbiota increased in the polluted organic layer after mulching.

Mulching the polluted forest floor was a successful remediation treatment although the mulch had not yet completely decomposed, and there is a need to study remediation for a longer time period. A possible disadvantage in the studied remediation treatment is the leaching of metals into groundwater. This might be possible to avoid by adding some chemical immobilisation agent to the mulch. Organic mulch addition has advantages, which might promote the vegetation recovery, compared to the addition of solely chemical agents.

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Paper I

I

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Short communication

Some observations on the copper tolerance of bacterial communities determined by the (^{3}H)-thymidine incorporation method in heavy metal polluted humus

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Abstract

Changes in pH after filtration of bacterial suspensions are important when applying the radioactive thymidine incorporation method to heavy-metal polluted soils with low microbial activity. In the original method (Bååth 1992; Soil Biology and Biochemistry 24, 1157–1165) the blended and centrifuged suspension was filtered through glass wool to remove humus particles from the suspension. When we filtered the bacterial suspension through glass wool the pH increased by 2 units and the thymidine incorporation rate decreased. This made the community copper tolerance measurement ambiguous. When using soil samples with very low activity, we recommend the use of acid-washed glass wool or polyester net filtration which eliminates changes in pH.

Keywords: Copper; Heavy metals; Microbial activity; Thymidine

The bacterial growth rate in soil has been studied on the basis of the incorporation of radioactive thymidine into the macromolecules of bacteria extracted from soil after homogenization-centrifugation (Bååth, 1992a). The technique has been modified for determining the heavy-metal tolerance of soil bacterial communities (Bååth, 1992b; Díaz-Raviña et al., 1994; Díaz-Raviña and Bååth, 1996; Pennanen et al., 1996; Pennanen et al., 1998). For the assay, the bacterial suspension was mixed with a range of heavy metal concentrations that gave no to complete inhibition of bacterial growth. Growth was estimated by adding (^{3}H)-thymidine and measuring its incorporation into the bacteria during a 2-h period. The IC_{50} value, i.e. the metal concentration resulting in 50% inhibition, was then calculated. The higher the IC_{50} value, the greater is the tolerance of the bacterial community to the metal in question. An overview of the suitability of the method for studying heavy-metal pollution has been given by Bååth et al. (1998).

We applied the (^{3}H)-thymidine incorporation method for measuring the heavy-metal tolerance of bacteria in a study on the microbial remediation of heavy-metal polluted humus. The polluted humus, which had a very low microbial activity, gave low mean incorporation values with high standard deviations. This made it difficult to assess a change in the IC_{50} values of Cu due to the addition of an organic top layer onto polluted humus. We therefore decided to reinvestigate the preparation of the bacterial suspension in order to raise the (^{3}H)-thymidine incorporation rate of coniferous humus with a high organic matter content and high metal concentrations.

Forest humus (F + H layer) from two different sites

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were used: unpolluted humus from a forest site in southern Finland, and heavy-metal polluted humus from a site located 2 km from a Cu-Ni smelter in southwestern Finland (Pennanen et al., 1996). The unpolluted and polluted humus contained 69% and 33% organic matter (loss in weight on ignition), and the respective basal respiration rates (14°C), describing microbial activity, were 26 and 5 μg CO_2 g^{-1} d.m. h^{-1}. The total copper concentration in the unpolluted humus was less than 100 mg and in the polluted humus 3000 mg kg^{-1} d.m. The pH_{H_2O} of the samples was ca 4. The samples were sieved (mesh size 2.8 mm) and stored at 4°C until analyzed. Copper tolerance measurement was determined as described by Bååth (1992a, b) using 1.0 g d.m. of fresh unpolluted humus or 3.0 g d.m. of polluted humus. The radioactivity was counted in a Wallac 1411 liquid scintillation counter using the fine-tuned external standard method. Two homogenization techniques for preparing the bacterial suspension and three filtration techniques were tested. The homogenization techniques were blending in a Sorvall Omnimixer at 80% of top speed for 1 min as used by Bååth (1992a, b) (B), and rotary shaking at 250 rpm for 1 h at 4°C (S). The filtration techniques were glass wool (Pyrex fiber glass, Sliver 8 micron; W), acid-washed (2% HCl) glass wool (AW), and a polyester net (mesh size 0.2 × 0.8 mm; N). We investigated the following four combinations: B–W, B–AW, B–N, and S–N, the B–W technique being the one originally published by Bååth (1992a).

The filtration technique affected the Cu tolerance value, whereas homogenization had only a minor effect. As an example, the results for the polluted humus are shown in Fig. 1. The difference in the IC_{50} values was mainly attributable to that between the B–W and the other treatments, the IC_{50} value for B–W being 0.00003 M Cu and for B–N 0.035 M Cu. For the unpolluted humus, the effect of the techniques was similar but smaller. Since replicates were not used, no statistical analysis could be performed on the data, but in repeated measurements the result was reproducible.

Possible explanations for the differences in IC_{50} values between the different homogenization and filtration techniques are (i) bacteria with different Cu tolerance were retained selectively in the suspension by the different filtration techniques, (ii) the isotope dilution increased with increasing copper concentrations due to the lysis of living cells, (iii) the copper was partially complexed with the humus, thereby lowering the effective metal concentration, or (iv) the pH values of the incubation suspension following filtration were different.

All four hypotheses were tested using polluted humus and the contrasting B–W and B–N treatments. If glass wool filtration retained different subsets of bacteria in the suspension compared to polyester net filtration, this should be revealed by microbial community structure analysis using, for instance, phospholipid fatty acid analysis (PLFA) (Frostegård et al., 1993). The bacteria for the PLFA analyses were harvested from the suspension by centrifugation (20 min; 47,000 × *g*). No clear differences between the treatments were detected. All the microbial PLFAs were at the same mol% level except for the fungal PLFA, 18:2ω6,9, which was slightly more abundant (2.0 mol%) in the B–N than in the B–W treatment (1.6 mol%). Fungi in general do not take up thymidine (Bååth, 1990) and thus do not contribute to these values. This hypothesis was therefore rejected.

The second hypothesis that the differences in the

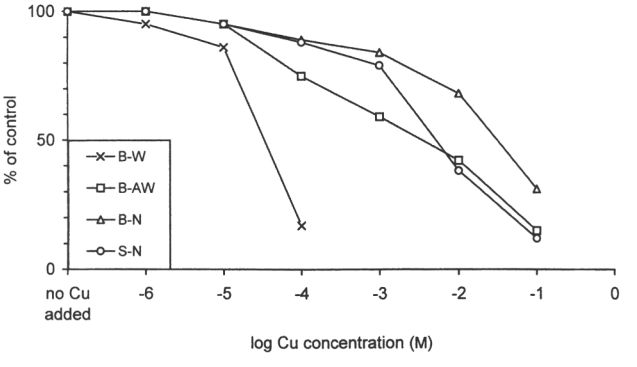

Fig. 1. Inhibition of bacterial growth by increasing copper concentrations as measured by the incorporation of (^{3}H)-thymidine into bacterial macromolecules. Extraction of the bacterial population from the heavy-metal polluted soil was performed either by blending with a Sorvall Omnimixer at 80% of top speed for 1 min (B) or rotary shaking at 250 rev min^{-1} for 1 h at 4°C (S). The filtration techniques were glass wool (Pyrex fiber glass, Sliver 8 micron; W), acid-washed (2% HCl) glass wool (AW), and a polyester net (mesh size 0.2 × 0.8 mm; N). The following four combinations were investigated: B–W, B–AW, B–N, and S–N, of which the B–W technique is the one in the original (Bååth, 1992a).

amount of isotope dilution were due to the lysis of living cells in the presence of high Cu concentrations was not valid. Isotope dilution was measured using unlabelled thymidine as described by Pollard and Moriarty (1984). The degree of isotope dilution was about the same irrespective of the copper concentration in the incubation suspension. At high copper concentrations the degree of isotope dilution was highly variable, but no trend could be detected.

The third hypothesis, the inactivation of added Cu due to complexation with humus, was tested by adding excess Cu to the B–W and B–N filtered suspensions. The B–W suspension had less humus (index 1.3) than the B–N suspension (index 1.5) when the color index of the liquid scintillation spectrometer was used to indicate the amount of dissolved humus. The amount of complexed copper in the suspension was determined by using an Amberlite IR-120 (plus) ion exchange resin. The amount of Cu in the solution, before and after passing the resin, was measured by atomic absorption spectrophotometry. The amount of complexed copper, 0.89 mg l^{-1} for B–W and 1.10 mg l^{-1} for B–N, was on the same level for the two techniques and the observed degree of complexation was so low, under 0.2% of the Cu added, that it cannot have had an influence on the IC_{50} value.

The last hypothesis was based on the difference in the pH of the suspension following filtration. In the original method by Bååth (1992a) the blended and centrifuged suspension was filtered through glass wool to remove humus particles from the suspension. However, our Pyrex fibre glass wool increased the pH of the suspension from ca 5 to 7, and gave a lower thymidine incorporation rate (0.8×10^{-11} mol TdR g^{-1} d.m.) than the unfiltered suspension (6.1×10^{-11} mol TdR g^{-1} d.m.). A high pH is a stress factor for bacteria adapted to pH 4 (Bååth, 1996), and thus the rate of thymidine incorporation remained low.

In conclusion we found one pitfall in the technique: the filtration of the soil suspension. The change in the pH of the soil suspension caused by the glass wool must be avoided when using soil samples of very low activity. We recommend the use of acid-washed glass wool or polyester net filtration.

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Paper II

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II

Organic material as a copper immobilising agent: a microcosm study on remediation

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Abstract

Remediation of heavy metal polluted forest soil was studied using nine different organic immobilising agents in a laboratory microcosm study. Composted sewage sludge, compost of organic household waste, a mixture of compost and woodchips, a mixture of compost and barkchips, garden soil, birch leaves, barkchips, humus or peat was applied on the surface of a polluted soil. Changes in the exchangeable Cu concentration, microbial respiration activity, microbial biomass and structure of the microbial community were assessed at four points during a 16-month period. Cu fractions, Cu^{2+} and complexed Cu, and toxicity of the soil solution to bacteria, bacterial growth rate, number of bacterial cells, and bacterial copper tolerance were determined on samples taken after 16 months. Sewage sludge, compost, the compost mixtures, and garden soil decreased the exchangeable Cu concentration in the polluted soil, but had no effect on microbial activities, bacterial tolerance to copper or on the structure of the microbial community directly attributable to remediation.

Zur Remediation von Schwermetall belasteten Waldböden wurden in einem Mikrokosmosversuch neun verschiedene Schwermetall immobilisierende organische Substanzen getestet. Klärschlamm, Haushaltskompost, eine Mixtur von Haushaltskompost und Holzspänen, eine Mixtur von Haushaltskompost und Rindenspänen, Gartenerde, Birkenblätter, Rindenspäne, Humus oder Torf wurde einem belasteten Waldboden als neue organische Schicht aufgetragen. Über den 16 Monate lang andauernden Laborversuch haben vier Beprobengun stattgefunden und die Konzentration von austauschbaren Cu, die Mikrobielle Respiration, die Mikrobielle Biomasse, und die Zusammensetzung der Bodenmikroflora wurden jeweils bestimmt. Zusätzlich wurden bei der letzten Beprobung (16 Monate) auch zwei verschiedene Cu Fraktionen, Cu^{++} und komplekziertes Cu, die Toksizität des Bodenwassers gegenüber Bakterien, die Bakterielle Wachstumsrate, die Bakterienanzahl und die Tolreranz der Bakterien gegen Cu bestimmt. Die Auftragung von Klärschlamm, Kompost, der beiden Kompostmixturen oder Gartenerde auf die Bodenoberfläche erniedrigte die Konzentration von austauschbaren Cu des Bodens aber hatte keine lang anhaltenden

Remedierungsefekte auf die Bodenbiologie des Schwermetal belasteten Waldbodens.

Key words: mulch, microbial activity, [^{3}H]-thymidine incorporation, PLFA, soil solution, copper fractionation, heavy metal, polluted soil

Introduction

Current approaches in the remediation of heavy metal polluted soil are often based on *in situ* immobilisation of the metals into less bioavailable forms. Several inorganic agents, such as apatite (Boisson et al. 1999), synthetic zeolites (Edwards et al. 1999), modified aluminosilicate, and manganese oxide (Mench et al. 1994) have been found to immobilise heavy metals. The potential offered by organic materials has received little attention in remediation studies. Immobilisation of heavy metals has been reported after an addition of a mixture of compost and beringite (Vangronsveld et al. 1996) or limed composted sewage sludge (Li et al. 2000) in the field, silage effluent, brewery residues and blood meal in the laboratory (Fisher et al. 1998). Organic matter is known to complex heavy metals (Alloway 1995), especially Cu (Baker & Senft 1995), into forms that are less available for microbiota (Hughes & Poole 1991). An increase in microbial activities was detected after application of a mixture of sewage sludge and fly ash (Kelly & Tate 1998), a mixture of compost and woodchips (Kiikkilä et al. 2001), or limestone (Mälkönen et al. 1999) to the surface of the polluted soil. Microbiological response to heavy metal immobilisation is still poorly studied. However, the recovery of microbiota is an important objective in remediation since nutrient cycling in polluted sites is often disturbed affecting nutrient deficiency to plants (Derome & Nieminen 1998).
The aim of the study was to provide a new microbiologically active organic layer onto podsolized mineral soil in boreal coniferous forest by adding mulch (organic remediation agent) onto the polluted soil surface.

We tested the effectiveness of nine organic materials to immobilise copper in a microcosm study. We assumed that immobilisation would be detected as a decrease in the exchangeable Cu concentration in the soil, as an increase in the complexed Cu concentration in the soil solution, and as a decrease in the toxicity of the soil solution to bacteria. We hypothesise that the decreasing toxic Cu concentration in the polluted soil would increase microbial respiration activity, bacterial growth rate and microbial biomass, and decrease bacterial tolerance to heavy metals and change the structure of the microbial community.

Materials and Methods

Experimental design

The organic layer (3 cm) of a polluted forest site was sampled at a distance of 2 km from a Cu-Ni smelter in south-western Finland (61°19'N 22°9'E). The site was a relatively infertile *Calluna* site type (Cajander 1949) with a tree cover consisting of Scots pine (*Pinus sylvestris* L.). The polluted organic layer had relatively high total heavy metal concentrations: Cu 1600 mg kg^{-1}, Ni 220 mg kg^{-1}, Fe 6100 mg kg^{-1}, Zn 160 mg kg^{-1}, Cd 2.1 mg kg^{-1} and Pb 130 mg kg^{-1} (Derome & Lindroos 1998). The organic matter content, determined as loss in weight on ignition (550°C), was 33%.

Table 1. The treatments, the origin, pH and C:N ratio of the mulch materials in the beginning of the microcosm experiment.

Mulch material		pH	C:N
Composted sewage sludge (SS)	Helsinki Metropolitan area sewage plant	7.7	16:1
Compost (C)	Mature compost from Ämmässuo Waste handling Centre, Helsinki Metropolitan area	7.7	11:1
A mixture of compost and woodchips (C-W)	The woodchips (diameter < 20 mm) from Scots pine and Norway Spruce (*Picea abies* (L.) Karsten) stemwood	6.4	17:1
A mixture of compost and barkchips (C-B)	The barkchips from Scots pine and Norway Spruce (Kekkilä Ltd) (diameter < 50 mm)	6.1	23:1
Garden soil (GS)	Commercial soil (Kekkilä Ltd)	6.7	29:1
Green leaves (L)	Downy birch (*Betula pubescens* Ehrh.)	5.8	11:1
Barkchips (B)		4.9	160:1
Humus (H)	F + H layers, mixed Scots pine and Norway Spruce stand	4.1	33:1
Peat (P)	low humufied *Sphagnum* peat (Kekkilä Ltd)	4.5	46:1
Control	polluted F + H layers	3.9	25:1

Undecomposed litter, dwarf shrubs and lichens were removed, and the organic layer was sampled (F and H layers). The samples were sieved through a 25 mm screen to separate out larger material and mixed thoroughly. The microcosms were established in 2 l plastic pots. A 50 mm layer of mulch was spread on the surface of the mixed organic layer sample (1 l). The ten treatments and their abbreviations are presented in Table 1. There were 12 replicates of each treatment, i.e. three replicates for each of the four samplings. The microcosms were kept at 20-28°C in the dark, and moistened with purified water to 50% of water holding capacity once a week. After 1, 4, 10 and 16 months of incubation the microcosms were destructively sampled. The mulch was discarded and the organic layer sample was homogenised by sieving (2.8 mm mesh), stored for one day at room temperature in order to stabilise 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 Cu analyses were air dried.

Mulch characterisation

Prior to the experiment the pH of the mulch was measured (mulch:water, 1:3, v/v), and the organic carbon (C) and nitrogen (N) content was determined by dry combustion with a LECO analyser. The PLFA pattern of the mulch extract was determined after 1 and 16 months from one composite sample prepared from the three replicates. 100 ml of fresh mulch was extracted with 150 ml of purified water on a shaker (250 rev min^{-1}) for 20 h at 14°C. The extract was filtered through quartz wool. The suspension was centrifuged for 30 min at 39000×g. The pellet was subjected to the same treatment as the soil samples in the PLFA analysis (described below).

Chemical analyses

Dry matter weight (d.m.) was determined by drying the soil samples overnight in an oven at 105°C, and the organic matter content (o.m.) as the loss in weight on ignition (550°C). Soil pH was measured from a water suspension (soil:water, 1:3, v/v). The exchangeable copper (Cu_{exc}) concentration was determined by extracting soil with $BaCl_2$ (Tamminen & Starr 1990). Soil solution was obtained by centrifugation from the fresh soil samples (Geisler 1996) taken after 16 months. Copper in the soil solution was fractionated into free Cu^{2+} ions and organically complexed Cu (Cu_{comp}) by passing the soil solution sample through a cation exchange column (Amberlite 120 Plus, Na^+ form; ICN Biomedicals Inc.) (Berggren 1989). 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 as the concentration of Cu^{2+} ions. The Cu concentration was determined by atomic absorption spectrometry (AAS). The pH of the soil solution was measured and the dissolved organic carbon (DOC) concentration was determined on a TOC analyser.

Microbiological analyses

Microbial respiration activity (BR) was measured as described by Pietikäinen & Fritze (1995). The microbial community structure was analysed as described in detail by Frostegård et al. (1993) 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. The individual PLFAs (a total of 39 PLFAs was identified) 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 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 & Bååth 1996). The amount of 18:2ω6,9 was used as an index of the fungal biomass ($PLFA_{fung}$) (Frostegård & Bååth 1996) and the ratio between fungal and bacterial PLFAs as an index of the fungal/bacterial ($PLFA_{fung}/PLFA_{bact}$) biomass in the soil. PLFA 16:1ω5 was used as an indicator of the amount of carbon available to the bacterial community (Frostegård et al. 1996).

The bacterial growth rate and copper tolerance were determined after 16 months by the [^{3}H]-thymidine incorporation technique as described by Bååth (1992a,b) and modified by Kiikkilä et al. (2000, 2001). The bacterial growth rate (TdR) was measured on the basis of the incorporation of radioactive thymidine into the macromolecules of bacteria extracted from soil after homogenisation and centrifugation. The ^{3}[H]-thymidine incorporation was calculated as mol TdR g^{-1} o.m. h^{-1}. In the copper tolerance assay different amounts of $CuSO_4$ was added to the bacterial suspension. The Cu concentration (M) giving a 50% reduction in [^{3}H]-thymidine incorporation was calculated (IC_{50}). The number of bacterial cells in the soil was determined after staining with acridine orange (AO) (Bååth & Arnebrandt 1994; Kiikkilä et al. 2001). The specific [^{3}H]-thymidine incorporation depicting the growth rate of the bacterial cells (TdR/AO) was calculated.

The toxicity of the soil solution (obtained by centrifugation as described above) to bacteria extracted from humus of an unpolluted forest site was studied on samples taken after 16 months by the [^{3}H]-thymidine incorporation technique (Kiikkilä et al. 2001). It was expected that the more toxic the soil solution, the less [^{3}H]-thymidine would be incorporated. The result was expressed as the inhibition percentage (DI; degree of inhibition) of the soil solution on the growth rate of bacteria.

Data analyses

The results of the soil analysis are expressed on the basis of the organic matter content (o.m.). The changes in the measured variables on the coarse of incubation were investigated by canonical correlation analysis (CCA) and multidimensional scaling procedure (MDS). Physico-chemical (pH, Cu_{exc}, d.m., o.m.) and microbiological variables (BR, $PLFA_{tot}$, $PLFA_{bact}$, $PLFA_{fung}$, $PLFA_{bact}$ /$PLFA_{fung}$, PLFA 16:1ω5) were subjected to CCA and the PLFA pattern to MDS.
CCA generates pairs of linear combinations from two sets of original variables such that the correlation is maximal between the pairs of new canonical variables (Gittins 1985). The new canonical variables are called CHEM and BIOL. Graphical presentations of CCA are scatter plot diagrams of the sample units (microcosms) on CHEM and BIOL. Canonical structure, i.e. correlations between the original variables and canonical variables, was applied to diagrams with the vectors. The length of the vector indicates the strength of the correlation. Redundancy analysis was used to determine the proportion of the variation that the canonical variables explain in their own data set (van den Wollenberg 1977).

The PLFA pattern of the soil in the microcosms was explored with global non-metric multidimensional scaling (MDS), using the program package PC-ORD (autopilot mode with medium thoroughness) (McCune & Mefford 1999). The pairwise dissimilarities were computed using a Bray-Curtis coefficient. The four sampling occasions resulted in 120 sample units (microcosms) and 32 variables (PLFAs). A total of 3 outlier microcosms were excluded. The mulch extracts (n = 18) that were prepared after 1 and 16 months of incubation were subjected to MDS. Prior to the MDS analyses, the mole percentages of the PLFAs (soil) and the amounts of PLFAs (mulch extract) were double-square root transformed ($y^{0.25}$) to down-weight the influence of very abundant PLFAs. Graphical presentation of MDS is in the form of scatter plot diagrams about the sample units. The final

ordination diagram is interpreted as follows: the closer the two sample units are on the ordination, the more similar is their PLFA pattern.

One-way analysis of variance (ANOVA) followed by Tukey's test was performed for the scores of the canonical variables CHEM and BIOL. The results from the 16 months sampling were subjected to a priori Dunnett's test to compare the treatments to the control. Pearson correlation coefficients were calculated, but Spearman correlation was used if the variable could not be normalised (DOC). Prior to the analyses, the necessary log, square root or exponential transformations were made to normalise the distribution of the variables.

Results

The mulch characterisation

The pH was highest (7.7) in the sewage sludge (SS) and compost (C), and decreased to a minimum value of 4.1 in the order: garden soil (GS) > compost and woodchips mixture (C-W) > compost and bark mixture (C-B) > leaves (L) > bark (B) > peat (P) > humus (H) (Tab. 1). In the control the pH of the polluted organic layer was 3.9. The C:N ratio was lowest in the L and C treatments (11:1), and increased in the order SS < C-W < C-B < GS < H < P < B (160:1). The C:N ratio of the control was 25:1.

The MDS of the PLFA pattern of the mulch extracts separated B and L from all the other treatments (data not shown). No significant correlations were found between the amounts of PLFAs in the mulch extract and the abundance of the PLFA in question in the underlying soil. After 16 months 16:1ω5 was more abundant in the GS, SS, C, C-W and C-B extracts (22, 27, 35, 48 and 57 pmol ml^{-1}, respectively) than in the B, P and H extracts (1, 6 and 6 pmol ml^{-1}, respectively).

Organic soil

In canonical correlation analysis (CCA) the first canonical variable, formed from the chemical data set (CHEM), explained 27% of the total variance in the chemical data set. The first biological canonical variable (BIOL) explained 30% of the total variation in the biological data set. The correlation between the first canonical variables CHEM and BIOL (canonical correlation) was 0.81. CCA, followed by Tukey's test ($p < 0.05$) for the scores of both CHEM and BIOL, clustered the treatments into two groups. The group A included the treatments mulched with SS, C, C-W, C-B and GS, and the group B included the control and treatments mulched with B, H, P, and L (Fig. 1). The treatments in the group B are characterised by a higher Cu_{exc} concentration, higher microbial biomass ($PLFA_{bact}$, $PLFA_{tot}$) and microbial respiration activity (BR), and a greater abundance of 16:1ω5 than the treatments in the group A (see vectors in Fig. 1). The data of the groups A and B were subjected separately to CCA to get a better understanding about the trends of the variables over the coarse of the incubation. The first canonical correlations were 0.81 and 0.80, the variance explained by CHEM of the chemical data set being 31% and 32%, and by BIOL of the biological data set 19% and 28%, for groups A and B, respectively. The soil pH decreased slightly in both groups during incubation, from 4.5 ± 0.2 (the grand mean of all microcosms ± SD) to 4.2 ± 0.2 during 16 months of incubation but other variables showed different trends between the groups (Fig. 2). The decreasing trend after 4 months of incubation in Cu_{exc} or BR was detected in group A but not in group B. In group B, $PLFA_{bact}$, $PLFA_{fung}$ and the abundance of 16:1ω5 decreased during incubation. In contrast in group A $PLFA_{tot}$ and $PLFA_{fung}$ increased during incubation when 16:1ω5 remained at a relatively constant level. 16:1ω5 followed approximately the same trend as $PLFA_{bact}$ and BR within the groups throughout the course of the incubation (Fig. 3).

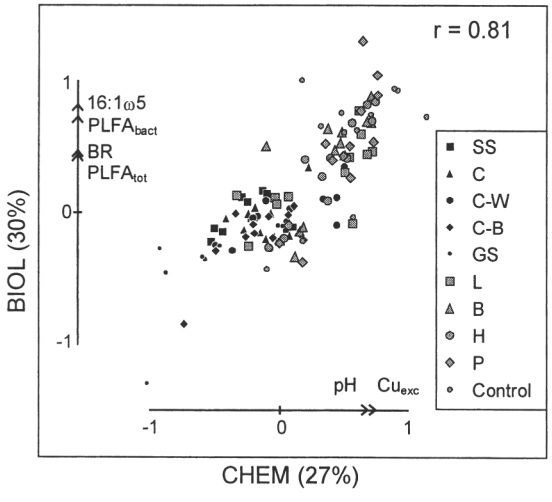

Figure 1. Plot of all the microcosms with respect to treatment along the first canonical variables, CHEM (the chemical data set) and BIOL (the biological data set) from CCA. The vectors indicate the correlation between the original variable and the canonical variable in question. The proportion of the variation that CHEM and BIOL explain is in the parentheses.

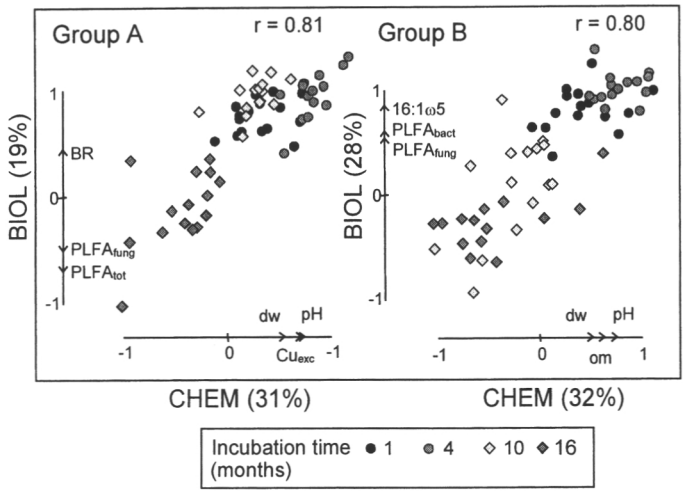

Figure 2. Plot of the microcosms in group A (SS, C, C-W, C-B, GS) and B (control, P, B, H, L) with respect to incubation time along CHEM and BIOL from CCA. See Figure 1. for the abbreviations and the vectors.

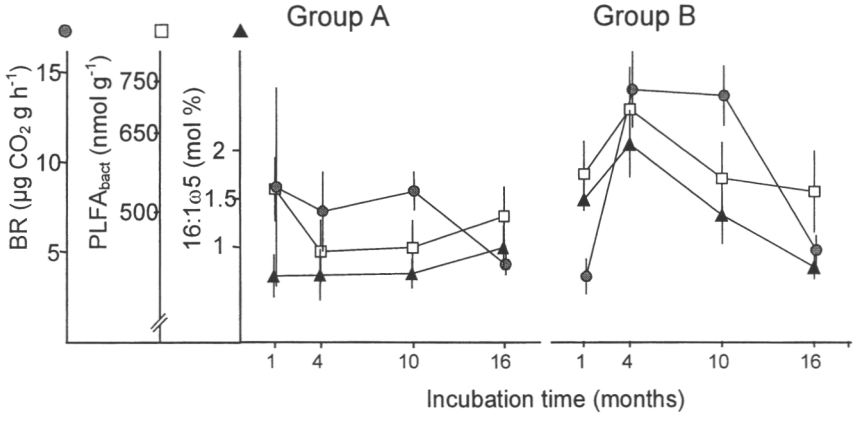

Figure 3. Microbial respiration activity (BR), bacterial biomass ($PLFA_{bact}$) and the proportion of 16:1ω5 after different incubation periods in group A and B (see Fig. 2). The dot and the bar indicate the mean and SD of all microcosms in group A or B (n = 15).

The Cu_{exc} concentrations in the soil were 25-39% lower ($p < 0.05$, Dunnett) after 16 months in the treatments mulched with SS, C, C-W, C-B, and GS than in the control (Tab. 2). The Cu_{comp} concentration was significantly lower in SS, C, C-B or GS than in the control. The distribution of the variable DOC could not be normalised. DOC was high in L and B compared to the control and other treatments. The toxicity of the soil solution (DI), was highest in L and lowest in the

control. In the other microbiological variables were no or only single significant differences after 16 months (Tab. 3).

After 16 months of incubation there was moderate positive correlations between pH of the soil solution and both Cu_{comp} and DOC, between Cu_{comp} in soil solution and Cu_{exc} in soil, and between both Cu_{exc} and Cu_{comp} and BR (Tab. 4). DI did not correlate with any of the other variables.

Table 2. The exchangeable Cu concentration (Cu_{exc}) in soil, pH, concentrations of dissolved organic carbon (DOC), Cu^{2+} and complexed Cu (Cu_{comp}) in the soil solution under the mulches after 16 months of incubation.

Mulch material	$pH_{soil\ solution}$	DOC (mg l^{-1})	Cu_{exc} (µg g^{-1})	Cu^{2+} (mg l^{-1})	Cu_{comp} (mg l^{-1})
SS	4.0 (0.1)	110 (20)	660 (20)*	3.1 (0.1)*	0.29 (0.01)*
C	4.1 (0.03)	100 (10)	570 (40)*	1.9 (0.1)	0.38 (0.03)*
C-W	3.9 (0.04)	130 (10)	590 (60)*	2.4 (0.2)	0.43 (0.04)
C-B	3.9 (0.03)	120 (10)	590 (20)*	2.3 (0.1)	0.35 (0.02)*
GS	4.1 (0.2)	120 (10)	540 (10)*	2.4 (0.2)	0.24 (0.01)*
L	4.2 (0.04)	390 (130)	750 (90)	1.8 (0.1)	0.49 (0.07)
B	4.3 (0.1)	270 (120)	870 (10)	2.3 (0.1)	0.66 (0.05)
H	4.3 (0.1)	180 (30)	780 (20)	1.6 (0.3)*	0.74 (0.14)
P	4.0 (0.1)	130 (30)	930 (50)	2.6 (0.3)	0.60 (0.04)
Control	4.2 (0.03)	220 (10)	880 (60)	2.1 (0.1)	0.57 (0.03)

See Table 1 for explanation of the symbols used for the mulches. The results are expressed as means (n = 3) and SEM (in parentheses). Means followed by * are significantly different from the control ($p < 0.05$) (Dunnett).

Table 3. Microbial respiration activity (BR), bacterial growth rate (TdR), number of bacterial cells (AO), toxicity of the soil solution to bacteria (DI), and bacterial copper tolerance (IC_{50}) of the polluted forest soil under the mulches after 16 months of incubation.

Mulch material	BR (µg CO_2 g h^{-1})	TdR (pmol h^{-1})	AO (cells × 10^9)	DI (%)	IC_{50} (mM Cu)
SS	4.3 (0.2)	31 (12)	64 (8)	74.8 (0.4)*	22 (10)
C	4.5 (0.1)	26 (9)	84 (7)	73.7 (0.9)*	6.1 (2.5)
C-W	4.1 (0.5)	20 (6)	59 (6)	73.5 (1.3)*	7.3 (0.8)
C-B	4.5 (0.1)	24 (8)	79 (14)	73.1 (1.5)*	7.3 (2.1)
GS	3.5 (0.1)*	14 (5)	55 (9)	73.6 (1.1)*	7.4 (1.4)
L	4.0 (0.5)	32 (12)	71 (3)	75.0 (0.5)*	13 (7.2)
B	5.3 (0.1)	26 (7)	78 (5)	73.9 (1.0)*	12 (7.5)
H	5.0 (0.4)	13 (1)	120 (20)*	74.1 (0.8)*	2.4 (1.0)
P	5.7 (0.1)	17 (1)	110 (8)	72.4 (0.9)	5.8 (0.6)
Control	4.8 (0.5)	26 (5)	72 (11)	68.7 (0.7)	9.7 (3.3)

See Table 1 for explanation of the symbols used for mulches.
The results are expressed as means (n = 3) and SEM (in parentheses). Means followed by * are significantly different from the control ($p < 0.05$) (Dunnett).

Table 4. The correlations ($r \geq 0.5$; $p < 0.01$; $n = 30$) between Cu^{2+}, complexed Cu (Cu_{comp}), pH and DOC in the soil solution, and exchangeable Cu (Cu_{exc}) and microbial respiration activity (BR) in the underlying polluted soil after 16 months of incubation.

	Cu_{exc}	Cu^{2+}	Cu_{comp}	$pH_{soil\ solution}$	DOC^a	BR
Cu_{exc}	1					
Cu^{2+}		1				
Cu_{comp}	0.70	-0.53	1			
$pH_{soil\ solution}$		-0.53	0.57	1		
DOC^a				0.60	1	
BR	0.61		0.61			1

[a]Spearman correlation coefficient

Structure of the microbial community in the polluted soil

The PLFA data was subjected to the multidimensional scaling procedure. The minimum stress value of 0.07 for MDS ordination was obtained by a three-dimensional solution. Two dimensions separated the incubation time (Fig. 4). Also the treatments separated along the dimensions after month 1, 4 and 10 months. The control was clustered with B, H, and P when SS, C, C-W, C-B, and GS were clustered together. After 16 months no clear separation was detected between the treatments.

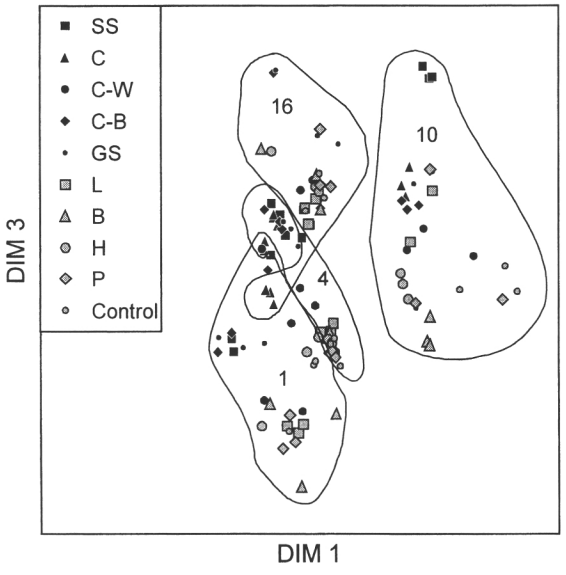

Figure 4. MDS ordination of the microcosms with respect to the treatments. Numbers in the encircled microcosms refer to incubation time. Black dots refer to group A, grey dots to group B.

Discussion

The exchangeable Cu concentration decreased during incubation in the treatments mulched with garden soil (GS), sewage sludge (SS), compost (C), and the compost mixtures (C-W, C-B), but not in the control or in the treatments involving barkchips (B), humus (H) or peat (P). The probable sink for the decreasing Cu_{exc} concentration was the formation of complexes with particulate organic matter (Baker & Senft 1995) carried down into the underlying soil from the mulch. This input of organic matter was measured as loss in weight on ignition, which is a very rough method, and as DOC in the soil solution but these variables seemed not to explain the decrease in Cu_{exc}. However, we assume that relatively large amounts of particulate material were released especially from the SS, C, C-W, C-B, and GS of which pH was over 6. The pH of the mulches did not reflect to the underlying heavy metal polluted soil. However, mulch pH may have affected the exchangeable Cu concentration since pH is related to organic matter quality (Bååth et al. 1995), which was assumed to be different in the SS, C, C-W, C-B, and GS than in P, B or H. We concluded that these treatments immobilised Cu.

The increase or decrease of bacterial tolerance to heavy metals has been studied earlier in laboratory conditions. An increase after the experimental contamination (Díaz-Raviña & Bååth 1996) and a decrease after the metal stress was removed (Díaz-Raviña & Bååth 2001) in the metal tolerance of the bacterial community was detected during 28 and 12 months period, respectively. These two experiments showed that the change in bacterial heavy metal tolerance could be studied in microcosms. Thus we followed the incubation procedure of Díaz-Raviña & Bååth (1996) to study the change in the Cu tolerance of the polluted soil after the remediation treatments. However, in contrast to our hypothesis, the decrease in the Cu_{exc} concentration was not reflected in bacterial copper tolerance or the microbial activities. Microbial respiration activity fluctuated during the incubation, but was in general lower under SS, C, C-W, C-B, and GS than in the control and the P, B and H treatments. Also the PLFA pattern fluctuated, but no new microbial community became established during the incubation in any of the treatments. The slight difference in the PLFA patterns between treatments disappeared by the end of the incubation. The fluctuation in microbial respiration activity and the PLFA patterns during the incubation may also be due to the successive breakdown of dead cells, and a concomitant increase in the microbiota feeding on them (Frostegård et al. 1996).

The microbes that were carried from the compost, sewage sludge or garden soil down into the underlying polluted soil were presumably not able to maintain their activity there since the PLFA pattern of the mulch extracts did not reflect to the PLFA pattern of the underlying polluted organic soil. The activity decreased gradually after one month's incubation owing to the toxicity of the organic soil, the loss of available carbon sources, or the stressful conditions in the microcosms. Frostegård et al. (1996) suggest that the abundance of the PLFA 16:1ω5 reflects the dynamics of organisms that are responding to changes in the carbon status of the soil. This interpretation is supported by present experiment where bacterial biomass and microbial respiration activity followed approximately the trends of 16:1ω5. Thus the decrease in the abundance of 16:1ω5 during incubation can be used as suggested by Frostegård et al. (1996) as an indication of the depletion of easily available carbon sources. The amount of 16:1ω5 was higher in the extract of SS, C,

C-W, C-B, and GS than in the extracts of B, P or H at the end of the experiment. We therefore suggest that the carbon sources were not depleted in the microcosms mulched with SS, C, C-W, C-B, or GS but the toxicity of the soil killed the microbes carried down from the mulches. This was confirmed by the fact that the number of bacterial cells and the toxicity of the soil solution were, in general, at the same level in all treatments after 16 months. In contrast, the sources of easily available carbon in the B, P or H treatments and in the control seemed to have become depleted by the end of the incubation. The stress conditions of the microcosm environment for microbiota are different from those in the field. The microbial activities were among the highest in the control, indicating stressful, maybe partly anaerobic, conditions in the mulched microcosms. The lack of living roots, destroyed physical structure of the soil and the limited amount of available carbon set limits when studying the microbiological properties of soil in a laboratory experiment.

In conclusion, in this microcosm study on the remediation of Cu polluted soil, the exchangeable copper concentration decreased during 16 months of incubation when the soil was mulched with compost, sewage sludge or garden soil. However, no microbial response directly attributable to remediation was detected.

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Paper III

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III

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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 [^{3}H]-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 Cu^{2+} 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 [^{3}H]-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 fractioned Cu in the soil solution into Cu^{2+} 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_{exc}, exchangeable copper concentration in soil; DM, dry matter weight; IC_{50}, inhibition concentration (i.e., bacterial copper tolerance); MDS, multidimensional scaling; Ni_{exc}, 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, [^{3}H]-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* $BaCl_2$ 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 Cu^{2+} 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 Cu^{2+} 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 Cu^{2+} 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 [^{3}H]-thymidine incorporation technique in order to measure the bacterial growth rates. The [^{3}H]-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 [^{3}H]-thymidine incorporation procedure was then performed as described below. It was expected that the more toxic the soil solution, the less [^{3}H]-thymidine would be incorporated.

Microbial Activities

Microbial activity was measured as basal respiration (BR). The CO_2 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 [^{3}H]-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 [^{3}H]-labelled thymidine. The growing bacteria incorporate [^{3}H]-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 (IC_{50}) was calculated as the log Cu concentration (*M*) giving a 50% reduction in [^{3}H]-thymidine incorporation. The lower the absolute IC_{50} 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 [^{3}H]-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

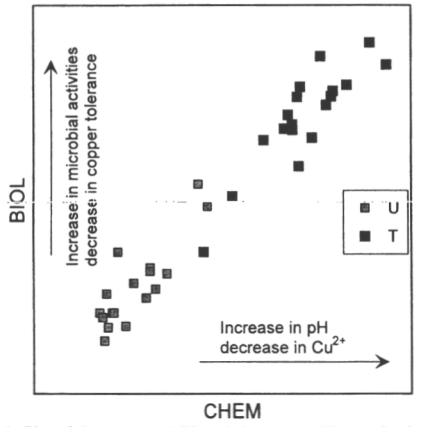

Fig. 1. Plot of the untreated (U) and the treated (T) sample plots of the last sampling date on the first canonical variables, CHEM (the chemical data set) and BIOL (the biological data set) from canonical correlation analysis. The arrows indicate the influence of the most important variables on formation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

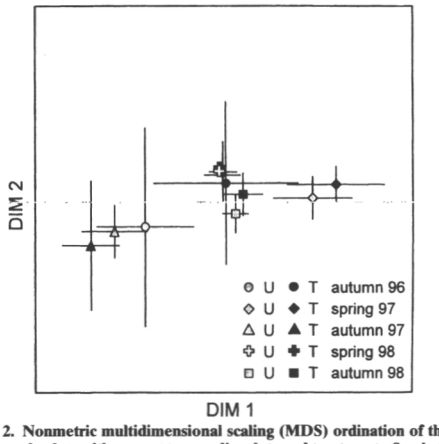

Fig. 2. Nonmetric multidimensional scaling (MDS) ordination of the sample plots with respect to sampling date and treatments. Symbols indicate the mean of 18 replicate untreated (U) and treated (T) plots, and bars indicate the standard deviation of the replicates.

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 CANCORR procedure (SAS Institute, 1996).

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

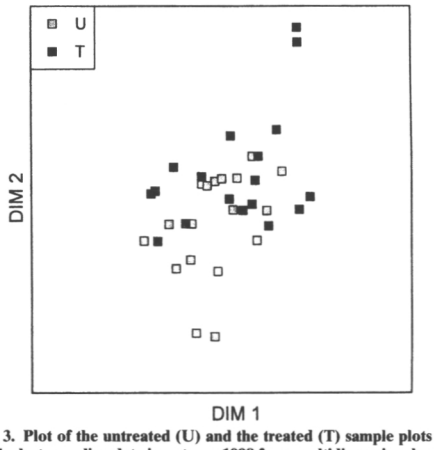

Fig. 3. Plot of the untreated (U) and the treated (T) sample plots of the last sampling date in autumn 1998 from multidimensional scaling (MDS) ordination in Fig. 2.

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).

	Autumn 1996		Spring 1997		Autumn 1997		Spring 1998		Autumn 1998	
Variable†	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 (20)	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	2.8 (0.2)	4.0 (0.4)	0.5 (0.0)	1.4 (0.1)	2.4 (0.2)	2.6 (0.2)	1.7 (0.1)	3.0 (0.2)
IC_{50}	nd	nd	−1.2 (0.1)	−1.5 (0.5)	−1.6 (0.1)	−3.0 (0.1)	−2.2 (0.3)	−2.6 (0.1)	−1.7 (0.1)	−2.6 (0.1)
AO (cell × 10^{10} g^{-1})	nd	nd	nd	nd	2.3 (0.2)	4.6 (0.4)	9.9 (1.0)	10.7 (0.7)	4.1 (0.5)	6.0 (0.4)
TdR/AO (mol × 10^{-22})	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)
$PLFA_{tot}$ (nmol g^{-1})	1790 (80)	1590 (80)	1950 (80)	1810 (60)	1260 (70)	1300 (60)	1580 (120)	1510 (120)	1610 (70)	1540 (80)
$PLFA_{bact}$ (nmol g^{-1})	684 (25)	594 (26)	708 (30)	647 (22)	541 (33)	553 (29)	579 (45)	544 (42)	634 (29)	597 (31)
$PLFA_{fung}$ (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 (0.00)	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; IC_{50}, bacterial copper tolerance; AO, number of bacterial cells; TdR/AO, specific bacterial growth rate; $PLFA_{tot}$, total microbial biomass; $PLFA_{bact}$, bacterial biomass; $PLFA_{fung}$, fungal 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 Cu^{2+} 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 [^{3}H]-thymidine incorporation for the T and U plots was 2.3 and 1.4 ± 0.1 × 10^{-11} mol [^{3}H]-thymidine g^{-1} h^{-1}, respectively (Table 2). There was strong correlation between [^{3}H]-thymidine incorporation and the Cu^{2+} 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 ± 0.3 μg CO_2 g^{-1} 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 ± 0.3% and on the U plots 46.5 ± 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 ± 0.1 log *M* 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) 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 ($p < 0.0001$).

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
		$PLFA_{bact}$	−0.17
		$PLFA_{fung}$	−0.23
		$PLFA_{bact}/PLFA_{fung}$	−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; $PLFA_{bact}$, bacterial biomass; $PLFA_{fung}$, fungal biomass; $PLFA_{fung}/PLFA_{bact}$, 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.

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 [^{3}H]-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)
16:1ω9	0.68 (0.02)	0.74 (0.02)
16:1ω7c	5.11 (0.07)	5.04 (0.08)
16:1ω7t	0.84 (0.02)	0.79 (0.02)
16:1ω5	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)
17:1ω8	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)
18:2ω6,9	1.98 (0.12)	2.12 (0.09)
18:1ω9	5.70 (0.10)	5.83 (0.11)
18:1ω7	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 [^{3}H]-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, 18:2ω6,9 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 18:2ω6,9 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 16:1ω5 and 20:4 increased and i15:0, 16:1ω7t, 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, 16:1ω5, 16:1ω9, 18:1ω7, 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. However, 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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Paper IV

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IV

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Copper mobility and toxicity of soil percolation water to bacteria in a metal polluted forest soil

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Key words: compost, Cu, [^{3}H]-thymidine incorporation, mulching, remediation, speciation

Abstract

In order to assess the success of *in situ* remediation of coniferous forest soil polluted by a Cu–Ni smelter, the total Cu concentration in soil percolation water, the fluxes of Cu down through the soil profile, and the toxicity of soil percolation water to soil bacteria were studied. Total Cu in percolation water was also fractionated into free ionic and complexed forms. The toxicity of the percolation water was measured by the [^{3}H]-thymidine incorporation method, which measures bacterial growth rates. Soil percolation water was collected during one growing season by zero tension lysimeters inserted at depths of 0.2 and 0.4 m in the soil. The treatments consisted of a control, mulch application to the forest floor (M) and mulch application after removing the polluted organic soil layer (MR). The mulch consisted of a mixture of compost and woodchips (1/1; vol/vol). Analysis of Cu species and dissolved organic carbon (DOC) indicated that DOC leached from the mulch and complexed Cu into forms that were less toxic to soil bacteria. At 0.2 m depth percolation water toxicity was 19% lower in the m and 42% lower in the MR treatment than in the control. Toxicity correlated with the Cu^{2+} concentration, which was 61 and 84% lower in the m and MR treatments, respectively, compared to the control. However, there were signs that total Cu had leached down through the soil profile, the leaching being more pronounced in the MR treatment.

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Abbreviations: DI – toxicity of the soil percolation water; DOC – dissolved organic carbon; Cu_{comp} – complexed copper; Cu_{tot} – total copper; M – the treatment in which the forest floor was mulched; MR – the treatment in which the exposed mineral soil was mulched after removal of the organic layer; TdR – [^{3}H]-thymidine incorporation

Introduction

One means of assessing the success of the remediation of heavy metal polluted soils is to study the effect of remediation measures on the toxicity and mobility of heavy metals. Although most heavy metals are considered to be relatively immobile in the soil (Ross, 1996), their mobility under certain conditions may increase and pose a significant threat even to groundwater quality (Karathanasis, 1999; McBride, 1998). The mobility can be enhanced when heavy metals form complexes with soluble organic matter (DOC) (Li and Shuman 1997a, b) in the soil percolation water. Complexed forms of heavy metals can be less toxic to bacteria (Menkissoglu and Lindow, 1991), since Cu^{2+} is generally thought to be the most toxic fraction of Cu (Giller et al., 1998). We used soil lysimeters to collect percolation water of a heavy metal polluted forest, and carried out speciation of the mobile Cu fraction and toxicity tests on soil bacteria.

This study is a part of a remediation project carried out close to the Harjavalta Cu–Ni smelter in southwestern Finland. The suitability of mulch, a mixture of compost and woodchips, to remediate coniferous forest soil polluted by the smelter was investigated. Soil fungi and bacteria are estimated to account for 99% of the total biomass of soil organisms, and about 96% of the heterotrophic metabolism in a Scots pine stand (Persson et al., 1980) comparable to that investigated in our study. At least 25% of that bio-

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mass is bacteria (Persson et al., 1980). Since heavy metals are toxic to soil bacteria (Doelman and Haanstra, 1984), nutrient cycling in the polluted sites has become heavily disturbed (Derome and Nieminen, 1998). The growth rate of the bacterial population in a coniferous forest soil, can be assessed by using the [^{3}H]-thymidine incorporation technique (Bååth 1992a, b). We used this approach to test the toxicity of the percolation water to soil bacteria after the remediation treatment. To our knowledge, this has not been studied earlier. We also fractionated total Cu in the percolation water into free ionic (Cu^{2+}) and complexed forms in order to determine the effects of mulching on Cu speciation. We hypothesised that the addition of mulch, which has an extremely high content of soluble organic compounds, would convert heavy metals in the soil into forms that were less toxic to soil bacteria.

Material and methods

Study site

The remediation experiment was established at a distance of 0.5 km from a Cu–Ni smelter in south-western Finland (61°19′N 22°9′E). The vegetation was originally typical of a xerophilous forest site, with a tree cover consisting of Scots pine (*Pinus sylvestris* L.). The study site was of the *Calluna* type, according to the Finnish forest site classification of Cajander (1949). The ground vegetation on the study sites has almost completely disappeared (Salemaa et al., 2001), the growth of trees is extremely poor (Mälkönen et al., 1999), and the overall microbial activity has decreased drastically (Fritze et al., 1989; Pennanen et al., 1996).

The experiment was established on a site with soil consisting of sorted fine or fine/coarse sand with almost no stones. The soil was classified as an orthic podzol (Anonymous, 1988). The original humus layer (A_{01}/A_{02}; F+H) (0-30 mm) on top of the podzolised mineral soil was mor with a clearly pronounced, dry, undecomposed litter layer (Mälkönen et al., 1999). The structure of the layer has changed, and is characterised by an extremely low amount of fine-root biomass (Helmisaari et al., 1999), high heavy metal concentrations (Derome and Nieminen, 1998) and an average organic matter content of 400 g kg^{-1} (Kiikkilä et al., 2001). Throughout this manuscript the polluted humus is called the organic soil layer. According to Derome and Nieminen (1998) the exchangeable Cu_{exc} concentration in the organic soil layer was 4700 μg g^{-1}. In the mineral soil at a depth of 0–50 mm Cu_{exc} was 230 μg g^{-1}, at 50-100 mm 27 μg g^{-1}, at 0.1–0.2 m 16 μg g^{-1} and at 0.2–0.3 m 12 μg g^{-1}. The respective Zn_{exc} concentrations were 130, 10, 2.9, 1.8 and 1.4 μg g^{-1} and Ni_{exc} concentrations 420, 25, 5.3, 3.2 and 2.1 μg g^{-1} (Derome and Nieminen, 1998).

Experimental design

There were three treatments: a control, a mulch application over the layer of undecomposed plant litter on the forest floor (M), and a mulch application on top of the exposed mineral soil after removing the polluted litter layer (0–150 mm) and organic soil layer (ca. 30 mm) (MR). The M and MR sample plots (5 × 5 m) were covered with a 50 mm thick layer of mulch (see below) in summer 1996 (M) or 1997 (MR). The replicate number of lysimeters at both 0.2 m and 0.4 m depth was 6 in the control treatment, 4 in the M treatment and 5 in the MR treatment. The control plots were of the same size as the treatment plots.

The plate lysimeters (0.40 × 0.25 m, stainless steel) used to collect percolation water were installed at depths of 0.2 and 0.4 m by pushing them with a pneumatic jack into the face of a trench dug at the edge of the plot. The plates were installed at a slight angle in order to ensure that all the percolation water ran into a collection bottle. The plates were pushed to a distance of at least 0.6 m into the wall of the trench (plate length 0.40 m), leaving a gap of about 0.20 m between the edge of the plate and the trench wall. Vertical plastic pipes (containing the collection bottles) were installed in the trench before filling the trench with soil.

At the start of the experiment the compost was 14 months old and had been produced in outdoor windrows from a mixture of organic household waste and coarse woodchips (dia. ca. 50 mm) at the Ämmässuo Waste Handling Centre (Espoo, Finland). According to Mäkelä-Kurtto and Sippola (1995, 1996, in Finnish), 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 0.37 g, NO_3-N 0.76 g, Ca 28 g, K 16 g, mg 3 g, P 2 g, Fe 7 g, Al 0.8 g, Mn 0.3 g, Cu 0.06 g, Zn 0.25 g, Ni 0.003 g, Cd 0.0006 g, Pb 0.05 g and Cr 0.003 g 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:N ratio of 11. The C:N ratio is used as a measure of compost maturity; in mature compost the ratio is 10–12 (Chefetz et al., 1996). The mulch was prepared one

week before spreading by mixing the compost with woodchips (dia. < 20 mm) of Scots pine and Norway spruce (*Picea abies* (L.) Karsten) 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:N ratio of 16, and the pH 6.3. The calculated amount of compost (excluding woodchips) added to the experimental plots was 5.4 kg m^{-2} (dry weight).

Percolation water analyses

Percolation water was collected seven times during the snowfree period in 1999. A total of 74 water samples were analysed from 29 lysimeters (Table 1). The theoretical number of samples in the three treatments at each depth is the number of sampling instances (7) × number of lysimeters installed, giving the values of 42, 28 and 35 for the control, M and MR treatments, respectively. However, the number of sampling instances when water was obtained during the growing season was 11 in the control, 9 in the M and 16 in the MR treatment at 0.2 m depth. The respective numbers at 0.4 m were 12, 6 and 20 (Table 1).

The amount of water and its pH was measured. Dissolved organic carbon (DOC) was determined on a TOC analyser, and the total concentrations of Zn_{tot} and Ni_{tot} by inductively coupled plasma atomic emission spectrometry (ICP/AES). Copper fractionation was performed on 46 samples, excluding the samples taken in October (Table 1). Fractionation into Cu^{2+} and complexed Cu_{comp} was performed by passing percolation water through a cation exchange column (Amberlite 120, Na^+ form, INC, Biomedicals Inc.) (Berggren, 1989). The column contained 5 mL of a slurry of the resin, and 50 mL of sample was passed through at a rate of 6.7 mL/min. Fresh resin was used for each sample. Calculations were also performed to ensure that the total amount of cations in the sample would not exceed the cation adsorption capacity of the resin. The Cu concentration of the sample was determined before and after passage through the column by atomic absorption spectrometry (AAS). 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 as the concentration of Cu^{2+} ions.

The toxicity of the percolation water to bacteria was studied by the [^{3}H]-thymidine incorporation technique (TdR) for measuring bacterial growth rates (Bååth, 1992a, b). Percolation water was mixed with a bacterial suspension extracted from humus from an unpolluted coniferous stand: fresh humus equalling 1.0 g of organic matter was shaken in 100 mL distilled water for one hour at 250 rpm. Percolation water (0.4 mL; $TdR_{soil\ percolation\ water}$) or distilled water (0.4 mL; TdR_{water}) was added to an aliquot of this bacterial suspension (1.6 mL), and the [^{3}H]-thymidine incorporation procedure then performed as described by Bååth (1992a, b). It was expected that the more toxic the soil percolation water, the less [^{3}H]-thymidine would be incorporated. The result was expressed as the inhibition percentage (DI; degree of inhibition) of the percolation water. DI was calculated as 1- $TdR_{soil\ percolation\ water}$ / TdR_{water}.

Table I. Number of samples collected during 22.4.–5.10.1999 in the control, and M and MR treatments. No water was obtained in September. The number in parentheses indicates the number of samples on which Cu fractionation was performed

	Depth (m)	Control	M	MR
Number of lysimeters	0.2	6	4	5
	0.4	6	4	5
April	0.2	6 (5)	4 (4)	5 (5)
	0.4	6 (6)	2 (2)	5 (5)
May	0.2	1 (1)	0 (0)	0 (0)
	0.4	2 (1)	0 (0)	4 (4)
June	0.2	0 (0)	0 (0)	0 (0)
	0.4	0 (0)	0 (0)	1 (1)
July	0.2	1 (1)	2 (0)	5 (3)
	0.4	2 (2)	1 (0)	5 (5)
August				
	0.2	0 (0)	0 (0)	1 (1)
	0.4	0 (0)	0 (0)	0 (0)
October	0.2	3 (0)	3 (0)	5 (0)
	0.4	2 (0)	3 (0)	5 (0)
Total	0.2	11 (7)	9 (4)	16 (9)
	0.4	12 (9)	6 (2)	20 (15)

Fluxes

The annual fluxes of DOC, Cu_{tot}, Zn_{tot}, Ni_{tot} and water down to 0.2 and 0.4 m depth were calculated by summing the amounts of individual metals or DOC

collected from each lysimeter (area 0.1 m^{-2}) at each sampling instance during the year:

$$\text{Flux} = \sum_{m=1}^{x} WmCm,$$

where m = collection period (April–October), w = the amount of water (l) collected from a lysimeter, and c = the concentration of DOC or metals ($g\ l^{-1}$). No percolation water was obtained from one of the lysimeters at 0.4 m depth in the M treatment. A value of 0 was used in the flux calculations since it was assumed that the lysimeter was functioning, but there was no water flow at that particular point.

Data analysis

DI, pH, the concentrations of DOC, Cu_{tot}, Cu^{2+}, Cu_{comp}, Zn_{tot}, and Ni_{tot} and the amount of water were subjected to two-factor analysis of variance (ANOVA) with depth and treatment as the main effects. The sample units (lysimeters, n= 29) were the means of all the sampling instances. Tukey's test ($p < 0.05$) was used for the comparison of the treatment means. Linear regression analyses of DI on Cu_{tot}, Cu^{2+}, Zn_{tot} or Ni_{tot} and of DOC on Cu_{comp} were performed. Logarithmic transformations were made for Cu_{tot}, Cu^{2+}, Zn_{tot}, and an exponential transformation for Cu_{comp}.

Results

Percolation water properties

The amount of water in the individual lysimeters did not correlate ($r < 0.5$) with any of the measured variables at either depth.

Control

In the control, the pH of the percolation water was 4.3 ± 0.2 (mean ± SE; n= 6) at 0.2 m and 4.4 ± 0.1 at 0.4 m depth (Table 2). The DOC ($p<0.05$; ANOVA), Cu_{tot}, Cu_{comp} ($p<0.05$) and Cu^{2+} ($p<0.05$) concentrations were higher at 0.2 m than at 0.4 m. The DOC concentration at 0.2 m was 23 ± 6 $mg\ l^{-1}$, and at 0.4 m 13 ± 1 $mg\ l^{-1}$. The respective values for Cu_{tot} were 1600 ± 600 $\mu g\ l^{-1}$ and 620 ± 150, for Cu_{comp} 110 ± 30 and 26 ± 5 $\mu g\ l^{-1}$, and for Cu^{2+} 2000 ± 780 and 560 ± 130 $\mu g\ l^{-1}$. Zn_{tot} and Ni_{tot} were, in contrast, lower at 0.2 than at 0.4 m. The respective values for Zn_{tot} were 550 ± 160 and 720 ± 140 $\mu g\ l^{-1}$, and for Ni_{tot} 780 ± 220 and 1130 ± 220 $\mu g\ l^{-1}$ (Table 2).

Effects of the treatments

The pH of percolation water at 0.4 m depth was slightly higher in treatments m and MR than in the control (Table 2). The DOC ($p<0.05$), Cu_{tot}, Cu_{comp} ($p<0.05$) and Cu^{2+} ($p<0.05$) concentrations were higher at 0.2 than at 0.4 m. There were no interactions in ANOVA between treatment and depth. The DOC concentration was the lowest and the Cu_{tot} concentration the highest in the control compared to the treated plots, but the differences were not significant (Table 2). The Cu_{comp} and Cu^{2+} concentrations differed significantly between the control and MR treatment (Tukey, $p<0.05$). Similar, but not significant, differences were found between the control and m treatment. The Cu_{comp} concentration was the highest and Cu^{2+} the lowest in the MR treatment (Table 2). The Cu^{2+} concentration in treatment m was 61% and in treatment MR 84% lower than that in the control at 0.2 m depth. At 0.4 m the Cu^{2+} concentration in treatment m was 66% and in treatment MR 54% lower than that in the control. The difference in the Cu_{comp} concentration was more pronounced at 0.4 m than at 0.2 m. At 0.4 m the Cu_{comp} concentration in treatment m was 200% and in MR 360% higher than in the control (Table 2).

The Zn_{tot} concentration was significantly lower in treatment M than in the control. The difference was greater at 0.4 m where the Zn_{tot} concentration was 71% lower in treatment m and 46% lower in MR than in the control (Table 2). The Zn_{tot} concentration was higher at 0.4 than at 0.2 m in the control, whereas in the m and MR treatments the concentrations at 0.4 m were slightly lower than at 0.2 m. However, there were no significant interactions between treatment and depth. The differences in the Ni_{tot}concentrations were similar to those for Zn_{tot} (Table 2).

Toxicity test

The toxicity of the percolation water to bacteria (DI) was higher ($p<0.05$) at 0.2 than at 0.4 m depth in all the treatments (Table 2). In the control, the DI was 36 ± 4% at 0.2 m and 25 ± 2% at 0.4 m. The respective values for the m treatment were 29 ± 3% and 23 ± 6%, and for MR 21 ± 3% and 17 ± 2%. The toxicity in the MR treatment was significantly lower ($p<0.05$) than that in the control at both depths.

Table 2. Mean and standard error of the amount of water, dissolved organic carbon (DOC), total Cu (Cu_{tot}) and complexed copper (Cu_{comp}) concentration, and the toxicity of the solution to bacteria (DI) in the three treatments on the soil percolation water at 0.2 and 0.4 m depths. Means followed by a different letter, treatments and depths, are significantly different (Tukey, $p<0.05$)

	Depth (m)	Control	M	MR
n	0.2	6	4	5
	0.4	6	3	5
Water (ml)	0.2	960 ± 302a	640 ± 300a	900 ± 210a
	0.4	1320 ± 320b	1530 ± 590b	1480 ± 210b
pH	0.2	4.3 ± 0.2	4.3 ± 0.1	4.3 ± 0.2
	0.4	4.4 ± 0.1	5.0 ± 0.3	4.9 ± 0.04
DOC ($mg\ l^{-1}$)	0.2	23 ± 6a	32 ± 4a	32 ± 3a
	0.4	13 ± 1b	18 ± 2b	20 ± 3b
Cu_{tot} ($\mu g\ l^{-1}$)	0.2	1600 ± 600	1100 ± 300	660 ± 200
	0.4	620 ± 150	420 ± 280	420 ± 160
Cu_{comp} ($\mu g\ l^{-1}$)	0.2	110 ± 30a	150 ± 40ab	250 ± 40b
	0.4	26 ± 5c	80 ± 25cd	120 ± 50d
Cu^{2+} ($\mu g\ l^{-1}$)	0.2	2000 ± 780a	790 ± 340ab	320 ± 110b
	0.4	560 ± 130c	190 ± 120cd	260 ± 100d
Zn_{tot} ($\mu g\ l^{-1}$)	0.2	550 ± 160a	250 ± 40b	400 ± 120ab
	0.4	720 ± 140a	210 ± 60b	390 ± 60ab
Ni_{tot} ($\mu g\ l^{-1}$)	0.2	780 ± 220	630 ± 200	650 ± 200
	0.4	1130 ± 220	440 ± 240	540 ± 80
DI (%)	0.2	36 ± 4a	29 ± 3ab	21 ± 3b
	0.4	25 ± 2c	23 ± 6cd	17 ± 2d

The toxicity of the percolation water decreased with decreasing Cu^{2+} or Cu_{tot} concentrations, but was not significantly dependent on the Zn_{tot} or Ni_{tot} concentrations. Cu^{2+} explained 42% of the variance of DI. Ni_{tot} or Zn_{tot} did not increase the explained variance (43%) when added to the regression model. Significant regression ($p<0.05$) was found between DI and Cu^{2+} ($r= 0.65$, $n=26$) (Figure 1), between DI and Cu_{tot} ($r= 0.59$, $n = 28$), and between Cu_{comp} and DOC ($r = 0.62$, $n = 26$) (Figure 2).

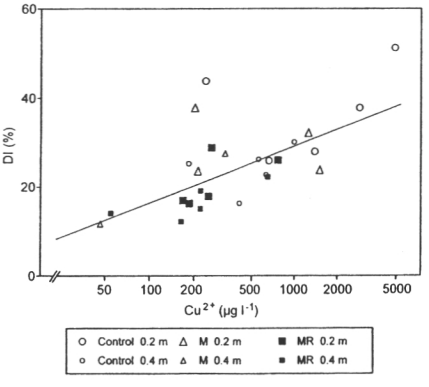

Figure 1. Regression ($r = 0.65$) between the toxicity and Cu^{2+} concentration of the percolation water at depths of 0.2 and at 0.4 m. Dots refer to the lysimeters.

Flux of water, DOC and metals

The amounts of water, DOC and Cu_{tot}, Zn_{tot} and Ni_{tot} passing down the soil profile to depths of 0.2 and 0.4 m were highly variable and therefore could not be analysed using statistical methods. The flux of water was higher at 0.4 than at 0.2 m in all the treatments and the highest in the MR treatment (Figure 3).

On the control plots, the flux of DOC at 0.2 m depth was 330 ± 190 (mean ± SE) and at 0.4 m 260 ± 110 mg $m^{-2}\ y^{-1}$(Figure 3). The respective values for Cu_{tot} were 23 ± 7 and 9.5 ± 3.3 mg $m^{-2}\ y^{-1}$, for Zn_{tot} 9.0 ± 2.9 and 15 ± 4, and for Ni_{tot} 12 ± 3 and 23 ± 5 mg $m^{-2}\ y^{-1}$ (Figure 4).

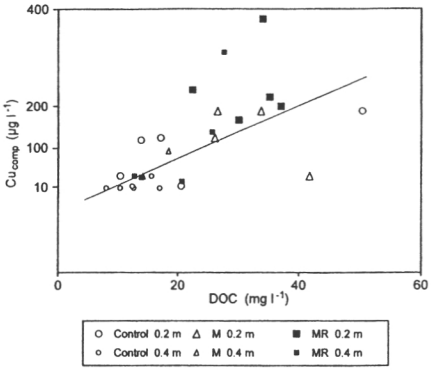

Figure 2. Regression line ($r = 0.62$) between the Cu_{comp} and DOC concentrations in percolation water at depths of 0.2 and at 0.4 m. Dots refer to the lysimeters.

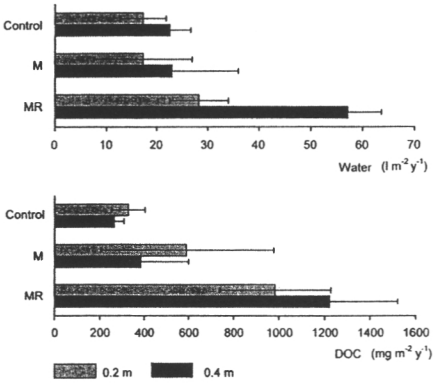

Figure 3. The amount of water and DOC in percolation water passing down to depths of 0.2 and 0.4 m. The bars indicate the standard error of the mean.

The amount of DOC passing down the soil profile was the highest in the MR treatment, where the flux of DOC also increased with increasing depth (Figure 3). In contrast, the flux of DOC in the m treatment and in the control decreased with increasing depth (Figure 3). The flux of Cu_{tot} at 0.2 m depth was similar in all the treatments (Figure 4). However, the amount of Cu_{tot} passing down to 0.4 m was the highest in the MR treatment, and the lowest in the control (Figure 4). The fluxes of Zn_{tot} increased with depth in all the treatments, with the lowest flux in the m treatment and the highest in the MR treatment (Figure 4).

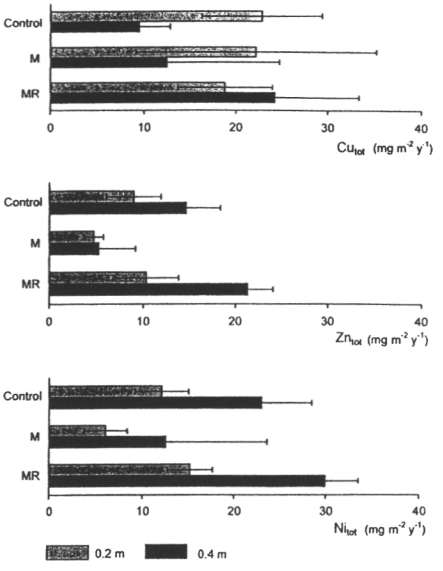

Figure 4. The amount of Cu_{tot}, Zn_{tot} and Ni_{tot} in percolation water passing down to a depth of 0.2 and 0.4 m. The bars indicate the standard error of the mean.

Discussion

Determining the toxicity of soil to bacteria as a means of assessing the success of remediation of heavy-metal polluted sites is a relatively neglected technique. Vangronsveld et al. (2000b) used bacteria-based, heavy metal-specific biosensors to measure the toxicity of Zn and Cd. They reported that the toxicity of soil to bacteria (*Alcaligenes eutrophus*) decreased after remediation treatment with cyclone ash. In addition to mineral compounds, organic substrates can also decrease the toxicity. Menkissoglu and Lindow (1991) showed that many organic compounds reduce the toxicity of a solution to bacteria (*Pseudomonas syringae*). In this study we tested the addition of mulch, a mixture of compost and woodchips, as a top dressing on heavy metal polluted coniferous forest soil. We found that the [^{3}H]- incorporation method, which measures the bacterial growth rate, was a suitable means of assessing the toxicity of percolation water to soil bacteria. The toxicity of the percolation water, expressed as the degree of inhibition of the bacterial growth rate (DI), decreased with decreasing Cu^{2+}concentrations when Zn_{tot} or Ni_{tot} did not correlate with DI. The

correlation was slightly stronger between DI and the Cu^{2+} concentration than between DI and the Cu_{tot} concentration. The Cu^{2+} concentration was thus a better variable describing toxicity. After mulching, part of the Cu^{2+} in the percolation water complexed with dissolved organic matter into less toxic forms. We assume that Cu^{2+} complexed with the DOC leached from the mulch. Our remediation treatments thus reduced the toxicity of the percolation water down to a depth of 0.4 m.

The addition of organic substances influences not only metal toxicity but also their mobility. However, the effects may vary according to the *in situ* soil conditions and the quality and quantity of the organic matter in the soil. Adding compost to remediate heavy metal polluted soil can immobilise metals (Vangronsveld and Clijsters, 1992), but it may also partly enhance their mobility. The mobility of heavy metals is known to increase in the presence of soluble organic matter due to the formation of soluble organo-metal complexes (chelates) (Ross, 1996). Increased mobility has been reported by Li and Shuman (1997a, b), who experimentally showed that Zn and Cd was leached from soil with a solution rich in DOC. Increased leaching of Cu_{tot}, Zn_{tot} or Ni_{tot} down the soil profile was found in soils amended with polluted sewage sludge (Brown et al., 1997; Jackson et al., 1999) or municipal compost (Giusguiani et al., 1992). In a semi-field experiment with *ex situ* lysimeters, Vangronsveld et al. (2000a) found a strong decrease in the Zn_{tot} and Cd_{tot} concentrations, but a strong increase in the Cu_{tot} concentrations, percolating out of polluted soils mixed with compost. The authors reported a slight reduction in percolated Cu_{tot} compared to untreated soil when a combination of compost, cyclone ash and steel shot were mixed with polluted soil.

In our experiment, we found that mulching the forest floor decreased the mobility of Zn_{tot} and Ni_{tot}. In contrast, the mobility of Cu_{tot} was higher in both of the mulch application treatments than in the control, i.e. larger amounts of Cu_{tot} at 0.4 m in the mulched treatments than in the control was observed. Removing the polluted organic soil and litter layer before mulching clearly increased the water, Cu_{tot}, Zn_{tot} and Ni_{tot} fluxes. Increased leaching of heavy metals is obviously an undesirable phenomenon in remediation, and requires further research. There is little information available about whether organo-metal complexes are leached into the groundwater. Increased DOC concentrations were found in groundwater after 15 years of heavy application of spent mushroom substrate, rich in DOC, on an agricultural field (Kaplan et al., 1995). However, the concentration of DOC in the groundwater was only 2–7% of that at a depth of 1 m. A laboratory study of Giusquiani et al. (1992) also showed that only 5–10% of the DOC extracted from compost leached down to a depth of 0.5 m, and 70–80% was retained in the upper 0.1 m layer. The soil therefore has a high absorption capacity for water-soluble organic matter and mulching with compost is not likely to markedly increase the leaching of heavy metals into the groundwater.

The concentration of Cu in different depths is regulated by the reservoir of Cu, the bio-physicochemical attributes of the soil and the flux of water, i.e. the amount of water passing down the soil profile. However, we observed no dilution effect since the amount of water in the lysimeters did not correlate with the Cu_{tot} concentration. Although the average amount of water did not differ significantly between the treatments the flux of water was highest in the MR treatment. Water was obtained more frequently in the MR treatment than in the control and m treatments, which resulted in a higher flux of Cu_{tot} in the MR treatment. We can therefore assume that removing the organic layer increased the flux of water.

In conclusion, the toxicity of soil percolation water to soil bacteria proved to be a suitable means of assessing the success of remediation. Mulching a heavy metal polluted forest soil with organic matter decreased the toxicity of the soil percolation water to bacteria. Part of the free Cu^{2+} ions was complexed into less toxic forms, but part was leached down the soil profile. Detoxification and decontamination were more pronounced when the polluted litter and organic soil layers were removed before mulching. However, this also increased the leaching of Cu_{tot}, Zn_{tot} and Ni_{tot}. Mulching the forest floor without removal of the polluted organic layer was therefore a more successful remediation technique.

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