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a soil microbiological study into the potential risk of cadmium release

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VANTAAN TUTKIMUSKESKUS – VANTAA RESEARCH CENTRE

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Faculty of Agriculture and Forestry
University of Helsinki

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Supervisor: Docent Hannu Fritze
Finnish Forest Research Institute
Vantaa Research Centre

Reviewers: Docent Laura Höijer
Maj and Tor Nessling Foundation
Helsinki, Finland

Docent Kirsten Jørgensen
Finnish Environment Institute
Helsinki, Finland

Opponent: Docent Håkan Wallander
Department of Ecology
Division of Microbial Ecology
University of Lund, Sweden

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Contents

ABSTRACT

Wood ash use in coniferous forests: a soil microbiological study into the potential risk of cadmium release

Wood ash fertilization could benefit the growth of forest vegetation and trees through increasing soil pH and mineral nutrient content. The use of wood ash in forestry has been questioned because the potential risk associated with its cadmium (Cd) content (1-30 mg kg^{-1}). In agriculture, wood ash is only allowed for use as fertilizer when its Cd content is below 3 mg kg^{-1}. This restriction has not been applied to forest soils and there is a lack of knowledge about the potential harmful effects of the Cd in wood ash on forest ecosystems. The main objective of this thesis was to test if the Cd in wood ash has the potential to affect the humus layer microflora of coniferous upland forests. This objective was tested both in laboratory and field experiments with ash and ash spiked with Cd (400 or 1000 mg Cd kg^{-1} as CdO or $CdCl_2$). In one study the dissolution of ash was accelerated by irrigating it with simulated acid rain (SAR).The form of the ash (loose or hardened) and dosage (3, 5 or 9 t ha^{-1}) also were investigated. In addition, the long-term (18-20 years) effects of wood ash on forest soil microbial community and decomposition rate (weight loss) of needle litter and thereby on tree growth were assessed. Also the potential of wood ash Cd to enter the human food chain were studied in the field. Finally, the potential use of wood ash as a remediation agent of heavy metal polluted soil was studied.

Wood ash increased humus layer pH and microbial activities (respiration or thymidine incorporation rates) and changed its microfloral community structure (Biolog®, PLFA, 16S or 18S rDNA PCR-DGGE) in all short-term and long-term laboratory and field experiments. Spiking ash with Cd induced no further changes in the above-mentioned variables as ash alone. The Cd added with wood ash did not become bioavailable as detected with a bacterial biosensor *Bacillus subtilis* BR151(pTOO24). The form and level of Cd added in the ash had no further effect on the microbiological variables studied. Irrigation of ash with SAR did not increase the amount of bioavailable Cd, although the dissolution rate of the ash was increased.

The results showed that, irrespective of the forest site fertility, ash fertilization induced similar chemical and microbiological responses in the humus layer. The changes were related to the dose and form of ash applied. The higher fertilization rate had stronger effects and applying loose wood ash at the same fertilization rate as hardened wood ash induced comparatively more changes, due to faster dissolution. Wood ash fertilization increased the decomposition rate of needle litter 19-20 years but not 1-4 years after treatment. On the poorer

forest site the enhancing effect of ash fertilization on needle mass loss and on Scots pine growth was more pronounced than on more fertile site.

The concentration of Cd in soil water and in *Vaccinium uliginosum* and *V. vitis-idaea* berries, and the amount of bioavailable Cd in the humus layer were not increased by the ash or Cd-spiked ash treatments in the 4 year field study. The only increase in Cd concentrations, significantly higher concentrations in the mushroom *Lactarius rufus* and a slight increase in the berries of *Empetrum nigrum* (first year only), were associated with the Cd-spiked ash treatment.

Humus layer that had been exposed to moderate amounts of continuous acid and metal (copper and nickel) deposition for nine growing seasons was used in a laboratory remediation experiment. Both acid and metal treatments changed the structure of the microbial community. Acid application decreased humus layer pH and base saturation and increased the amounts of both extractable and bioavailable Cu measured with a bacterial biosensor *Pseudomonas fluorescens* DF57-Cu15. Metal application increased the concentration of humus layer extractable Ni and changed the fungal community structure. When this humus was irrigated with water the above-mentioned treatment effects were still seen except for the acid and metal effects on microbial and fungal community structures. After treatment with wood ash, none of the acid or metal effects could be detected.

In conclusion, the Cd in wood ash did not become bioavailable and harmful to forest soil microbes or leach through the humus layer, even when treated with simulated acid rain. Neither did the concentration of Cd in the studied mushroom and berries increase with the unspiked "normal" wood ash treatment. It is thus safe to use wood ash as a vitality fertilizer in upland forests. Nevertheless it would be prudent not to fertilize the same sites with wood ash more than once during a tree stand generation. The effect of wood ash (3 t ha^{-1}) on upland forest soil microbes lasts for at least 20 years, and probably longer if higher doses and/or hardened ash are applied. In addition, wood ash can be used to remediate sites with acidified and metal polluted humus layers.

Keywords: biosensor, cadmium bioavailability, copper bioavailability, DGGE, *Empetrum nigrum,* hardened ash, *Lactarius rufus*, litter decomposition, loose ash, metal pollution, microbial activity, microbial community structure, PCR, *Pinus sylvestris*, PLFA, remediation, stem volume, thymidine incorporation, *Vaccinium uliginosum, Vaccinium vitis-idaea*

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I Fritze H., Perkiömäki J., Saarela U., Katainen R., Tikka P., Yrjälä K., Karp M., Haimi J. and Romantschuk M. 2000. Effect of Cd-containing wood ash on the microflora of coniferous forest humus. FEMS Microbiology Ecology 32: 43-51.

II Fritze H., Perkiömäki J., Petänen T., Pennanen T., Romantschuk M., Karp M. and Yrjälä K. 2001. A microcosmos study on the effects of Cd-containing wood ash on the coniferous humus fungal community and the Cd bioavailability. Journal of Soils & Sediments 1: 146-150.

III Perkiömäki J., Kiikkilä O., Moilanen M., Issakainen J., Tervahauta A. and Fritze H. 2003. Cadmium-containing wood ash in a pine forest: effects on humus microflora and cadmium concentrations in mushrooms, berries and needles. Canadian Journal of Forest Research 33: 2443-2451.

IV Perkiömäki J. and Fritze H. 2002. Short and long-term effects of wood ash on the boreal forest humus microbial community. Soil Biology & Biochemistry 34: 1343-1353.

V Perkiömäki J., Levula T. and Fritze H. 2004. A reciprocal decomposition experiment of Scots pine needles 19 yr after wood ash fertilization. Soil Biology & Biochemistry 36: 731-734.

VI Perkiömäki J. and Fritze H. 2003. Does simulated acid rain increase the leaching of cadmium from wood ash to toxic levels to coniferous forest humus microbes? FEMS Microbiology Ecology 44: 27-33.

VII Perkiömäki J., Tom-Petersen A., Nybroe O. and Fritze H. 2003. Boreal forest microbial community after long-term field exposure to acid and metal pollution and its potential remediation by using wood ash. Soil Biology & Biochemistry 35: 1517-1526.

Jonna Perkiömäki performed part of the experimental work, and calculation and interpretation of the results for papers I and II. In the papers III, IV, V, VI and VII she performed most of the experimental work, and calculation and interpretation of the results. Andreas Tom-Petersen performed the measurements of bioavailable Cu in paper VII. Jonna Perkiömäki wrote the papers III, IV, V and VII, and she has participated in the preparation of the manuscripts of papers I and II. Jonna Perkiömäki wrote the paper VI together with her supervisor Hannu Fritze.

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1 INTRODUCTION

1.1 Wood ash production and quality

Today about 15% of the primary energy production in Finland is from wood (Pingoud and Lehtilä 1997). Wood combustion produces ca. 250 000-300 000 t ash annually (Hytönen and Nurmi 1997). The aim of the Finnish government is that 25% of the primary energy production will be from wood and peat by the year 2005 (Hakkila and Fredriksson 1996). Thus, the amount of wood ash produced will increase in the future. The use of wood for energy production has a number of advantages over other sources - it is renewable, domestic and local, creates work and promotes silviculture (Hakkila and Fredriksson 1996). The most important justification for the use of wood bioenergy, however, is that it could significantly reduce the use of fossil fuels and their carbon dioxide emissions (Pingoud and Lehtilä 1997). The ash that is produced is often stored in waste dumps but could be recycled back to the forest ecosystem. Wood ash contains a considerable amount of mineral nutrients which could benefit the growth of forest ground vegetation and trees (Silfverberg 1996b). In order to preserve the vitality and productivity of soil the nutrients removed from a site by tree harvesting need to be returned and wood ash fertilization is one way to do this.

During combustion most of the inorganic nutrients and trace elements in wood are retained in the ash. Many factors affect the quality of wood ash, including: tree species, fraction of tree burnt, degree of processing of the tree before combustion, burning temperature and type of filters used in the incineration system, and proportion of bottom and fly ash in the end product. Hardwood ash contains significantly more phosphorus and potassium than softwood ash, which contains more calcium (Hakkila and Kalaja 1983). Bark ash has higher contents of calcium and unburnt charcoal and lower amounts of potassium, phosphorus and magnesium than stem wood ash (Hakkila 1986). The ashes of pulp and paper have lower amounts of nutrients than ashes from unprocessed wood (Demeyer et al. 2001). Residual charcoal in the ash reflects inefficient combustion which results in lower acid neutralization capacity when applied to the soil (Khanna et al. 1994). Fly ash has more charcoal, but less insoluble sand than bottom ash (Hakkila and Kalaja 1983). Dynamic separators recover 50–90%, and electric filters 90–99% of the fly ash (Hakkila and Kalaja 1983, Hakkila 1986) and the more effective the filtration of the combustion gases the higher is the proportion of heavy metals in the fly ash (Hakkila 1986, for information about wood ash Cd see chapter 1.3). The alkanity (Demeyer et al. 2001) and leachability of some heavy metals (Ramesh and Koziñski 2001, Zhang et al. 2001) of ash decreases with increasing com-

bustion temperature. Elemental concentrations in wood ash therefore show great variation.

The most abundant element in Finnish wood ash on a mass concentration basis is calcium followed by potassium and magnesium (Table 1). There is more variation in the concentrations of other elements, but usually wood ash contains more phosphorus, iron and manganese than zinc, copper and boron. The amounts of sulphur and aluminum can be relatively high when the ash originates from paper and pulp industry. The amount of sulphur in wood ash is seldom measured/reported, however, probably because it is assumed that most of the sulphur is lost from the ash to the air with combustion gases (Hakkila and Fredriksson 1996, Silfverberg 1996a, Eriksson 1998a). However, according to Obernberger (1998), 40-90% of the total S in biofuel remains in the ash and the rest is emitted as SO_2 and SO_3 with the flue gas. The amounts of carbon and nitrogen are negligible in wood ash because they are generally oxidized and transformed to gaseous constituents during combustion (Demeyer et al. 2001). Khanna et al. (1994) grouped the elements in wood ash to those that rapidly dissolve (>50% of total content) (K, B, and S), those that dissolution increases with increasing dilution of ash with water (Ca, Mg, Si, Fe, and Al) and those that are quite insoluble (P).

During the combustion of wood, organic compounds are mineralized and the base cations are transformed to their oxides which then slowly hydrate and subsequently carbonate under atmospheric conditions (Demeyer et al. 2001). Oxides and hydroxides are the more reactive while carbonates the more slowly reactive fractions of wood ash (Steenari and Lindqvist 1997). During spreading fluffy loose wood ash its reactive fractions are detrimental to human health (Juntunen 1982), and they can erode parts of the spreading machines (Hakkila and Kalaja 1983), cause salt effects in soils and burn damage to plant tissues (see chapters 1.2.1 and 1.2.2 below). In order to reduce these harmful effects

Table 1. Element concentrations in Finnish wood ashes.

Nutrient	Content in ash (kg t^{-1}) (means± SD; n = 130-199)
Ca	226 (81)
K	47.8 (33.8)
Mg	32.7 (15.5)
Fe	22.9 (34.6)
P	15.5 (9.7)
Mn	14.9 (8.0)
Zn	1.66 (1.65)
B	0.27 (0.25)

Data from Silfverberg 1996a.

of wood ashes one can stabilize them before spreading in forest by self-hardening (compacting in a pile), pelletizing (pressing to form pellets) or granulating (granulating in a drum or a disc) (Holmberg and Claesson 2001). In all these stabilization techniques ash is mixed first with water and in pelletation or granulation a binder (e.g. dolomite) is used (Holmberg and Claesson 2001). Self-hardening is the cheapest technique to stabilize wood ash. In hardening process the reactivity of wood ash is reduced by hydroxide formation and carbonation of hydroxides ($Ca(OH)_2 \rightarrow CaCO_3$) (Steenari and Lindqvist 1997). Calcite ($CaCO_3$) is approximately a hundred times less soluble than calcium oxide (CaO) and calcium hydroxide ($Ca(OH)_2$) (Steenari et al. 1999). Carbonation also reduces the alkanity of the ash (Steenari and Lindqvist 1997).

1.2 Wood ash effects in the coniferous forest ecosystem

The following chapters summarize the knowledge about the effects of wood ash fertilization in the coniferous forest ecosystem (shortly summarized in Table 2).

1.2.1 Soil and water chemistry

The most commonly reported changes on humus layer chemistry following wood ash fertilization are a decrease in extractable Al concentrations, a rise in pH and in the concentrations of base cations (Khanna et al. 1994, Bramryd and Fransman 1995, Kahl et al. 1996, Levula et al. 2000, Saarsalmi et al. 2001, Ludwig et al. 2002).

For N and P the effects of wood ash fertilization are not so clear. Wood ash application was found to increase the amount of total N and extractable P in the humus layer only in one of the four studied forests 16 years after application (Saarsalmi et al. 2001). Levula et al. (2000) and Tamminen (1998) also observed an increase in extractable P contents after wood ash treatment. Fritze et al. (1995) and Arvidsson and Lundkvist (2003) did not observe an ash effect on the amount of humus layer N contents and Kahl et al. (1996) and Fransson et al. (1999) did not observe an increase for extractable P contents. The amount of total N in the humus layer has sometimes been shown to decrease after wood ash treatment (Fritze et al. 1994b, Haimi et al. 2000).

Because nutrient concentrations in the groundwater of peatlands have been shown to increase after wood ash treatment (Nilsson and Lundin 1996, Piirainen 2001, Moilanen et al. 2002), there has been concern about a possible threat that applying wood ash could cause to watercourses. The following summarizes the knowledge about the effects of wood ash fertilization on min-

eral (upland) soil water quality. In the studies by Fransman and Nihlgård (1995) and Ring et al. (1999), wood ash application did not change pH or the concentrations of Ca, SO^{2-}_4, NO^-_3 and Al in soil water. Neither did Fransson et al. (1999) find increase in soil water pH or concentration of P nor Arvidsson (2001) recorded an increase in soil water pH or the concentrations of NO^-_3 and Al. However, there are also studies where a rise in soil water pH and the concentrations of these ions has been observed due to wood ash application. In the study by Kahl et al. (1996) there was a rise in pH and Ca, SO^{2-}_4 and NO^-_3 concentrations after wood ash fertilization (24 and 38 t ash ha^{-1}). All the changes, except the rise in solution pH and SO^{2-}_4 concentrations at the application level of 38 t ash ha^{-1} were transient. In the study by Lundell et al. (2001) there was a clear increase of Ca and a slight increase in the leaching of SO^{2-}_4 and Al after wood ash addition even though the application level was quite low (4 t ha^{-1}). Arvidsson (2001) also observed increased levels of Ca in soil water after low application of wood ash (3 t ha^{-1}), but they decreased to control levels within five years after application at most of the studied sites. When Ludwig et al. (2002) applied 4.8 t $ha^{-1}$of wood ash to one forest plot (no replications), they observed an increase in the concentrations of Ca, SO^{2-}_4, NO^-_3 and Al in soil water, but these changes were absent after two years.

Fransman and Nihlgård (1995) studied the quality of runoff water from forested areas for 4 years after wood ash treatment (2.2 t ha^{-1}) and the only change they observed was an increase in the concentration of K. In the study by Tulonen et al. (2002), performed in a forest growing on mineral soil, 12% of the catchment area was treated with wood ash (6.1 t ha^{-1}) and resulted in a slight increase in runoff (annual average) and recipient lake water pH and K concentrations, but no increase in Ca, P and N concentrations occurred. Wood ash fertilization thus does not appear to lead to the eutrophication of watercourses as there is negligible leaching of N and P. Wood ash treatment has, however, been found to increase dissolved organic carbon (DOC) concentrations in some studies (Weber et al. 1985, Khanna et al. 1994, Ludwig et al. 2000, Chirenje et al. 2002). Yet Parkman and Munthe (1998) did not observe increased DOC concentrations in runoff water from a study area treated with granulated wood ash (2.2 t ha^{-1}) 4-5 years earlier. There is a lack of knowledge about the effects of wood ash on upland forest groundwater quality.

A salt-effect is sometimes observed after wood ash application (Eriksson et al. 1998), whereby soil pH initially (during the first month) decreases with increasing level of ash because of increased ionic strength and displacement of H and Al from exchange sites by other cations dissolving from the ash. Later on, when the carbonates and oxides dissolve to a higher degree than neutral salts, the ash has a more direct alkalizing effect (Eriksson et al. 1998). A temporary decrease in soil water pH has also been observed in the first year after wood ash addition (4.8 t ha^{-1}) (Ludwig et al. 2002).

In summary, the effects of wood ash fertilization on the chemical properties of upland forest soils are beneficial - soil acidity is decreased and base cation

concentrations, which are important plant nutrients, increased. Eutrophication of watercourses related to wood ash treatment has not been observed.

1.2.2 Forest floor and ground vegetation

The effect of wood ash on vegetation depends on the development stage of the forest stand (Pihlström et al. 2000), site fertility (Silfverberg and Huikari 1985, Arvidsson et al. 2002), form (Rühling 1996, Kellner and Weibull 1998) and dosage (Silfverberg and Huikari 1985, Ferm et al. 1992, Jacobson and Gustafsson 2001) of the ash applied. Although the effects of wood ash are not consistent in all studies performed in upland forests, some generalization can be made.

The coverage of herbs such as *Epilobium angustifolium (*Pihlström et al. 2000, Arvidsson et al. 2002, Olsson and Kellner 2002), *Trientalis europaea* (Gyllin and Kruuse 1996, Arvidsson et al. 2002), *Melampyrum* (Gyllin and Kruuse 1996, Pihlström et al. 2000, Jacobson and Gustafsson 2001) and *Taraxacum* (Rühling 1996, Olsson and Kellner 2002), grasses such as *Deschampsia flexuosa* (Silfverberg and Issakainen 1991, Gyllin and Kruuse 1996, Arvidsson et al. 2002) and *Luzula pilosa* (Gyllin and Kruuse 1996, Olsson and Kellner 2002), and shrubs such as *Rubus ideaus* (Rühling 1996, Arvidsson et al. 2002) increased. The increase in the coverage of *E. angustifolium* and *D. flexuosa* has been attributed to higher nitrogen availability in the soil (Arvidsson et al. 2002).

The coverage of dwarf shrubs decreased in some studies (e.g. *Vaccinium vitis-idaea* - Levula et al. 2000, Jacobson and Gustafsson 2001; *Vaccinium myrtillus* - Jacobson and Gustafsson 2001 and *Calluna vulgaris* - Jacobson and Gustafsson 2001, Arvidsson et al. 2002) while increased in other (e.g. *V. vitis-idaea* and *V. myrtillus* - Pihlström et al. 2000). The berry yields of *V. vitis-idaea* and *V. myrtillus* have also been shown to increase after wood ash fertilization (Silfverberg and Issakainen 1991), but the berry yield of *V. myrtillus* decreased in the study by Moilanen and Issakainen (2000).

Wood ash has caused salt-burn damage to bryophytes *Dicranum polysetum*, *Pleurozium schreberi* and *Hylocomium splendens* (Kellner and Weibull 1998, Jacobson and Gustafsson 2001). Despite of the burn damage on *H. splendens*, no changes in its coverage were observed (Kellner and Weibull 1998, Jacobson and Gustafsson 2001), but that of *D. polysetum* (Jacobson and Gustafsson 2001) and *P. schreberi* (Kellner and Weibull 1998) decreased. In the study by Kellner and Weibull (1998) a significant negative relationship between burn damage and the photosynthetic capacity of the bryophytes was found. One bryophyte, the calciphilic *Pohlia nutans*, benefited from wood ash fertilization (Gyllin and Kruuse 1996, Jacobson and Gustafsson 2001). Lichens *Cladonia* (Gyllin and Kruuse 1996, Pihlström et al. 2000) and *Cladina* (Gyllin and Kruuse 1996, Jacobson and Gustafsson 2001) suffer from wood ash treatment. There are also studies were no changes in the cover of bryophytes (Levula et

al. 2000, Arvidsson et al. 2002) or lichens (Kellner and Weibull 1998, Levula et al. 2000) were found.

In many long-term wood ash fertilization studies performed on peatland an increase in the coverage of herbs and grasses and colonization by nitrophiles (e.g. *E. angustifolium*) has also been observed (Lukkala 1951, Silfverberg and Huikari 1985, Silfverberg and Hotanen 1989, Ferm et al. 1992, Moilanen et al. 2002). In addition, *Sphagnum* mosses are substituted by forest mosses. These changes in vegetation could result in increased decomposition of needle litter and improvement in nutrient cycling and thereby increased tree growth (Silfverberg and Huikari 1985).

In conclusion, in upland forests the changes in the composition of the forest floor and ground vegetation after wood ash treatment were quite small (Levula et al. 2000, Jacobson and Gustafsson 2001, Arvidsson et al. 2002). In the study by Pihlström et al. (2000) the changes in the vegetation following wood ash application were mainly changes in the abundance of existing species and not in the appearance of the new species and disappearance of the old ones. However, Gyllin and Kruuse (1996) and Arvidsson et al. (2002) observed a slight increase in species richness after wood ash application. In the peatland forests the changes in the vegetation were more pronounced.

1.2.3 Trees

1.2.3.1 Growth

In the study by Silfverberg (1995) soaking (one day or week) Scots pine (*Pinus sylvestris)* seeds in a wood ash solution decreased their germination, while Rikala and Jozefek (1990) did not find an ash effect on the germination capacity of Scots pine or Norway spruce (*Picea abies*) on peat. Wood ash treatment on peat increased the number (Silfverberg 1995) and growth (Kaunisto 1987, Rikala and Jozefek 1990) of Scots pine seedlings and decreased their growth disturbances caused by nutrient imbalances (Kaunisto 1987). The higher number of surviving seedlings indicates that wood ash improves the forest regeneration.

Wood ash has also been shown to increase the growth of Scots pine on peatlands in many long-term (9-47 years) experiments (Lukkala 1951, Silfverberg and Huikari 1985, Silfverberg and Hotanen 1989, Silfverberg 1991, Ferm et al. 1992, Moilanen and Issakainen 2000, Moilanen et al. 2002, Hytönen 2003). Usually the ash effect was stronger when the used dose was higher (Silfverberg and Huikari 1985, Silfverberg and Hotanen 1989), but not always (Silfverberg and Huikari 1985). In addition, the growth increment of trees was higher on peat with higher nitrogen content (Lukkala 1951, Silfverberg and Huikari 1985, Silfverberg 1991). According to Moilanen and Issakainen (2000), the increase in the growth increment appeared sooner (2-3

years after application) on sites with higher nitrogen contents than on poorer sites (7-8 years after application).

There are few publications in Finland concerning wood ash fertilization effects on tree growth on mineral soils. A Finnish review article (Saarsalmi and Mälkönen 2001) concluded that wood ash does not increase the growth of coniferous upland forests on mineral soils. According to a Swedish review (Nohrstedt 2001), wood ash stimulated forest growth on sites with a humus C-to-N ratio below 30, while on N-poor sites the effect could be reduced growth. When Tamminen (1998) studied the effect of wood ash fertilization (3 t ha^{-1}) on the height growth of 8-15-years old Scots pines he observed an increase in growth 2-5 years after fertilization (2 replications). Sikström (1992) did not observe increased conifer tree (Scots pine and Norway spruce) growth during 5 year study period in stands fertilized with quite low doses (0.3 and 0.5 t ha^{-1}) of wood ash. Jacobson (2001) found the same result 7-11 years after fertilization but with higher doses (1, 3, 6, 9 t ha^{-1}) unless nitrogen (150 kg ha^{-1}) was also applied. However, in the study by Jacobson (2001) there was an increasing, although not significant, trend in tree growth on fertile sites treated with wood ash. Levula (1991) found that when nitrogen (180 kg N ha^{-1}) was added with wood ash (2 t ha^{-1}) the volume increment of Scots pine 9 years after ash application was ca. 73%. In the study by Saramäki and Susila (1991) the increase in Scots pine volume growth after wood ash application (5 t ha^{-1}) with nitrogen (urea 400 kg ha^{-1}) during 10 years was ca. 60%, and without nitrogen it was 17% (no replications). In another Finnish study, Moilanen and Issakainen (2000) found that wood ash treatment (3.6 and 7.2 t ha^{-1} 10 years earlier) did not increase the growth of Scots pine, except on the plot where N in addition to ash (4.5 t ha^{-1} 19 years earlier) also have been applied.

The effects of peat ash on tree growth have also been studied. However, the acid neutralization capacity (Saarela 1991) and nutrient contents (especially K) are not as high as in wood ash, but it has been considered as a phosphorus fertilizer (Hakkila 1986, Saarela 1991, Silfverberg and Issakainen 1991, Issakainen et al. 1994, Oikarinen and Pasanen 1994). Thus, more peat ash than wood ash is needed to achieve the same growth increment for Scots pines (Kaunisto 1987, Saramäki and Susila 1991, Issakainen et al. 1994, Moilanen and Issakainen 2000, Hytönen 2003).

In summary, while wood ash fertilization has been shown to generally increase the growth of coniferous trees on peatlands forests this is not the case with upland forests unless extra nitrogen is also applied.

1.2.3.2 Foliar chemistry

In tree stands growing on mineral soils P and especially N deficiency is quite common (Raitio et al. 2000). A boron deficiency also sometimes occurs. While wood ash treatment has not been shown to increase foliar N concentrations in conifer trees growing on mineral soils (Vuorinen and Kurkela 2000, Jacobson 2001), it has been shown to increase B concentrations (Moilanen and

Issakainen 2000, Jacobson 2001, Nohrstedt 2001). However, Arvidsson and Lundkvist (2002) have shown increased Norway spruce needle N concentrations after ash treatment, and attributed it to increased mineralization of soil N (see below chapter 1.2.4.1). Also needle P (Kaunisto 1987, Jacobson 2001, Arvidsson and Lundkvist 2002) and K (Kaunisto 1987, Moilanen and Issakainen 2000, Jacobson 2001, Arvidsson and Lundkvist 2002) concentrations have been shown to increase after wood ash treatment. In the studies by Vuorinen and Kurkela (2000) and Ludwig et al. (2002), wood ash fertilization was found to not affect the nutrient concentrations of Scots pine needles.

In peatland forests P and K (Silfverberg and Huikari 1985, Silfverberg and Hotanen 1989, Moilanen et al. 2002), and B (Kaunisto and Paavilainen 1988) deficiency may occur. Studies in peatlands have found that wood ash treatment sometimes increases nutrient concentrations in Scots pine needles and sometimes has no effect. These effects were not consistent for all nutrients, but generally needle concentrations of K (Silfverberg and Hotanen 1989, Ferm et al. 1992, Silfverberg et al. 1994, Moilanen and Issakainen 2000, Moilanen et al. 2002, Hytönen 2003) and B (Silfverberg and Issakainen 1987, Ferm et al. 1992, Moilanen and Issakainen 2000, Hytönen 2003) have been found to increase after wood ash fertilization. Increases in needle P concentrations have also been observed (Silfverberg and Hotanen 1989, Silfverberg et al. 1994, Moilanen and Issakainen 2000, Moilanen et al. 2002). These increases in needle P, K and B concentrations have been found to be long-term; lasting for 51, 51 and 26 years, respectively (Moilanen and Issakainen 2000). Long-term increases in needle N concentrations on peatlands after wood ash treatment have also been recorded (Silfverberg and Hotanen 1989). Wood ash fertilization has no effect on needle nutrient concentrations in stands with originally good nutrient status (Silfverberg and Huikari 1985, Silfverberg 1991).

In summary, wood ash fertilization has often been shown to increase concentrations of P, K and B in Scots pine needles, both on uplands and peatlands.

1.2.3.3 Diseases

In upland forests wood ash treatment has been shown to have little or no effect on the occurrence of tree diseases. Wood ash fertilization did not affect the *Lophodermella sulcigena* infection of Scots pine needles, but nitrogen fertilization increased it (Vuorinen and Kurkela 2000). The infection frequency of *Heterobasidion annosum*, the causative agent of root rot in Scots pine roots, was not higher and average mycelia growth was lower on plots treated with wood ash (Piri 2000). In the study by Tamminen (1998), wood ash had no significant effect on the occurrence of Scots pine flatbug infection (*Aradus cinnamomeus*) although a slight decreasing trend was seen. Also in peatland forests wood ash fertilization has been shown to increase the proportion of healthy trees (Kaunisto 1987, Ferm et al. 1992, Hytönen 2003).

In conclusion for the chapter 1.2.3 (effects on trees)., wood ash can be successfully be used to remediate soils by reducing soil acidity and the depletion of nutrients rather than a fertilizer to increase the growth of coniferous trees directly.

1.2.4 Decomposers

1.2.4.1 Microbes

Microbial biomass has commonly been measured using substrate-induced respiration (SIR), fumigation-extraction (FE) and the ATP content of the soil. The PLFA method has also become popular. In this method the phospholipid fatty acid content of the microbial cell membranes is measured. PLFA analysis can also be used to reveal changes in the structure of the microbial community and signature fatty acids can be used to indicate of certain groups of microorganisms (see below).

In general wood ash treatment has been shown to have no effect on the microbial biomass of the boreal forest humus layer in a number of field studies. Thus, no change in SIR (Priha and Smolander 1994) or FE (Fritze et al. 1994b, Priha and Smolander 1994), or ATP content of the soil (Bååth and Arnebrant 1994) was reported. However, Bååth and Arnebrant (1994) observed an increase in SIR after wood ash fertilization. Bååth and Arnebrant (1994) also observed an increase in the amount of culturable bacteria, but the acridine-orange stained direct counts (AODC) did not change. In contrast, Bååth et al. (1995) observed a decrease in total amounts of phospholipid fatty acids (PLFAs) after wood ash fertilization, but this decrease was only significant at the highest application level (5 t ha^{-1}). In addition to application level of wood ash, also the exposure time to ash has been shown to affect on the strength of microbial biomass response. Frostegård et al. (1993a) did not find an ash effect on the total amounts of PLFAs 5 years after the treatment at the same site where Bååth et al. (1995) found an ash effect two years after treatment. When bacterial and fungal biomasses in the humus layer after wood ash treatment have been measured separately with PLFA analysis Frostegård et al. (1993a) found no change in either while Bååth et al. (1995) found them both to decrease. When fungal biomass was measured as ergosterol content, no change following ash fertilization was observed by Fritze et al. (1994b).

Although the microbial biomass may not change after ash treatment its activity may. In most of the wood ash studies performed in boreal forests, microbial activity in humus layer, measured as either mineralization activity (basal respiration rate measured as CO_2 production; Bååth and Arnebrant 1994, Fritze et al. 1994b, Fritze et al. 1995) or growth rate (thymidine incorporation rate; Bååth and Arnebrant 1994, Bååth et al. 1995, Hagerberg and Wallander 2002), increased. However, in the study by Priha and Smolander (1994), wood ash treatment (2.5 t ha^{-1}) did not have an effect on respiration rates one year after application.

Raison and McGarity (1980) observed that ash addition to soil (sandy podzolic soil) increased the respiration rate (measured as CO_2 production), compared to a sterilized control soil. This indicates that ash has a direct effect on microbial activity. In addition, they also showed that the elevation of soil pH was the major factor, which led to the increased respiration rate as neutralized ash did not give a respiration response. An increased respiration rate is usually taken to indicate increased mineralization and nutrient cycling, but it may also indicate a stress response by soil microbes due to increase in soil pH. Bacterial communities in ash treated soils have, however, been shown to be able to adapt to increased humus layer pH (Bååth and Arnebrant 1994, Bååth et al. 1995).

The mineralization activity of microbes can also be measured as the mass loss of needle litter. In the study by Smolander et al. (1996), wood ash fertilization increased the mass loss of Scots pine needle litter.

Wood ash fertilization has also been shown to increase net nitrification (Martikainen 1984) and the numbers of autotrophic nitrifiers (NH_4 and NO_2 oxidizers) (Martikainen 1985a) in humus layer samples one and two years after application. Five years after wood ash application nitrification rates were no longer elevated above controls, unless ammonium had also been added with ash (Martikainen 1985b). Neither did Fritze et al. (1994b) observe increased nitrification 2 years after wood ash application. Increased nitrification may lead to nitrate leaching from the soil (see chapter 1.2.1).

The effects of wood ash fertilization on soil microbes have also been studied in peatland forests. Microbial activity has been measured as respiration rate (Silvola et al. 1985, Ferm et al. 1992), decomposition rate of needles (Silfverberg and Huikari 1985, Silfverberg and Hotanen 1989) and cellulose (Karsisto 1979, Weber et al. 1985, Moilanen et al. 2002), and all have increased following ash application. Also the abundance of many bacterial groups were increased by wood ash. Karsisto (1979) observed an increase in the total number of bacteria, starch decomposing, glucose fermenting and lipolytic bacteria 53 years after wood ash treatment and Weber et al. (1985) in the numbers of aerobic, amylolytic, denitrifying and clostridial bacteria 2 years after wood ash treatment. In both these studies the numbers of proteolytic, ammonifying and ureolytic bacteria and the net mineralization of N increased. In the study by Huikari (1953) wood ash application increased the amounts of aerobic bacteria and yeasts in the surface layer of peat, but decreased the amounts of moulds.

PLFA analysis, which has been shown to be a sensitive measure of environmental changes (Pennanen et al. 1996, Pennanen et al. 1998, Bååth et al. 1998), has been used to study the effects of wood ash on forest soil microbial community structure. In the study by Frostegård et al. (1993a), wood ash treatment induced a change in humus layer PLFA pattern but not in microbial biomass five years after treatment, which indicated that some species suffered from wood ash treatment while others benefited and compensated for the loss in biomass caused by more sensitive species. Also Bååth et al. (1995) observed

a change in humus layer microbial community structure two years after wood ash fertilization. In both these studies the changes in microbial community structures correlated with humus layer pH. Fritze et al. (1994a) found that humus layer quality analyzed with near-infrared spectroscopy (NIR) was not significantly different between wood ash treated and control samples. However, when Bååth et al. (1995) performed an infrared spectroscopy (IR) analysis of humus layer samples they did find difference between wood ash treated and control samples. Bååth et al. (1995) concluded that the changes in humus layer microbial community structure were related to humus layer quantity (availability of organic matter), quality and pH. The availability of organic matter may be related to increased amounts of dissolved organic carbon (DOC) that has sometimes been observed after wood ash application (see chapter 1.2.1). In addition to increased amounts of DOC, the amount of dissolved organic nitrogen (DON) has been shown to increase after wood ash addition (Ludwig et al. 2000). N is an important macro nutrient for microbes, although microbial growth in soils is generally C limited (Aldén et al. 2001).

A deeper sight into the changes of microbial community with PLFA analysis may be achieved by using signature fatty acids specific for certain groups of microorganisms. For example, there are signature fatty acids for fungi (18:2ω6; Federle 1986, Frostegård and Bååth 1996), arbuscular mycorrhiza (AM) fungi e.g. *Glomus* species (16:1ω5; Graham et al. 1995, Olsson et al. 1995, Larsen et al. 1998) and actinomycetes (10Me16:0, 10Me17:0, and 10Me18:0; Kroppenstedt 1985). In their AM fungal studies performed in the laboratory, Larsen et al. (1998) and Olsson et al. (1998) used 18:2ω6 as an indicator of saprophytic fungi, while Olsson and Wallander (1998; laboratory study) and Hagerberg and Wallander (2002; field study) used it as an indicator of ectomycorrhizal (EM) fungi. Since mycorrhizal hyphae form a substantial part of fungal biomass in soil (Finlay and Söderström 1989), the amount of 18:2ω6 in field soil sample probably indicates the amount of EM fungi. EM are mainly associated with woody plants, including the genera *Pinus* and *Picea*, while AM are associated with herbs and grasses. It is noted that the coverage of herbs and grasses often increases after wood ash fertilization (see chapter 1.2.2). In the studies by Frostegård et al. (1993a) and Bååth et al. (1995), the amount of PLFAs 16:1ω5 and 10Me18:0 increased after ash application. Frostegård (1993a) did not observe change in the amount of PLFA 18:2ω6, but Bååth et al. (1995) observed a significant decrease at the highest dose of wood ash (5 t ha^{-1}).

EM fungi play an important role in the nutrition of trees because they can transfer organic and inorganic nutrients from soil to the colonized tree roots. Nitrogen deficiency is common in boreal forest ecosystems because only a small fraction of total soil N is available to plants. EM fungi infection have been shown to improve the nitrogen acquisition of trees through providing access to organic N (France and Reid 1983, Finlay and Söderström 1989, Chalot and Brun 1998) and increase the uptake of ammonium (NH_4^+) (France and Reid 1983, Rygiewicz et al. 1984a, Wallander et al. 1999) and nitrate

(NO_3^-) (France and Reid 1983, Rygiewicz et al. 1984b). The response of EM fungi to wood ash fertilized soil is thus important, as wood ash contains negligible amounts of N itself.

As EM fungi have been shown to colonize (Mahmood et al. 2002) and solubilize wood ash (Mahmood et al. 2001, 2003) they may affect the acquisition of N and other nutrients in wood ash treated forests. Phosphorous in wood ash is primarily bound in apatite and other compounds of low solubilities (Steenari and Lindqkvist 1997, Nieminen 2003). EM fungi may be expected to play an important role in mobilizing the P in wood ash (Mahmood et al. 2001, 2003). In wood ash treated soil microbial biomass (including mycorrhizal extramatrical hyphae) has been found to store P and it could be released for use of trees (Clarholm 1998). EM fungi have also been shown to release Ca from the wood ash and transport it to the roots of Norway spruce (Hagerberg 2003, Wallander et al. 2003, Mahmood et al. 2003).

A third wood ash nutirient possibly affected by EM fungi is K, which significant fraction is rapidly released from wood ash (Eriksson 1998b, Steenari et al. 1998, Steenari et al. 1999, Holmberg et al. 2000, Hagerberg and Wallander 2002, Nieminen 2003). As some EM fungi have been shown to have high capacity to accumulate K (Kottke et al. 1998, Wallander et al. 2003), they could play an important part in hindering the leaching of K.

Studies have shown that wood ash treatment does not have detrimental effects on EM fungi. Indeed, wood ash has been found to stimulate EM mycelium production (e.g. Ohtonen and Tuohenmaa 1999, Mahmood et al. 2001, Hagerberg and Wallander 2002). Wood ash treatment has been shown to have no (Erland and Söderström 1991) or only minor effects (Mahmood 2000) on EM community structure in coniferous tree forests. However, some negative effects on EM fungi have been observed after wood ash treatment. Rühling (1996) found that while the occurrence of fruiting bodies of saprophytic fungi (e.g. *Clitocybe*, *Lycoperdon*) increased those of mycorrhiza-forming species (*Russula, Boletus* s.l.) decreased after ash application. Erland and Söderström (1991) observed a decrease in the number of mycorrhizal root tips per meter root in Scots pine seedlings 4 months after wood ash fertilization. But Mahmood et al. (2002) did not observe a change in the number of mycorrhizal root tips of Norway spruce seedlings 7 years after ash application.

In summary, the effects of wood ash on forest soil microbes are stimulative. Mineralization activity is increased leading to improved nutrient cycling. Because wood ash does not contain nitrogen, microbes and especially mycorrhizal fungi play an important role in improving the acquisition of soil nitrogen for transfer to trees.

1.2.4.2 Fauna

Although microbes play a major role in decomposition processes in forest soils also soil fauna either directly or indirectly increase organic matter decomposition and nutrient mineralization (Faber and Verhoef 1991, Heneghan and

Bolger 1998, Edsberg 2000), and subsequently coniferous tree seedling growth (Jentschke et al. 1995, Setälä 1995). The dominant soil animal groups in coniferous forest soils in terms of biomass are enchytraeids and earthworms, followed by mites, spiders, beetles, nematodes, collembolas, protozoans, rotifers and dipterous larvae (Huhta et al. 1998). Enchytraeids and earthworms influence more the soil processes in boreal forest soil than microarthropods (mites; Oribatida, Mesostigmata, Prostigmata, and collembolas) and nematodes (Huhta et al. 1998).

The effect of wood ash fertilization on the abundance of enchytraeids has varied. Lundkvist (1998) and Liiri et al. (2002b) did not find a reaction to wood ash treatment while Huhta et al. (1986) observed a decrease in the biomass of enchytraeids following forest wood ash application and Haimi et al. (2000) found the numbers of *Cognettia sphagnetorum*, which was the only enchytraeid worm species, decreased. *C. sphagnetorum* is the dominant enchytraeid worm species and forms the main part of the soil animal biomass in northern European coniferous forests (see review Huhta et al. 1998). In addition *C. sphagnetorum* may be a keystone species in these soils (Huhta et al. 1998). Lundkvist (1998; ash with NH_4 addition) found an increased abundance of earthworms in wood ash treated soil while Huhta et al. (1986) found no earthworms in control soils and only a few in ash treated soil. Both Huhta et al. (1986) and Liiri et al. (2002b) found an increase in the number of nematodes, especially bacterial feeders related to wood ash treatment.

Huhta et al. (1986) and Haimi et al. (2000) observed a decrease in the total abundance of microarthropods following forest wood ash fertilization, but this was not found by Liiri et al. (2002a, b), probably because of the lower increase in humus layer pH. The effect of wood ash fertilization on the number of collembolas has been neutral (Haimi et al. 2000), positive (Huhta et al. 1986) or negative (Liiri et al. 2002a). The reaction of mites to wood ash fertilization has also been varied with stimulation (Huhta et al. 1986; Prostigmata), inhibition (Huhta et al. 1986; Mesostigmata, Oribatida, Haimi et al. 2000; Oribatida) or no reaction (Haimi et al. 2000; Mesostigmata, Liiri et al. 2002a; Mesostigmata, Oribatida) in their abundance being reported.

All the above-mentioned changes observed by Haimi et al. (2000) were caused with an ash application dose of 5 t ha^{-1}, but not with 1 t ha^{-1}. In addition to the changes in the abundance of some soil fauna groups, changes in the vertical distribution have also been observed after wood ash doses of 5 (Haimi et al. 2000), 7 (Huhta et al. 1986) and 8 t ha^{-1} (Lundkvist 1998), but not with lower doses. All these studies were performed in the field. In the study by Huhta et al. (1986) wood ash effect on soil fauna was stronger in the laboratory than in the field, but the results were generally in good accordance. Neither did Liiri et al. (2002a) find a discrepancy between the wood ash effects on soil fauna in their field and laboratory experiments.

In summary, although some soil fauna groups and species suffer from wood ash treatment, soil fauna in general show little response to wood ash fertilization (Haimi et al. 2000, Liiri 2001). Even in the study by Huhta et al. (1986),

Table 2. A summary of general effects of wood ash fertilization on coniferous, upland forests from the publications cited in this thesis

Compartment of ecosystem	Effect of wood ash fertilization
Trees	- little effect on growth - increase in needle P, K and B content - little effect on disease occurrence
Forest floor and ground vegetation	- a slight increase in abundance of herbs and grasses and decrease of lichens and bryopytes
Humus layer	- rise in pH and base cation content - increase in carbon mineralization - change in microbial community structure - little response on faunal community - stimulation of EM mycelium production - nutrient solubilization from ash by EM fungi which then accumulate nutrients or transport them to tree roots
Soil water	- little effect on soil water quality
Runoff and lake water	- a slight increase in pH and K content
Groundwater	- lack of knowledge

where the effect of ash on certain soil fauna was negative, the biomass of soil fauna was much higher in ash treated soil than in controls if earthworms, which have a strong influence on soil processes, were included in the calculation. This was attributed to the rise in soil pH brought about by the wood ash.

1.3 Wood ash harmful substances

Although wood ash has many beneficial effects when spread in forest ecosystems, as stated in chapters above, it does contain toxic substances, which may be harmful. These toxic substances include organic compounds (polyaromatic hydrocarbons, chlorobenzenes, and chlorophenols), ^{137}Cs activity and heavy metals (Cu, Zn, Mn, Pb, Cd, Cr, Hg, and Ni).

The levels of polyaromatic hydrocarbons (PAH) in wood ash granules were below 0.16 mg kg^{-1} in stored granules and 6.7 mg kg^{-1} in fresh granules in the study by Holmberg et al. (2000). Ash granules are sometimes dried in order to increase their hardening rate and this drying process affects the concentration of PAHs in granules. When dried with flue gas at a heating plant, PAH concentrations were 0.8 mg kg^{-1} compared to 1.4 mg kg^{-1} when dried under hot air (Holmberg et al. 2003). However, PAH concentrations in wood ash can be high. In the study by Bundt et al. (2001), wood ash total PAH concentration was 16.8 mg kg^{-1} and one year after its application (8 t ha^{-1}) in the forest, con-

centrations in the organic horizon were up to six fold higher than those in controls. According to Danish legislation, the content of PAH in wood ash fertilizer should not exceed 3 mg kg^{-1} d.m. (Møller and Ingerslev 2001). Currently, there are no limits set in Finnish legislation.

The Chernobyl nuclear accident in 1986 resulted in the deposition of radioactivity to forest ecosystems in Finland and Sweden. This has led to increased radioactivity of wood ash. The Swedish Radiation Protection Institute recommends that no wood ash with radioactivity exceeding 5 kBq kg^{-1} should be applied to forests (Högbom and Nohrstedt 2001). In the study by Högbom and Nohrstedt (2001) the application of wood ash (3 t ha^{-1}) contaminated with ^{137}Cs (0 - 4.8 kBq kg^{-1}) did not change or even decreased the ^{137}Cs activity within forest soil and vegetation at one studied site. One explanation for the decrease in radioactivity given was the antagonistic effect of K occurring in wood ash. In another Swedish study, part (11 - 24%) of the ^{137}Cs (1.9 - 2.1 kBq kg^{-1}) in ash granules (4 t ha^{-1}) leached into the soil and transferred from there to trees during 5 year period (Ravila and Holm 1996). However, no ^{137}Cs activity was observed in the soil water at depths of 20 and 50 cm. In Finland, Levula et al. (2000) studied the effect of wood ash (5 t ha^{-1}) having ^{137}Cs concentrations of 5 - 10 kBq kg^{-1}, and they found that wood ash application decreased the ^{137}Cs activity of lingonberries (*Vaccinium vitis-idaea*).

Because of its severe toxicity and relatively high mobility, cadmium (Cd) is considered to be the most harmful heavy metal in the wood ash. When Wang et al. (2001) studied the partitioning of Cd during combustion of municipal solid waste they observed that about 80% of Cd was partitioned in flue gas, about 20% in fly ash and none in bottom ash. Because these partitioning measurements of Cd are from incineration of municipal waste they are not strictly comparable to incineration of wood. Cd content of wood ash tends to increase with decreasing particle size (Zhan et al. 1996, Obernberger et al. 1997) and thus Cd concentrates in fine fly ash fraction (Narodoslawsky and Obernberger 1996). Fly ash forms the major part of the ash produced in modern incineration systems (circulating fluidized bed boilers; Eriksson et al. 1998) used by Finnish forest companies. In addition, today the amounts of heavy metals in wood ash have increased due to better filtering in the incineration systems. The Cd concentration of wood ash (varying between 1 - 30 mg kg^{-1} ash; Steenari and Lindqvist 1997) is generally higher than is allowed for fertilizers use in agriculture; the limit being in Finland d" 3 mg kg^{-1}. There is currently not enough knowledge available to recommend restrictions on the Cd concentration of wood ash for use as forest fertilizer in Finland. In Sweden, the National Board of Forestry has recommended that it should not be > 30 mg Cd kg^{-1} ash (Arvidsson 2001) and the Danish Environmental Protection Agency recommended that maximum Cd level should be 15 mg Cd kg^{-1} ash and the load should not exceed 0.5 t ha^{-1} per 10 years (Pedersen 2003).

The adsorptive capacity of ash has been used to adsorb Cd from contaminated water (Iyer and Scott 2001, Ricou-Hoeffer et al. 2001). There have also been tests done to remove Cd from wood ash (Pedersen 2003, Pedersen et al.

2003). This electrodialytic process removed about 70% of the Cd (28 mg kg^{-1}) from wood ash but the technology is still at a too early stage to make reasonable cost calculations, but the aim is to make the costs competitive with the costs for land filling. Another way to reduce the amount of Cd in wood ash is to concentrate it in a fine ash fraction (e.g. filter fly ash or condensation sludge), which could be separated from that part of the ash used for fertilization. This could be achieved by new furnace technology (Narodoslawsky and Obernberger 1996, Obernberger 1998).

1.4 Overall conclusion

The use and benefits of wood ash as a forest fertilizer depends on its quality, in particular unburnt carbon, nutrient, heavy metal and organic compound contents. If the wood ash contains high concentrations of harmful substances, it should not be used at all. Because elemental concentrations in wood ash show great variation its quality should always be determined. Where wood ash is used in upland forests it should preferably be supplemented with N in order to maintain a balanced nutrient status. To avoid potential drastic pH effects of wood ash, hardened rather than not loose ash should be used.

2 AIMS OF THE THESIS

My main objective was to find out if the cadmium (Cd) content of wood ash cause harm to boreal, coniferous forest soil humus layer microbes (I-III, VI). In addition, the long-term effects of wood ash on forest soil humus layer microbes (IV) and decomposition rate of needle litter and thereby on tree growth (V) were assessed. Also the potential of wood ash as a remediation agent of heavy metal polluted soil was studied (VII). More precisely, the questions that I have tried to answer were:

1) Is Cd-containing wood ash harmful to forest soil microbes? (I, II, III)

2) Does wood ash Cd transfer to food chains? (III)

3) Does wood ash exposure to simulated acid rain change the bioavailability of Cd and thereby affect forest soil microbes? (VI)

4) Does the form (loose vs. hardened) and the application dose of wood ash affect the magnitude of response of the measured forest soil chemical and microbial variables? (IV)

5) Does wood ash fertilization affect the forest soil microbes and needle litter decomposition rate still after 18-20 years of the treatment? (IV, V)

6) Is wood ash a potential remediation agent for acidified and metal polluted forest soil? (VII)

3 MATERIALS AND METHODS

3.1 Experimental designs

The experimental designs are presented in Table 3 and the details are given in papers I-VII. The wood ash used in studies I-III, IV (experiment 1), VI and VII, was fly ash from burning of softwood (26%) and hardwood bark (66%). Fiber and mineral containing waste sludge (8%) from wastewater cleaning process of paper and pulp mill was also co-burned. A different wood ash was used in the long-term studies IV (experiment 2) and V. The element content of the ashes used in experiments is presented in paper IV.

In papers I and II, I used a laboratory microcosms approach to investigate the combined and separate effects of Cd and wood ash on the humus microflora and the Cd bioavailability. In addition, the effect of different forms (water-soluble $CdCl_2$ and insoluble CdO) and application levels of Cd (0, 400 and 1000 mg kg^{-1} ash or pumice) were studied.

The objectives of paper III were to test in field conditions, if the Cd of wood ash has the potential to affect the coniferous forest humus microflora and if Cd becomes enriched in the food chain 1-4 years after fertilization. These objectives were tested with wood ash and wood ash spiked with extra Cd (400 mg kg^{-1} ash) applied onto the forest floor. Cd concentrations of different compartments (humus, soil percolation water, mushrooms, fruits and leaves of berries, and needles) of the forest ecosystem were determined.

There were two aims for paper IV. The first aim was to compare the effects of loose and hardened ashes on humus layer microbes in the field 1-3 years after fertilization. This experiment was performed using two application levels of the ashes and repeated in two forest stands of different fertility. The second aim was to study the long-term effects of loose ash in four forest stands of different site fertility 18 years after ash application.

Field study V aimed to examine if the wood ash treatment is reflected in the decomposition rate of Scots pine needle litter and Scots pine stem volumes, and if wood ash affects the quality of the Scots pine needle litter, which is then reflected in the decomposition rate. To achieve these objectives the layout of our experiment was as follows: Scots pine (*Pinus sylvestris*) needle litter samples from control plots and plots that had been fertilized with wood ash 19 years earlier were exposed in a reciprocal experimental design to detect the effects of ash fertilization and needle litter origin on the decomposition rate (Fig. 1 in V). The experimental design was repeated in two Scots pine forest stands of different fertility.

In paper VI, I tried to elucidate if simulated acid rain increases the leaching of Cd from wood ash and does it become bioavailable and harmful to co-

Table 3. Experimental designs in the studies.

Study	Symbol	Ash dose t ha^{-1}	Cd addition mg kg^{-1}	Irrigation[d]	Duration of ash treatment	Study type	Replicates	Site type of study/humus sample forest[e]	Location
I, II[a]	P	0	0	H_2O	2 months	laboratory microcosm	10	EVT	64°43'N/26°02'E
	PLO	"	400	"	"	"	5	"	"
	PHO	"	1000	"	"	"	"	"	"
	PLC	"	400	"	"	"	"	"	"
	PHC	"	1000	"	"	"	"	"	"
	A	5	10	"	"	"	10	"	"
	ALO	"	400	"	"	"	5	"	"
	AHO	"	1000	"	"	"	"	"	"
	ALC	"	400	"	"	"	"	"	"
	AHC	"	1000	"	"	"	"	"	"
III	C	0	0	natural conditions	1-4 years	field	3	"	"
	A	3	15	"	"	"	"	"	"
	ACd	"	400	"	"	"	"	"	"
IV[b]	C	0	0	"	1-3 years	"	"	ECT, VMT	64°43'N/26°02'E, 65°01'N/25°07'E
	A3	3	15	"	"	"	"	"	"
	A9	9	"	"	"	"	"	"	"
	HA3	3	13	"	"	"	"	"	"
	HA9	9	"	"	"	"	"	"	"
	C-18	0	0	"	18 years	"	4	CT, VT, MT, OMT	62°03'N/24°51'E,62°16'N/24°20'E
	A3-18	3	ND	"	"	"	"	"	61°00'N/24°45'E, 61°02'N/24°39'E
V	C	0	0	"	19-20 years	"	"	CT, VT	62°03'N/24°51'E,62°16'N/24°20'E
	A	3	ND	"	"	"	"	"	"
VI	C	0	0	H_2O or H_2SO_4 (=SAR)	3 months	field microcosm	"	MT	60° 07'N/23° 18'E
	A	5	10	"	"	"	"	"	"
	ACd	"	1000	"	"	"	"	"	"
VII[c]	Control	0 or 5	0 or 15	H_2O	2 months	field+laboratory microcosm	5	CT-VT	69°45'N/27°01'E
	Acid	"	"	" +H_2SO_4	"	"	"	"	"
	CuNi	"	"	" +$CuSO_4$+$NiSO_4$	"	"	"	"	"
	CuNi+Acid	"	"	" +H_2SO_4+$CuSO_4$+$NiSO_4$	"	"	"	"	"

[a] P = pumice, O = Cd form CdO, C = Cd form $CdCl_2$. [b] A = loose wood ash, HA = hardened wood ash, study IV comprises short- and long-term experiments, experiment I (1-3 years) and 2 (18 years), respectively; ND = not determined; SAR = simulated acid rain; [c] Humus from field was collected after 9 years exposure to acid and/or metal irrigation. Remediation study with wood ash done in laboratory microcosms; [d] In all studies, the irrigation were done at 2- to 3-days intervals; [e]EV=*Empetrum-Vaccinium*, EC=*Empetrum-Calluna*, VM=*Vaccinium-Myrtillus*, C=*Calluna*, V=*Vaccinium*, M=*Myrtillus*, OM=*Oxalis-Myrtillus*.

niferous humus microbes. The effect of Cd spiked into the ash (1000 mg Cd kg^{-1}ash) in response to the treatments was also determined. The simulated acid rain (SAR; pH 3.1) plots received a sulphur load of 3.64 g S m^{-2}, which was 15 times more than the S deposition on the water irrigated control plots.

In paper VII, I have assessed the effects of moderate amounts of continuous acid and Cu-Ni deposition on humus layer microbial community in the field after nine growing seasons. To determine whether these contaminated soils could be remediated, samples from the field were placed in laboratory microcosms and water irrigation combined with wood ash fertilization remediation treatment was evaluated. Microcosms that were only irrigated with water served as a control. The cumulative S, Cu and Ni load over the period 1991 – 2000 were for the CuNi plots 1220, 160 and 100 mg m^{-2}, and for CuNi+Acid plots 18 860, 160 and 100. The cumulative S load for the Control and Acid plots was 1080 and 18730 mg m^{-2}, respectively. The pH values for the irrigation in Control, Acid and CuNi plots were 5.5, 3.1 and 5.7, respectively.

3.2 Samplings

In all the studies, samples were taken from the organic, humus (F/H) layer, hereafter referred to as soil samples. At least 21 cores (usually 40 mm in diameter) were taken; that is, seven cores from three lines, and then combined to form one composite, bulk sample. The samples were sieved (2.8 mm mesh), visible plant material was removed, and then stored at +4°C before analyses were conducted.

In paper III, the wood ash was spread on the study plots in autumn 1997. Soil samples were collected in August 1998, November 1999, September 2000 and 2001. In addition needle (*Pinus sylvestris* L.), berry (*Empetrum nigrum*, *Vaccinium uliginosum*, *Vaccinium vitis-idaea*) and mushroom (*Lactarius rufus*) samples were collected. *P. sylvestris* needles from the previous year were collected each autumn, always from eight trees and bulked to make composite sample for the plot. Unfortunately, it was not possible to take an adequate mushroom and berry sample each year, that is, 6-20 mushroom specimens (upper half of stipe and cap) and 1.5 decilitres of berries per sample plot. Soil water was sampled with suction-cup lysimeters installed at depths of 5 and 20 cm below the soil mineral layer surface six times during the snow free period in 1998 and 2001, and seven times in 1999 and 2000. In 2000 also the leaves of *V. uliginosum* and *V. vitis-idaea* were collected from all over each study plot to form a composite sample of ca. two decilitres (over 2 g dry matter). Litterbags for mass loss determination were sampled in November 1999, 2000 and 2001 after 2, 3 and 4 years exposure in the field, respectively.

In paper IV, the wood ash in the short-term study (experiment 1) was spread in May-July 1997 and soil samples collected in August 1998, June 1999 and

September 2000. In the long-term study (experiment 2), the wood ash was spread in July-August 1982 and soil samples collected in September 2000.

In paper V, the needle litterbags were retrieved in autumn of 2001 and 2002 after 4 and 16 months exposure in the field, respectively. Scots pine stem volumes were measured in autumn 1998 (ash was spread in July-August 1982).

3.3 Methods

The various methods used are listed in Table 4. A brief summary is given here; for details refer to papers I-VII. Soil pH was measured in a water suspension. Soil dry matter weight was determined after drying at 105°C and organic matter content determined after furnacing subsamples at 550°C. Total organic carbon and nitrogen content were determined by dry combustion. Total and extractable contents of elements were determined with ICP-AES. In paper III, dissolved organic carbon (DOC) concentrations in soil water was determined with a TOC analyser and NH_4^+ and NO_3^- concentrations determined with a flow injection analyser. Concentrations of Ca and Cd in soil water were analysed by ICP-AES. After collection of the mushroom, berry, leaf and needle samples (III), they were cleaned, air dried and concentrations of Ca and Cd determined by ICP-AES after wet digestion (HNO_3+H_2O_2).

Bioavailability of Cd (extraction with deionized water) and Cu (extraction with 50 mM $CaCl_2$) to bacteria in the air dried soil were determined with two biosensor bacteria that emit light specifically in the presence of Cd or Cu. The enumeration of culturable bacteria was done on agar nutrient plates. Colony forming units (cfu) were counted after 12 days of incubation in darkness at room temperature. Bacterial Cd tolerance was also estimated on nutrient agar plates to which 5 or 20 mg Cd per liter (as $CdCl_2$ x 2.5 H_2O) was added.

Microbial activities measurements were basal respiration, [^{3}H]-thymidine incorporation and needle mass loss rates. Basal respiration rates were determined as the amount of CO_2-C evolved over 23-26 h. Bacterial growth rate and Cd tolerance were determined using the [^{3}H]-thymidine incorporation technique. For the bacterial growth rate, the bacterial cells were extracted from soil, after sample homogenization and centrifugation. In the Cd tolerance assay, different amounts of $CdCl_2$ were added to the bacterial suspension. The Cd concentration (mM) giving a 50% (or 30%) reduction in [^{3}H]-thymidine incorporation was calculated (IC_{50} or IC_{30}). The higher the IC_{50} value, the greater is the tolerance of the bacterial community to Cd. In the determination of the mass loss rate of needles in the papers III and V, the contents of needles remaining in the litterbags were weighed.

The microbial community profiling analyzes were made using the Biolog®, phospholipid fatty acid (PLFA) method, and 16S or 18S ribosomal DNA (rDNA) targeted single step polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). The Biolog® technique used in paper I deter-

Table 4. Methods used in this thesis.

Analysis	Method	Used in study	Reference
Total Cd in humus [a]	wet digestion, HNO_3+H_2O_2 extraction	I, II, III, VI	Tamminen and Starr 1990
Extractable Cd in humus [b]	1.0 M CH_3COONH_4 extraction (pH 7.0) [c]	I, III, VI	Tamminen and Starr 1990
Bioavailability of Cd to bacteria	Bioluminescence of *Bacillus subtilis* BR 151/pTOO24	II, III, VI	Young et al. 1969, Tauriainen et al. 1998
Bioavailability of Cu to bacteria	Bioluminescence of *Pseudomonas fluorescens* DF57-Cu15	VII	Tom-Petersen et al. 2001
Microbial activity	CO_2 evolution	I, III, IV, VI, VII	Pietikäinen and Fritze 1995
Mass loss of needles	litter bags	III, V	
Bacterial growth rate	[^{3}H]-thymidine incorporation	I, III	Bååth 1992a, Kiikkilä et al. 2000
Cd tolerance of bacteria	[^{3}H]-thymidine incorporation	I, III	Bååth 1992b, Kiikkilä et al. 2000
Cd tolerance of bacteria	cfu	I	
CLPP	Biolog EcoPlates	I	Insam 1997
Microbial community structure	PLFA pattern	I, III, IV, VI, VII	Frostegård et al. 1993b, Pennanen et al. 1999
Bacterial community structure	PCR-DGGE, primer pair F984GC+R1378	VI	Heuer et al. 1997
Fungal community structure	PCR-DGGE, primer pair FF390+FR1GC [d]	II, VI, VII	Vainio and Hantula 2000, Pennanen et al. 2001
Total microbial biomass	PLFA analysis	I, III, IV, VI, VII	Frostegård and Bååth 1996
Bacterial biomass	PLFA analysis	I, III, IV, VI, VII	Frostegård and Bååth 1996
Fungal biomass	PLFA analysis	I, III, IV, VI, VII	Frostegård and Bååth 1996
Culturable bacteria	cfu	I	

For the closer description of materials and methods, see papers I-VII

[a, b)] Other elements (Ca, Mg, K, Al, Cu and Ni) were also analyzed with these methods; [c)] In study VI, 0.1 M $BaCl_2$ extraction was used; [d)] In study II primer pair NS1+FR1GC was also used; cfu = colony forming units; CLPP = community level physiological profiling; PLFA = phospholipid fatty acid; PCR = polymerase chain reaction; DGGE = denaturing gradient gel electrophoresis; F = forward primer, R = reverse primer, GC = G+C rich sequence attached at 5´ end.

mines the community level physiological profile (= CLPP), that is, substrate utilization potential, of the bacterial community. The EcoPlates used contained 31 different carbon sources and they were inoculated with 10^{-3} diluted soil suspension. The EcoPlates were incubated at 20°C and the absorbances read every day until the 120 h was reached. The PLFA method was used to detect changes in microbial community structure. Lipids were extracted from the soil with a one-phase mixture (1:2:0.8 v/v/v) of chloroform, methanol and citrate buffer (0.15 M, pH 4.0), and fractionated into neutral, glyco- and phospholipids on a silicic acid column. By using nonadecanoate (19:0) as an internal standard the total amount of PLFAs in the soil sample were calculated and this value used as an indicator of soil total 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 used as an index of bacterial biomass ($PLFA_{bact}$) and the quantity of 18:2ω6 was used as an indicator of fungal biomass ($PLFA_{fung}$). The Biolog® and PLFA methods target mainly bacteria. Treatment-induced changes in fungal community were detected using fungal 390 (II, VI, VII) or 1650 (II) basepair (bp) rDNA fragment (18S) DGGE patterns. Bacterial 434 bp rDNA fragment (16S) DGGE patterns were also used to detect changes in bacterial community in paper VI. The advantage of both the PCR-DGGE and PLFA methods is that they do not rely on the culturability of the microbes.

3.4 Data analyses

Analysis of variance (ANOVA) was used to compare the effects of different treatments. In ANOVA the differences between variable means were tested using Tukey's (II, V) or LSD (III, IV, VI) test. Differences between means were considered statistically significant when $p < 0.05$ (I, II, V, VI) or $p < 0.10$ (III, VII). The mol % and area % values from the PLFA (I, III, VI, VII) and Biolog® data (I), respectively, were standardised by dividing by the standard deviation (correlation matrix) before being subjected to principal component analysis (PCA). In paper IV, the PLFA pattern was explored with global non-metric multidimensional scaling (MDS) (Clarke 1999). In paper VI MDS was used to analyse the data matrix of DGGE gel bands. In papers II and VI the Kruskall-Wallis non-parametric ANOVA followed by a mean ranks test was performed for the biosensor data because the assumptions of normal distribution and equality of variances were not met. Canonical correlation analysis (CCA) (Gittins 1985) was used to investigate the relationships between chemical and biological variables (III, IV). The scores from PCA and CCA were also tested with ANOVA. The data were log-transformed where necessary. Pearson correlation tests were used to evaluate the relationships between some individual variables. The software used for ANOVA, PCA and CCA were Statistix 7 (Analytical Software 2000), PC-ORD version 4 (MjM Software Design 1999) and SAS (Institute Inc. 1996), respectively.

4 RESULTS AND DISCUSSION

4.1 Cd effects on forest soil microbes

The addition of Cd to the soil can disturb the nutrient cycling of forest ecosystems because of its potential toxic effects on microbes. Cadmium amendments have been shown to inhibit phosphatase, sulphatase and respiration activities (Speir et al. 1999), change microbial community structure (Frostegård et al. 1993b) and reduce microbial biomass (Frostegård et al. 1993b) and dehydrogenase activity (Welp 1999). Cadmium concentrations as low as 1-5 mg Cd kg^{-1} humus have been found to inhibit the activity and change the community structure of forest soil microbes (see review by Bååth 1989). Microbes have been shown to have a number of mechanisms to prevent Cd toxicity, including the exclusion of Cd by an energy-dependent pump situated in the cell membranes of resistant stains and the production of Cd-binding proteins in microbes that accumulate Cd (Trevors et al. 1986).

In order to estimate the possible effect of Cd not associated with ash, Cd with pumice was spread onto the soil (I, II). Pumice proved to be an ideal agent to distribute the small amounts of Cd evenly over the soil surface, because it has no effects on the microbes or pH of the soil compared to untreated soil (I). The different forms of Cd (CdO vs. $CdCl_2$) had no effect on the soil respiration rate, but the level of Cd applied (0, 400 or 1000 mg kg^{-1} pumice) influenced the respiration rate. The highest application of Cd (5 t pumice ha^{-1} of 1000 mg Cd kg^{-1} pumice) decreased respiration rates compared to lower Cd levels (I; Fig. 1a, page 34). The form and level of Cd had no effect on the bacterial growth rates, bacterial and fungal biomasses, culturable bacteria, tolerance of the bacterial community to Cd measured with thymidine incorporation or with a more conventional cfu method (I). Neither did the substrate utilisation pattern of bacterial community (I; Biolog®) nor fungal community structure (II; PCR-DGGE) change.

When Yrjälä et al. (2004) analyzed the *Archaea* community from paper I microcosm soil with the PCR-DGGE method, they detected no Cd effect. Neither did Cd have an effect on community structure of *Actinomycetes* (unpublished result). Díaz-Raviña et al. (1994) tested the development of community tolerance to Cd (added as $CdSO_4$) in agricultural soil over a 5- to 8-month incubation period. The addition of 896 mg Cd kg^{-1} dry soil resulted in the development of a Cd-tolerant community, whereas the addition of 448 mg Cd kg^{-1} dry soil gave minor differences compared to the unpolluted control. In paper I the addition of Cd with pumice to the soil surface at the highest doses, resulted in the amount of Cd to 171 mg kg^{-1} and 182 mg kg^{-1} soil for CdO and $CdCl_2$, respectively. These levels did not exceed the threshold level needed to induce tolerance of the bacterial community to Cd.

The addition of Cd with pumice altered the microbial community structure as detected by the changed PLFA pattern (I; Fig. 1a, page 34). The ANOVA performed on the PC1 scores of principal component analysis (PCA), using the PLFA data, revealed that the high Cd (added as $CdCl_2$) treatment differed significantly from the other treatments, including the control, and the scores correlated significantly ($r = 0.58$) with the total amount of Cd in the soil. The relative mol % of the methylated PLFAs increased with increasing Cd concentration. Fatty acids with a methyl group in the tenth carbon atom from carboxyl end of the chain (10Me16:0, 10Me17:0, 10Me18:0) are found exclusively in actinomycetes (Kroppenstedt 1985). Thus, the result of paper I suggests that Cd application caused a relative increase in the number of actinomycetes. In contrast, the relative mol % of PLFAs 18:2w6 and 20:4, which are found in fungi (Frostegård and Bååth 1996, Stahl and Klug 1996), decreased as a result of Cd addition. The changes observed in the PLFA pattern of the pumiced samples due to Cd addition are consistent with the results of Frostegård et al. (1993b). At the beginning of a 6-month incubation they added solutions of $CdSO_4$ to humus layer samples to give comparable Cd concentrations (56, 112 and 224 mg kg^{-1} dry humus) to those used in study I ($\leq$ 182 mg kg^{-1} dry humus).

In all soil samples treated with Cd spiked pumice, the *Bacillus subtilis* biosensor detected an increased amount of bioavailable Cd when compared to the unspiked pumice controls (II; Fig 1a, page 34). The biosensor detected significantly higher Cd amounts in the soil of the PHC and PHO treatments than in the PLC and PLO treatments. The highest amount of bioavailable Cd (20.2 ± 1.0 (SE) mg Cd kg^{-1} soil) was detected in the PHC treatment. The percentage of bioavailable Cd in soil was only 4.7–11% of the total Cd content. In general, a higher availability of Cd was detected in the soil samples that received the water soluble form of Cd, but the difference between PHC and PHO, and between PLC and PLO, were statistically insignificant.

In conclusion, both Cd application levels (400 and 1000 mg Cd kg^{-1} pumice) increased the amount of bioavailable Cd to bacteria, and highest Cd application level (both CdO and $CdCl_2$) reduced the microbial mineralization activity measured as respiration rate. In addition, the highest application of water soluble Cd changed the microbial community structure measured with PLFA method.

4.2 Wood ash effects on forest soil microbes

The application of ash increased soil pH (I-IV, VI-VII). The pH effect of the ash increased microbial activities as measured by soil respiration (I; Fig. 1a, III; Fig. 1b, IV, VI, VII) and thymidine incorporation rates (I, III). These results are in accordance with investigations where higher soil respiration (Bååth and Arnebrant 1994, Fritze et al. 1994b, Khanna et al. 1994, Fritze et al. 1995) and

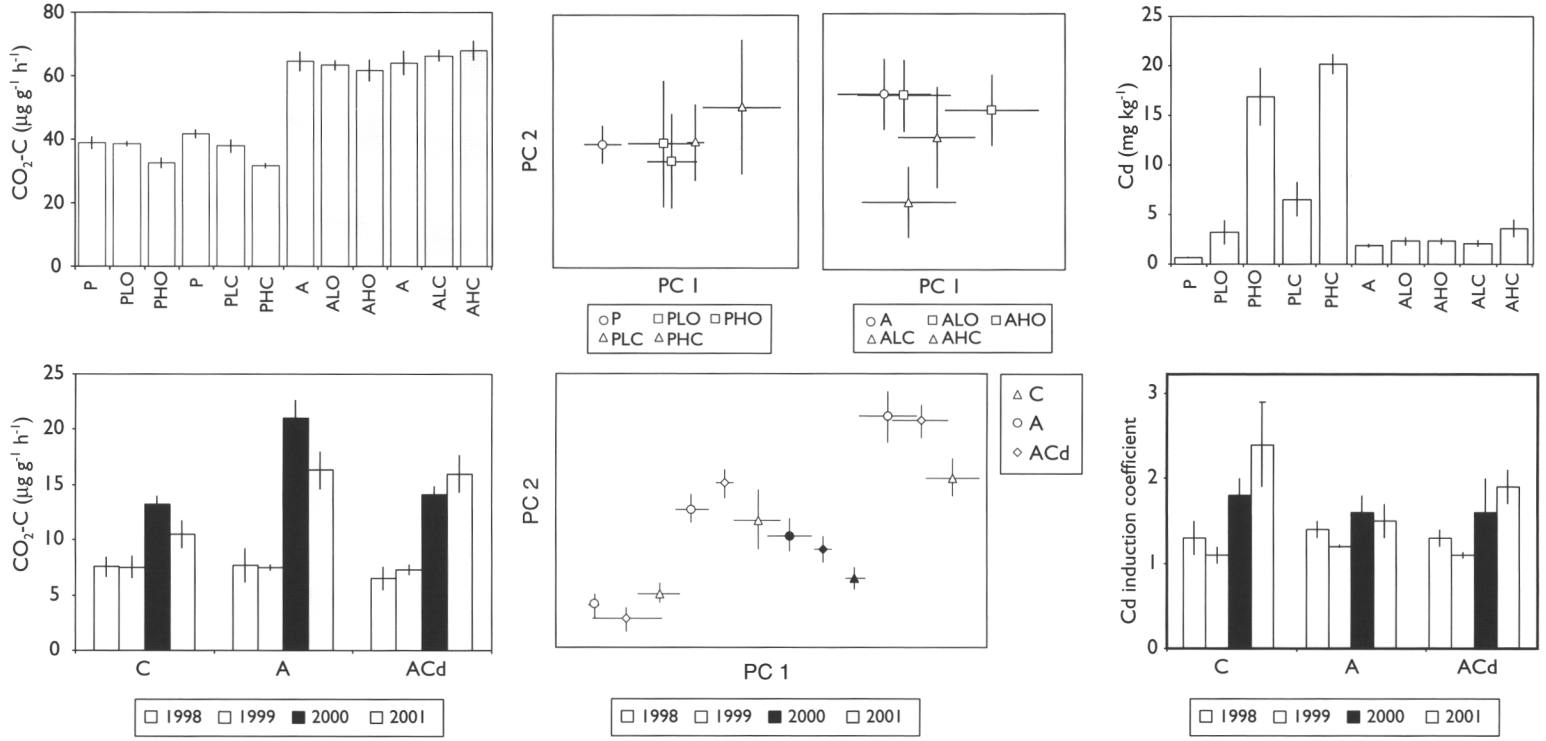

Fig. 1. Respiration rates, PLFA patterns and reactions of Cd-biosensors in the studies I-II (a) and III (b). In study III, the majority of samples exhibited bioavailable Cd-levels below the reliable quantification level and the induction coefficients were not transformed to Cd concentrations. Bars indicate standard error. See Table 3 for treatment symbols.

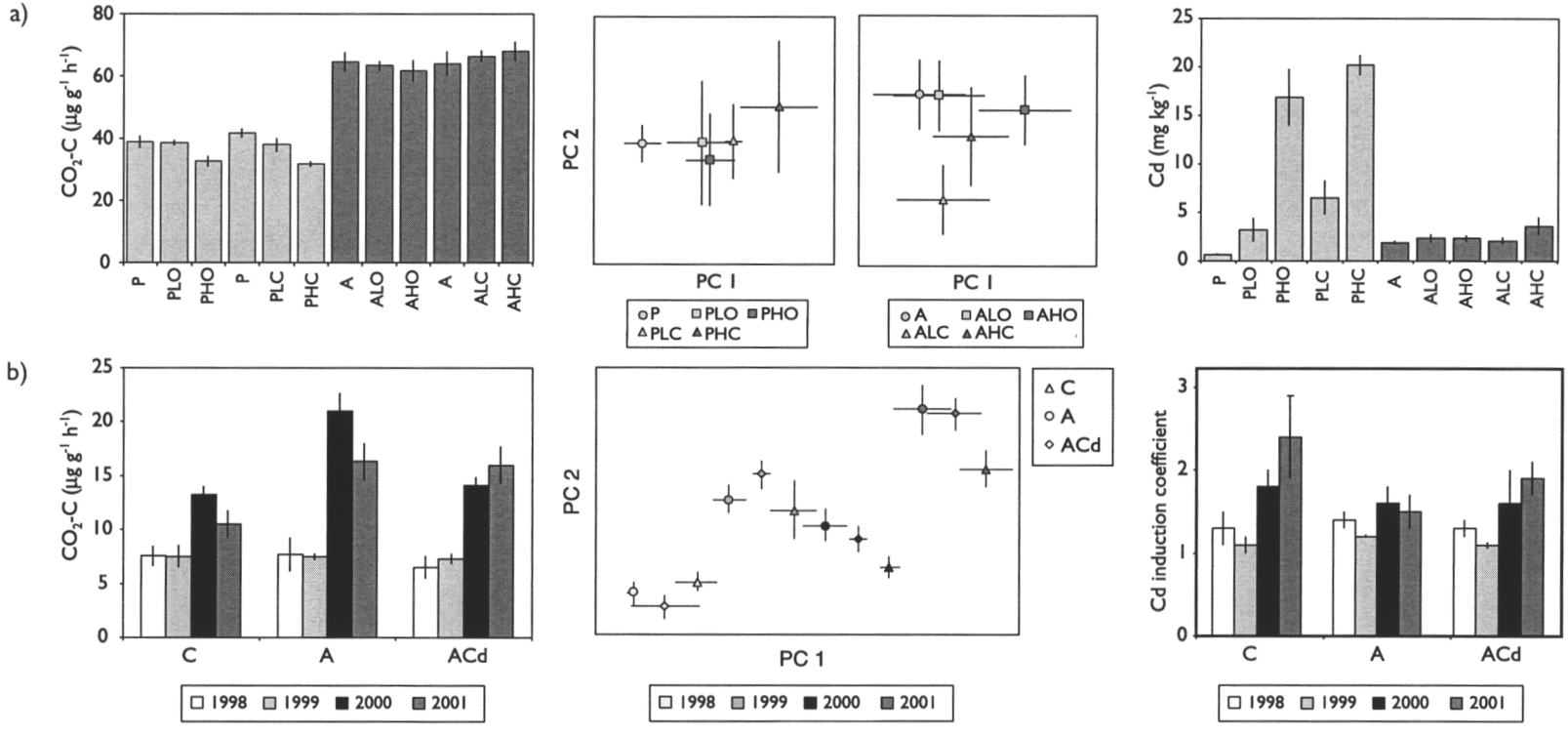

Fig. 1. Respiration rates, PLFA patterns and reactions of Cd-biosensors in the studies I-II (a) and III (b). In study III, the majority of samples exhibited bioavailable Cd-levels below the reliable quantification level and the induction coefficients were not transformed to Cd concentrations. Bars indicate standard error. See Table 3 for treatment symbols.

thymidine incorporation rate (Bååth and Arnebrant 1994, Bååth et al. 1995, Hagerberg and Wallander 2002) were detected after ash fertilization. According to Khanna et al. (1994), much of the respired C is derived from soil sources rather than the C added in the ash. In many studies, wood ash fertilization resulted in increased soil water dissolved organic carbon (DOC) concentrations (see chapter 1.2.1), which could act as a source of carbon for microbes and explain the rise in respiration and thymidine incorporation.

In accordance with the activity measurements, the number of culturable bacteria also increased (I). Similar increase in cfu values due to wood ash fertilization at levels of 5 t ha^{-1} have been reported for coniferous forest humus by Bååth and Arnebrant (1994). The increased microbial activity of the ash treated samples was also accompanied by a change in the bacterial substrate utilisation pattern (I; Biolog®), and in the microbial (I; Fig. 1a, III; Fig. 1b, IV, VI, VII; PLFA), bacterial (VI; PCR-DGGE), fungal (II, VI; PCR-DGGE), archaeal (Yrjälä et al. 2004) and actinomycetal (unpublished result) community structure.

The Biolog® results revealed that the bacterial community in the ash treated soil was able to use other C sources compared to the bacteria community in the pumice treated samples (I). The ash application was therefore great enough to change the microbial community structure (PLFA) to such an extent that different subsets of the bacterial community became enriched in the Biolog® plate, being capable of utilizing different C-sources than the subsets of the untreated or pumiced soil samples (I).

A change in the PLFA pattern due to increased humus pH has been demonstrated earlier in an area subjected to alkaline pollution (Bååth et al. 1992), forest liming (Frostegård et al. 1993a) and wood ash fertilization (Frostegård et al. 1993a, Bååth et al. 1995). In a 3-year field mesocosm study by Liiri et al. (2002b), wood ash treatment changed the microbial PLFA and bacterial substrate utilization pattern. Changes in humus quality with increasing ash fertilization levels have been measured using infrared spectroscopy (Bååth et al. 1995). These authors showed that the humus quality changed but were only partly successful in their attempts to correlate the changes in the PLFA pattern with soil pH or substrate quality. Consequently, they concluded that changes in the PLFA pattern of the soil organisms were related to an altered substrate quantity (availability of substrates), quality and pH.

The results from paper VII showed a slight increase in the abundance of actinomycetes (indicator PLFAs 10Me16:0, 10Me17:0 and 10Me18:0 see chapter 4.1) and decrease that of fungi (indicator PLFA 18:2ω6) following ash fertilization. Similar changes in fungal and actinomycetal PLFAs after wood ash treatment in the microcosm experiment were found in paper I. In the field studies by Frostegård et al. (1993a), Bååth et al. (1995), III and IV, the amount of indicator PLFA (16:1ω5) for arbuscular mycorrhiza (AM) fungi increased after ash application. In these field studies the amount of PLFAs 10Me16:0 and 18:2ω6 either decreased or did not change, and that of PLFAs 10Me17:0 and 10Me18:0 either increased or did not change. However, in the long-term study

IV (experiment 2) the abundance of fungal PLFA increased, and that of PLFAs 10Me16:0 and 10Me17:0 decreased in the wood ash treated plots. The most striking difference between the results from these field studies and microcosm studies I and VII was that the amount of the fatty acid 16:1ω5 decreased after ash amendment in microcosm studies. One explanation for this difference may be related to the absence of plants in microcosms. Herbs and grasses thrive after ash fertilization (see chapter 1.2.2) and the amount of fatty acid 16:1ω5 increases when the amount of herbs and grasses increase (Pennanen et al. 1999, Saetre and Bååth 2000).

The results of bacterial and fungal biomass measurements in this thesis, were not consistent with each other. Mean bacterial (I, IV (experiment 1); $PLFA_{bact}$) and fungal (I, IV (experiment 1), VI; $PLFA_{fung}$) biomasses either decreased or did not react to wood ash treatment (VI; $PLFA_{bact}$, III, IV (experiment 2),VII). These kinds of contradictory results have also been observed by others. For example, Bååth et al. (1995) observed a decrease in bacterial and fungal biomasses in a field study with ash treatment, but Frostegård et al. (1993a) did not find a significant ash effect on bacterial and fungal biomasses.

In papers II and VI, fungal 18 S rDNA primer pair detected an ash effect but in paper VII no ash effect on fungal community was detected. It can be argued that at the time of sampling these fungal species were the most dominant growing. The fungal species diversity as indicated by spores is probably not detected by the PCR-DGGE method. In a soil microfungal study (Fritze and Bååth 1993), the fungal species composition growing on agar plates was not different if the isolation was performed from vegetative hyphaea or from the spores. Microcosms in papers II, VI and VII did not include plant seedlings and, thus mycorrhizal fungi were probably excluded, leaving only saprophytic fungi that could be detected. Using isolation techniques it has been shown that ash fertilization (Bååth and Arnebrant 1993) and fly ash deposition (Fritze and Bååth 1993) changes the composition of the microfungal flora inhabiting the humus layer. The same conclusion was obtained by using the PCR-DGGE method (II, VI).

Experiment 1 of paper IV compared two forms, loose and hardened wood ash (abbreviations A and HA, respectively) at two fertilization levels, 3 and 9 tons per hectare (these numbers follow the abbreviations). The results showed that ash of both types decreased soil extractable Al, increased soil pH and exchangeable base cation concentrations; reactions to ash amendment that have been widely reported in the literature (see chapter 1.2.1). Hardened ash did not have as strong effects on above-mentioned soil properties as loose ash did. Increase in soil extractable Ca and Mg concentrations was dependent on the dose and form of the ash applied, with A9 treatment having the highest values, followed by HA9 and A3, and then the HA3 treatment (IV; experiment 1).The lowered leaching rate of base cations due to hardening has also been confirmed in laboratory studies by Eriksson (1998b) and Ring et al. (1999). The hardening of ash by granulation helps avoid the rapid dissolution of the ash and thereby avoiding drastic increases in humus pH (Eriksson 1998b), burning

damage to mosses (Kellner and Weibull 1998) and changes in the coverage of fungi and vascular plants (Rühling 1996). However, the nutritional response with hardened, granulated and pelletized wood ash on the forest vegetation was found to be similar to that with loose wood ash (Moilanen and Issakainen 2000, Hytönen 2003).

Due to its slower dissolution rate the effects of hardened ash on soil microbes may be expected to be less than loose ash. Both multivariate statistical approaches supported the above hypothesis. The canonical correlation analysis (CCA) separated the ash treatments according to the form and level of ash used. The vectors that best explained the separation of the treatments were increases in bacterial growth rate, microbial respiration activity, and soil extractable Ca concentration (Fig. 2 in IV). The A9 treatment that has highest values for these variables separated from other treatments. Treatments A3 and HA9, and HA3 and C clustered into same group. The multidimensional scaling (MDS) procedure, using the eluted phospholipid fatty acids (PLFAs) data, separated the A9 treatment from the other treatments from the first year onwards. In the third year, the HA9 and the A3 treatments started to separate from the control and remained separated from the A9 treatment (Fig. 3 in IV).

The aim of experiment 2 in paper IV was to estimate the duration of the ash effect on upland soil microbes. The fact that the microbial community structure (PLFA pattern) and activity (respiration and thymidine incorporation rates) were still different from the control after 18 years of ash fertilization points toward a very long-term effect of wood ash on soil microbes. However, there were no clear long-term ash effect on the biomass indicators except that the $PLFA_{fung}/PLFA_{bact}$ ratio was slightly higher in the ash treated (A3-18) than in the control plots (C-18). The CCA separated the old ash plots according to microbial activity (respiration and thymidine incorporation rates) and soil extractable Ca concentration that are the same variables as in the short-term field experiments in papers III (see chapter 4.3 below) and IV. These findings provide new information about the longer-term effects of wood ash on upland forest soil microbes. Previous studies have only studied these effects for short time periods of 1-6 years after application.

One of the oldest documented ash fertilization trial in Finland was performed in a forest growing on peat and therefore not directly comparable to study IV. The trial on peat (no treatment replications) showed elevated peat pH (Silfverberg and Huikari 1985), Scots pine stem volume growth and decomposition rate of cellulose strips (Moilanen et al. 2002) after 30, 47 and 50 years of fertilization, respectively, with rather high amounts of loose ash (8 and 16 t ha^{-1}). In study IV humus pH (highest difference between pH of C-18 and A3-18 plots was 1.2 units) was also an important discriminating variable in the CCA. From these results one can speculate that the microbiological effects of loose ash on upland soil can persist for 50 years, and the effects of hardened ash can persist for longer.

The needle decomposition experiment in paper V was performed on the same sites as in paper IV that had been treated with wood ash 19-20 years ear-

lier. The litterbags were retrieved after 4 and 16 months. The loss of needles mass was significantly larger on the ash treated plots than on control plots. The increased microbial activity and the changed microbial community structure shown in paper IV were thus reflected in enhanced needle decomposition. On drained peatland sites, Silfverberg and Hotanen (1989) have observed a similar increase in needle decomposition rate as a result of long-term ash effects. However, in a 4-year long field study III, no significant increase in the mass loss of needles was detected although rise in respiration and thymidine incorporation rates were detected. It is difficult to explain the lack of a wood ash effect on the mass loss of needles. Most likely, the time was not too short for a fertilization effect to occur, because Smolander et al. (1996) found increased mass loss of Scots pine needle litter on study plots fertilized with wood ash (2.5 t ha^{-1}) only 8 months prior to the start of the decomposition experiment. One possible explanation could be the unfavourable quality of needles in study III.

The ash fertilization induced changes in the ecosystem may be reflected in the quality of the coniferous trees needles. In both sites in paper V, needles originating from the ash fertilized plots had lower C-to-N ratios. The origin, and thus the quality of needles, did not significantly influence the decomposition rate although ash needles exposed on ash plots (AA) had a slightly higher decomposition rate than control needles exposed on ash plots (CA) during the first 4 months (Fig. 2 in V). Further details about the quality of needles on the decomposition rate are discussed in paper V.

Scots pine stem volumes increased due to ash fertilization (V). The enhancing effect of ash fertilization on both needle mass loss and tree growth was more pronounced in the less fertile CT than the VT site. This difference was accompanied by a significant increase in concentrations of P in the humus layer of CT site. Phosphorus and especially N deficiency is quite common in Finnish tree stands growing on mineral soils, and B deficiency also sometimes occurs (Raitio et al. 2000). Humus layer N concentrations did not respond to wood ash fertilization and the amount of B was not analyzed. Usually, in contradiction to the findings in study V, the effect of wood ash fertilization on the growth of coniferous trees on mineral soils has been insignificant (see chapter 1.2.3.1). However, Tamminen (1998) did observe an increase in the height growth of 8-15-years old Scot pines 2-5 years after wood ash fertilization (3 t ha^{-1}).

One negative effect following wood ash application was the increase in NO_3^- concentrations in the soil water sampled from the ash treated plots (A) at 0.2 m depth (III). This increase in NO_3^- concentrations could be due to an increase in the nitrification rate in response to the increased soil pH (Martikainen 1984, Khanna et al. 1994, Paavolainen and Smolander 1998, Priha and Smolander 1999). Kahl et al. (1996) and Ludwig et al. (2002) also observed increased NO_3^- leaching after wood ash application on sandy soil. Increased NO_3^- leaching after ash application, however, is not a rule since many other wood ash studies have not detected it (see chapter 1.2.1).

In conclusion, the microbiological variables that responded to wood ash fertilization were: respiration and thymidine incorporation rates, cfu values, substrate utilisation pattern of the bacterial community, and the structure of microbial, bacterial and fungal communities. In some studies, fungal and bacterial biomasses have also decreased. In the field studies, the activity measurements reacted earlier (1-3 years after ash treatment) than community structure changes (3-4 years after ash treatment) and the response did not differ between forest site types. The degree of change on the measured soil chemical and microbial properties depended on the time since application and the level and form of ash applied. Increased soil microbial activity and changed community structure were still detected 18 years after wood ash treatment and Scots pine needles on treated sites decomposed faster 20 years after treatment.

4.3 Wood ash Cd effects on forest soil microbes

Cadmium in unspiked or Cd spiked wood ash had no significant effect on respiration (I; Fig. 1a, III; Fig. 1b) and thymidine incorporation rates (I, III), needle litter weight loss (III), cfu counts (I), total ($PLFA_{tot}$), bacterial ($PLFA_{bact}$) and fungal ($PLFA_{fung}$) biomasses (I, III) or on the substrate utilisation pattern of the bacterial community (I), or microbial (I; Fig. 1a, III; Fig. 1b), bacterial (VI), fungal (II, VI), archaeal (Yrjälä et al. 2004) and actinomycetal (unpublished result) community structure in the ash treated soil samples. Neither did the amount of bioavailable Cd to bacteria in the soil increase (II; Fig. 1a, III; Fig. 1b). The results of microcosm experiments reflected the same trends than the field experiment.

In study II, *Bacillus subtilis* biosensor values of Cd spiked ash treatments did not differ from their unspiked ash controls and the mean level of bioavailable Cd was 2.4 mg kg^{-1} soil in all the ash treatments. Thus, the soil samples receiving pumice spiked with Cd (see chapter 4.1 above) had significantly higher amounts of bioavailable Cd than the ash treated samples. In study III, the total Cd concentrations in wood ash treated soil were about ten times lower than in the microcosms study II and the amount of bioavailable Cd to bacteria were in many samples below a reliable quantification level.

Fritze et al. (1995) have shown earlier that the soil respiration rate decrease when Cd is added to wood ash treated humus. Humus layer samples from a field experiment given 5 t ha^{-1} of wood ash required more than 4000 mg Cd kg^{-1} humus to decrease the respiration rate by 50 %, whereas the unfertilized control humus required 1570 mg Cd kg^{-1} humus to reach the same degree of inhibition (Fritze et al. 1995). Their study indicated that wood ash can to some extent reduce the toxicity of Cd, but levels of Cd given were unnaturally high. The experiment performed in paper I, is in this respect more realistic because the Cd was first mixed with the wood ash to give a theoretical maximum level

of 1000 mg Cd kg^{-1} ash before spreading it onto the surface of the soil to imitate a fertilization load of 5 t ha^{-1}. Converting this to the amount of Cd per soil dry weight used in this experiment, 196 mg Cd kg^{-1} humus entered the soil system. This was enough to reduce the soil respiration rate by 20 % in the absence of ash (see chapter 4.1 above). In the presence of ash, even the highest addition of Cd had no effect on soil respiration (I; Fig. 1a).

Principal component analysis (PCA) of the PLFAs separated, but not significantly, the Cd-treated ash samples from the untreated ash samples along the first PC (I; Fig. 1a). Actinomycetes (representative PLFAs 10Me16:0 and 10Me17:0), which benefited from the addition of ash or Cd alone, suffered from the combined effect of ash and Cd. The third methylated PLFA 10Me18:0 did not react markedly to the Cd treatments in the ash treated soil. Since the differences among the treatments were not statistically significant and the PCA axis 1 scores did not significantly correlate with the measured Cd concentration in the soil, it can be concluded that the presence of ash countered the toxic action of Cd on the microbial community. This was probably due to the capacity of the ash to adsorb the Cd and to increase soil pH. The hypothesis that wood ash can protect bacteria from Cd toxicity was supported by the finding that the treatment with just wood ash did not show the effects on Cd bioavailability, microbial respiration rate and community structure that the Cd-alone treatment had.

Bacterial growth rate measured by the thymidine incorporation method in all ash treated samples was more sensitive to Cd additions than the pumice treated samples (I). On average, only 0.0023 ($\pm$ 9.1 x 10^{-5}) mM Cd (= 0.26 mg Cd l^{-1}) was needed to decrease the thymidine incorporation rate by 30%, whereas for the pumice treated samples 0.21 ($\pm$ 2.6 x 10^{-2}) mM Cd (= 24 mg Cd l^{-1}) was required. This result indicated that the bacterial community of the ash treated soil, which was isolated into the water solution before the addition of Cd in the tolerance test, was more sensitive to external Cd than the respective bacterial community of the pumice treated controls. In the field study III, cadmium tolerance IC_{50} values indicated an ash but not a Cd effect two and three years after wood ash addition. Indeed the bacteria in control soil were more tolerant to Cd than bacteria in the ash treated (A and ACd) soils. Thus, according to thymidine incorporation method, ash treatments did not induce bacterial tolerance to Cd. The bacteria that could be cultivated on nutrient media, however, reacted in the opposite (I). The addition of 5 mg Cd to 1 liter of nutrient agar decreased the cfu of the pumice treated samples by nearly 100%, whereas the cfu of the ash treated samples decreased by between 20 to 70%. The traditional plate count method supports the hypothesis that ash can protect bacteria from Cd toxicity. There are many possible reasons for the discrepancy between the results given by these two methods. One of them could be the different incubation times with external Cd that were used, 12 days in the plate count method and 2 hours in the thyminidine incorporation method. Thus, the thymidine incorporation method detects Cd-tolerance of fast growing bacteria and plate count method of slower growing bacteria. One can hy-

pothesise that the decreased IC_{50} value may have been due to increases in bacterial growth rather than a decreased tolerance, since rapid growing bacteria are, in general, more sensitive to external disturbances.

In paper III, the chemical and microbiological data were summarised using a multivariate statistical approach (Canonical Correlation Analysis, CCA; Fig. 1 in III). Accordingly the A and ACd treatments started to separate from controls, but not from each other from the third year onwards. The higher Cd additions in the ACd treatment did not produce any effect on the microbiological and chemical variables differing from that produced by the ash only (A). This is verified by the fact that neither total nor extractable Cd levels were selected as key variables in the formation of the CCA. In contrast, the increase in soil pH, respiration and thymidine incorporation rates and concentrations of total and extractable Ca were selected as key variables that best explained the separation of treatments as they all increased due to wood ash treatment. In addition, the changes in PLFA pattern caused by wood ash affected the formation of the CCA.

In general, the liberation of Cd from different kind of ashes is low (Eriksson 1998b, Ring et al. 1999, Steenari et al. 1999, Richards et al. 2000, Ramesh and Koziñski 2001, Praharaj et al. 2002, Nieminen 2003). A laboratory study showed that solution pH has to be lower than 4.0 before Cd is released from wood ash (Hansen et al. 2001). Zhan et al. (1996) showed that, at solution pH values lower than 6.0, which are normal for rain water, there is a sharp increase in the amount of dissolved Cd from wood ash. Holmberg et al. (2000) showed that almost 15% of the Cd in wood ash granules leached during a 7 months exposure in the field. In paper III, the ACd and A treatments respectively added 128 and 4.5 mg Cd m^{-2} to the forest floor, which theoretically could leach when the ash-induced increase in pH effect stops and pH declines. In the last sampling, that is four years after wood ash treatment, soil pH was 5.6 in A and 5.5 in ACd plots (III).

Little is known about the effect of acid rain on microbial function and community structure of the humus layer fertilized with wood ash. As acidic deposition is still a threat to the terrestrial environment (Alewell et al. 2000) and this could affect the liberation of Cd from the ash when applied to the forest. In paper VI, watering the microcosms in the field with simulated acid rain (SAR pH 3, H_2SO_4 acid, total 3.64 g S m^{-2}) over the whole growing season decreased the soil pH but did not change the results achieved with water irrigation, that is, change in microbial, bacterial and fungal community structures due to wood ash application. Although there was probably a higher dissolution rate of the ash due to the SAR treatment, Cd in the wood ash was not liberated into bioavailable forms as measured with the biosensor *B. subtilis*. The higher dissolution of the ash induced a higher basal respiration rate.

Neither the Cd occurring naturally in wood ash nor that added to wood ash, had any effect on the measured microbial variables. In addition, irrigation with SAR and the level and form of added Cd had no effects. In conclusion, wood ash protected the microbes from the harmful effects of Cd.

4.4 Wood ash Cd transference into food chains

Cadmium may affect human health through carcinogenity, renal and bone effects, and diet is the main source of human exposure to Cd. There is a potential risk that Cd from wood ash enters into the human food chain directly through the eating of mushrooms and berries or indirectly through eating game animals. Ground water contaminated with Cd may also be a source.

The soil total and extractable Cd concentrations in the ACd plots increased, but the increases were not reflected in Cd concentrations in the soil water collected at 5 and 20 cm depth below the soil mineral layer surface or in the Cd concentration of berries and leaves of *Vaccinium uliginosum* and *Vaccinium vitis-idaea* or *Pinus sylvestris* needles (III). In the study by Bramryd and Fransman (1995), wood ash fertilization did not increase EDTA-extractable Cd in the humus or mineral soil layers 10 years after application. A similar result was obtained by Arvidsson and Ludkvist (2003) 6 years after ash application. Wood ash treatment was also shown to not increase Cd concentrations in soil water (Ring et al. 1999), runoff water (brook) (Tulonen et al. 2002), ground water (Piirainen 2001) nor in Norway spruce needles (Arvidsson and Lundkvist 2002).

Higher Cd concentrations in the mushroom *Lactarius rufus* and in the berries of *Empetrum nigrum* growing on ash spiked with Cd treated plots was found in paper III. The Cd concentrations in *L. rufus* correlated with soil total ($r = 0.90$) and extractable ($r = 0.84$) Cd concentrations. This indicated that Cd could enter the human food chain. Even though the ACd treatment added 28 times more Cd to the soil than the A treatment, the Cd concentration in *L. rufus* was roughly only doubled.

There are few studies concerning Cd concentrations in edible mushrooms and berries after wood ash fertilization. The results from paper III described above are in accordance with the few such studies where the Cd concentrations in mushroom *L. rufus* (Rühling 1996, Moilanen and Issakainen 2000, Lodenius et al. 2002) and in berries (Silfverberg and Issakainen 1991, Levula et al. 2000, Rühling 1996, Moilanen and Issakainen 2000, Nilsson 2001) did not increase after wood ash treatment. Moilanen and Issakainen (2000) found, however, that Cd concentrations in *L. rufus* growing on one upland site 19 years after wood ash fertilization increased and on one peatland site it decreased significantly. Lodenius et al. (2002) reported an increase in Cd concentrations in mushroom *Russula emetica*, which is mordant but edible like *L. rufus*, 2 years after the wood ash treatment. Moilanen and Issakainen (2000) reported no change in Cd concentrations in the popular edible mushroom *Russula paludosa* (14 months after ash treatment) and *Lactarius trivialis* (13-52 years after ash treatment) on peatland.

In conclusion, wood ash fertilization did not increase the Cd concentrations in mushroom, berries or in soil water.

4.5 Wood ash as a potential remediation agent for acidified and metal polluted forest soil

Remediation of polluted soils aims to remove or immobilize the pollutants, making them less mobile and available for plants and microbiota. Because wood ash seemed to render the Cd added in wood ash unavailable (II, III, VI), the study VII aimed to test if ash has the same effect on other heavy metals (Cu and Ni). To determine whether samples of humus layer that had been exposed to continuous acid and Cu-Ni deposition for nine growing seasons could be remediated by treatment with wood ash, a microcosm laboratory experiment was carried out. Microcosms that were only irrigated with water served as a control. The wood ash treatment removed all the acid (decrease in pH and base saturation and increase in extractable and bioavailable copper concentrations (Fig. 2), and change in microbial PLFA pattern; (Fig. 1 in VII)) and metal (increase in extractable nickel concentrations, and change in microbial PLFA and fungal DGGE patterns) treatment effects observed in the field. The detoxifying effect of ash on Cu may be due to several mechanisms and they are speculated about in article VII.

In conclusion, it may be possible to use wood ash to remediate acid and metal polluted upland forest sites. Its use should be tested on the polluted soils in the surroundings of industrial smelting plants.

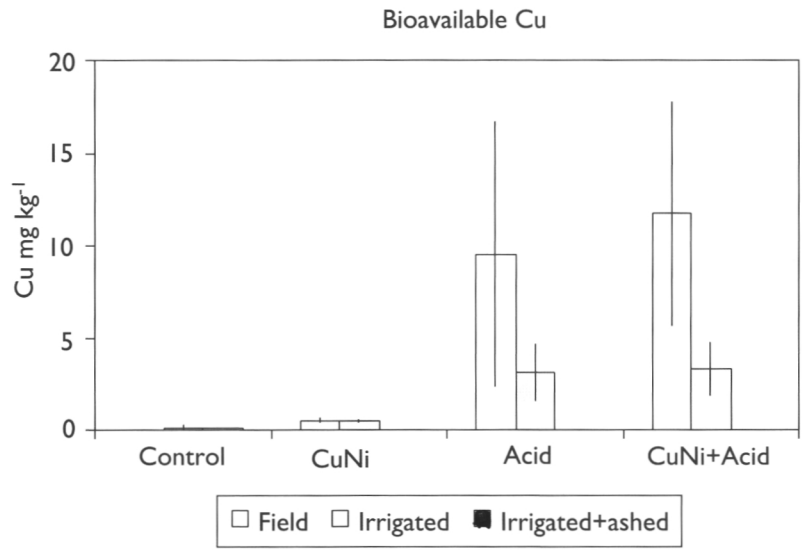

Fig. 2. The amount of bioavailable copper measured with the Cu-sensor *Pseudomonas fluorencens* of the study plots. Bars indicate standard error. See Table 3 for treatment symbols.

5 CONCLUSIONS

Spiking wood ash with Cd at levels that exceed the natural concentrations did not cause harm to forest soil microbes, although Cd addition without ash did. In addition, Cd added with wood ash was not in the bioavailable form, but part of the Cd added without ash was bioavailable to bacteria. Ash thus counteracted the toxic effect of Cd. It can therefore be concluded that the much lower Cd content of the wood ash itself would have no harmful effect on humus layer microflora. A potential risk for Cd to enter the human food chain is through uptake and concentration in mushrooms and berries which was only observed when Cd was added to the ash at unnaturally high levels. The effects of wood ash on soil microbes appeared to be long lasting, with increased needle litter decomposition still detectable 20 years after ash fertilization. The hardening of ash decreased its dissolution rate and therefore hardened ash effects on soil chemical and microbial variables were not as strong as loose ash. Increasing the rate of ash dissolution with simulated acid rain did not increase the bioavailability of Cd to bacteria in the humus layer. The benefits of wood ash fertilization (increase in soil pH and mineral nutrient contents) to microbes, plants and trees far outweigh any risk of the Cd in the ash having harmful effects on the humus microflora or entering the human food chain. However, since wood ash contains between 1 – 30 mg Cd kg^{-1} ash, it would be prudent not to apply ash more than once during a tree generation in the same site, as there remains the possibility that the Cd could be released after the effect of ash on pH levels off. In addition, wood ash can be used for remediation of acid and metal polluted humus due to its capacity to adsorb metals and increase soil pH.

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Paper I

I

Fritze H., Perkiömäki J., Saarela U., Katainen R., Tikka P., Yrjälä K., Karp M., Haimi J. and Romantschuk M. 2000. Effect of Cd-containing wood ash on the microflora of coniferous forest humus. FEMS Microbiology Ecology 32: 43-51.

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Effect of Cd-containing wood ash on the microflora of coniferous forest humus

Hannu Fritze [a,*], Jonna Perkiömäki [a], Ulla Saarela [b], Riikka Katainen [b], Päivi Tikka [a], Kim Yrjälä [b], Matti Karp [c], Jari Haimi [d], Martin Romantschuk [b]

[a] *Vantaa Research Center, Finnish Forest Research Institute, P.O. BOX 18, FIN-01301 Vantaa, Finland*

[b] *Department of Biosciences, Division of General Microbiology, Helsinki University, P.O. Box 56, FIN-00014 Helsinki, Finland*

[c] *Deparment of Biotechnology, University of Turku, Tykistökatu 5, FIN-20520 Turku, Finland*

[d] *Department of Biological and Environmental Science, University of Jyväskylä, P.O. Box 35, FIN-40351 Jyväskylä, Finland*

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Abstract

The use of wood ash in forestry has been questioned because the cadmium (Cd) concentration of ash, which varies between 1 and 20 mg kg^{-1} ash, exceeds the level allowed for fertilizers (3 mg kg^{-1}) used in agriculture. To investigate the combined and separated effects of Cd and ash on the forest humus microflora, pumice or wood ash, spiked with a water-soluble ($CdCl_2$) or -insoluble (CdO) form of Cd at three levels (0, 400 and 1000 mg kg^{-1}), were applied at a fertilization level of 5000 kg ha^{-1} in a laboratory microcosm study. The trial consisted of 60 microcosms (five replications per treatment), which were incubated in darkness at +20°C and a constant relative air humidity of 60%. After two months the humus in the microcosms was sampled. Analyses of CO_2 evolution to measure the overall microbial activity and of phospholipid fatty acid (PLFA) pattern to measure microbial community structure were performed. The substrate-use patterns of Biolog EcoPlates were analyzed as a measure of bacterial functionality. Finally the bacterial ^{3}H-thymidine incorporation in the presence of different concentrations of Cd and the number of colony forming units (cfu) of bacteria on nutrient agar in the presence of 0, 5 and 20 mg Cd l^{-1} agar were applied to measure Cd tolerance. The use of pumice (pH of humus under the pumice 4.0) did not induce any changes in the above variables compared to two untreated microcosms (humus pH 3.9). Pumice was therefore used to distribute the Cd evenly over the humus surface in order to estimate the possible effect of Cd without ash (pH of humus under the ash 7.0). The application of ash increased the microbial activity, changed the PLFA and substrate-use patterns and increased cfu compared to the humus under pumice. The form and level of Cd in the ash had no further effect on this result. In the humus under pumice the level, but not the form of Cd decreased the microbial activity and changed the PLFA pattern compared to the unspiked pumice. None of the treatments induced bacterial tolerance to Cd. Ash thus protected the humus microflora from the harmful effects of Cd.

Keywords: Cadmium; Coniferous forest humus; Heavy metal tolerance; Microbial community structure; Wood ash

1. Introduction

The Finnish wood industry annually produces approximately 150 000 t of wood ash that could be recycled in forest ecosystems in order to maintain the long-term base cation and plant mineral nutrient status of the soil. During combustion most of the inorganic nutrients and trace elements in wood are retained in the ash. Recently the use of wood ash with a cadmium (Cd) concentration that normally varies between 1 and 20 mg kg^{-1} ash has been questioned because the measured Cd concentrations in the ash in most cases exceed the level allowed for fertilizers (3 mg kg^{-1}) used in agriculture. This restriction on fertilizer use does not apply to forest soil and there is a lack of knowledge about the harmful effects of Cd in wood ash on forest ecosystems [1]. The present study focuses on humus microbes which, being responsible for the mineralization of organic material, play an important role in the functioning of the whole ecosystem.

We used a laboratory microcosm approach to investigate the combined and separate effects of Cd and ash on the soil microflora. Wood ash and pumice were spiked with a water-soluble ($CdCl_2$) and -insoluble (CdO) form of Cd at three levels (0, 400 and 1000 mg kg^{-1} ash) and

* Corresponding author. Fax: +358 (9) 8572575; E-mail: hannu.fritze@metla.fi

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Table 1
Characterization of the humus before the start of the two-month incubation experiment

Parameter	Result
d.m.	32%
C	52%
N	1.36%
pH	3.95
CO_2-evolution	84.9 μg g^{-1} h^{-1}
$PLFA_{bact}$	788 nmol g^{-1}
$PLFA_{fung}$	419 nmol g^{-1}

The dry matter (d.m.) percentage is calculated from the humus fresh weight. Carbon (C) and nitrogen (N) are given in percentage of d.m. All other results are presented as g d.m.$^{-1}$

used at a fertilization level of 5000 kg ha^{-1}. As the pH of pumice is close to that of humus, pumice eliminates the ash-induced increase in soil pH but at the same time ensures the even dispersion of Cd over the surface of the humus. After two months the microcosms were destructively sampled and the humus was analyzed for basal respiration activity, microbial community structure using phospholipid fatty acid analyses [2], the Cd tolerance of the bacterial community using both the ^{3}H-thymidine incorporation method [3] and the colony forming units (cfu) technique involving growing bacteria on nutrient agar in the presence of 0, 5 and 20 mg Cd l^{-1} agar, and the community-level, carbon source utilization pattern [4] using Biolog® Ecoplates.

2. Materials and methods

2.1. Humus collection and preparation

Approximately 150 dm^3 of frozen forest humus was collected in November 1998 from a Scots pine (*Pinus sylvestris* L.) stand growing on an *Empetrum–Vaccinium* (EVT) type site near the Finnish Forest Research Institute in Muhos, Central Finland (63°43′N 26°02′E). After thawing at room temperature the lichen and moss layer and dwarf shrub vegetation, which consisted mainly of *Empetrum nigrum*, *Vaccinium vitis-ideae* and *V. uliginosum*, was removed from the samples. The humus was then passed through a 2.8 mm sieve by hand and stored at 4°C. The physico-chemical and microbiological properties were determined two weeks after sieving (Table 1). For description of the methods see below. The humus was kept at 4°C for a total of one month before the experiment was started.

2.2. Treatments

Powdered wood ash and finely ground (< 0.2 mm) pumice (Riedel de Haën 31802, Bimsstein für Analyse) were spiked with CdO or $CdCl_2$ in order to give final Cd concentrations of 400 and 1000 mg kg^{-1}. Before spiking the $CdCl_2$ was ground in a mortar to produce a powder as fine as the CdO. The Cd was mixed into the ash and pumice by rotation over night before determining the Cd concentrations of the mixtures. The treatments, their abbreviations and the results of the Cd spiking are presented in Table 2.

For the laboratory incubation experiment, 60 pots were filled with 90 g of humus each and watered to 50% of their water holding capacity (WHC). The pot diameter at the humus surface was 12 cm. Randomly chosen sets of five pots ($n = 5$) were treated according to the treatments listed in Table 2. This resulted in 12 treatments, ten of which are presented in Table 2. Unspiked controls were prepared for each of the water-soluble and -insoluble forms of Cd in order to give a full statistical layout. The fertilization level of the treatments corresponded to a dose of 5000 kg ha^{-1}. The pumice and wood ash were spread evenly over the

Table 2
Cadmium concentration of the treatments

Treatment	Abbreviation[a]	Cd		
		Cover[b] layer	Humus[c]	
		mg kg^{-1}	mg kg^{-1}	extractable %
Pumice	P	BD	0.94 ± 0.08	BD
Pumice+400 mg kg^{-1} CdO	PLO	362 ± 7	68.9 ± 2.56	43.6 ± 1.33
Pumice+1000 mg kg^{-1} CdO	PHO	923 ± 15	171 ± 3.04	45.0 ± 0.56
Pumice+400 mg kg^{-1} $CdCl_2$	PLC	388 ± 15	70.8 ± 0.68	42.8 ± 0.53
Pumice+1000 mg kg^{-1} $CdCl_2$	PHC	977 ± 63	182 ± 6.04	45.1 ± 0.47
Ash	A	10.6 ± 0.11	2.04 ± 0.09	15.4 ± 1.50
Ash+400 mg kg^{-1} CdO	ALO	380 ± 1.8	51.8 ± 3.64	18.4 ± 1.14
Ash+1000 mg kg^{-1} CdO	AHO	980 ± 21	127 ± 10.6	20.3 ± 0.66
Ash+400 mg kg^{-1} $CdCl_2$	ALC	344 ± 8.5	45.4 ± 2.78	16.0 ± 1.05
Ash+1000 mg kg^{-1} $CdCl_2$	AHC	914 ± 32	104 ± 11.3	18.1 ± 1.31

BD = below the detection limit ($n = 5$, except P and A where $n = 10$)
[a] In the abbreviations P represents pumice, A ash, L low, H high, O the water-insoluble CdO and C the water-soluble $CdCl_2$.
[b] Pumice or ash.
[c] At the end of the experiment.

surface of the humus using a spoon. The 60 pots were incubated in darkness at +20°C and a constant relative humidity of 60%. In addition to the 60 pots, two untreated (no cover layer) microcosms were prepared. The pots were watered at 2- to 3-day intervals in order to keep the humus water content at 50% WHC. If water passed through the microcosm it was poured back onto the humus. After two months the pots were destructively sampled.

2.3. Chemical analyses

Total organic carbon and nitrogen content were determined by dry combustion (Leco CHN-600). The total Cd concentrations were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES, ARL 3580) after dry digestion (550°C) and extraction with concentrated HCl. Cd extractable at pH 7 was determined using buffered 1 M ammonium acetate at a matrix:extractant ratio of 1:10. The dry weight was determined by drying duplicate subsamples at 105°C overnight and the organic matter content was obtained as the loss in weight on ignition (550°C for 4 h). The humus (and pumice) pH was measured in a water suspension (1:15; w/v).

2.4. Microbial analyses

The basal respiration rate was measured as the amount of CO_2 evolved in 25 h as described by Pietikäinen and Fritze [5]. Fresh humus samples, equaling 2 g dry weight, were used in the analyses.

The phospholipid extraction and analysis of phospholipid fatty acids (PLFAs) were carried out as described by Frostegård et al. [2]. Briefly, 0.5 g of fresh humus was extracted with a 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 detected by gas-chromatography (flame ionization detector) using a 50-m HP-5 capillary column.

Fatty acids are designated in terms of the total number of carbon atoms:number of double bonds, followed by the position of the double bond from the methyl end of the molecule. The prefixes, br, a, i, cy and Me indicate different kinds of branching of the carbon chain; for details see Frostegård et al. [6]. The prefix C (e.g. C16:0) indicates that the PLFA has 16 carbon atoms and no double bonds, but the arrangement of the atoms (branching) is not confirmed. The total amount of PLFAs was used to indicate the total microbial biomass and 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) were chosen as an index of the bacterial biomass ($PLFA_{bact}$) [7]. The amount of 18:2ω6 was used as an indicator of fungal biomass ($PLFA_{fung}$), since 18:2ω6 is suggested to be mainly of fungal origin in soil [8] and it is known to correlate with the amount of ergosterol [7].

For the thymidine incorporation and Biolog measurements, fresh soil (equaling 1 g dry weight) was homogenized by shaking (200 rpm) with 100 ml distilled water for 1.5 h at +4°C. The suspension was centrifuged for 10 min at $750 \times g$ and passed through glass wool (Pyrex fiber glass, silver 8 μm). Subsamples of this supernatant were used for the growth rate determinations.

Bacterial growth rates were estimated by the thymidine incorporation technique as described by Bååth [3]. Aliquots (2 ml) of the supernatant were incubated for 2 h with 100 nM [methyl-^{3}H]thymidine (925 GBq mmol^{-1}, Amersham) at 22°C. Growth was stopped by adding 1 ml of 5% formalin. ^{3}H-Thymidine containing cells were then precipitated as cold-acid-insoluble material with 80% ethanol and 5% TCA and harvested using glass-fiber filters. The macromolecules were solubilized with NaOH and the radioactivity was measured with a Wallac 1411 scintillation spectrometer using the fine-tuned external standard method.

Biolog® EcoPlates (Biolog Inc., Hayward, CA, USA) containing 31 different carbon sources and a redox dye [9] were inoculated with 150 μl volume of 10^{-3} diluted humus suspension. The EcoPlates were incubated at 20°C and the absorbances were read every day with a Labsystem Multiscan plate reader using a 590-nm filter until the 120 h was reached.

The Cd tolerance of the bacteria was determined by adding different concentrations of $CdCl_2$, ranging from 10^{-7} to 10^{-3} M (final concentrations), to the humus solution before ^{3}H-thymidine was added [3]. The heavy metal tolerance of the community was expressed as a percentage of the growth in samples without metal addition. The Cd tolerance of the bacterial communities was then estimated by calculating the concentration of added metal resulting in a 30 and 50% decrease in incorporation into the bacterial suspension compared with the growth without metal addition (IC_{30} and IC_{50}).

For the enumeration of bacteria on agar plates the bacteria were extracted by mixing (30 min, 250 rpm horizontally, room temperature) 2.5 g of fresh humus with 22.75 ml of 0.9% NaCl, 0.11% $Na_5P_3O_{10}$ and 0.02% Tween 80 [10] in a 100-ml infusion bottle. Following serial dilution, 100-μl aliquots were spread on nutrient agar (1 l of deionized water: 0.075 g beef extract, 0.125 peptone, 0.05 g cycloheximide and 15 g agar) plates. Colony forming units (cfu) were counted after 12 days of incubation in darkness at room temperature. The bacterial Cd tolerance was also estimated in parallel on nutrient agar plates to which 5 or 20 mg Cd l^{-1} (as $CdCl_2 \times 2.5\ H_2O$) was added.

2.5. Data analysis

The results were presented per dry matter (d.m.) of humus. The individual PLFAs were expressed as mole per-

Table 3
Analysis of variance for soil respiration

Source	DF	Mean square	F
Ash (a)	1	8782	658[a]
Form (b)	1	12.4	0.93
Level (c)	2	63.0	4.73[b]
a×b	1	0.82	0.06
a×c	2	187	14.05[a]
b×c	2	2.08	0.16
a×b×c	2	15.4	1.16
Residual	48	13.3	

[a] $P < 0.001$
[b] $P < 0.05$

centage (mol%) of the total PLFA content of the samples. For the Biolog data, individual substrates were corrected for background absorbance by subtracting the absorbance of the control (water) well. The blank-corrected absorbance values of the subsequent readings were used to calculate the total area under the absorbance curve. The calculated values (area under the curve) were summed for each sample and the proportions of individual substrates out of the total area were expressed as area%. The mol% and area% values from the PLFA and Biolog data, respectively, were standardized by dividing by the standard deviation (correlation matrix) before being subjected to principal component analysis (PCA). All the results, including the scores of the multivariate analysis (PLFA and Biolog measurements), were subjected to analysis of variance (ANOVA). Log transformed IC_{30} and IC_{50} values were used. Three main effects and their interactions were tested. The main effects were ash contra pumice, soluble contra insoluble form of Cd and the three Cd levels. A full statistical layout for the soil respiration rate is presented in Table 3. All biological variables measured were subjected to this ANOVA test. In the case of the PLFA and Biolog data, the ashed sample scores were also separated from the pumiced sample scores before ANOVA. Pearson correlation tests were also performed between the PCA scores and the Cd concentration of the humus. Interactions are discussed only when they are statistically significant. Standard error of means are reported.

3. Results and discussion

3.1. The effect of pumice

Ground pumice stone was used to ensure even dispersion of the small amounts of Cd over the surface of the humus. We had two untreated microcosms in our experimental set-up to determine the effect of pumice on the humus microbiota. We have therefore used the results of the pumice (P) microcosms ($n = 10$) for comparison purposes (see Table 4 for respective treatment means; $n = 5$). At the start of the experiment the humus pH was 3.9. After two months of incubation the pH of the two untreated microcosms was 3.9 and that of the pumice-treated microcosms 4.0 (±0.0). The pH of the ground pumice/water suspension was 5.4. Application of the pumice had therefore changed the humus pH by only 0.1 unit. The mean respiration activity of the untreated microcosms was 43.1 μg CO_2 g^{-1} h^{-1} and the respective value for the P microcosms was 40.3 (±1.2) μg g^{-1} h^{-1} (see Table 4 for treatment respective means; $n = 5$). Furthermore, as the microbial community structure of the untreated humus resembled that under the pumice (Fig. 1a) and the utilizations of the C sources of the Biolog plates were similar (Fig. 2), we conclude that the use of pumice had no effect on the microbiota of the humus and can be regarded as a true control in this experiment.

3.2. The effect of ash

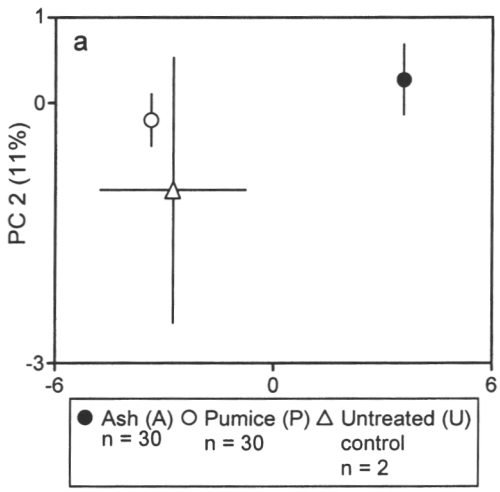
The application of ash ($n = 30$) increased the humus pH

Table 4
Treatment respective mean and S.E. of pH and the biological measurements

Treatment	pH	CO_2 (μg g^{-1} h^{-1})	TdR incorporation mol×10^{-10} g^{-1} h^{-1}	$PLFA_{bact}$ (nmol g^{-1})	$PLFA_{fung}$ (nmol g^{-1})	CFU×10^7 ml^{-1}	% CFU growing on 5 mg Cd l^{-1} agar	% CFU growing on 20 mg Cd l^{-1} agar	$IC_{30}\times10^{-6}$ (Cd; M)	$IC_{50}\times10^{-6}$ (Cd; M)
P	4.1[a] ± 0.03	38.8[a] ± 1.96	0.23[a] ± 0.018	790[a] ± 22	260[a,b] ± 11	0.86[a] ± 0.17	2.43[a] ± 1.93	0.02	221[a] ± 26	ND
PLO	4.1[a] ± 0.02	38.5[a] ± 0.82	0.19[a] ± 0.016	834[a] ± 22	240[a,b] ± 16	3.00[a] ± 1.48	0.16[a] ± 0.03	0.23 ± 0.22	253[a] ± 12	ND
PHO	4.1[a] ± 0.02	32.5[b] ± 1.73	0.10[a] ± 0.030	919[a] ± 121	253[a,b] ± 31	2.47[a] ± 1.14	0.70[a] ± 0.35	ND	215[a] ± 47	ND
P	4.0[a] ± 0.02	41.8[a] ± 1.40	0.25[a] ± 0.013	910[a] ± 57	295[a] ± 27	2.68[a] ± 1.08	0.33[a] ± 0.19	0.56 ± 0.55	268[a] ± 81	ND
PLC	4.0[a] ± 0.01	37.9[a] ± 2.19	0.50[a] ± 0.195	894[a] ± 44	249[a,b] ± 16	1.66[a] ± 0.74	1.44[a] ± 1.34	0.04	114[a] ± 30	ND
PHC	4.0[a] ± 0.02	31.8[b] ± 0.87	0.23[a] ± 0.020	836[a] ± 56	221[a,b] ± 11	2.72[a] ± 0.98	0.27[a] ± 0.12	0.02	181[a] ± 28	ND
A	7.1[b] ± 0.04	64.5[c] ± 3.09	1.43[b] ± 0.051	715[b] ± 23	215[a,b] ± 11	153[b] ± 75	51.1[b] ± 18.2	0.50 ± 0.15	2.26[b] ± 0.22	4.80[b] ± 0.55
ALO	7.0[b] ± 0.07	63.3[c] ± 1.59	1.33[b] ± 0.111	712[b] ± 48	228[a,b] ± 17	172[b] ± 84	68.0[b] ± 31.8	0.48 ± 0.10	2.25[b] ± 0.22	4.68[b] ± 0.48
AHO	7.1[b] ± 0.04	61.8[c] ± 3.38	1.12[b] ± 0.159	730[b] ± 34	212[a,b] ± 16	218[b] ± 97	80.3[b] ± 33.4	0.70 ± 0.24	2.70[b] ± 0.29	6.42[b] ± 1.17
A	7.0[b] ± 0.04	64.1[c] ± 3.94	1.77[b] ± 0.237	703[b] ± 48	204[b] ± 24	328[b] ± 125	27.6[b] ± 9.40	0.85 ± 0.55	2.37[b] ± 0.14	4.81[b] ± 0.30
ALC	7.0[b] ± 0.03	66.4[c] ± 1.75	1.19[b] ± 0.066	624[b] ± 22	185[b] ± 4.91	214[b] ± 86	38.9[b] ± 8.73	0.46 ± 0.10	2.13[b] ± 0.11	4.49[b] ± 0.19
AHC	7.0[b] ± 0.03	68.1[c] ± 3.14	1.53[b] ± 0.095	687[b] ± 26	206[b] ± 17	144[b] ± 64	58.1[b] ± 16.3	0.49 ± 0.20	2.33[b] ± 0.31	5.49[b] ± 0.91

Values indexed with the same letter are not significantly different from each other. ND = not detected

by 3 units from 4.0 (±0.0; all pumice; $n=30$) to 7.0 (±0.0). The pH effect of the ash increased the microbial activity of the humus as measured by the soil respiration and thymidine incorporation rate from 36.9 (±0.89) to 64.7 (±1.16) μg CO_2 g^{-1} h^{-1} and from 0.27×10^{-10} ($\pm0.03\times10^{-10}$) to 1.40×10^{-10} ($\pm0.06\times10^{-10}$) mol thymidine g^{-1} h^{-1}, respectively (see Table 4 for respective treatment means; $n=5$). In accordance with the activity measurements, the number of cultivable bacteria also increased from 2.23×10^{7} ($\pm4.02\times10^{6}$) to 2.05×10^{9} ($\pm3.56\times10^{8}$) cfu due to the ash application. Similar increases in activity and cfu values due to ash fertilization at a level of 5000 kg ha^{-1} have been reported for coniferous forest humus by Bååth and Arnebrant [11]. According to the PLFA analyses, the increased microbial activity of the ashed humus samples was accompanied by a change in the microbial community structure (Fig. 1a). The ashed samples had positive and the pumiced samples negative loadings on PC 1. The PLFAs responsible for the change in the community structure are shown in Fig. 1b. The change in the PLFA pattern was accompanied by a change in substrate utilization (Fig. 2).



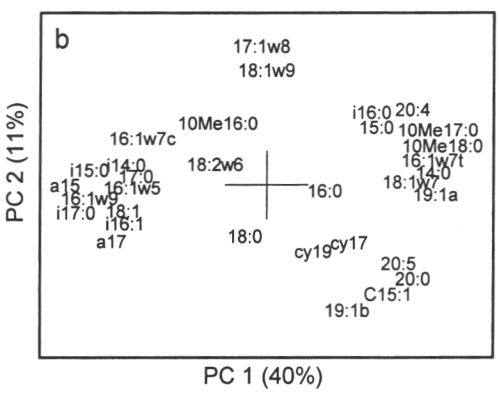


Fig. 1. Principal component analysis (PCA) using the mol% of the phospholipid fatty acids (PLFAs) from all the 62 microcosms. In the score plot (a) the mean (±S.E.) of all the ashed (A; $n=30$), the pumiced (P; $n=30$) and the untreated (U; $n=2$) samples for the first two principal components are shown. The loading plot (b) identifies the PLFAs most responsible for the separation.

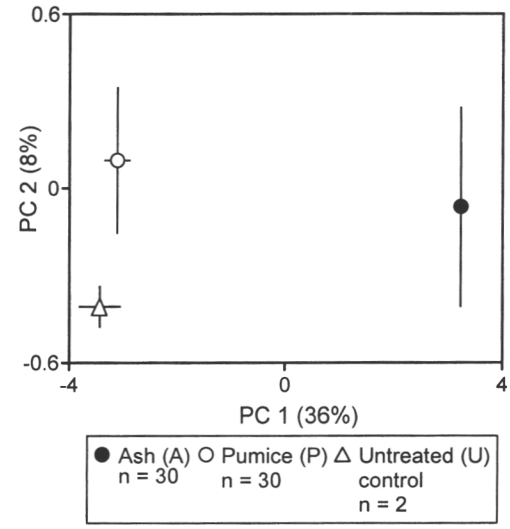


Fig. 2. Score plot from PCA performed on the area% of the C sources from the Biolog EcoPlates for all 62 microcosms. See Fig. 1 for further information.

A change in the PLFA pattern due to treatments that increase the pH of coniferous forest humus has earlier been demonstrated in field studies carried out by Bååth et al. [12] in an area subjected to alkaline pollution, forest liming [2] and wood ash fertilization [13]. In the field study using wood ash at a level of 5000 kg ha^{-1} the bacterial and fungal biomass levels were lower than those on the untreated control plots [13]. This was also observed in our microcosm experiment, the ash application decreasing the mean bacterial biomass ($PLFA_{bact}$) from 864 (±25) to 695 (±14) nmol g^{-1} and the mean fungal biomass ($PLFA_{fung}$) from 253 (±8.5) to 208 (±6.4) nmol g^{-1} (see Table 4 for respective treatment means; $n=5$).

Smalla et al. [14] showed, using molecular techniques, that the structure of the microbial communities in various Biolog wells were not identical despite having received the same inoculum. It is apparent that different subsets of populations from the inoculated microbial community are favored by the different C sources. The bacterial community in the ashed humus samples were able to use other C sources than the bacteria from the pumiced samples (Fig. 2). The ashed samples were characterized by extensive utilization of the following C sources: phenylethylamine, itacinic acid, g-hydroxybutyric acid, a-D-lactose and D-xylose. The pumiced samples extensively utilized Tween 40, L-threonine, pyruvic acid methyl ester, L-phenylalanine and *N*-acetyl-D-glucosamine. The effect of ash application onto the surface of the humus was therefore

Table 5
Treatment-induced changes in the humus PLFAs[a]

PLFA	mol% of PLFAs in P humus	Ratio to P treatment								
		PLO	PHO	PLC	PHC	A	ALO	AHO	ALC	AHC
i14:0	0.58 ± 0.01	0.90	0.97	1.02	0.94	0.79	0.76	0.80	0.78	0.78
14:0	1.98 ± 0.05	0.99	0.99	1.08	1.03	1.50	1.61	1.68	1.67	1.48
i15:0	9.52 ± 0.12	1.03	1.07	1.09	1.08	0.87	0.81	0.85	0.82	0.83
a15:0	3.88 ± 0.04	1.00	1.05	1.03	1.03	0.69	0.64	0.70	0.68	0.70
C15:1	0.66 ± 0.09	1.21	1.16	1.18	1.50	1.42	1.82	1.64	2.02	1.91
15:0	1.24 ± 0.02	1.00	1.03	1.10	1.08	1.13	1.12	1.16	1.14	1.14
i16:1	0.29 ± 0.03	1.02	1.06	0.91	0.81	0.30	0.38	0.35	0.28	0.14
i16:0	2.50 ± 0.04	1.06	1.11	1.10	1.14	1.30	1.17	1.16	1.20	1.21
16:1ω9	0.95 ± 0.01	1.01	1.03	0.97	0.94	0.64	0.58	0.73	0.63	0.66
16:1ω7c	6.72 ± 0.06	1.03	1.02	1.01	1.00	0.99	0.93	0.96	0.94	0.98
16:1ω7t	0.92 ± 0.03	1.04	1.01	1.08	1.21	1.24	1.21	1.25	1.29	1.31
16:1ω5	4.23 ± 0.05	1.04	1.06	0.94	0.83	0.82	0.90	0.94	0.82	0.80
16:0	16.4 ± 0.09	1.01	0.99	1.00	1.02	1.01	1.01	1.04	1.02	1.01
10Me16:0	4.19 ± 0.08	0.93	1.06	1.06	1.05	1.07	0.97	0.99	0.97	1.01
i17:0	1.03 ± 0.01	1.04	1.11	1.02	0.98	0.83	0.82	0.83	0.78	0.80
a17:0	1.38 ± 0.02	1.03	1.01	1.00	0.97	0.87	0.91	0.86	0.88	0.86
17:1ω8	0.91 ± 0.02	0.95	0.94	0.98	1.00	0.98	1.01	0.98	0.95	0.96
cy17:0	1.80 ± 0.03	1.07	1.07	1.06	1.07	1.05	1.09	1.13	1.10	1.10
17:0	0.81 ± 0.01	1.02	1.02	1.02	1.02	0.93	0.95	0.93	0.95	0.92
10Me17:0	0.34 ± 0.005	1.03	1.06	1.06	1.11	1.73	1.59	1.46	1.59	1.59
18:2ω6	10.4 ± 0.2	0.91	0.89	0.90	0.86	0.88	0.94	0.86	0.88	0.90
18:1ω9	11.8 ± 0.2	0.98	0.94	0.96	1.00	0.99	0.99	1.00	0.95	0.98
18:1ω7	4.27 ± 0.05	0.99	0.97	0.97	0.94	1.21	1.31	1.26	1.28	1.31
18:1	0.73 ± 0.01	0.98	0.97	0.92	0.63	0.61	0.51	0.34	0.76	0.56
18:0	2.86 ± 0.1	0.99	0.98	0.91	0.89	0.94	0.94	0.89	1.01	0.94
19:1a	0.52 ± 0.009	1.07	1.06	1.03	1.01	1.37	1.64	1.68	1.50	1.48
10Me18:0	0.57 ± 0.01	0.96	1.02	1.06	1.16	1.30	1.35	1.36	1.32	1.35
19:1b	1.14 ± 0.09	1.18	1.14	1.12	1.30	1.19	1.28	1.13	1.48	1.39
cy19:0	3.92 ± 0.07	1.06	1.02	1.03	1.08	1.04	1.04	1.03	1.03	1.08
20:5	0.56 ± 0.03	1.12	0.96	1.07	1.12	1.30	1.25	1.24	1.48	1.32
20:4	1.38 ± 0.05	0.87	0.89	0.84	0.79	1.55	1.44	1.38	1.33	1.38
20:0	1.44 ± 0.09	1.07	0.98	0.96	1.09	1.28	1.26	1.12	1.47	1.30

[a]In the humus samples of the P treatment the proportions of the PLFAs, expressed as means ± S.E. ($n = 10$), are given. To show the effect of treatments, the ratios of the means of individual PLFAs found in the treated humus samples to the means ($n = 5$, except A where $n = 10$) of the P treatment are presented.

great enough to change the microbial community structure (PLFA) to such an extent that different subsets of the bacterial community became enriched in the Biolog plate being capable of utilizing different C-sources than the subsets of the untreated or pumiced humus samples (Figs. 1a and 2).

In our microcosm experiment, the microbial activity, the cultureable bacterial population, the community structure and the microbial biomass results reflected the trends that have been observed in field trials using wood ash in coniferous stands. Therefore our two-month microcosm incubation was successful with respect to the ash fertilization and can be used to identify the effects of Cd.

3.3. The effect of cadmium

The different forms of Cd had no effect on the soil respiration rate. In contrast, the Cd level influenced the respiration rate, and there was significant interaction between the ash treatment and the Cd level (Table 3). This is due to the fact that Cd decreased the soil respiration in the pumiced samples but not in the ashed ones (Table 4). The ash thus counteracted the toxic effect of Cd. We have earlier shown that the soil respiration rate decreases when Cd is applied at high doses to wood ash fertilized humus [15]. Humus samples from a field experiment given 5000 kg ha^{-1} wood ash required more than 4000 mg Cd kg^{-1} humus to decrease the respiration rate by 50%, whereas the unfertilized control humus required 1570 mg Cd kg^{-1} to reach the same degree of inhibition [15]. This showed that wood ash can to some extent reduce the toxicity of Cd. Fritze et al. [15] applied completely unrealistic amounts of Cd to give Cd levels of up to 4000 mg kg^{-1} dry humus. The experiment performed in this study is in this respect realistic because the Cd was first mixed with the wood ash (and pumice) to give a theoretical maximum level of 1000 mg kg^{-1} ash (see Table 2) before spreading it onto the surface of the humus to imitate a fertilization load of 5000 kg ha^{-1}. Converting this to the amount of Cd per humus dry weight used in this experiment, only

196 mg Cd kg^{-1} humus entered the soil system. This was enough to reduce the soil respiration rate by 20% in the absence of ash counteracting the toxicity of Cd (Table 4). In the presence of ash not even the highest Cd addition had any effect. The respiration rate correlated well with the thymidine incorporation ($r = 0.89$), but the form and level of Cd had no detectable effect with the method used for determining the growth rate in this study (Table 4).

The addition of Cd onto the surface of the humus changed the microbial community structure as shown by the PLFA pattern. In general, the Cd-induced changes in the mol% of the individual PLFAs within the pumiced ($n = 30$) or ashed ($n = 30$) samples were small compared to the differences between these two groups (for the effect of ash; see Fig. 1). This is demonstrated by calculating the ratios of the means of the individual PLFAs in the treated humus samples to the respective means of the P treatment (Table 5). Within the P samples ($n = 30$) there were 15 PLFAs out of 32 whose ratio changed by more than 0.1 units due to the PLO, PHO, PLC or PHC treatments (Table 5). The highest observed change was 0.5 units due to the PHC treatment (see PLFA C15:1; Table 5). Even this change in the ratio is small compared to the change reported for field studies in heavy metal polluted sites [16]. Eight of these fifteen PLFAs (15:0, i16:1, 16:1ω7t, 16:1ω5, 10Me17:0, 18:1, 18:0 and 10Me18:0) reacted to the soluble form of Cd. C15:1, i16:0, 18:2ω6, 19:1b, 20:4 and 20:5 reacted to both forms of Cd, whereas i17:0 reacted only to the PHO treatment.

Principal component analysis (PCA) performed on the pumiced samples showed that the effect of increasing Cd addition explained 24% of the data variation on the first PC axis and the water-soluble form of Cd was more toxic than the insoluble one (Fig. 3a). ANOVA on the scores of PC 1 revealed that the PHC treatment differed significantly from the other treatments, including the control and the scores correlated significantly ($r = 0.58$) with the total amount of Cd present in the humus. The PLFAs responsible for the separation in the PCA are shown in Fig. 3b. The relative mol% of the methylated PLFAs increased with increasing Cd concentration. These PLFAs are located on the right of the center in the plot (Fig. 3b). Tuberculostearic acid (10Me18:0) occurs almost exclusively in actinomycetes [17–18]. 10Me16:0 and 10Me17:0 are also produced by a number of actinomycete genera [19]. Thus, the result suggests that Cd pollution caused a relative increase in the number of actinomycetes. In contrast, the relative mol% of PLFAs 18:2ω6 and 20:4, which are of fungal origin [7,20], decreased as a result of Cd addition (Fig. 3b). The changes observed in the PLFA pattern of the pumiced samples due to Cd addition are consistent with the results of Frostegård et al. [6]. At the beginning of a 6-month incubation they added solutions of $CdSO_4$ to humus samples to give comparable Cd concentrations (56, 112 and 224 mg kg^{-1} dry humus) to those used in our study (theoretically 74.8 and 196 mg kg^{-1} dry humus, see measured values in Table 2). This provides additional evidence that the use of pumice in this experiment had no effect on the result.

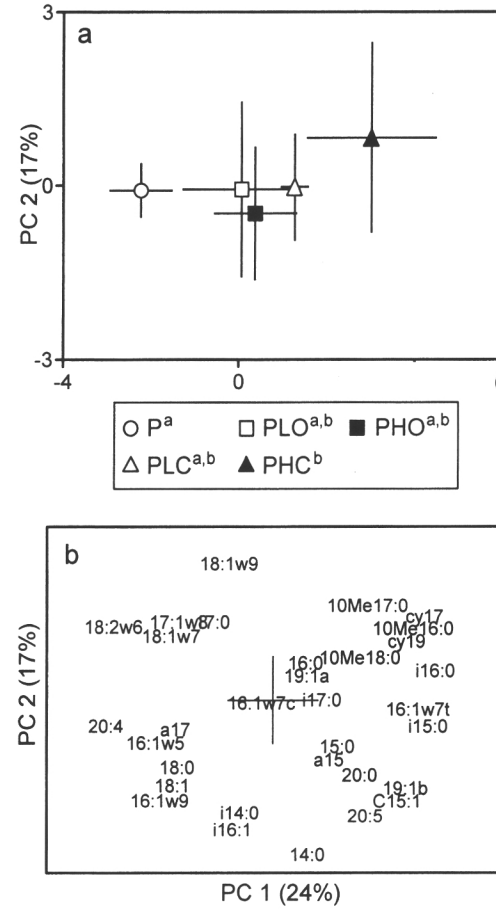


Fig. 3. Score (a) and loading (b) plots from PCA performed on the PLFAs of the pumiced samples. See Table 2 for the abbreviations.

In the ashed samples ($n = 30$) there were thirteen PLFAs whose ratio changed by more than 0.1 units due to the Cd addition compared to the ratio of the A treatment (Table 5). PLFA i16:1 reacted to the soluble form of Cd and 18:1 to the insoluble form. The PLFAs C15:1, i16:0, 10Me16:0, 10Me17:0, 18:1ω7, 19:1a, 19:1b and 20:4 reacted to both forms of Cd. PLFAs 14:0, 20:5 and 20:0 reacted to the ALC treatment.

The Cd-treated ash samples also separated from the untreated ash along the first PC and explained 19% of the variation (Fig. 4a). Actinomycetes, which benefited from the addition of ash or Cd alone, suffered from the combined effect of ash and Cd. This is because the relative mol% of their representative PLFAs, 10Me16:0 and 10Me17:0, are to be found on the left side of the origon (Fig. 4b), thereby characterizing the A, ALO and ALC treatments. The third methylated PLFA, 10Me18:0, did

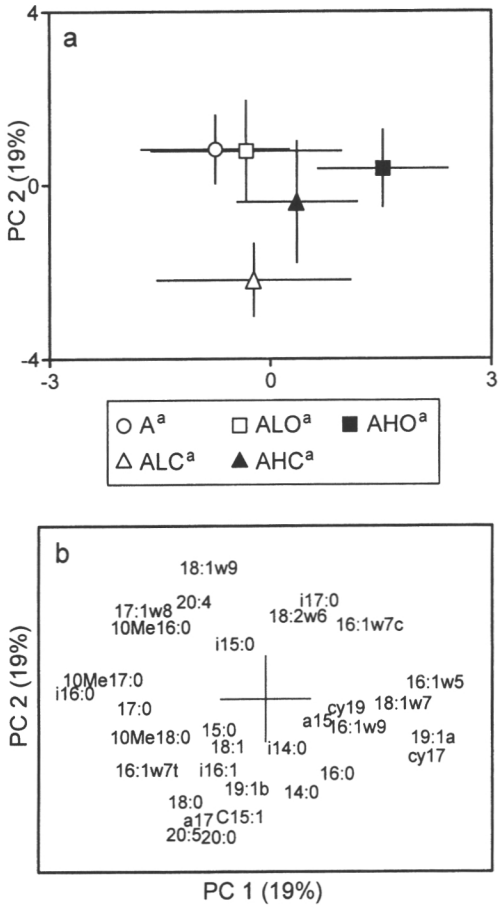


Fig. 4. Score (a) and loading (b) plots from PCA performed on the PLFAs of the ashed samples. See Table 2 for the abbreviations.

not react markedly to the Cd treatments in the ashed humus (Table 5). However, since the differences among the treatments were not statistically significant and the PC scores did not significantly correlate ($r = 0.25$) with the measured Cd concentration in the humus, it can be concluded that the presence of ash counteracted the toxic action of Cd.

The level and form of Cd had no significant effect on the cfu counts, the bacterial ($PLFA_{bact}$) or fungal ($PLFA_{fung}$) biomass in the pumiced or ashed humus samples (Table 4) or on the pattern of substrate utilization (Biolog data not shown). Thus, the change in the microbial community structure (PLFA) of the pumiced samples resulting from the level and form of Cd was not sufficiently large to lead to the enrichment of certain bacterial subsets in the wells of the Biolog plates.

The soil bacterial growth rate was studied using radioactive thymidine incorporation into macromolecules of the bacteria. The same method has been used to determine the heavy metal tolerance of the soil bacterial population [3]. For this assay the bacterial suspension obtained from soil homogenization–centrifugation is incubated with water (control) and a range of heavy metal concentrations giving a range of inhibitions from none to complete inhibition of bacterial growth. The growth is estimated by measuring ^{3}H-thymidine incorporation into the bacteria. Setting the control sample to represent 100% growth, the IC_{50} value, that is the metal concentration resulting in 50% inhibition, is calculated. A higher IC_{50} (and IC_{30} as in our case) concentration indicates a more tolerant bacterial community. The form and level of Cd had no effect on the tolerance of the bacterial community to Cd (Table 4) and there was no interaction between ash addition and the Cd level. Díaz-Raviña et al. [21] tested the development of community tolerance to Cd (added as $CdSO_4$) in agricultural soil during a 5- to 8-month incubation period. The addition of 896 mg Cd kg^{-1} dry soil resulted in the development of a Cd-tolerant community, whereas the addition of 448 mg Cd kg^{-1} dry soil gave minor differences compared to the unpolluted control. This means that the addition of Cd via wood ash or pumice to the humus, even at the highest doses (theoretical humus Cd concentration 196 mg kg^{-1}), did not exceed the threshold level needed to induce tolerance of the bacterial community to Cd. The same result was obtained with a more conventional method. The cfu was also counted on nutrient media supplemented with 5 or 20 mg Cd l^{-1}. The addition of Cd to the media reduced the cfu (Table 4), but the reduction with 20 mg Cd l^{-1} of agar was of similar magnitude from both the pumiced and the ashed samples. Thus there was no elevation of community Cd tolerance, as measured by cfu, at the Cd concentrations used.

The results given by the thymidine incorporation and the plate count method for the ashed and pumiced samples showed significant differences (Table 4). All the ashed samples were more sensitive to external Cd than the pumiced samples when measured by the thymidine incorporation method. On average only 0.0023 ($\pm 9.1 \times 10^{-5}$) mM Cd ($= 0.26$ mg l^{-1} Cd) was needed to decrease the thymidine incorporation rate by 30%, whereas the pumiced samples required 0.21 ($\pm 2.6 \times 10^{-2}$) mM Cd ($= 23.6$ mg l^{-1} Cd). This result indicated that the bacterial community of the ashed humus, which was isolated into the water solution before the addition of Cd in the tolerance test, was more sensitive to external Cd than the respective bacterial community of the pumiced controls. The bacteria that could be cultivated on nutrient media reacted in an opposite fashion. The addition of 5 mg Cd to 1 l of nutrient agar decreased the cfu of the pumiced samples by nearly 100%, whereas the cfu of the ashed samples decreased by between 20 to 70% (Table 4). The traditional plate count method supports the hypothesis that ash can protect the bacteria from Cd toxicity, but the discrepancy between the results given by the two methods cannot be explained.

In conclusion, spiking wood ash with Cd at levels that exceed the natural concentrations by 100-fold, did not

result in a change in the biological variables, e.g. microbial activity, colony forming units, microbial community structure, appearance of bacterial Cd resistance, as measured on the humus after two months laboratory incubation, compared to the unspiked ash treatments. When the same amounts of Cd were applied without ash there was a decrease in soil respiration and a change in community structure. Therefore we conclude from our laboratory experiment that the naturally occurring Cd content of wood ash has no harmful effect on the humus microflora. A field experiment including ALO treatment will be conducted to verify these results.

Acknowledgements

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Paper II

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II

Research Articles

A Microcosmos Study on the Effects of Cd-containing Wood Ash on the Coniferous Humus Fungal Community and the Cd Bioavailability

Hannu Fritze[1], Jonna Perkiömäki[1], Tiina Petänen[2], Taina Pennanen[1], Martin Romantschuk[2], Matti Karp[3] and Kim Yrjälä[2]

[1] Finnish Forest Research Institute, P.O. Box 18, FIN-01301 Vantaa, Finland
[2] Department of Biosciences, Division of General Microbiology, P.O. Box 56 (Biocenter 1 C), FIN-00014 University of Helsinki, Finland
[3] Department of Biotechnology, University of Turku, Tykistökatu 5, FIN-20520 Turku, Finland

Corresponding author: Hannu Fritze; e-mail: Hannu.Fritze@metla.fi

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Abstract. Background and Aims. The use of wood ash in forestry has been questioned because the cadmium (Cd) concentration of ash, which varies between 1 and 20 mg kg^{-1} ash, exceeds the level allowed for fertilizers (3 mg kg^{-1}) used in agriculture. To investigate the effects of Cd and ash on the fungal community composition and Cd bioavailability of the humus layer of boreal, coniferous forests, pumice or wood ash, spiked with a water soluble ($CdCl_2$) or insoluble (CdO) form of Cd at three levels (0, 400 and 1000 mg kg^{-1}), were applied at a fertilization level of 5000 kg ha^{-1} in a laboratory microcosm study.

Methods. After 2 months, the humus in the microcosms was sampled and extracted for total DNA to detect changes in the fungal community by using polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) techniques. PCR was performed using the fungal 18S rDNA primers FR1 + FF390 and FR1 + NS1. The bioavailability of Cd was measured with a bacterial biosensor (*Bacillus subtilis* BR 151/pT0024) emitting light in the presence of Cd.

Results. Using the primer pairs FR1 + FF390 and FR1 + NS1, resulted in over 35 and 15 DGGE bands, respectively. Both primer pairs detected an ash, but no Cd effect. When using FR1 + FF390, a higher fluorescence was observed in one DGGE band of all ashed samples compared to the pumiced samples. With the primer pair FR1 + NS1, the ashed samples had a DGGE band which was not visible or only faintly visible in the pumiced samples. In addition, one DGGE band disappeared from the ashed samples. Humus layer water extracts showed that the Cd added with the pumice was in a bioavailable form. The luminescence intensity of the biosensor was dependent on the form and level of the Cd added. No luminescence was detected when the Cd was added with the ash.

Conclusions. Ash fertilization altered the humus layer fungal community, whereas the level and form of additional Cd in the ash had no influence because it was not bioavailable.

Keywords: Biosensor; cadmium; coniferous forest humus; DGGE; fungal community structure; luminescence; PCR; wood ash

Introduction

The Finnish wood industry annually produces approximately 150 000 t of wood ash that could be recycled in forest ecosystems in order to maintain the long-term base cation and plant mineral nutrient status of the soil. During combustion most of the inorganic nutrients and trace elements in wood are retained in the ash. Recently, the use of wood ash with a cadmium (Cd) concentration that normally varies between 1 and 20 mg kg^{-1} ash has been questioned because the measured Cd concentrations in the ash in most cases exceed the level allowed for fertilizers (3 mg kg^{-1}) used in Finnish agriculture (Levula et al. 2000). This restriction on fertilizer use does not apply to forest soil, and there is a lack of knowledge about the harmful effects of Cd in wood ash on forest ecosystems.

This prompted us to study the effect of Cd and ash on the forest humus microflora (Fritze et al. 2000). In that study, pumice and wood ash were spiked with a water soluble ($CdCl_2$) or insoluble (CdO) form of Cd at three levels (0, 400 and 1000 mg kg^{-1}) and applied in the laboratory microcosm study at a fertilization level corresponding to 5000 kg ha^{-1}. Pumice could be used to distribute the Cd evenly over the humus surface in order to estimate the possible effect of Cd without ash. The application of ash increased microbial activity and changed the phospholipid fatty acid (PLFA) pattern, which reflects the bacterial community structure, compared to the humus of the pumice treatment. The form and level of Cd in the ash had no further effect on this result. In the humus under pumice, the higher level of Cd decreased the microbial activity and changed the PLFA pattern compared to the unspiked pumice (Fritze et al. 2000).

Although the PLFA approach gives information about differences in the bacterial community it does not work with fungi since the analysis detects only one PLFA present solely in fungi. To detect differences in the humus fungal community molecular techniques can be used, bypassing, like the PLFA approach, the steps of isolation and cultivation. For example, denaturing gradient gel electrophoresis (DGGE)

can be used to separate DNA-fragments according to their sequences, thus allowing the simultaneous analyses of several different sequences PCR-amplified from a single sample (Muyzer et al. 1993).

Recently, 18S fungal primers suitable for a single step PCR-DGGE approach have been published (Vainio and Hantula 2000) and their suitability to detect coniferous forest humus fungal communities has been tested (Pennanen et al. 2001). The aim of this study was to use the 18S PCR-DGGE approach to reveal the effects of wood ash with and without Cd additions on the fungal part of the microbial community in humus layer samples taken from a coniferous, boreal forest. In addition, the effect of wood ash on the bioavailability of Cd was determined using a bacterial biosensor strain that emits light in the presence of Cd (Tauriainen et al. 1998). The same humus samples were used from which the changes in bacterial community, including PLFA profiles, have previously been reported (Fritze et al. 2000).

1 Materials and Methods

1.1 Treatments

For sample collection and preparation, consult Fritze et al. (2000), since the same soil samples from these treatments were used in this work. To improve the understanding, the treatments are described here once again.

Powdered wood ash and finely ground (<0.2 mm) pumice (Riedel de Haën 31802, Bimsstein für Analyse) were spiked with solid CdO or $CdCl_2$ in order to give final Cd concentrations of 400 and 1000 mg kg^{-1}. The $CdCl_2$ was ground in a mortar to produce a powder as fine as the CdO before spiking. The Cd was mixed into the ash and pumice in glass jars by rotation over night before determining the Cd concentrations of the mixtures by inductively coupled plasma atomic emission spectrometry (ICP-AES, ARL 3580) after dry digestion (550°C) and extraction with conc. HCl (**Table 1**).

Sixty pots were filled with 90 g of humus material and each watered to 50% of their water holding capacity (WHC). Randomly chosen sets of 5 pots (n = 5) were treated. Unspiked controls were prepared for each of the water-soluble and insoluble forms of Cd in order to give a full statistical layout. This resulted in 12 treatments, 10 of which are presented in Table 1. The pumice and wood ash (a total of 5.65 g per pot) were spread evenly over the surface of the humus (top dressing) using a spoon at a fertilization level of 5000 kg ha^{-1}. The 60 pots were incubated in darkness at +20°C and a constant relative humidity of 60%. In addition to the 60 pots, two untreated (no cover layer) microcosms were prepared. All pots were watered at 2 to 3-day intervals in order to keep the humus water content at 50% WHC. If water passed through the microcosm, it was poured back onto the humus. After two months the microcosms were destructively sampled. The whole material was mixed, transferred to plastic bags and kept at –20°C until analysed. The treatments, their abbreviations, and the resulting Cd concentrations in the humus are presented in Table 1.

1.2 DNA isolation, PCR and DGGE analysis

Humus DNA extraction followed the protocol of Pennanen et al. (2001). The DNA was diluted 10-fold and subjected to PCR amplification of 390 bp and 1650 bp products using FR1 + FF390 and FR1 + NS1 primer pairs, respectively, targeting fungal 18S (SSU) rDNA. Fungal DNA isolated from pure cultures obtained from humus samples was used as

Table 1: Cadmium concentration of the treatments from Fritze et al. (2000)

		Cd	
		Cover[b] layer	Humus[c]
Treatment	Abbreviation[a]	(mg kg^{-1})	(mg kg^{-1})
Pumice	P	n.d.	0.94 ± 0.08
Pumice + 400 mg kg^{-1} CdO	PLO	362 ± 7	68.9 ± 2.56
Pumice + 1000 mg kg^{-1} CdO	PHO	923 ± 15	171 ± 3.04
Pumice + 400 mg kg^{-1} $CdCl_2$	PLC	388 ± 15	70.8 ± 0.68
Pumice + 1000 mg kg^{-1} $CdCl_2$	PHC	977 ± 63	182 ± 6.04
Ash	A	10.6 ± 0.11	2.04 ± 0.09
Ash + 400 mg kg^{-1} CdO	ALO	380 ± 1.8	51.8 ± 3.64
Ash + 1000 mg kg^{-1} CdO	AHO	980 ± 21	127 ± 10.6
Ash + 400 mg kg^{-1} $CdCl_2$	ALC	344 ± 8.5	45.4 ± 2.78
Ash + 1000 mg kg^{-1} $CdCl_2$	AHC	914 ± 32	104 ± 11.3

n.d. = below the detection limit (n = 5, except P and A where n = 10 due to 2 separate P and A treatments to have a full statistical design for the two forms of Cd)

[a] In the abbreviations P represents pumice, A ash, L low, H high, O the water insoluble CdO, and C the water soluble $CdCl_2$

[b] Pumice or ash

[c] at the end of the experiment

positive controls. The resulting amplification products were checked for size, purity and product yield in 1% agarose gels. In order to obtain efficient separation in DGGE, a GC clamp was attached to the FR1 primer. The primer pairs, the GC clamp, and the exact DGGE conditions have been published by Vainio and Hantula (2000). The wells of the DGGE gels were loaded with approximately the same amount of DNA as judged from the product yield in the agarose gels.

1.3 Bioluminescence measurement

The bioavailability of Cd in the humus samples after the treatments was determined using the *Bacillus subtilis* strain BR151 (Young et al. 1969) containing the Cd sensor plasmid pTOO24 (Tauriainen et al. 1998) to control the expression of firefly luciferase. This biosensor emits light specifically in the presence of Cd.

Cadmium in air-dried subsamples of humus material (2.5 g) was extracted by adding 20 ml of deionised water followed by shaking for 30 min (250 rpm) at room temperature. After shaking, the suspensions were centrifuged for 8 min (3000 rpm) and filtered through a Whatman 3 (pore size 6 mm) filter paper. This extract was of the same colour for all treatments and used in the luminescence measurement.

B. subtilis was grown at 28°C in LB medium (10 g Tryptone, 5 g yeast extract, 5 g NaCl to a litre, pH 7.0; supplemented with 30 µg ml^{-1} Kanamycin) on a rotary shaker (250 rpm). The culture was harvested during the exponential growth phase between optical densities of 0.3 to 0.8 at 600 nm. After centrifugation (5 min, 3000 rpm, +4°C), the supernatant was removed and the cell pellet was suspended in PBS (8.5 g NaCl, 1.34 g $Na_2HPO_4 \times 2H_2O$, 0.39 g $NaH_2PO_4 \times 2H_2O$ to a litre, pH 7.1).

To obtain the relationship between bioluminescence and Cd concentration a 1 mM $CdCl_2$ solution was prepared in Milli-Q-purified (Millipore, Bedford, Massachusetts) water. A range of dilutions (1 pM–1 mM) of this solution were prepared and added in 50 µl aliquots to the wells of a 96-well white microtiter plate. To these wells, 50 µl of the biosensor suspension (containing ca. 1.7×10^7 *B. subtilis* cells) was added, and the plate was incubated without shaking at room temperature for 2 h. Then 100 µl of luciferase substrate (0.1 mM D-luciferin in 1 M Na-citrate buffer, pH 5.0; D-luciferin from Promega) was added. After 20 min, the plate was moved to a Wallac Victor² 1420 Multilabel counter and bioluminescence values were measured. Induction coefficients were calculated with different amounts of Cd as follows: induction coefficient $I = L_i/L_b$, where L_i is the light emitted by an induced sample (containing Cd) and L_b is the light emitted by the non-induced sample (containing Milli-Q water). The induction coefficient was then plotted against Cd concentration resulting in a typical standard curve for metal biosensors having a maximum I value (I_{max}) at a certain metal concentration where after the I value decreases again with increasing metal concentration.

The same procedure was performed for the undiluted (see above) water extracts of the humus samples from the treatments. The light emission was converted to Cd concentrations using the linear part of the standard curve before reaching I_{max}.

1.4 Data analysis

The luminescence results are presented in mg bioavailable Cd kg^{-1} dry matter of humus material and these results were subjected to analysis of variance (ANOVA) followed by Tukey's test. Three main effects and their interactions in the ANOVA were tested. The main effects were ash contra pumice, soluble contra insoluble form of Cd, and the three Cd levels.

2 Results and Discussion

There are a limited number of publications, where the use of primer pairs designed for the PCR amplification of fungal 18S (SSU) rDNA from environmental samples is described. Kowalchuk et al. (1997) analysed fungi associated with the roots of dune grasses and Smit et al. (1999) reported amplification of fungal 18S rDNA fragments from wheat rhizosphere soil. In both publications, a nested PCR approach was used and the detection of different PCR fragments was performed using DGGE (Kowalchuk et al. 1997) or temperature gradient gel electrophoresis (TGGE; Smit et al. 1999). We used two primer pairs, FR1 + FF390 and FR1 + NS1, for PCR amplification of fungal DNA. These primers were tested to amplify a broad spectrum of fungal species without amplifying plant or bacterial DNA (Vainio and Hantula 2000). Pennanen et al. (2001) tested further to see that these primers did not amplify DNA from soil animals common in a coniferous forest humus layer.

All the samples were initially screened by PCR-DGGE using the FR1 + FF390 primer pair. Since no Cd effect at the lower level could be detected, the PLO, PLC, ALO and ALC treatments were omitted from the final gels. As in our DGGE, only 20 wells could be loaded with a sample. We randomly selected three of the five treatment replicates in order to be able to run the final comparisons in the same gel. The PCR-DGGE with the FR1 + FF390 produced over 35 bands of which several were very weak (**Fig. 1**). With the FR1 + NS1 primer pair, only the U, P, PHO, PHC, A, AHO, and AHC treatments were analysed. The PCR-DGGE produced over 15 bands (**Fig. 2**). Both primer pairs detected an ash effect, but no Cd effect was obtained, implying that the form and level of Cd in combination with or without ash had no detectable effect on fungal species composition. Using the FR1 + FF390 primer pair, the ash effect was seen in one DGGE band, where a higher fluorescence was obtained in all the ashed samples (Fig. 1; band indexed as no. 1) implying a higher amount of template DNA of this fungus. This increase may be due to the relative increase in the presence of mycelia of this fungus in response to the ash induced rise in humus pH from 4 to 7. With the FR1 + NS1 primer pair, the ashed samples had a band in the DGGE that was not visible or only faintly visible with the pumiced samples (Fig. 2; band indexed no. 2). In addition, one DGGE band was absent in the ashed samples (Fig. 2; band indexed no. 3). The pumiced samples did not differ from the untreated controls in both PCR-DGGE approaches.

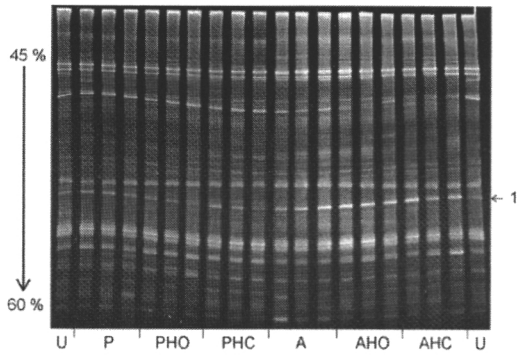

Fig. 1: DGGE of the fungal PCR products amplified using the FR1 + FF390 primer pair. The numbered band indicated with an arrow is discussed in the text. Abbreviations: U represents untreated, P pumice, A ash, H high, O the water insoluble CdO, and C the water soluble $CdCl_2$

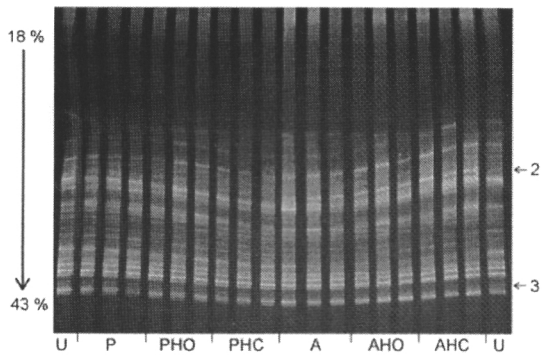

Fig. 2: DGGE of the fungal PCR products amplified using the FR1 + NS1 primer pair. The numbered bands indicated with arrows are discussed in the text. See Figure 1 for an explanation of the abbreviations of the treatments

It can be argued that at the time of sampling these were the most dominant growing fungal species. The fungal species diversity, hidden in spores, was probably not detected by the PCR-DGGE method. From a soil microfungal study, however, we know that the fungal species composition growing on agar plates was not different from each other when the isolation was performed from vegetative hyphaea or from the spores inhabiting the sample (Fritze and Bååth 1993). Our microcosms did not include plant seedlings and, thus, mycorrhizal fungi were probably excluded, leaving only saprophytic fungi that could be detected. It has been shown using isolation techniques that ash fertilization (Bååth and Arnebrant 1993) or fly ash deposition (Fritze and Bååth 1993) of coniferous forests changes the composition of the microfungal flora inhabiting the humus. Using the PCR-DGGE method, we came to the same conclusion.

The use of the *B. subtilis* biosensor showed that the Cd added with the ash was not in a bioavailable form in the humus (**Fig. 3**). The Cd spiked ash treatments did not differ from their unspiked ash controls and the biosensor detected a mean bioavailable level of 2.4 mg Cd kg^{-1} humus in all the ash

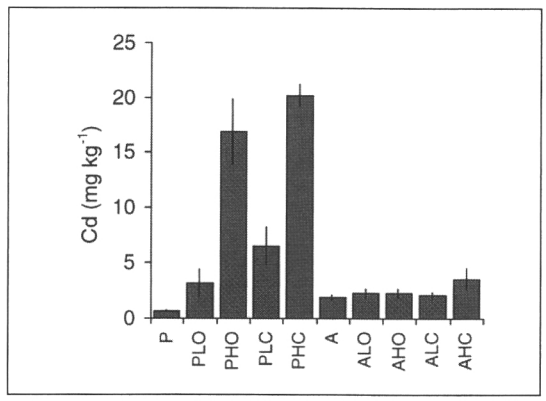

Fig. 3: Amount of bioavailable Cd in the humus of the different treatments detected using the biosensor bacteria *Bacillus subtilis* BR151. See Figure 1 for explanations of the abbreviations of the treatments

treatments. The pumiced humus samples differed significantly from the ash treatments ($F = 10.94$, $p < 0.05$). In all humus samples treated with Cd spiked pumice, the biosensor detected an increased amount of bioavailable Cd when compared to the unspiked pumice controls (Fig. 3). The biosensor detected significantly higher Cd amounts ($F = 30.93$, $p < 0.001$) in the humus of the PHC and PHO treatments than in the PLC and PLO treatments. The highest amount of bioavailable Cd, 20.2 ± 1.0 (SE) mg Cd kg^{-1} humus, was detected in the PHC treatment. When calculating the percentage of bioavaivable Cd out of the total amount of humus Cd (see Table 1) it can be seen that only 4.7%–11% of the total Cd content was in a bioavailable form when the Cd was applied with pumice. In general, a higher availability of Cd was detected in the humus samples that received the water soluble form of Cd (Fig. 3), but the difference between the pairs, PHC versus PHO and PLC versus PLO, were statistically insignificant.

Forest humus of pH 4 thus needs a bioavailable Cd concentration above 20 mg kg^{-1} (Fig. 3) to induce changes in the bacterial part of the microflora (Fritze et al. 2000), although, according to this study, this dose had no influence on the fungal community. It might also be that the 18S PCR-DGGE approach is not sensitive enough to measure the possible changes. The experiment performed in this study is realistic because the Cd was first mixed with the wood ash (and pumice) to give a theoretical maximum level of 1000 mg kg^{-1} ash (see Table 1) before spreading it onto the surface of the humus to imitate a fertilization load of 5000 kg ha^{-1}. Converting this to the maximal amount of Cd per humus dry weight used in this experiment, only 196 mg Cd kg^{-1} humus could have entered the soil system. During the two months incubation and watering period the Cd dispersed well into the humus when adding the Cd with pumice. This can be judged from the total Cd amount present in the humus after the experiment (Table 1). Under pumice, the measured amounts of Cd, 171 mg kg^{-1} and 182 mg kg^{-1} for CdO and $CdCl_2$ (Table 1), respectively, came close to the theoretical maximum amount of 196 mg of Cd kg^{-1} of humus, which

could have entered the humus ecosystem. It also has to be noted that the spiking of the pumice with Cd came only close to the theoretical maximum level of 1000 mg kg^{-1} pumice (Table 1), which is also a reason that the measured humus Cd values are below 196 mg kg^{-1}. The ash fertilization yielded contrasting results. The Cd values of the humus under ash, 127 mg kg^{-1} and 104 mg kg^{-1} for CdO and $CdCl_2$ (Table 1), respectively, were not close to the maximum Cd value, which could have entered the humus. The explanation for this is probably as follows. Loose wood ash forms crumbs of different sizes when watered and dried at different intervals. Smaller crumbs enter the soil ecosystem while large crumbs stay on the surface. The higher standard error of our Cd measurements in the humus under the ash, as compared to the humus under pumice (Table 1), point to the fact that the Cd in the humus under ash in unevenly distributed. As the biosensor measurement showed that the Cd added with the ash was not in the humus, we conclude that the Cd is still in the ash crumbs of different sizes.

3 Conclusion

In Finland, ash fertilization at a maximum level of 5000 kg ha^{-1} (equaling 0.5 kg m^{-2}), is allowed on mineral forest soil once within a time span of 30 years. The use of wood ash in forestry has been questioned because the Cd concentration of ash, which varies between 1 and 20 mg kg^{-1} ash, exceeds the level allowed for fertilizers (3 mg kg^{-1}) used in agriculture. If ash would have the theoretical value of 20 mg Cd kg^{-1} then only 10 mg Cd are added on top of the humus of 1 m^2 in real forestry. Through mineralisation, this Cd can be distributed into the humus and will thereby be diluted by the amount of humus present in 1 m^2. Combining the results from this study and Fritze et al. (2000), the microcosm approach could show that only 4.7 to 11% of the Cd added without ash was bioavailable. The amount of bioavailable Cd kg^{-1} humus has to be over 20 mg before reducing the microbial activity (soil respiration) and changing the bacterial community structure (PLFA pattern) whilst still having no effect on the structure of the fungal community (PCR-DGGE). Furthermore, this study showed that the Cd, when added with ash, was not bioavailable. The results of this study imply that, while treatment with wood ash affects the fungal community of the humus layer, the changes are unrelated to the levels of the Cd it contains. A field experiment to verify these results has been started.

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Note added in proof. In addition to the current topic, we investigate, 1. the effect of ash fertilization on CH_4 emission and consumption rates of boreal forest soils, 2. the long term effect of ash fertilization on microbial activity and community structure in a range of forest soils with different fertility, 3. the use of ash in remediation treatments of polluted forest soil, and 4. the persistance of the ash fertilization effect under simulated acid rain treatments.

Paper III

Perkiömäki J., Kiikkilä O., Moilanen M., Issakainen J., Tervahauta A. and Fritze H. 2003. Cadmium-containing wood ash in a pine forest: effects on humus microflora and cadmium concentrations in mushrooms, berries and needles. Canadian Journal of Forest Research 33: 2443-2451.

III

Cadmium-containing wood ash in a pine forest: effects on humus microflora and cadmium concentrations in mushrooms, berries, and needles

Jonna Perkiömäki, Oili Kiikkilä, Mikko Moilanen, Jorma Issakainen, Arja Tervahauta, and Hannu Fritze

Abstract: The cadmium (Cd) concentration of wood ash (1–30 mg·kg^{-1}) is higher than allowed for agriculture fertilizers (3 mg·kg^{-1}). Therefore, the objectives of this field study were to test if the Cd of wood ash has the potential to affect the coniferous forest humus microflora and if Cd enters the human food chain. These objectives were tested with ash (A) and Cd-spiked ash (ACd, 400 mg Cd·kg^{-1}) at a fertilization rate of 3 t·ha^{-1}. Microbial community structure, respiration, needle litter decomposition, growth rates and Cd tolerance of bacteria, and the bioavailability of Cd were measured. Also, Cd concentrations of humus, soil percolation water, mushrooms, fruits and leaves of berries, and needles were determined. The amount of Cd in the percolation water or bioavailable Cd, measured with a bacterial biosensor, and Cd tolerance of bacteria did not increase, although the ACd treatment increased the amount of humus total and extractable Cd. Only the ACd and not the A treatment caused Cd concentration increment in *Lactarius rufus* and berries of *Empetrum nigrum*. In spite of the high Cd concentration of the spiked ash, it did not have harmful effects on humus microorganisms during this 4-year study. Thus, wood ash is safe to use as a fertilizer in forests.

Résumé : La concentration de cadmium (Cd) (1–30 mg·kg^{-1}) dans la cendre de bois est plus élevée que la limite permise dans les fertilisants agricoles (3 mg·kg^{-1}). Par conséquent, l'objectif de cette étude au champ consistait à vérifier si le Cd présent dans la cendre de bois peut affecter la microflore de l'humus dans les forêts de conifères et si on le retrouve dans la chaîne alimentaire de l'homme. Ces hypothèses ont été testées avec de la cendre seule (C) et de la cendre additionnée de Cd (CCd, 400 mg Cd·kg^{-1}) en fertilisant au taux de 3 t·ha^{-1}. La structure de la communauté microbienne, la respiration, la décomposition de la litière d'aiguilles, le taux de croissance et la tolérance au Cd des bactéries et le Cd biodisponible ont été mesurés. La concentration de Cd dans l'humus, dans l'eau de percolation dans le sol, dans les champignons, dans les fruits et les feuilles des petits fruits et dans les aiguilles a également été mesurée. La quantité de Cd dans l'eau de percolation ou le Cd biodisponible, mesurée à l'aide d'un biocapteur bactérien, et la tolérance des bactéries au Cd n'ont pas augmenté bien que le traitement CCd a entraîné une augmentation des quantités de Cd total et extractible dans l'humus. Seul le traitement CCd et non le traitement C a entraîné une augmentation de la concentration de Cd chez *Lactarius rufus* et dans les baies de *Empetrum nigrum*. Malgré la concentration élevée de Cd dans la cendre additionnée de Cd, aucun effet néfaste n'a été observé chez les microorganismes de l'humus durant les 4 années de l'étude. Par conséquent, la cendre de bois peut sans danger être utilisée comme fertilisant en forêt.

[Traduit par la Rédaction]

Introduction

The use of wood ash as a forest fertilizer has been increased in Finnish forestry because of its capacity to reduce acidity and to increase nutrient supplies of forest soils. Ash fertilization of forests is also a potential way to avoid the waste storage problem of wood ash, since new landfill directives have become operational. The cadmium (Cd) content of wood ash exceeds (1–30 mg·kg ash^{-1}; Steenari and Lindqvist 1997) the level that is allowed for agriculture fertilizers (3 mg·kg^{-1}) and there is not enough knowledge to define restrictions for Cd concentrations of wood ash in forest fertilization. Cadmium may affect human health, e.g., carcinogenity and renal and bone effects. Diet is the main source of human exposure to Cd. There is a potential risk that Cd from wood ash is enriched in the human food chain directly by eating mushrooms and berries or indirectly through eating game animals. Also, groundwater could become contaminated with Cd.

In addition to harmful effects on humans, Cd application could disturb the nutrient cycling of a forest because of its possible harmful effects on microbes. Cadmium amendments have resulted in inhibition of phosphatase, sulphatase, and respiration activities (Speir et al. 1999). Cadmium has also caused changes in microbial community structure (Frostegård et al. 1993*b*; Fritze et al. 2000) and reduction of

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J. Perkiömäki,[1] **O. Kiikkilä, A. Tervahauta, and H. Fritze.** Finnish Forest Research Institute, P.O. Box 18, 01301 Vantaa, Finland.
M. Moilanen and J. Issakainen. Finnish Forest Research Institute, Kirkkosaarentie 7, 91500 Muhos, Finland.

[1]Corresponding author (e-mail: Jonna.Perkiomaki@metla.fi).

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microbial biomass (Frostegård et al. 1993*b*) and dehydrogenase activities of soil microbes (Welp 1999).

In earlier studies, no increased concentration of Cd in mushrooms (Moilanen and Issakainen 2000; Lodenius et al. 2002 (except in *Russula emetica*)) and berries (Silfverberg and Issakainen 1991; Levula et al. 2000; Moilanen and Issakainen 2000) was detected after wood ash application. Instead, higher respiration (Fritze et al. 1994, 1995; Khanna et al. 1994) and bacterial growth rates (Bååth and Arnebrant 1994; Fritze et al. 2000; Perkiömäki and Fritze 2002) were determined after ash application. Additionally, in laboratory studies with Cd-spiked (400 or 1000 $mg \cdot kg^{-1}$) wood ash, no harmful effects on microbes were found (Fritze et al. 2000), and Cd added to ash was not bioavailable (Fritze et al. 2001).

The objectives of this study were to test in field conditions (*i*) if the Cd of wood ash has the potential to affect the coniferous forest humus microflora and (*ii*) if Cd becomes enriched in the food chain. These objectives were tested with wood ash and wood ash spiked with extra Cd (400 $mg \cdot kg\ ash^{-1}$) applied onto the forest floor. The measurements taken over a 4-year study period were humus respiration and bacterial growth rates, Cd tolerance of the bacterial community, mass loss percentage of pine needles, bioavailability of Cd to bacteria, and microbial community structure. Also, Cd concentrations of different compartments (humus, soil percolation water, mushrooms, fruits and leaves of berries, and needles) of the forest ecosystem were determined.

Materials and methods

Study site

The study plots were established in the autumn 1997 in a 65-year-old Scots pine (*Pinus sylvestris* L.) stand in central Finland (64°43′N, 26°02′E) growing on a podzolized sandy soil with a 30- to 50-mm-thick humus layer. The study site was of the dry *Empetrum–Vaccinium* site type and mean stem volume was 80 $m^3 \cdot ha^{-1}$. The mean annual temperature and precipitation for the years 1998, 1999, 2000, and 2001 were 2.0, 2.7, 4.2, and 2.5 °C and 535, 500, 618, and 479 mm, respectively. Long-term (1971–2000) mean annual values for temperature and precipitation for this site were 2.4 °C and 446 mm, respectively. The following treatments were performed: control, loose wood ash at a fertilization rate of 3 $t \cdot ha^{-1}$ (A), and the same fertilization rate of wood ash spiked with Cd (ACd). For the ACd treatment, ash was spiked with CdO to give a Cd concentration of 400 $mg \cdot kg^{-1}$. CdO is used for spiking because it is the dominant form of Cd, in addition to silicates, that has been observed in wood ash (Hansen et al. 2001). The plot size was 30 m × 30 m and the treatments were replicated three times. Wood ash was spread by hand with a spade evenly over the soil surface.

Sampling for chemical and microbiological analyses

Humus (F–H layer) samples were collected in August 1998, November 1999, and September 2000 and 2001 using a soil corer (40 mm in diameter). To obtain one composite sample per plot, seven cores at least 2 m distance from three lines were taken and the 21 cores from each plot were then combined. The composite samples were sieved (2.8-mm mesh) and visible plant material was removed and then stored at 4 °C for 1–4 weeks before analyses were conducted.

Table 1. Elemental contents of the wood ash used in the experiment.

Element	Wood ash ($mg \cdot kg^{-1}$)
Ca	350 000
K	28 000
Mg	18 000
Fe	11 000
Mn	10 000
P	9 000
Al	8 000
Zn	2 400
B	200
Cu	80
Ni	69
Cr	64
Cd	15*

*The Cd content of the spiked ash was 427 $mg \cdot kg^{-1}$.

Two suction-cup lysimeters were installed on each plot in 1997 at depths of 0.05 and 0.2 m below the surface of the mineral soil layer. Percolation water was collected six times during the snow-free period in 1998 and 2001 and seven times in 1999 and 2000.

Edible mushroom sporocarps and berries were collected yearly during the study period (1998–2001) if the following conditions were met to get an adequate sample for chemical analyses: 6–20 mushroom specimens (upper half of stipe and cap) and 1.5 dL of berries per sample plot. Rufous milkcap (*Lactarius rufus*) was the only mushroom meeting the criteria during the study period and could be collected in autumn 1998 and 2000. Crowberry (*Empetrum nigrum*) samples were collected in autumn 1998, 2000, and 2001, whortleberry (*Vaccinium uliginosum*) in 1998, and lingonberry (*Vaccinium vitis-idaea*) in 1998–2001.

Leaves of *V. uliginosum* and *V. vitis-idaea* were collected from all over each study plot to form a composite sample of approximate 2 dL (over 2 g of dry matter). These leaves were not collected systematically and sampling was only done in 2000. *Pinus sylvestris* needles from the growth of the previous year were collected in the autumn between 1998 and 2001, always from eight trees to form a composite sample of the study plot. The needles were collected from twigs pointing in a southerly direction and branching 1–1.5 m below the top.

Chemical analyses

The chemical composition of the ash (Moilanen and Issakainen 2000) was determined after HCl digestion by atomic absorption spectrophotometry (Table 1). Humus samples were analysed using the techniques described by Tamminen and Starr (1990). Humus pH was measured in water (15 cm^3 of humus plus 25 mL of water). Humus dry mass was determined after drying overnight at 105 °C. Organic matter was determined after furnacing samples at 550 °C for 4 h. Total organic C and N were determined by dry combustion (Leco CHN-600). Total Ca and Cd were determined

Table 2. Chemical properties of humus as means of three replicate plots (SE in parentheses).

Sampling year	Treatment	pH	Ca_{tot}	Ca_{ext}	Cd_{tot}	Cd_{ext}	Organic matter	C/N
1998	C	4.1 (0.03)	5 400 (400)	1 700 (400)	0.64 (0.12)a	bdl	32 (2)	39 (1)
	A	4.2 (0.1)	6 600 (900)	2 200 (600)	0.60 (0.09)a	bdl	29 (1)	40 (1)
	ACd	4.3 (0.1)	6 000 (800)	1 900 (700)	1.1 (0.1)b	bdl	37 (9)	40 (2)
1999	C	3.6 (0.03)a	3 500 (400)a	2 000 (300)a	0.56 (0.03)a	bdl	90 (1)	35 (1)
	A	4.6 (0.1)b	9 000 (1200)b	4 900 (800)b	0.36 (0.18)a	bdl	85 (3)	35 (2)
	ACd	4.7 (0.1)b	9 400 (1500)b	4 900 (700)b	2.1 (0.4)b	0.85 (0.14)	85 (1)	35 (1)
2000	C	3.8 (0.1)a	2 800 (200)a	1 700 (200)a	bdl	bdl	93 (1)	41 (1)
	A	6.1 (0.3)b	15 000 (1000)b	11 000 (900)b	bdl	bdl	90 (1)	40 (1)
	ACd	5.2 (0.1)c	11 000 (60)c	6 700 (80)c	5.0 (0.5)	1.7 (0.2)	92 (0.3)	40 (1)
2001	C	3.7 (0.02)a	3 200 (200)a	2 100 (100)a	0.42 (0.03)a	bdl	89 (2)	37 (0.4)a
	A	5.6 (0.4)b	20 000 (4000)b	13 000 (1900)b	0.60 (0.08)a	bdl	85 (1)	38 (1)b
	ACd	5.5 (0.4)b	20 000 (4000)b	12 000 (2000)b	10 (2)b	3.9 (1.0)	86 (2)	36 (0.4)a

Note: Treatments of the plots are coded as C (control), A (ash), and ACd (ash spiked with Cd). Calcium and Cd are expressed as milligrams per kilogram organic matter. Organic matter is given as a percentage of dry matter. Means followed by a different letter are significantly different (LSD, $P < 0.10$). bdl, below detection limit.

after wet digestion (HNO_3 plus H_2O_2) with inductively coupled plasma – atomic emission spectroscopy (ICP–AES) (TJA, Iris Advantage, Franklin, Mass.). For extractable Ca and Cd analyses, humus was air dried for 48 h at 40 °C and then extracted with 1 mol/L ammonium acetate (pH 7.0) using a soil to solution volume ratio of 1:10. This suspension was analysed with ICP–AES (ARL 3580, Applied Research Laboratories, Ecublens, Switzerland). The results are given in Table 2.

The amount of dissolved organic C was determined on a TOC analyser. NH_4-N and NO_3-N were determined with a flow injection analyser (FIA 5012, Tecator, Höganäs, Sweden). Concentrations of Ca and Cd in percolation water were analysed by ICP–AES (TJA, Iris Advantage).

After collection, the mushroom, berry, leaf, and needle samples were cleaned, air dried for 48 h at 40 °C, and concentrations of Ca and Cd determined by ICP–AES (TJA, Iris Advantage) after wet digestion (HNO_3 plus H_2O_2).

Microbial analyses

The humus respiration rate was determined as the amount of CO_2-C evolved in 24–25.5 h from field moist humus incubated at 14 °C (Pietikäinen and Fritze 1995). Fresh humus samples, equalling 2 g dry mass, were used in the analyses. CO_2 was measured with a gas chromatograph (Hewlett Packard 6890) equipped with a TC detector and a Megapore GS-Q column (J & W Scientific, Folsom, Calif.). Injector, column, and detector temperatures were 120, 30, and 150 °C, respectively. Helium (5 mL·min^{-1}) was used as a carrier gas.

Bacterial growth rate and Cd tolerance were determined using the [^{3}H]thymidine incorporation technique as described by Bååth (1992*a*, 1992*b*) and modified by Kiikkilä et al. (2000). The bacterial growth rate was measured by incorporating radioactive thymidine into the macromolecules of bacteria extracted from soil, after sample homogenization and centrifugation. The final results are expressed as moles of radioactive thymidine per gram of organic matter per hour. In the Cd tolerance assay, different amounts of $CdCl_2$ were added to the bacterial suspension. The Cd concentration (millimoles per litre) giving a 50% reduction in [^{3}H]thymidine incorporation was calculated (IC_{50}). The higher the IC_{50} value, the greater the tolerance of the bacterial community to Cd.

The extraction of phospholipid fatty acids (PLFAs) was conducted with the technique described by Frostegård et al. (1993*b*) and modified by Pennanen et al. (1999). Fresh mass (0.5–1 g) of humus was extracted with 1.9 mL of chloroform, 3.75 mL of methanol, and 2 mL of Blight and Dyer mixture (Blight and Dyer contains chloroform – methanol – citrate buffer (0.15 mol/L, pH 4) at a volume ratio of 1:2:0.8) and the lipids were separated into neutral lipids, glycolipids, and phospholipids in a silicic acid column. The phospholipids were subjected to mild alkaline methanolysis, and the fatty acid methyl esters were separated by gas chromatography (Hewlett Packard 5890) equipped with a flame ionization detector and an HP-5 (phenylmethyl silicone) capillary column (50 m in length) using He (30 mL·min^{-1}) as a carrier gas. The peak areas were quantified by adding methyl nonadecanoate fatty acid (19:0) as an internal standard.

The total amount of PLFAs ($PLFA_{tot}$) was used to indicate the total microbial biomass, and the sum of PLFAs was 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, 18:1 ω7, and cy19:0) and chosen as an index of bacterial biomass ($PLFA_{bact}$) (Frostegård and Bååth 1996). The quantity of 18:2ω6 was used as an indicator of fungal biomass ($PLFA_{fung}$), as it was suggested to be mainly of fungal origin in soil (Federle 1986) and is known to correlate well with the amount of ergosterol (Frostegård and Bååth 1996). The ratio $PLFA_{fung}/PLFA_{bact}$ was used as an index of the ratio of fungal to bacterial biomass in the soil.

Induction caused by bioavailable Cd in soil samples was determined using the genetically modified *Bacillus subtilis* strain BR151 (Young et al. 1969) containing the Cd sensor plasmid pTOO24 (Tauriainen et al. 1998) to control the expression of firefly lusiferase. Preparation of soil suspension and the growing of *B. subtilis* were performed as previously described by Fritze et al. (2001). Briefly, 50 µL of supernatant (obtained from 2.5 g of air-dried soil plus 20 mL of deionized water) or standard $CdCl_2$ solutions (1 pmol/L – 1 mmol/L Cd) was mixed with a 50 µL of biosensor suspension and incubated for 2 h at room temperature and then,

100 µL of lusiferase substrate was added. After 20 min, induction bioluminescence was measured with a Wallac Victor[2] 1420 multilabel counter. Induction coefficients were calculated with different amounts of Cd as follows: induction coefficient $I = L_i/L_b$, where L_i is the light emitted by an induced sample (containing Cd) and L_b is the light emitted by the noninduced sample (containing Milli-Q water). The induction coefficient was then plotted against Cd concentrations resulting in a typical standard curve for metal biosensors having a maximum I value (I_{max}) at a certain metal concentration, after which the I value decreases again with increasing metal concentration. The light emission of humus water extracts was converted to Cd concentration using the linear part of the standard curve before reaching I_{max}.

Litter bags

Pinus sylvestris needles from 1996 growth were collected in August 1997 from the crown of local youngish trees that were felled in early spring 1997. In the laboratory, the needles were washed with distilled water and dried overnight at 60 °C. Needles (1 g) were weighed into litter bags (80 mm × 80 mm, mesh size about 1.0 mm × 0.5 mm) made of polyester net. All together, 60 litter bags were placed onto the litter layer of each study plot in September 1997. Twenty bags were sampled in November 1999, 2000, and 2001. Litter bags were transported to the laboratory and cleaned of root and soil remnants. To determine the mass loss, the remaining contents were weighed individually after drying overnight at 60 °C.

Data analysis

The results are calculated on the basis of organic matter content except for bioavailable Cd, since conversion of light emission to Cd concentrations resulted in very high variation between samples. This was probably due to the low amount of bioavailable Cd in many samples, which was below reliable quantification level. To diminish this uncertainty, we used the induction coefficients of the humus samples in statistical analyses and did not convert them to Cd concentrations. The results of the percolation water are averaged over the sampling year and are presented as milligrams per litre.

The mole percent of individual PLFAs was subjected to principal components analysis (PCA) with correlation matrix. The data having measurements from each of the four samplings, including the scores of the PCA, were summarized by a canonical correlation analysis (CCA) to form two combined variables. CCA generates pairs of linear combinations from two sets of original variables so 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 data set consisted of pH, C/N ratio, total and extractable Ca and Cd in the humus layer, Ca and Cd concentration of needles, and the amount of dissoved organic C, NH_4-N, NO_3-N, and Ca in percolation water of 0.05-m lysimeters; the biological data set consisted of humus respiration, thymidine incorporation, IC_{50}, $PLFA_{tot}$, $PLFA_{bact}$, $PLFA_{fung}$, $PLFA_{bact}/PLFA_{fung}$ ratio, induction coefficient (I), mass loss of needles, and PLFA scores of PC1 and PC2. The new canonical variables were called CHEM and BIOL. Graphical presentation of CCA was a scatterplot diagram of the sample plots on CHEM (x axis) and BIOL (y axis). The canonical structure, i.e., correlations between the original variables and canonical variables, was applied to the figure with the vectors of the original variables indicating the influence of the most important original variables on the formation of the new canonical variable. The length of the vector indicates the strength of the correlation, and the direction indicates the direction in which the variable increases. A 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 in their own data set (Van den Wollenberg 1977). Prior to the CCA test, the relationships between individual variables were examined by plotting the variables against each other. The canonical correlation analyses were performed with SAS using the CANCORR procedure (SAS Institute Inc. 1996). ANOVA followed by an LSD test was performed with scores of the canonical variables CHEM and BIOL to detect the effect of the treatments. ANOVA was also performed with individual variables. The significant differences between treatments are reported on the $P < 0.10$ level. Pearson correlation tests were used to evaluate relationships between variables.

Results

Humus layer chemical analyses

Humus pH and concentrations of total Ca (Ca_{tot}) and extractable Ca (Ca_{ext}) were not different between treatments during 1998, but in the subsequent 3 years, they were higher than in controls as a result of both ash treatments (A and ACd) (Table 2). The concentration of total Cd (Cd_{tot}) in the humus layer was always highest in the ACd treatment. During the whole study period, the concentration of extractable Cd (Cd_{ext}) in the humus layer was below the detection limit (<0.002 mg·L^{-1}) in control and A plots, but its concentration increased in ACd plots from 1999 onwards. There were no differences between treatments in the amount of organic matter. In 2001, the C/N ratio was highest in A plots.

Percolation water chemical analyses

The amount of dissolved organic C did not vary between treatments, except in 1998 when it was higher in the A treatment at 0.05 m depth compared with the control (Table 3). Neither were there treatment effects in the amounts of NH_4-N and NO_3-N, aside from the amount of NO_3-N at 0.2 m depth in 1999 and 2001 when it was highest in the A treatment. The concentration of Ca in percolation water was highest in the A plots at 0.05 m depth in 1998 and 2001, but its amount did not differ at this depth in other years and in any sampling at 0.2 m depth. The concentration of Cd was below the detection limit (<0.001 mg·L^{-1}) at both depths in all other years, except in 1998 when there was no treatment effect.

Microbial analyses of the humus layer

There were no treatment effects in the microbial variables during the first year (Table 4). From 2000 onwards, respiration and thymidine incorporation rates were higher in A plots compared with controls, and by 2001, ACd plots also had higher values of these (Table 4). In the data as a whole (1998–2001), both respiration ($r = 0.78$) and thymidine in-

Table 3. Properties of soil percolation waters at 0.05 and 0.2 m depths.

		Dissolved organic C		NH_4-N		NO_3-N		Ca		Cd × 10^{-3}	
Sampling year	Treatment	0.05 m	0.2 m	0.05 m	0.2 m	0.05 m	0.2 m	0.05 m	0.2 m	0.05 m	0.2 m
1998	C	61 (7)a	52 (3)	0.21 (0.08)	0.21 (0.12)	0.021 (0.006)	0.025 (0.010)	1.7 (0.6)a	1.4 (0.1)	0.85 (0.25)	0.70
	A	130 (23)b	70 (25)	0.47 (0.35)	0.43 (0.18)	0.029 (0.003)	0.050 (0.019)	10 (3)b	14 (8)	1.5 (0.4)	2.3 (0.7)
	ACd	97 (19)ab	71 (26)	1.0 (0.2)	0.45 (0.28)	0.030 (0.003)	0.024 (0.009)	3.6 (1.2)ab	5.4 (0.7)	1.3 (0.2)	1.2 (0.4)
1999	C	63 (15)	39 (10)	0.42 (0.21)	0.056 (0.008)	0.015 (0.006)	0.012 (0.003)a	1.9 (1.0)	0.96 (0.31)	bdl	bdl
	A	70 (20)	36 (7)	0.77 (0.27)	0.47 (0.09)	0.030 (0.009)	0.11 (0.02)b	6.3 (3.0)	2.6 (0.5)	bdl	bdl
	ACd	61 (12)	31 (4)	0.59 (0.27)	0.42 (0.22)	0.016 (0.005)	0.034 (0.025)a	5.0 (2.2)	4.1 (2.9)	bdl	bdl
2000	C	82 (21)	64 (16)	0.18 (0.11)	0.30 (0.16)	0.012 (0.005)	0.0047	2.0 (1.4)	1.7 (0.9)	bdl	bdl
	A	53 (18)	33 (15)	0.43 (0.15)	0.13 (0.11)	0.022 (0.008)	0.22 (0.13)	5.9 (2.2)	1.9 (0.9)	bdl	bdl
	ACd	52 (8)	64	0.28 (0.08)	0.067	0.015 (0.007)	bdl	2.2 (0.1)	1.6	bdl	bdl
2001	C	61 (8)	36 (4)	0.59 (0.23)	0.18 (0.07)	0.024 (0.005)	0.0060a	1.2 (0.6)a	1.1 (0.4)	bdl	bdl
	A	100 (40)	45 (16)	0.74 (0.44)	0.30 (0.28)	0.016 (0.008)	0.10 (0.01)b	9.3 (2.2)b	5.3 (4.5)	bdl	bdl
	ACd	64 (1)	44 (16)	0.93 (0.58)	0.30 (0.14)	0.020 (0.004)	0.009 (0.005)a	4.6 (1.7)ab	1.8 (1.0)	bdl	bdl

Note: All results are expressed as milligrams per litre. bdl, below detection limit. SE is in parentheses; where there is no SE, the other replicates are missing or the values are under the detection limit. Means followed by a different letter are significantly different (LSD, $P < 0.10$). Treatment symbols as in Table 2.

Table 4. Microbiological properties of humus as means (SE in parentheses) of three replicate plots.

Sampling year	Treatment	CO_2 ($\mu g \cdot g^{-1} \cdot h^{-1}$)	[^{3}H]thymidine incorporation ($\times 10^{-11}$ $mol \cdot g^{-1} \cdot h^{-1}$)	IC_{50} (mmol/L)	Needle mass loss (%)	Cd induction coefficient	$PLFA_{tot}$ ($nmol \cdot g^{-1}$)	$PLFA_{bact}$ ($nmol \cdot g^{-1}$)	$PLFA_{fung}$ ($nmol \cdot g^{-1}$)	$PLFA_{fung}$/ $PLFA_{bact}$
1998	C	7.5 (0.9)	7.6 (1.5)	5.4 (1.2)	nd	1.3 (0.2)	3300 (300)	1100 (100)	390 (50)	0.36 (0.02)
	A	7.7 (1.6)	5.1 (0.9)	3.2 (1.3)	nd	1.4 (0.1)	2700 (200)	840 (70)	330 (50)	0.39 (0.02)
	ACd	6.5 (1.1)	5.1 (0.9)	3.9 (0.9)	nd	1.3 (0.1)	2800 (500)	860 (190)	360 (40)	0.44 (0.09)
1999	C	7.5 (1.0)	2.3 (0.5)	0.3 (0.03)a	54 (1)	1.1 (0.1)	1700 (300)	550 (140)	210 (10)	0.43 (0.11)
	A	7.5 (0.3)	4.1 (1.1)	0.1 (0.01)b	53 (1)	1.2 (0.02)	2100 (100)	690 (60)	240 (5)	0.35 (0.02)
	ACd	7.3 (0.5)	4.9 (1.1)	0.1 (0.08)ab	52 (1)	1.1 (0.04)	1900 (100)	640 (30)	220 (20)	0.34 (0.02)
2000	C	13 (1)a	2.4 (0.7)a	0.5 (0.09)a	68 (1)	1.8 (0.2)	1600 (100)	420 (50)	350 (20)	0.85 (0.06)
	A	21 (2)b	5.0 (0.3)b	0.02 (0.01)b	67 (4)	1.6 (0.2)	1400 (50)	370 (20)	320 (10)	0.88 (0.06)
	ACd	14 (1)a	3.7 (0.7)ab	0.02 (0.01)b	65 (2)	1.6 (0.4)	1500 (20)	380 (9)	310 (30)	0.92 (0.09)
2001	C	10 (1)a	2.0 (0.03)a	0.07 (0.01)	75 (1)	2.4 (0.5)	2100 (200)	540 (50)	450 (60)	0.84 (0.05)
	A	16 (2)b	8.3 (3.5)b	0.04 (0.02)	70 (5)	1.5 (0.2)	1700 (80)	450 (30)	330 (20)	0.76 (0.06)
	ACd	16 (2)b	6.6 (1.5)b	0.04 (0.02)	69 (2)	1.9 (0.2)	1700 (40)	450 (10)	350 (10)	0.77 (0.04)

Note: All results are expressed as per gram organic matter. nd, not determined. Means followed by a different letter are significantly different (LSD, $P < 0.10$). Treatment symbols as in Table 2.

Table 5. Calcium and Cd concentrations in *L. rufus*, berries, and needles of treatments (C, A, ACd), 1998–2001.

		1998			1999	
Species	Source	C	A	ACd	C	A
L. rufus	Cap and stipe					
	Ca	70 (12)	640 (240)	400 (120)	nd	nd
	Cd	0.85 (0.03)a	0.69 (0.13)a	1.33 (0.06)b	nd	nd
E. nigrum	Berries					
	Ca	630 (30)	780 (140)	570 (40)	nd	nd
	Cd	bdl	bdl	0.046 (0.023)	nd	nd
V. uliginosum	Berries					
	Ca	1300 (80)	1200 (40)	1100 (80)	nd	nd
	Cd	0.13 (0.01)	0.12 (0.01)	0.14 (0.02)	nd	nd
	Leaves					
	Ca	nd	nd	nd	nd	nd
	Cd	nd	nd	nd	nd	nd
V. vitis-idaea	Berries					
	Ca	1500 (80)	1400 (60)	1500 (30)	1200 (20)a	1100 (30)b
	Cd	bdl	bdl	bdl	bdl	bdl
	Leaves					
	Ca	nd	nd	nd	nd	nd
	Cd	nd	nd	nd	nd	nd
P. sylvestris	Needles					
	Ca	1800 (70)a	2300 (70)b	2300 (100)b	1500 (90)a	1700 (30)ab
	Cd	0.082 (0.008)	0.090 (0.011)	0.095 (0.014)	0.11 (0.02)	0.11 (0.02)

Note: All results are expressed as milligrams per kilogram dry mass. nd, not determined; bdl, below detection limit. SE is in parentheses; where there $P < 0.10$). Treatment symbols as in Table 2.

corporation rates ($r = 0.58$) correlated with humus layer pH. These correlations with pH occurred from 2000 and 1999 onwards for respiration and thymidine incorporation, respectively. IC_{50} had an ash but not a Cd effect in 1999 and 2000 (Table 4). Mass loss of needles, Cd induction coefficient (bioavailable Cd), and the amount of the PLFA-derived microbial biomass measures $PLFA_{tot}$, $PLFA_{bact}$, $PLFA_{fung}$, and $PLFA_{fung}/PLFA_{bact}$ ratio did not show ash or Cd effects at any sampling occasion (Table 4). The $PLFA_{fung}/PLFA_{bact}$ ratio was higher for 2000 and 2001 than for 1998 and 1999. PCA of individual PLFAs separated sampling occasions along PC1. PC2 is a combination of differences between 2000 and 1998 and between 1999 and 2001 and a separation of the two ash treatments from the respective controls on the last sampling (data not shown). For the last two samplings years, 2000 ($r = 0.78$) and 2001 ($r = 0.83$), PC2 correlated with soil pH. Both PC1 and PC2 were taken as a part of biological data set of CCA.

Calcium and Cd concentrations in *L. rufus*, berries, and needles

The A treatment increased Ca concentration of *L. rufus* significantly in 2000 (Table 5). Calcium concentrations were not raised significantly at any particular sampling in the berries of *E. nigrum* or in the berries and leaves of *V. uliginosum* (Table 5). The A treatment raised the Ca concentrations in the berries of *V. vitis-idaea* only in 1999 and in leaves after both ash treatments (A and ACd) in 2000 (Table 5). Both ash treatments induced higher Ca concentrations in *P. sylvestris* needles in the first two years, 1998 and 1999, with this effect levelling off in the two subsequent years (Table 5). When detectable (detection limit <0.001 $mg \cdot L^{-1}$), higher Cd concentrations resulting from the ACd treatment could be measured in *L. rufus* and the berries of *E. nigrum* (Table 5). Cadmium concentrations in *L. rufus* correlated with humus Cd_{tot} ($r = 0.90$) and Cd_{ext} ($r = 0.84$). Concentrations of Cd in the berries and leaves of *V. uliginosum* and *V. vitis-idaea* and *P. sylvestris* needles were not different between the ACd and control treatments (Table 5). The A treatment did not induce increased Cd concentrations in any measurement.

CCA: summarizing the chemical and biological data

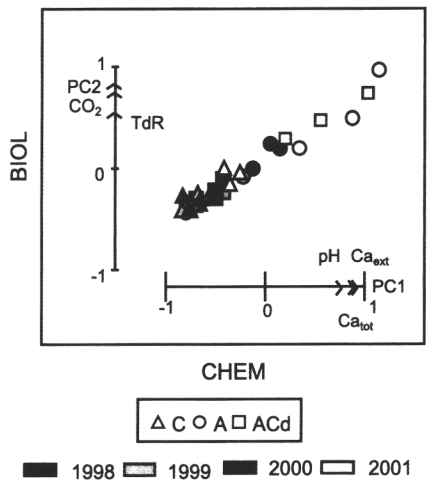
All of the data having continuous measurement values throughout the study period, except properties of percolation water at 0.2 m depth, were included in the CCA data set. Applying CCA, the chemical and microbial variables could be diminished to only two variables, CHEM and BIOL (Fig. 1). The first chemical canonical variable (CHEM) explained 26% of the total variance in the chemical data set. The first biological canonical variable (BIOL) explained 24% of the total variation in biological data set. The correlation between the first canonical variables CHEM and BIOL (canonical correlation) was 0.98 ($P < 0.0001$).

In CCA, CHEM and BIOL separated the A plots from controls from 2000 onwards, and also, the ACd plots of the third sampling (2000) differed from control plots along the CHEM axis but not along the BIOL axis (Fig. 1). In 2001, both ash treatments (A and ACd) differed from the control but not from each other and are situated in the right upper corner of the graph (Fig. 1). They are characterized by a high humus layer pH, concentrations of Ca_{tot} and Ca_{ext}, and

	2000			2001		
ACd	C	A	ACd	C	A	ACd
nd	58 (5)a	210 (70)b	150 (20)ab	nd	nd	nd
nd	0.62 (0.10)a	0.77 (0.17)a	1.3 (0.02)b	nd	nd	nd
nd	480 (20)	440	460 (40)	530 (7)	570 (30)	540 (10)
nd	bdl	bdl	bdl	bdl	bdl	bdl
nd	nd	nd	nd	nd	nd	nd
nd	nd	nd	nd	nd	nd	nd
nd	6900 (60)	6400 (200)	7000 (300)	nd	nd	nd
nd	0.41 (0.06)	0.35 (0.04)	0.56 (0.07)	nd	nd	nd
1200 (20)a	1400 (80)ab	1300 (20)a	1500 (40)b	1300 (40)	1400 (20)	1300 (40)
0.023	bdl	bdl	bdl	bdl	bdl	bdl
nd	5100 (30)a	5400 (100)b	5600 (60)b	nd	nd	nd
nd	bdl	bdl	bdl	nd	nd	nd
1900 (100)b	2000 (20)	2300 (60)	2100 (200)	2300 (100)	2400 (300)	2300 (200)
0.12 (0.01)	0.038	bdl	0.034	0.075 (0.01)	0.076 (0.011)	0.072 (0.061)

is no SE, the other replicates are missing or the values are under the detection limit. Means followed by a different letter are significantly different (LSD,

Fig. 1. Plot of the treatments (C, A, and ACd) with respect to incubation time (1998–2001) along CHEM and BIOL from canonical correlation analysis. Treatment symbols are as defined in Table 2.



respiration and thymidine incorporation rates (Fig. 1). The effect of PC2 on the CCA is a combination of both treatment and sampling occasion effects. Plots of the first and second sampling years (1998 and 1999) are mostly situated in left bottom corner and neither of these samplings had a treatment effect (Fig. 1).

Discussion

Rise in humus respiration (Fritze et al. 1995, 2000; Perkiömäki and Fritze 2002) and bacterial growth rates (Bååth et al. 1995; Fritze et al. 2000; Perkiömäki and Fritze 2002) and changes in microbial community structure as determined with the PLFA method (Frostegård et al. 1993*a*; Fritze et al. 2000; Perkiömäki and Fritze 2002) are commonly observed after wood ash application. This study confirms these results. All three microbial variables correlated with humus pH, which generally rises after wood ash amendment (Unger and Fernandez 1990; Bramryd and Fransman 1995; Saarsalmi et al. 2001). The Ca concentration in humus, percolation water, *L. rufus*, berries, and needles was used as an indicator of wood ash fertilization success. The aim of this field study was to identify potential risks of Cd concentrated in the wood ash to humus microbes using the same methods that have been shown to react separately to both Cd and ash in a laboratory experiment (Fritze et al. 2000). Therefore, the Cd concentration of ash was artificially increased to reach 400 mg Cd·kg ash^{-1} (ACd treatment) and compared with untreated control and wood ash (A) treatments. During the 4-year field study, the ACd treatment was not different from the A treatment with respect to all of the microbial measurements taken. Both induced the same ash effect as presented above.

As a result of the ACd treatment, the humus Cd_{tot} and Cd_{ext} concentrations increased but the bacterial biosensor did not react to that increase, nor was Cd tolerance (IC_{50}) of soil bacteria increased as a result of the treatment. The rise in humus Cd concentration was not reflected in the amount of Cd in percolation water collected at two depths, 5 and

20 cm, under the humus layer or in the Cd concentration of berries and leaves of *V. uliginosum* and *V. vitis-idaea* and *P. sylvestris* needles. Only the concentrations of Cd in *L. rufus* and berries of *E. nigrum* increased due to the ACd treatment. The ACd treatment thus shows the potential flow path of Cd in the forest environment. Even though the ACd treatment added 28 times more Cd to the soil than the A treatment, the increment of Cd in *L. rufus*, which was the only biological variable showing a significant Cd effect in this study, was roughly doubled. There are not many studies concerning the Cd concentrations in mushrooms and berries after wood ash fertilization. Our results are in accordance with earlier studies showing that the effects of wood ash fertilization on the Cd concentrations of *L. rufus* (Moilanen and Issakainen 2000; Lodenius et al. 2002) and berries (Silfverberg and Issakainen 1991; Levula et al. 2000; Moilanen and Issakainen 2000) are practically nonexistent. Higher amounts of Ca in the ectomycorrhizal fungus *L. rufus* on wood ash treated plots could be an indication of the ability to dissolve wood ash like the ectomycorrhizal fungus *Piloderma* sp. 1 in the study of Mahmood et al. (2003).

The chemical and biological data were summarized in a multivariate statistical approach (CCA). According to CCA, the A and ACd treatments started to separate from controls but not from each other from the third sampling (2000) onwards. Thus, the higher amount of Cd in the ACd treatment did not cause different effects on biological and chemical variables than the A treatment. This is verified by the fact that Cd_{tot} and Cd_{ext} were not the key variables in the formation of CCA. In contrast, the rise in humus pH, respiration and thymidine incorporation rates, and concentrations of Ca_{tot} and Ca_{ext} were the key phenomena that best explained the separation of treatments. In addition, the changes in microbial community structure along PC2 affected the formation of CCA, which was a result of both ash treatment and sampling occasion effects.

In general, concentrations of Cd in the leachates of different types of ash are low (Steenari et al. 1999; Richards et al. 2000; Ramesh and Kozinski 2001; Praharaj et al. 2002). A laboratory study showed that pH must be lower than 4.0 before Cd is released from wood ash (Hansen et al. 2001). Zhan et al. (1996) showed that at solution pH values lower than 6.0, which are normal rainwater values, there is a sharp increase in the amount of dissolved Cd from wood ash. The ACd and A treatments add 128 and 4.5 mg $Cd \cdot m^{-2}$ onto the forest floor, respectively, which theoretically could leach into the environment when the ash-induced pH effect stops. There are no investigations on the duration of the humus pH wood ash effect in the forest growing on mineral soil, but it must be a considerable time, since ash-induced effects on humus pH and microflora are still observed 18 years after fertilization (Perkiömäki and Fritze 2002).

One negative effect after wood ash application was the increase in NO_3-N concentration in the percolation water of the A treatment at 0.2 m depth. This rise in NO_3-N could be caused by an increase in nitrification rate that has been shown to be elevated at higher soil pH values (Khanna et al. 1994; Paavolainen and Smolander 1998; Priha and Smolander 1999). Also, Kahl et al. (1996) observed increased leaching of NO_3-N after wood ash application on sandy soil. This phenomenon was not observed with the ACd treatment and the reason for this is unclear. Increased NO_3-N after ash application is not a rule, since other wood ash studies have not detected it (Weber et al. 1985; Fransman and Nihlgård 1995; Eriksson 1998; Lundell et al. 2001).

Conclusions

It can be concluded that the natural Cd concentration of wood ash, which varies between 1 and 30 mg·kg ash^{-1}, is not harmful to the coniferous humus microflora, since even the much higher (28 times) Cd concentration of the spiked ash had no effect. The potential enrichment of Cd in the food chain is through concentration increment in mushrooms and berries. But this was only observed when Cd in the ash was increased to unnaturally high levels. Since wood ash adds Cd to the environment, we strongly recommend that the same sites should, if at all, be fertilized with wood ash only once.

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Paper IV

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Short and long-term effects of wood ash on the boreal forest humus microbial community

Jonna Perkiömäki*, Hannu Fritze

Vantaa Research Centre, Finnish Forest Research Institute, P.O. Box 18, FIN-01301 Vantaa, Finland

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Abstract

The short-term effects of loose and hardened wood ash on the coniferous forest humus layer microbes were studied 1–3 years after fertilization. The experiment was performed using two fertilization levels (3 and 9 t ash ha^{-1}) and repeated in two coniferous forest stands of different site fertility. It was hypothesized that the effects of hardened wood ash on soil microbes are of less magnitude when compared to loose ash due to the slower dissolution rates. The long-term effects of loose ash (3 t ash ha^{-1}) were studied in four forest stands of different site fertility 18 years after ash application. In order to study ash effects, the microbial activity (basal respiration- and thymidine incorporation rates) and microbial community structure (PLFA pattern) were determined. The results showed that irrespective of the forest site fertility, ash fertilization induced the same responses in the humus layer. It raised the microbial activity and changed the community structure. The changes were related to the dose and form of ash applied. Applying the same fertilization rate induced comparatively more changes to the loose wood ash sites than hardened wood ash sites, due to the detected slower dissolution of hardened ash into the humus. The effects of wood ash were long-term. Changes in the humus microbial activity and PLFA pattern were still detectable after 18 years.

Keywords: Hardened ash; Loose ash; Microbial activity; Microbial community structure; Phospholipid fatty acid; Respiration; Thymidine incorporation

1. Introduction

To compensate for nutrient losses and to reduce soil acidity in Finland, wood ash fertilization can be performed on mineral forest soils once every 30 years at a rate of 5 t ash ha^{-1}. Mineral soils cover 17 million hectares of the total 20 million hectares of Finnish forests, and with approximately 250–300 000 t of wood ash produced each year (Hytönen and Nurmi, 1997), so potentially 50–60 000 ha could be fertilized annually.

The effects of loose wood ash on soil microbes have been studied for only short time periods of 1–6 years from the fertilization. Earlier investigations have shown higher soil respiration (Bååth and Arnebrant, 1994; Fritze et al., 1994, 1995, 2000; Khanna et al., 1994) and thymidine incorporation rate (Bååth and Arnebrant, 1994; Bååth et al., 1995; Fritze et al., 2000) after wood ash application. Changes in microbial species composition following ash application have been shown using the phospholipid fatty acid (PLFA) technique to measure the microbial community structure (Frostegård et al., 1993a; Bååth et al., 1995; Fritze et al., 2000).

Field studies showed that the application of loose ash, which contains reactive oxides and hydroxides, cause damage to bryophytes (Kellner and Weibull, 1998; Moilanen and Issakainen, 2000; Jacobson and Gustafsson, 2001). Granulated or hardened ashes are less reactive and easier to handle during spreading. Granulation of ash helps to avoid rapid dissolution and therefore reduces any drastic pH effects (Eriksson, 1998a,b). During granulation, reactive oxides and hydroxides in the ash are converted to carbonates or other solid compounds. Kellner and Weibull (1998) have shown that the initial damage to mosses caused by loose ash was more severe than the initial damage caused by hardened ash. However, in contrast, it can be postulated that the effects of hardened ash can persist much longer than loose ash. Little is known about the long-term effects of ash on soil microbes due to the lack of experiments in forests growing on mineral soil. In addition, the effects of loose ash against the effects of hardened ash on soil microbes have never been compared.

There were two aims for this study. The first aim was to compare the effects of loose and hardened ashes on humus

* Corresponding author. Tel.: +358-9-857051; fax: +358-9-8572575.
E-mail address: jonna.perkiomaki@metla.fi (J. Perkiömäki).

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Table 1
Selected properties of the forest stands at the year of fertilization

Location	Site type	Humus layer (cm)	Main three species	Age
Muhos, Lummenlampi 64°43′N 26°02′E	ECT	3.6	Scots pine	ca. 60
Oulu, Sadinselkä 65°01′N 25°47′E	VMT	6.5	Scots pine	ca. 50
Keuruu, Vuorisjärvi 62°03′N 24°51′E	CT	1.8	Scots pine	5
Keuruu, Yltiä 62°16′N 24°20′E	VT	2.5	Scots pine	6
Janakkala, Harviala 61°00′N 24°45′E	MT	2.3	Scots pine	6
Janakkala, Harviala 61°02′N 24°39′E	OMT	1.6	Norway spruce	5

layer microbes 1–3 years after fertilization. We hypothesized that due to the slower dissolution of hardened ash the effects on soil microbes are of less magnitude when compared to loose ash. This experiment was performed using two application levels of the ashes and repeated in two forest stands of different fertility. The second aim was to study the long-term effects of loose ash in four forest stands of different site fertility 18 years after ash application. We assumed that if soil microbes still responded to the treatment then according to the hypothesis, the effects of hardened ash can be traced in nature for a long time period.

To test these hypotheses, we measured the basal respiration rate, the thymidine incorporation rate and the PLFA pattern. These have been proved to be very sensitive indicators in detecting the changes in microbes caused by wood ash fertilization.

2. Materials and methods

2.1. Study sites and sampling

2.1.1. Experiment 1

The study which compared loose and hardened wood ash at different fertilization levels was replicated in two forests with different site fertility: *Empetrum-Calluna* (ECT) and *Vaccinium-Myrtillus* (VMT) site types (Table 1). In these study areas, the ash was spread by hand with a spade evenly over the soil surface in May–July, 1997 using a randomized block design. The treatment in each of the four blocks were: control (C); loose wood ash at a fertilization rate of 3 t ha^{-1} (A3); loose wood ash at a fertilization rate of 9 t ha^{-1} (A9); and hardened wood ash, at the same fertilization level as for loose ash, (HA3 and HA9). The ash was collected from one company as fly ash. Hardened ash was made from fly ash by adding water to finally increase the ash weight by ca. 30%. After 1 month of hardening the ash was crushed and sieved to retain particles of 10–40 mm in size. The plot size was 30×30 m^2 and we sampled three of the four blocks. Humus samples were collected in August, 1998, June, 1999, and September, 2000.

2.1.2. Experiment 2

The long-term effects of loose wood ash were studied in four forest stands of different site fertility: *Calluna* (CT), *Vaccinium* (VT), *Myrtillus* (MT) and *Oxalis-Myrtillus* (OMT) forest site types (Table 1). In these study areas, the wood ash was spread by hand with spade evenly above soil surface in July–August, 1982 using a randomized block design. The treatments in each of the four blocks were: Control (C-18) and loose wood ash (A3-18) at an application rate of 3 t ha^{-1}. The number following the abbreviations C and A3 refer to the year of sampling after the start of the experiment. The plot size was 25×25 m^2. Humus samples were collected in September, 2000.

In both experiments, humus (the F/H layer) samples were taken using a soil corer (40 mm dia.). To obtain one composite sample per plot, seven cores from three lines were taken and the 21 cores from each plot were then combined. The composite samples were sieved (2.8 mm mesh), visible plant material was removed, and then stored at +4 °C for 1–5 weeks before analyses were conducted.

2.2. Physicochemical analyses

The chemical composition of the new ash (Moilanen and Issakainen, 2000) and old ash (Saarsalmi et al., 2001) were determined after HCl digestion by atomic absorption spectrophotometry (AAS, Table 2).

Humus samples from experiment 1 were analyzed using the techniques described by Tamminen and Starr (1990). Humus pH was measured in water (15 cm^3 humus + 25 ml water). Humus dry weight was determined after drying overnight at 105 °C. Organic matter (o.m.) was determined after furnacing samples at 550 °C for 4 h. Total organic carbon and nitrogen were determined by dry combustion (Leco CHN-600). For the nutrient analyses, humus was air dried for 48 h at 40 °C and then extracted with 1 M ammonium acetate (pH 7.0) using a soil/solution volume ratio of 1:10. The suspension was analyzed for Ca, K, Mg, and Al with an inductively coupled plasma emission spectrometer (ICP-AES, ARL 3580). The results are given in Table 3. For experiment 2 the humus nutrient data were obtained from Saarsalmi et al. (2001); Table 4.

2.3. Microbial analyses

The basal respiration rate was determined as the amount

Table 2
Approximate elemental contents of the ashes ($n = 1$) used in the experiments

Ash	Ca ($g\ kg^{-1}$)	K ($g\ kg^{-1}$)	Mg ($g\ kg^{-1}$)	Al ($g\ kg^{-1}$)	Fe ($g\ kg^{-1}$)	Mn ($g\ kg^{-1}$)	P ($g\ kg^{-1}$)	Zn ($mg\ kg^{-1}$)	B ($mg\ kg^{-1}$)	Cu ($mg\ kg^{-1}$)	Cr ($mg\ kg^{-1}$)	Ni ($mg\ kg^{-1}$)	Cd ($mg\ kg^{-1}$)
Loose new	351	28	18	8	11	10	9	2390	197	80	64	69	15
Hardened	323	25	19	10	10	9	9	2447	191	88	67	73	13
Loose old	250	42	23	nd	nd	16	13	376	nd	23	nd	nd	nd

nd: not determined.

of CO_2–C evolved in 23–26 h from field moist humus incubated at 14 °C (Pietikäinen and Fritze, 1995). Fresh humus samples, equaling 2 g dry weight, were used in the analyses. CO_2 was measured with a gas chromatograph (Hewlett Packard 6890) equipped with a TC detector and a Megapore GS-Q column (J&W Scientific). Injector, column, and detector temperatures were 120, 30, and 150 °C, respectively. He (5 ml min^{-1}) was used as a carrier gas.

The bacterial growth rate was determined using the [^{3}H]-thymidine incorporation technique as described by Bååth (1992) and modified by Kiikkilä et al. (2000). The bacterial growth rate (TdR) was measured by incorporating radioactive thymidine into the macromolecules of bacteria extracted from soil, after sample homogenization and centrifugation. The final results are expressed as mol TdR g^{-1} o.m. h^{-1}.

The extraction of PLFAs was conducted as by the technique described by Frostegård et al. (1993b). Fresh weight of 0.5 g of humus was extracted with 1.9 ml chloroform, 3.75 ml methanol and 2 ml Blight&Dyer mixture (Blight&Dyer contains chloroform/methanol/citrate buffer [0.15 M; pH 4] at volume ratios 1:2:0.8), and the lipids were separated into neutral lipids, glycolipids, and phospholipids in a silicic acid column. The phospholipids were subjected to mild alkaline methanolysis, and the fatty acid methyl esters were separated by gas chromatography (Hewlett Packard 5890) equipped with a flame ionization detector and a HP-5 (phenylmethyl silicone) capillary column, 50 m in length, using He (30 ml min^{-1}) as a carrier gas. The peak areas were quantified by adding methyl nonadecanoate fatty acid (19:0) as an internal standard.

The total amount of PLFAs, $PLFA_{tot}$ was used to indicate the total microbial biomass, and the sum of PLFAs was 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) and chosen as an index of bacterial biomass ($PLFA_{bact}$) (Frostegård and Bååth, 1996). The quantity of 18:2ω6 was used as an indicator of fungal biomass ($PLFA_{fung}$), as it is suggested to be mainly of fungal origin in soil (Federle, 1986) and is known to correlate well with the amount of ergosterol (Frostegård and Bååth, 1996). The ratio of $PLFA_{bact}/PLFA_{fung}$ was used as an index of the ratio of fungal/bacterial biomass in the soil.

2.4. Statistical analyses

The results are calculated on the basis of organic matter content (o.m.). Instead of performing ANOVA separately on each variable, a canonical correlation analysis (CCA) was used to form two combined variables. CCA generates pairs of linear combinations from two sets of original variables, so 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

Table 3
Treatment respective mean ($n = 3$) and standard error of chemical variables of the humus of the two forest stands (Experiment 1)

Site type and sampling year	Treatment	K	Mg	Al	o.m.	C/N
ECT 1998	C	460(38)	155(5.8)	23.5(5.0)	21.0(4.1)	43.6(1.7)
	A3	707(64)	776(124)	16.4(6.1)	24.0(6.8)	44.6(2.0)
	A9	867(79)	1381(254)	9.2(5.7)	24.6(7.7)	43.8(1.9)
	HA3	591(33)	241(21)	19.2(2.0)	22.6(4.2)	43.7(1.1)
	HA9	814(58)	403(32)	19.1(4.2)	22.8(3.6)	45.3(1.1)
1999	C	637(7.1)	187(17)	9.6(0.5)	40.3(4.4)	42.9(0.9)
	A3	834(47)	869(215)	bdl	34.0(7.7)	44.6(0.8)
	A9	872(92)	1496(154)	bdl	23.5(6.9)	42.7(0.6)
	HA3	687(43)	269(24)	8.2(4.1)	21.8(3.7)	43.4(1.6)
	HA9	814(75)	499(17)	1.9	46.6(4.2)	43.8(0.3)
2000	C	588(33)	172(3.2)	12.6(3.1)	88.8(2.3)	42.9(0.4)
	A3	584(22)	754(42)	bdl	83.5(1.8)	45.0(1.3)
	A9	573(22)	1008(92)	1.4	79.1(1.2)	41.4(1.5)
	HA3	625(34)	350(75)	11.6(1.4)	85.3(2.2)	42.8(1.2)
	HA9	696(35)	692(38)	6.06(0.1)	78.8(2.4)	43.7(1.9)
VMT 1998	C	816(52)	394(4.5)	10.1(1.9)	38.4(7.4)	37.5(3.1)
	A3	939(80)	736(90)	17.4(4.9)	41.9(11)	36.3(2.4)
	A9	1163(20)	1938(147)	bdl	55.5(11)	35.8(0.3)
	HA3	843(35)	495(63)	5.2(2.7)	48.6(9.5)	36.0(2.1)
	HA9	1319(199)	713(86)	12.4(3.2)	23.0(7.2)	39.6(5.1)
1999	C	924(10)	417(19)	6.4	47.8(1.8)	33.7(2.0)
	A3	959(63)	779(34)	2.5	70.7(4.3)	33.7(1.3)
	A9	1102(115)	1540(201)	bdl	55.3(8.8)	33.4(0.6)
	HA3	1060(35)	568(33)	3.3	53.5(8.5)	34.2(1.5)
	HA9	1258(65)	792(140)	5.0	45.0(5.1)	34.5(2.0)
2000	C	866(97)	348(7.5)	1.8	85.3(2.3)	37.0(1.5)
	A3	878(61)	717(21)	2.0	82.0(3.1)	37.0(0.9)
	A9	901(39)	1313(93)	bdl	74.5(5.9)	37.4(0.8)
	HA3	795(14)	509(45)	6.1(0.5)	79.7(5.3)	36.6(1.0)
	HA9	873(46)	889(14)	2.1	79.1(2.9)	37.5(0.9)

Nutrients are expressed as mg kg^{-1} per organic matter. Organic matter (o.m.) is given as % of dry matter. bdl, below detection limit. Detection limit for Al is 0.32 mg l^{-1} extraction liquid.

variables (Gittins, 1985). The chemical dataset consisted of: pH; extractable nutrients Ca, K, Mg, and Al; and the biological dataset of basal respiration, thymidine incorporation, $PLFA_{tot}$, $PLFA_{bact}$, $PLFA_{fung}$, and $PLFA_{bact}/PLFA_{fung}$. Two thymidine analysis samples (A9) from the first VMT sampling were excluded as outliers. 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). The canonical structure, i.e. correlations between the original variables and canonical variables, was applied to the figure with the vectors of the original variables indicating the influence of the most important original variables on the formation of the new canonical variable. The length of the vector indicates the strength of the correlation, and the direction indicates the direction in which the variable

Table 4
Treatment respective mean ($n = 4$) and standard error of chemical variables of the humus of the four forest stands (Experiment 2)

Site type	Treatment	K	Mg	Al	o.m.	C/N
CT	C-18	423(86)	167(50)	518(84)	37.1(4.4)	33.2(0.4)
	A3-18	384(100)	186(31)	32(7.8)	35.4(1.6)	33.8(0.8)
VT	C-18	531(95)	265(59)	224(72)	41.7(8.2)	29.1(2.0)
	A3-18	660(94)	356(44)	18(8.7)	27.5(1.7)	29.2(1.5)
MT	C-18	394(21)	254(7.6)	67(17)	25.0(2.9)	22.0(1.0)
	A3-18	573(47)	578(48)	0.3	24.7(1.8)	19.7(0.5)
OMT	C-18	355(47)	333(19)	28(10)	19.0(3.1)	20.4(0.4)
	A3-18	376(44)	391(31)	2.2(1.2)	16.8(0.4)	20.3(0.7)

Nutrients are expressed as mg kg^{-1} per organic matter. Organic matter (o.m.) is given as % of dry matter.

increases. A redundancy analysis, which can be seen as a part of the CCA, was used to determine the proportion of the variation the canonical variables explain in their own data set (Van den Wollenberg, 1977). Prior to the CCA test, the relationships between individual variables were examined by plotting the variables against each other. A log transformation was made for variable Al in order to make the relationships between the variables linear. The canonical correlation analyses were performed on SAS using the CANCORR procedure (SAS Institute, 1996). A two-way analysis of variance (ANOVA) followed by LSD test was performed for scores of the canonical variables CHEM and BIOL to detect the effect of the treatments (C, A3, A9, HA3, HA9, C-18, A3-18) and the forest site types (ECT, VMT, CT, VT, MT, OMT). ANOVA was performed both to the whole data and separately to each sampling occasion and experiments 1 and 2.

The PLFA pattern was explored with global nonmetric multidimensional scaling (MDS) procedure using the program package PC-ORD (McCune and Mefford, 1999), which measures the rank order of distances (Minchin, 1987). In experiment 1, the three sampling occasions resulted in 90 sample units and 34 variables (PLFAs). A total of five outlier samples, of which three were from the second sampling and two from the third sampling, were excluded from the experiment 1. In experiment 2, the sampling occasion resulted in 31 sample units and 39 variables. Prior to the MDS analyses, the mole percentages of the PLFAs were double-square root transformed ($y^{0.25}$) to down-weight the influence of very abundant PLFAs. The PC-ORD autopilot mode with medium thoroughness was used to compute the ordination.

The purpose of using MDS is to find a representation of the data in a limited number of dimensions, and to establish the ordination distances of the sample units that reflect the (dis)similarities between the respective PLFA patterns. The pairwise dissimilarities were computed using a Bray–Curtis coefficient, which is adopted widely in community-level studies (Clarke, 1999), and has been found to be a robust measure of quantitative dissimilarity (Faith et al., 1987). The scatter plot diagrams are a graphical presentation of MDS in the form of 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. Pearson correlation tests were used to evaluate the relationships between some individual variables.

3. Results

3.1. Physicochemical analyses

3.1.1. Experiment 1

The physicochemical analyses results of experiment 1 are presented in Table 3 and Fig. 1. Humus pH increased with time in the ash treated plots but remained more or less constants in the controls (Fig. 1). Application of ash increased pH in all treatments with the effect increasing with the rate of application and being greatest for the loose ash. Extractable Ca followed a similar pattern as pH, and was higher in amount on all ash-applied plots than on C plots, in the second and third samplings (Fig. 1). This rising trend of Ca towards the third sampling was most remarkable, as also for the pH, due to the A9 treatment. In the first sampling, the amount of extractable K varied between treatments, and was highest in the higher fertilization level plots, but the differences leveled out in the third sampling. The amount of extractable Mg was highest for the A9 plots during the whole experiment, although the amount had a decreasing trend. At the third sampling, all the ashed plots still had more Mg than the C plots. During the whole experiment, the loose ash treated plots had more Mg than hardened ash treated plots. The amount of extractable Al was highest during the first sampling. Due to the A9 treatment, Al concentrations decreased below the concentrations measured in the control plots. On the second and third sampling occasions, the loose ash treated plots had less Al than the hardened ash treated plots. The Al concentration did not decrease due to HA3 treatment, instead the HA3 plots had the same amounts of Al as the controls. There was no treatment related changes in the amount of organic matter and C/N ratio. The amounts of extractable K and Mg were higher in the VMT site type than in the ECT site type. In contrast, the ECT site type had more extractable Al than VMT site type.

3.1.2. Experiment 2

The physicochemical analyses results of experiment 2 are presented in Table 4 and Fig. 1. Humus pH was higher in the A3-18 plots than in the C-18 plots, and was higher in the higher fertility (OMT and MT) site types than in the lower fertility (CT and VT) site types (Fig. 1). In all four studied forest site types, the A3-18 plots had more extractable Ca than the C-18 plots (Fig. 1), the situation being opposite with the amount of extractable Al. The concentrations of extractable K and Mg increased due to ash treatment, but not notably, except in the MT site. The lower fertility site types had more Al than the higher fertility site types. In contrast, the amount of Mg was higher in the higher fertility site types than in the lower fertility site types. The amount of organic matter and C/N ratio were higher in the lower fertility site types than in the higher fertility sites, but there were no treatment related changes in them.

3.2. Microbial analyses

3.2.1. Experiment 1

The respiration rate and [^{3}H]-thymidine incorporation rate were higher in the A9 plots than in the other plots (Fig. 1). They were also higher in VMT than in ECT site. The increase in the respiration and thymidine incorporation rates

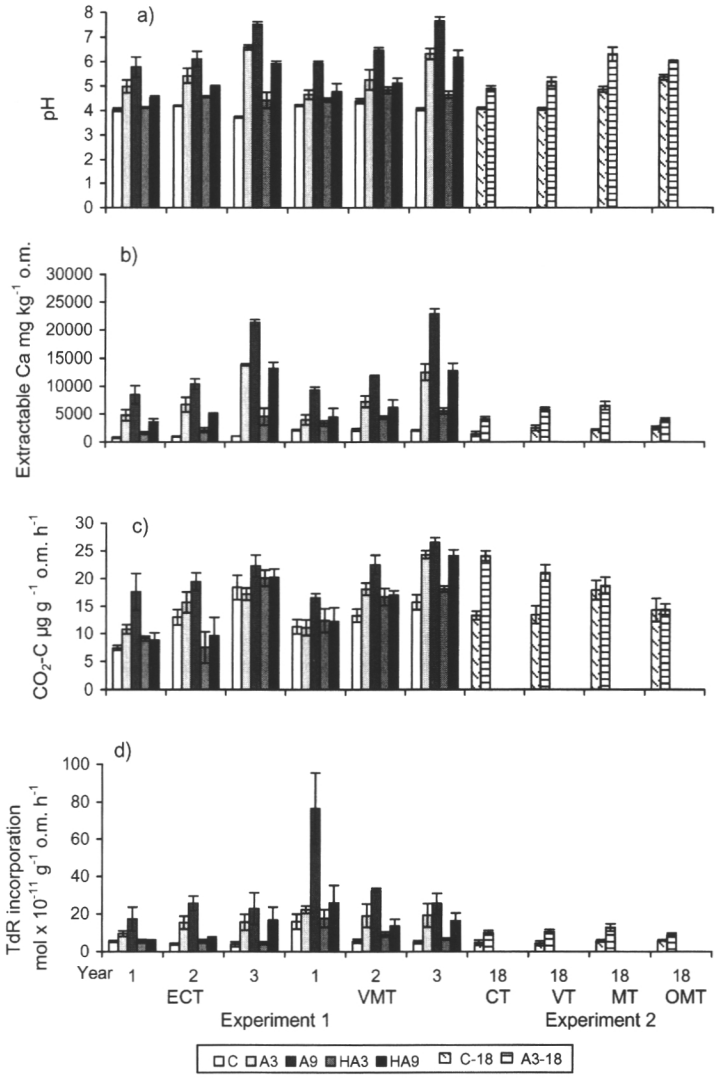

Fig. 1. The key variables measured from the humus layer of experiment 1 (ECT and VMT site types) and 2 (CT, VT, MT, and OMT site types); (a) pH, (b) extractable Ca, (c) respiration rate and (d) thymidine incorporation rate. The treatments for experiment 1 were control (C), loose wood ash at a fertilization rate of 3 and 9 t ha^{-1} (A3 and A9, respectively) and hardened wood ash (HA3 and HA9) at the same fertilization levels as for loose ash. The treatments for experiment 2 were Control (C-18) and loose wood ash (A3-18) at a fertilization rate of 3 t ha^{-1}. Year refers to the year of sampling after the start of the experiment. Bars show standard error of the means.

was clearer in the loose ash fertilized than in hardened ash fertilized plots, and more pronounced with the higher fertilization level. Both respiration ($r = 0.68$) and thymidine incorporation ($r = 0.66$) correlated with soil pH. There were no treatment effects in any of the biomass indicators for the first and second sampling occasions (Table 5). For the third sampling, $PLFA_{tot}$ and $PLFA_{fung}$ were lower in the loose ash treated plots than in the control and hardened ash treated plots. In addition, $PLFA_{bact}$ was lower in the A3 plots than in the controls, and the $PLFA_{fung}/PLFA_{bact}$ ratio was lower in the loose ash treated plots than in the hardened ash treated plots.

3.2.2. Experiment 2

The respiration rate was higher in A3-18 plots than in C-18 plots for the CT and VT sites types (Fig. 1). The [^{3}H]-thymidine incorporation was higher in all ashed plots compared to the control plots (Fig. 1). The thymidine incorporation correlated with soil pH ($r = 0.69$) but did not correlate with respiration. There was no clear treatment

Table 5
Treatment respective mean ($n = 3$) and standard error of biological variables of the two forest stands (Experiment 1)

Site type and sampling year	Treatment	$PLFA_{tot}$ (nmol g^{-1})	$PLFA_{bact}$ (nmol g^{-1})	$PLFA_{fung}$ (nmol g^{-1})	Fung/Bact
ECT 1998	C	2393(280)	693(85)	294(29)	0.428(0.01)
	A3	2322(218)	637(54)	346(54)	0.540(0.05)
	A9	2471(331)	734(63)	345(42)	0.469(0.04)
	HA3	2350(188)	637(57)	327(30)	0.514(0.007)
	HA9	2181(163)	599(56)	320(20)	0.539(0.03)
1999	C	1411(174)	410(44)	158(20)	0.386(0.03)
	A3	1409(192)	429(55)	167(19)	0.391(0.007)
	A9	1584(138)	472(37)	200(26)	0.420(0.02)
	HA3	1504(99)	460(43)	171(4.3)	0.376(0.03)
	HA9	1309(110)	401(36)	149(10)	0.373(0.01)
2000	C	1783(120)	480(37)	37327)	0.778(0.004)
	A3	1395(64)	379(16)	272(24)	0.718(0.04)
	A9	1429(67)	403(20)	258(29)	0.639(0.06)
	HA3	1689(117)	465(37)	35714)	0.778(0.08)
	HA9	1613(82)	431(30)	346(10)	0.807(0.03)
VMT 1998	C	2912(244)	871(74)	337(34)	0.386(0.007)
	A3	3322(255)	1093(130)	352(77)	0.346(0.11)
	A9	3035(375)	879(93)	402(64)	0.452(0.03)
	HA3	3067(320)	934(128)	363(22)	0.397(0.03)
	HA9	3894(172)	1179(13)	493(88)	0.420(0.08)
1999	C	3268(631)	943(229)	480(48)	0.544(0.08)
	A3	2419(189)	637(86)	421(13)	0.684(0.08)
	A9	2532(242)	707(59)	361(57)	0.504(0.04)
	HA3	2649(261)	702(74)	494(74)	0.710(0.10)
	HA9	3319(683)	855(142)	388(18)	0.542(0.02)
2000	C	1477(71)	429(31)	286(8.3)	0.677(0.07)
	A3	1363(186)	384(58)	287(19)	0.768(0.07)
	A9	1391(192)	414(67)	232(21)	0.590(0.11)
	HA3	1499(107)	416(42)	313(9.8)	0.770(0.09)
	HA9	1537(102)	422(47)	337(19)	0.822(0.12)

All results are expressed per gram organic matter.

effect in the $PLFA_{tot}$, $PLFA_{bact}$ or $PLFA_{fung}$ values. The $PLFA_{fung}/PLFA_{bact}$ ratio was a bit higher in the ashed plots (Table 6). This ratio correlated with the C/N ratio ($r = 0.60$) and was higher in CT than in the MT and OMT sites types.

3.3. Canonical correlation analysis

Applying the CCA, we diminished the large chemical and microbial data of both experiments to two variables, CHEM and BIOL (Fig. 2). The first canonical variable (CHEM) explained 59% 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 (28%) of the total variation in biological data set. The correlation between the first canonical variables CHEM and BIOL (canonical correlation) was 0.83 ($p < 0.0001$).

Table 6
Treatment respective mean ($n = 4$) and standard error of biological variables of four forest stands (Experiment 2)

Site type	Treatment	$PLFA_{tot}$ (nmol g^{-1})	$PLFA_{bact}$ (nmol g^{-1})	$PLFA_{fung}$ (nmol g^{-1})	Fung/Bact
CT	C-18	3868(1146)	1186(372)	567(158)	0.497(0.06)
	A3-18	2246(77)	690(31)	395(28)	0.576(0.05)
VT	C-18	2227(273)	761(97)	300(16)	0.401(0.03)
	A3-18	2586(115)	892(46)	366(35)	0.408(0.04)
MT	C-18	2512(262)	917(91)	228(30)	0.247(0.01)
	A3-18	2250(81)	820(30)	260(22)	0.316(0.02)
OMT	C-18	2515(145)	957(42)	249(20)	0.260(0.02)
	A3-18	2818(397)	928(72)	408(132)	0.421(0.10)

All results are expressed per gram organic matter.

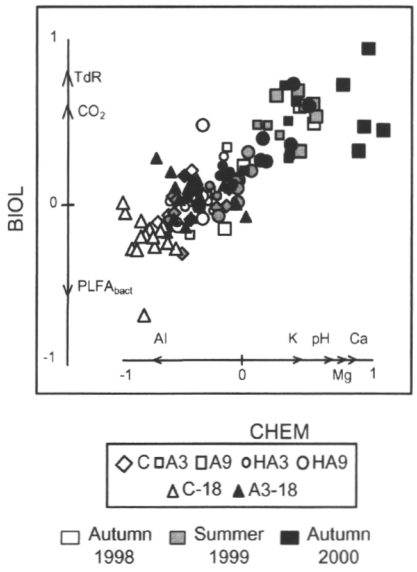

Fig. 2. Plot of the samples of the experiment 1 and 2 on the first canonical variables, CHEM (the chemical data set) and BIOL (biological data set) from CCA. The vectors indicate the correlation between the original variable and the canonical variable in question. See Fig. 1 for the abbreviations used.

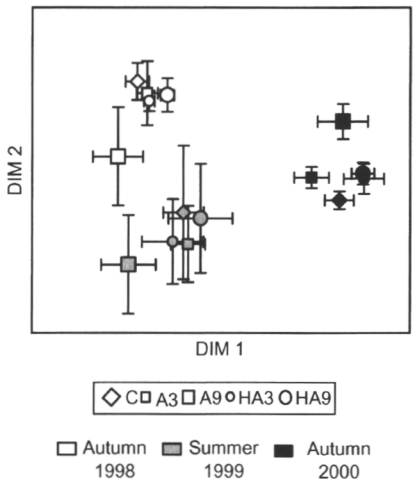

Fig. 3. MDS ordination of the samples of experiment 1. Symbols indicate the mean of three replicate and bars indicate the standard error of the replicates. See Fig. 1 for the abbreviations used.

In the CCA, the A9 plots of the second and third samplings are mostly situated in the right upper corner and according to the canonical structure are characterized by high pH and base cation (Ca, K, Mg) concentrations, low Al concentrations, and high thymidine incorporation and respiration rates. The HA9 and A3 plots of the third sampling are also situated in the right upper corner. The control plots of experiment 1 (C) and experiment 2 (C-18) are characterized with a lower base cation concentration, pH and lower thymidine incorporation and respiration rates. In addition to the control plots, the HA3 and A3 plots of the first sampling are also situated in the left bottom corner.

ANOVA followed by a LSD test ($p < 0.05$) was performed to the scores of the canonical variables CHEM and BIOL of both experiments. The LSD test divided the treatments into four groups. The treatments A9 and C-18 formed their own groups. The treatments A3 and HA9 clustered into same group and the treatments HA3, A3-18 and C into another group. The scores of variable CHEM did not differ between forest site types ECT and VMT in experiment 1, whereas the scores of variable BIOL did (data not shown). The scores of CHEM and BIOL clustered forest site types CT, VT, MT, and OMT into same group, and were separated from the experiment 1 forest site types (data not shown).

Separate ANOVA was performed to variables CHEM and BIOL for both experiments and each sampling occasion of experiment 1. The following results were obtained. In the first sampling, A9 was the only treatment, which differed from other treatments along the CHEM axis, and there were no treatment effects for scores along the BIOL axis. In the second sampling, variable CHEM clustered treatments A9 and A3, A3 and HA9, HA9 and HA3, and, HA3 and C in the two treatments groups. Variable BIOL produced the same results as variable CHEM but did not separate HA9 into a different group than C. For the third sampling, A9 was the treatment that differed from treatment HA3 along the CHEM axis and differed from treatment C along the BIOL axis. Two forest types in the experiment 1 differed from each other along CMEM axis only in the second sampling. In experiment 2, variables CHEM and BIOL separated treatments A3-18 and C-18 into different groups. The MT and VT sites were separated from CT site along the CHEM axis and the OMT site was classified into the same group with all the other forest site types.

3.4. Structure of microbial community (PLFA pattern)

3.4.1. Experiment 1

The PLFAs from all the samplings and the two forest site types were subjected to multidimensional scaling procedure (MDS). The minimum MDS ordination stress value of 0.13 (autopilot mode in program PC-ORD) was obtained by a two-dimensional solution for experiment 1. The MDS formed three groups, which indicates that the PLFA pattern is different between the sampling occasions (Fig. 3). Within the groups, the PLFA pattern of the A9 plots differed from the other treatments for every sampling occasion, although the differences were not so clear on the first two samplings. The A3 plots differed from HA3 and HA9 plots along DIM 1, for the last sampling. The PLFA pattern of the two forest site types ECT and VMT differed from each other only for the first two samplings (data not shown). DIM 1 correlated

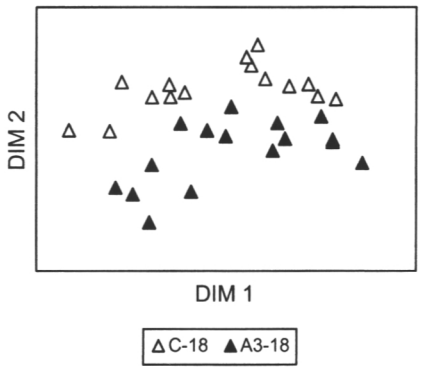

Fig. 4. MDS ordination of the samples of experiment 2. See Fig. 1 for the abbreviations used. The number following the abbreviations C and A3 refer to the year of sampling after the start of the experiment.

with the respiration rate ($r = -0.61$), $PLFA_{fung}/PLFA_{bact}$ ratio ($r = -0.82$) and o.m. ($r = -0.66$). No significant correlations were obtained for DIM 2.

When each sampling was studied separately there were no correlations between either dimensions or environmental variables of first sampling. Instead, DIM 1 correlated with $PLFA_{fung}/PLFA_{bact}$ ratio ($r = -0.76$), and there is a correlation between o.m. ($r = 0.57$) and C/N ratio ($r = 0.77$) with DIM 2, for the second sampling. In addition, DIM 1 correlated with the $PLFA_{fung}/PLFA_{bact}$ ratio ($r = -0.57$), and there is correlation between the $PLFA_{fung}/PLFA_{bact}$ ratio ($r = 0.61$), Ca ($r = -0.67$), Mg ($r = -0.60$), o.m. ($r = 0.51$) and pH ($r = -0.64$) with DIM 2, for the third sampling.

3.4.2. Experiment 2

For experiment 2, a two-dimensional solution was selected for MDS ordination and the minimum stress value obtained was 0.12 (Fig. 4). The PLFA pattern differed between treatments C-18 and A3-18 along DIM 2, which correlated with the $PLFA_{fung}/PLFA_{bact}$ ratio ($r = -0.70$). DIM 1 separated CT and VT forest site types on the left from the MT and OMT forest site types on the right (data not shown). DIM 2 also separated CT site types from the others. DIM 1 correlated with the $PLFA_{fung}/PLFA_{bact}$ ratio ($r = -0.80$), and with the following environmental variables pH ($r = 0.70$), C/N ratio ($r = -0.79$), Mg ($r = 0.71$) and Al ($r = -0.62$).

4. Discussion

All the forest site types reacted similarly with respect to the response of humus chemistry, microbial activity and community structure to wood ash fertilization. This implies that ash fertilization overrules the biological variation detected between forest site types of coniferous forests (Pennanen et al., 1999). In this study, we could verify all results obtained by Pennanen et al. (1999) by analyzing only the results of the control plots, and from now on, only the wood ash fertilization effects are discussed.

In wood ash, K is the most soluble nutrient, followed by Ca and Mg (Khanna et al., 1994; Kahl et al., 1996; Holmberg et al., 2000). As K is weakly adsorbed in organic soil, most of the liberated amounts are quickly leached from the humus layer of a coniferous forest (Eriksson, 1998b). In contrast, Ca and Mg are concentrated in the humus layer. Our experiment compared two forms, loose and hardened wood ash (abbreviations A and HA, respectively) at two fertilization levels (3 and 9 t ha^{-1}; these numbers follow the abbreviations), and confirm these results. Our results showed decreased extractable Al, increased pH and exchangeable base cations in humus after ash amendment, and these are phenomena that have been reported several times in the literature (Unger and Fernandez, 1990; Khanna et al., 1994; Bramryd and Fransman, 1995; Kahl et al., 1996; Eriksson, 1998a). The rate of their appearance in the humus was dependent on the level and form of ash applied. This complies with the literature, where the hardening of wood ash changes its mineral composition (Steenari et al., 1998; Steenari et al., 1999; Holmberg and Claesson, 2001); and the most important reaction during wetted loose ash agglomeration is the transformation of $Ca(OH)_2$ into $CaCO_3$, which lowers the calcium leaching rate (Steenari et al., 1999). The lowered leaching rate of base cations due to hardening has also been confirmed in other laboratory studies (Eriksson, 1998; Ring et al., 1999).

In our experiment, the increase in humus Ca and Mg was dependent on the rate and form of the ash applied with A9 treatment having the highest values, followed by HA9 and A3, and then by the HA3 treatment. The loose ash dissolved faster into the humus than the hardened ash. The amount of Ca in the humus can therefore be used to test the experimental hypothesis—the effects of hardened ash on soil microbes are of less magnitude when compared to loose ash due to the slower dissolution rate

Both the multivariate statistical approaches supported the experimental hypothesis. The canonical correlation analyses (CCA) separated the treatments according to the form and level of ash used (Fig. 2). The vectors that best explained the separation of the treatments were increases in humus bacterial growth rate, microbial respiration activity, and Ca concentration. The multidimensional scaling (MDS) procedure separated the A9 treatment from the other treatments from the first year onwards (Fig. 3). The MDS used the PLFAs eluted from the treatment plots. A change in PLFA pattern indicated a change in microbial, especially the bacterial, community structure. In the third year, the HA9 and the A3 treatments started to separate from the control and remained separated from the A9 treatment.

The second aim of the study was to give an estimate for the duration of the ash effect. Thirteen years after wood ash application to a Scots pine plantation on peat tree growth disturbance and mortality decreased and height growth and

stem volume production increased due ash application (Ferm et al., 1992) implying a more effective nutrient cycling after ash fertilization. The fact that the PLFA pattern was still different from the control (Fig. 4) after 18 years of an ash fertilization, which equals the form and dose of A3, points toward a very long-term effect of wood ash on soil microbes. The CCA also separated the old ash plots according to the same variables humus bacterial growth rate, microbial respiration activity, and Ca concentration as told earlier (Fig. 2). These estimations are an improvement on what is known on the duration of ash effect in coniferous forests. The oldest documented ash fertilization trial in Finland was performed in a forest growing on peat and therefore not directly comparable to our trials. This experiment showed an elevated pH after 40 years of fertilization with loose ash (Silfverberg and Huikari, 1985; Silfverberg and Hotanen, 1989). Since, the pH was also an important discriminating variable in the CCA (Fig. 2), one can speculate that the biological effects of loose ash can persist for at least 40 years, and the effects of hardened ash can persist much longer.

There seems to be no concise trend in the relation between wood ash fertilization and PLFA derived humus biomass measures $PLFA_{tot}$, $PLFA_{fung}$, $PLFA_{bact}$ or the $PLFA_{fung}/PLFA_{bact}$ ratio. At a fertilization rate of 5 t ash ha^{-1}, no (Frostegård et al., 1993a) or a decreasing effect (Bååth et al., 1995; Fritze et al., 2000) were reported on these biomass values. In this study, the loose ash treatment, at a dose of 9 t ha^{-1} lowered the humus $PLFA_{tot}$ and $PLFA_{fung}$ amounts for the third sampling. The $PLFA_{fung}/PLFA_{bact}$ ratio was lower in loose ash treated plots than in hardened ash treated plots, but neither of them differed remarkably from controls. There were no clear long-termed ash effects for any biomass indicators, except that the $PLFA_{fung}/PLFA_{bact}$ ratio was a bit higher in ash treated plots. The reason for the strong effect of $PLFA_{bact}$ on the CCA (Fig. 2) was not a treatment effect, rather than the decreasing trend of this biomass during the experiment.

The application of an ash cover stimulated the soil respiration and the [^{3}H]-thymidine incorporation rates, and both correlated with soil pH. These results are in accordance with earlier investigations where higher soil respiration (Bååth and Arnebrant, 1994; Fritze et al., 1994, 1995, 2000; Khanna et al., 1994) or thymidine incorporation rate (Bååth and Arnebrant, 1994; Bååth et al., 1995; Fritze et al., 2000), were detected following ash fertilization. However, some of the soil respiration might be attributed to ash cover, which could have contained organic carbon due to incomplete combustion (Khanna et al., 1994). But nevertheless, the ash stimulated respiration occurred most in soils with a high organic matter content, and this suggests that much of the respired C was derived from soil sources rather than the C added in the ash (Khanna et al., 1994). In addition, Weber et al. (1985) measured increased water-soluble organic-C (DOC) after ash fertilization. Ludwig et al. (2000) measured a manifold increase in the production of DOC following the addition of wood ash to the forest floor. The DOC originated from the humus and not from the straw added with the ash. The DOC could also act as source of carbon for microbes explaining the rise in respiration and thymidine incorporation.

The application of ash to coniferous forest soils has resulted in changes in microbial species composition in studies that have applied the PLFA technique (Frostegård et al., 1993a; Bååth et al., 1995; Fritze et al., 2000). A correlation between soil pH and a change in bacterial PLFA composition was observed by Frostegård et al. (1993a). The change in humus quality with increasing ash fertilization levels was measured using infrared spectroscopy by Bååth et al. (1995). These authors showed that the humus quality changed but were only partly successful in their attempts to correlate the changes in the PLFA pattern with soil pH or substrate quality with multivariate statistics. Consequently, they hypothesized that changes in the PLFA pattern of the soil organisms were related to an altered substrate quantity, that is the availability of substrates after treatments. The increase in DOC content of the soil water following ash fertilization could reflect that change in humus quality.

5. Conclusions

In this study we have shown that the application of wood ash onto the forest floor of coniferous forests changed the microbial activity and community structure. The changes were related to the dose and form of ash applied. At the same application rate, loose wood ash induced comparatively more changes than hardened wood ash due to the slower dissolution of hardened ash into the humus. The effects of wood ash appeared to be long term since 3 t ash ha^{-1} continued to induce changes in the soil microflora after 18 years of application. If these long-term changes in microbial activity and community structure affect the decomposition of needle litter and therefore the forest nutrient cycling is investigated in a current study.

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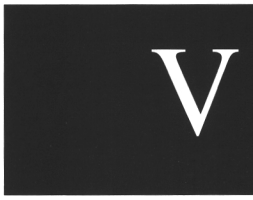
Paper V

Perkiömäki J., Levula T. and Fritze H. 2004. A reciprocal decomposition experiment of Scots pine needles 19 yr after wood ash fertilization. Soil Biology & Biochemistry (in press; http://dx.doi.org/10.1016/j.soilbio.2003.11.008).



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Short Communication

A reciprocal decomposition experiment of Scots pine needles 19 yr after wood ash fertilization

Jonna Perkiömäki[a,*], Teuvo Levula[b], Hannu Fritze[a]

[a]*Finnish Forest Research Institute, Vantaa Research Centre, P.O. Box 18, Vantaa 01301, Finland*
[b]*Finnish Forest Research Institute, Kaironiementie 54, Parkano 39700, Finland*

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Abstract

Scots pine (*Pinus sylvestris*) needle litter originating from control plots and plots that had received a wood ash fertilization (3 t ha^{-1}) 19 yr earlier were allowed to decompose in a reciprocal experimental design to detect the effects of ash fertilization and needle litter origin on the decomposition rate. The experimental design was repeated in two Scots pine forest stands of different fertility and the litterbags were harvested after 4 and 16 months. Ash fertilization resulted in a higher needle litter decomposition rate but the needle origin did not influence the results. Stand fertility correlated positively to the decomposition rate.

Keywords: Fertilization; Litter decomposition; *Pinus sylvestris*; Stem volume; Wood ash

A higher humus layer pH and microbial activity (basal respiration rate), and changed microbial community structure (phospholipid fatty acid, PLFA pattern) have been observed as long as 18 yr after wood ash fertilization (Perkiömäki and Fritze, 2002). The decomposition rate of needle litter depends primarily on microbial activity and therefore also on factors controlling microbial activity such as the chemical composition of the needles. In a field study we aimed to examine if the wood ash treatment: (1) is reflected in the decomposition rate of Scots pine needle litter and (2) affected the quality of the Scots pine needle litter, which is then reflected in the decomposition rate. To achieve these objectives the layout of our experiment was as follows: Scots pine (*Pinus sylvestris*) needle litter samples from control plots and plots that had been fertilized with wood ash (3 t ha^{-1}) 19 yr earlier were exposed in a reciprocal experimental design to detect the effects of ash fertilization and needle litter origin on the decomposition rate (Fig. 1). The experimental design was repeated in two Scots pine forest stands of different fertility and the litterbags were harvested 4 and 16 months after the start of the experiment.

Scots pine needles (Table 1) were obtained in October 2000 by shaking branches and collecting the litter on tarpaulins spread under the trees of two upland forest site types, *Calluna* (CT) and *Vaccinium* (VT). Both experimental areas had four control and four wood ash treated (fertilization rate 3 t ha^{-1}) plots. Wood ash contained in total amounts P 13, K 42, Ca 250, Mg 23, Mn 16, Cu 0.023 and Zn 0.38 g kg^{-1} on a dry matter basis (Saarsalmi et al., 2001). Both sites were situated in southern Finland, in Keuruu, Vuorisjärvi (CT: 62°03′N, 24°51′E) and Yltiä (VT: 62°16′N, 24°20′E). At the time of ash fertilization in 1982 the trees of the CT and VT sites were 5 and 6 yr old, respectively.

The needles were dried at 40 °C and 1 g was weighed into polyester net bags (80 × 80 mm^2, mesh size about 1.0 × 0.5 mm^2). In May 2001 litterbags originating from control plots were placed onto the litter layer of both control (CC) and ash (CA) plots (total 32 bags per plot × 8 plots = 256 bags). In addition also 256 bags of needles originating from ash-fertilized plots were placed in the same way on control (AC) and ash (AA) plots (Fig. 1). This made a total of 1024 litterbags of which 512 were placed in the CT and VT site type forest stands, respectively. The samplings were performed in the September of 2001 and 2002. At both dates 16 control and 16 ash litterbags were sampled from

* Corresponding author. Tel.: +358-10 010 2111; fax: +358-10 211 2206.
E-mail address: jonna.perkiomaki@metla.fi (J. Perkiömäki).

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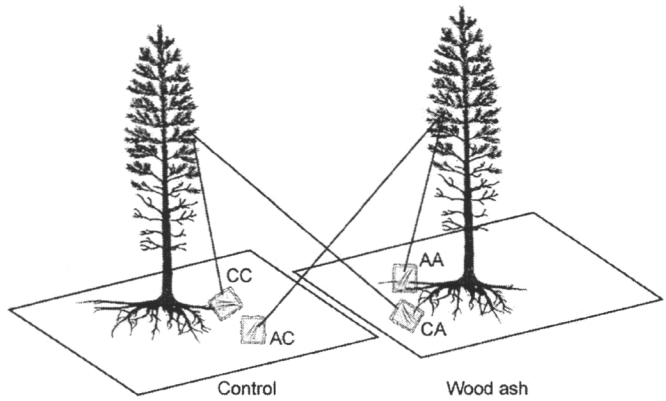

Fig. 1. The layout of the reciprocal needle litter field exposure. The first symbol (C or A) refers to the plot of needle origin, and second (C or A) to the plot where the samples were exposed.

each of four control and ash plots, transported to the laboratory, and cleaned of root and soil remnants. To determine the mass loss, the remaining contents were weighed individually after drying at 40 °C. The needle litter of the 16 replicate bags was combined before C and N were determined by dry combustion (Leco CHN-600). The background information of humus layer chemistry and stem volume of trees after wood ash fertilization is presented in Table 2. According to Berg and Söderström (1979) the amount of N in needles is expressed in two ways. The relative amount expresses the N in the remaining needle litter mass and the absolute amount expresses the N in the initial needle mass. Also both relative and absolute needle litter C are presented.

The results were subjected to analysis of variance (ANOVA) followed by Tukey's test ($P < 0.05$) for comparison of means. Four main effects and their interactions were tested. The main effects were the origin of needle litter, the site of field exposure, forest site type and sampling date. If significant interactions were found (needle N concentration and C-to-N ratio), then the effect of sampling date was tested separately with a one-way ANOVA.

The mass loss of needles was significantly larger on ash-treated than on control plots (Fig. 2a). This result gave a clear answer to our first aim: the increased microbial activity and the changed microbial community structure of wood ash fertilized coniferous forest stands is reflected in an enhanced decomposition rate of needle litter at these sites. On drained peatland sites, Silfverberg and Hotanen (1989) have observed the same kind of long-term ash effects on the needle decomposition rate. Following wood ash fertilization increased soil respiration rates have often been measured in laboratory conditions (Bååth and Arnebrant, 1994; Fritze et al., 1994, 1995, 2000; Khanna et al., 1994; Perkiömäki and Fritze, 2002; Zimmermann and Frey, 2002) but seldom in the field (Zimmermann and Frey, 2002). Here we showed

Table 1
Initial concentrations of C and N and their ratio in the CT and VT site needles

	Origin[a]	CT	VT
C	C	53.9	53.3
	A	54.1	53.1
N	C	0.37	0.45
	A	0.43	0.50
C-to-N ratio	C	145.7	118.4
	A	125.8	106.2

[a] C (control) and A (ashed) refer to the plots of origin of needle litter. C and N are given as % of dry matter.

Table 2
Humus layer properties and stem volume of trees of C (control) and A (wood ash) plots in CT and VT sites expressed as means ± S.E. ($n = 4$)

	Treatment	CT	VT
CEC[a]	C	110 (12)	118 (11)
	A	169 (13)	223 (14)
BS[a]	C	49 (3)	76 (4)
	A	95 (1)	95 (1)
C-to-N ratio[a]	C	33 (0.4)	27 (2)
	A	34 (1)	29 (2)
N[a]	C	6.9 (0.9)	8.7 (0.6)
	A	5.6 (0.8)	7.4 (0.9)
P[a]	C	0.58 (0.04)	0.66 (0.04)
	A	0.81 (0.05)	0.70 (0.08)
pH[b]	C	4.1 (0.04)	4.1 (0.1)
	A	4.9 (0.1)	5.2 (0.2)
o.m[b]	C	37 (4)	42 (8)
	A	35 (2)	27 (2)
Stem V[c]	C	18 (1)	91 (7)
	A	31 (1)	105 (3)

Cation exchange capacity (CEC) is given in mmol kg^{-1} o.m. and base saturation (BS) as % of Ca, Mg, K and Na of CEC. N and P are expressed as g kg^{-1} o.m. Organic matter (o.m.) is given as % of dry matter. Stem volume (V) of trees is given in m^3 ha^{-1}.

[a] 16 (Saarsalmi et al., 2001).

[b] 18 (Perkiömäki and Fritze, 2002).

[c] 16 yr after ash fertilization.

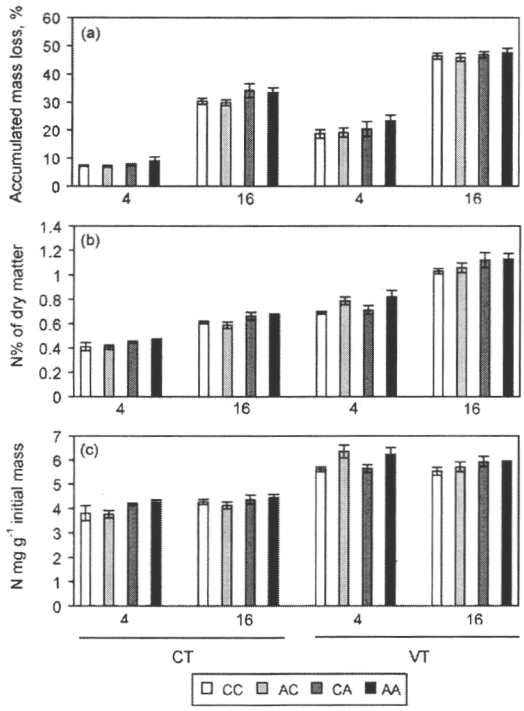

Fig. 2. Accumulated mass loss (% initial mass) of needle litter (a), the amount of N in needles as % of dry matter (relative to remaining needle mass) (b) and the amount of N as absolute to initial needle mass (c) in CT and VT site types. The numbers 4 and 16 refers to the exposure time in months. Values are means ± SE of four plots. For explanation of treatment symbols, see Fig. 1.

that the decomposition rate of needle litter in the field was increased following wood ash fertilization. Therefore respiration rates, measured in laboratory conditions are reflecting the mineralization rate of organic matter, as Prescott and Parkinson (1985) have also shown. Overall, decomposition was faster in the more fertile VT than CT sites.

The ash fertilization induced quality change can be reflected in many biochemical and chemical fractions of the needle litter tissue. In both forest sites the needles originating from the ash-fertilized plots had a lower C-to-N ratio (Table 1). Our second aim of interest the origin, and thus the quality of needles, did not significantly influence the decomposition rate although ash needles exposed on ash plots (AA) had a slightly higher decomposition rate than control needles exposed on ash plots (CA) during the first four months.

According to Berg (1986), the decomposition of Scots pine needle litter can be divided into at least two phases. In the early phase (comparable to our study) high amounts of nutrients (e.g. N, P, S) enhance the mass loss rate of non-lignified compounds. In the late phase the lignin fraction becomes rate controlling. High N concentration is an important factor, which retards decomposition rate of lignin (Berg, 1986). After four months the needles originating from ash-treated plots had more N (both relative and absolute N) than control plot needles and this effect was clearer in the VT site (Fig. 2b and c). The field plot of exposure did not significantly affect this result although in the CT site the amount of needle N (both relative and absolute N) was slightly higher when exposed on ash plots compared to control plots. After 16 months the needles exposed on ash plots had significantly higher amounts of N (both relative and absolute N) than those exposed on control plots.

By comparing the initial amount of N (Table 1) and absolute amount of N in needles after four months field exposure (Fig. 2c) one can see that in the CT site N was released from ash needles exposed on control plots (AC). As Berg and Söderström (1979) we also observed an increase for N in decomposing Scots pine needle litter between 4 and 16 months in the CT site. According to them, fungi played a crucial role in this N accumulation process probably by importing N from the surrounding and the net release of N does not begin before the net release of lignin begins (Berg and McClaugherty, 1989). During the first four months VT site needles accumulated N, but after that no accumulation was seen. During the whole time of exposure VT site needles had more N (both relative and absolute N) than CT site needles. Ash fertilization and litter origin did not affect the relative amount of needle C, but needles exposed on control plots had more absolute C (Table 3). This indicates that on the ash plots more C is released from needles

Table 3
C and C-to-N ratio of needles expressed as means ± S.E. ($n = 4$)

Site	Months of incubation	Treatment	C relative	C absolute[a]	C-to-N ratio[a]
CT	4	CC	53.8 (0.1)	498.3 (0.8)	133.5 (9.8)
		AC	53.6 (0.2)	498.0 (2.0)	132.9 (4.9)
		CA	53.6 (0.1)	495.5 (1.2)	119.2 (1.8)
		AA	54.1 (0.1)	492.0 (6.8)	115.8 (2.3)
	16	CC	54.2 (0.2)	377.2 (6.6)	88.5 (1.6)
		AC	54.5 (0.2)	382.3 (5.6)	93.0 (4.5)
		CA	54.4 (0.03)	357.6 (13)	82.2 (3.5)
		AA	54.4 (0.2)	361.8 (9.4)	81.3 (1.5)
VT	4	CC	54.2 (0.1)	440.4 (7.5)	78.5 (1.3)
		AC	54.1 (0.2)	437.4 (7.3)	69.0 (2.5)
		CA	54.3 (0.3)	431.5 (12)	76.6 (3.3)
		AA	54.6 (0.3)	418.6 (9.8)	67.7 (4.7)
	16	CC	54.9 (0.4)	294.9 (6.5)	53.4 (1.2)
		AC	55.1 (0.1)	298.0 (7.9)	52.3 (1.9)
		CA	54.8 (0.2)	291.0 (6.0)	49.3 (2.5)
		AA	54.5 (0.2)	285.9 (9.0)	48.6 (2.0)

[a] Means are different between exposing plots C and A (Tukey, $P < 0.05$). Means of underlined variables are significantly different between both two forest sites and incubation times. For explanation of treatment symbols, see Fig. 1. C relative is given as % of dry matter (d.m.) and absolute as mg C g^{-1} dry matter of initial needle mass.

and this increased C mineralization is also seen as an increase in soil respiration rate. As a result of these N and C dynamics, the C-to-N ratio was higher in the needles exposed on control plots and it decreased during the exposure period.

Stem volume of trees increased due to ash fertilization (Table 2). The enhancing effect of ash fertilization on both needle mass loss and tree growth was more pronounced in CT than VT site. This was accompanied with the rise in amounts of humus layer total P (Table 2). P deficiency occurs, in addition to N, in Finnish upland forests (Raitio et al., 2000). The amount of humus layer N did not respond to wood ash fertilization (Table 2). Usually the effect of wood ash fertilization on tree growth on upland coniferous forest soils has been insignificant (Saarsalmi and Mälkönen, 2001) or minor when the humus layer C-to-N ratio was below 30 (Nohrstedt, 2001).

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Paper VI

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VI

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Does simulated acid rain increase the leaching of cadmium from wood ash to toxic levels to coniferous forest humus microbes?

Jonna Perkiömäki, Hannu Fritze *

Finnish Forest Research Institute, Vantaa Research Center, PO Box 18, 01301 Vantaa, Finland

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Abstract

Wood ash contains Cd in concentrations not permitted for fertilization use in agriculture (>3 mg kg^{-1}). It has been shown that spiking ash with Cd to concentrations of 1000 mg kg^{-1} induced no further changes in humus microbial activity and community structure as ash alone. To accelerate the weathering process and thus to liberate the spiked Cd from the ash, three treatments – wood ash (A), Cd spiked wood ash (ACd, 1000 mg Cd kg^{-1} ash), both applied at a fertilization rate of 5000 kg ha^{-1}, together with a control (C) – were performed in microcosms and incubated in field condition under two types of irrigation – water and simulated acid rain. During the incubation period of one growing season the simulated acid rain plots received a sulfur load of 3.64 g S m^{-2}, which was 15 times more than the S deposition on the water irrigated plots. The treatments resulted in a mean Cd increase of the humus from 0.23 mg kg^{-1} of the C treatment to 0.52 and 39.5 mg kg^{-1} of the A and ACd treatments, respectively. The irrigation had no further effect on the result. The microbial activity, measured as soil basal respiration, and the microbial community structure, measured as humus phospholipid fatty acid and 16S and 18S polymerase chain reaction/denaturing gradient gel electrophoresis patterns, changed only due to the ash (A and ACd treatments) fertilization irrespective of the irrigation. The bacterial biosensor, emitting light in the presence of bioavailable Cd, did not react to any of the treatments. This result shows that Cd in ash was not leached into the humus due to increased deposition of acidified rain.

Keywords: Cadmium; Denaturing gradient gel electrophoresis profiling; Humus; Multivariate analysis; Phospholipid fatty acid; Wood ash

1. Introduction

During the last decade, there has been increasing interest in using logging residues for bioenergy in Scandinavia. The use of such residues for energy production generates large amounts of wood ash. Intensive harvesting increases nutrient export and soil acidification [1] and therefore it has been suggested to recycle the nutrients contained in the wood ash. Due to improved technology in cleaning exhausts from power plants the resulting fly ash, originating from bark or stems, has become enriched with heavy metals. The use of this wood ash in forest fertilization has therefore been questioned on the ground of the cadmium (Cd) concentration of the ash, which varies between 1 and 30 mg kg^{-1} ash [2], thereby exceeding the level allowed for fertilizers (3 mg kg^{-1}) used in agriculture. Therefore the impact of this ash on the forest ecosystem has to be investigated.

Humus microbes, being responsible for the mineralization of organic material, play an important role in the functioning of the forest ecosystem. Cd has been shown to inhibit forest soil microbial activity and to change the microbial community structure already at very low levels with the range from 1 to 5 mg Cd kg^{-1} humus being the lowest toxicity inducing values reported for heavy metals in general [3]. In a recent microcosm study we could show that wood ash added onto the humus layer as top dressing and artificially enriched with Cd up to a level of 1000 mg kg^{-1} ash induced the same changes to the humus microflora than unspiked ash addition itself. Ash thus protected the microflora from the harmful effects of Cd since the same amount of Cd added onto the humus layer without ash decreased the soil respiration and changed the bacterial community structure [4]. Furthermore Cd was bioavailable only when ash was not added to the microcosm [5]. This is due to the fact that ash fertilization increases

* Corresponding author. Tel.: +358 (9) 85705407; Fax: +358 (9) 8572575.
E-mail address: hannu.fritze@metla.fi (H. Fritze).

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the pH of the humus layer [6], which in turn affects the solubility of Cd.

Wood ash dissolves more rapidly when treated with acidified water [7]. As acidic deposition is still a threat to the terrestrial environment [8] this could affect the liberation of Cd from the ash into the soil. In general nothing is known about the effect of simulated acid rain (SAR) on the microbial function and community structure of coniferous forest humus fertilized with wood ash. The aims of this study were therefore to elucidate the effects of wood ash fertilization under water and acidified water irrigation and to determine the effect of Cd spiked into the ash in response to the treatments. In order to determine treatment induced microbial responses basal respiration, microbial community structure and Cd bioavailability were determined.

2. Materials and methods

2.1. Microcosms and treatments

Coniferous forest humus was collected in May 2001 from a Norway spruce (*Picea abies*) stand growing on a *Myrtillus* site type in southern Finland. The humus was passed through a 2.8-mm sieve by hand and stored at 4°C. The physico-chemical properties were determined from air-dried samples prepared within two weeks after sieving. The humus was characterized to have a pH value of 4.1, a cation-exchange capacity of 30.2 cmol kg^{-1}, a base saturation of 25.1% and a C/N ratio of 18 (see Section 2.2). The humus was kept at 4°C for a total of 4 weeks before the experiment was started.

All together 120 pots were filled with 110 g of humus. Randomly chosen sets of four pots were either treated with wood ash (A), Cd spiked wood ash (ACd) or left untreated (C). For the ACd treatment wood ash was spiked with CdO to give a Cd concentration of 1000 mg kg^{-1}ash. The Cd was mixed into the ash by rotation over night before determining the Cd concentration of the mixture (Table 1). To mimic a dose of 5000 kg ash ha^{-1}, 3.6 g of ash was added to the humus as top dressing. The microcosms were then placed outdoors in a field experiment consisting of, respectively, five treatment plots receiving either water or SAR (pH 3, sulfuric acid). Always four replicate treatment microcosms were placed on one field plot on 18 June. This experimental area is situated near the Kevo Subarctic Research Station (69°45′N, 27°01′E) in northern Finland where the growing season is 110–125 days. The irrigation in the field was started on 15 June and ended on 13 September. During this period the plots were irrigated 40 times and the SAR plots received a sulfur load of 3.64 g S m^{-2}, which was 15 times more than the S deposition on the water irrigated plots.

The 120 microcosms were destructively sampled on 22 September and brought to the laboratory within three days. Of the four treatment replicates per irrigation plot always two were combined to give a total of 60 samples. From these the respiration and biosensor measurements were taken. Finally all treatment replicates per irrigation plot were combined for the microbial community structure analyses and the physico-chemical measurements ($n = 30$).

2.2. Chemical analyses

The dry matter weight (d.m.) was determined by drying duplicate subsamples at 105°C overnight. Total organic carbon and nitrogen content were determined by dry combustion (Leco CHN-600). Cd and other total elemental concentrations were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES, ARL 3580) after wet digestion and extraction with HNO_3–H_2O_2. In the case of original humus the extractable elements were eluted 0.1 M $BaCl_2$ before measuring with ICP-AES. The humus pH was measured in a water suspension (1:15, w/v). See Table 1 for the characterization of the humus and ash used.

2.3. Microbial analyses

The basal respiration rate was measured as the amount of CO_2–C evolved in 25 h [9]. Fresh humus samples, equalling 2 g dry weight, were used in the analyses.

The phospholipid fatty acids (PLFAs) were extracted and analyzed from 1 g fresh weight of humus [10,11]. The total amount of PLFAs ($PLFA_{tot}$) was used to indicate the total microbial biomass, and 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

Table 1
Characterization of the humus and ash used in this experiment

	Humus (mg kg^{-1}; extractable)	Humus (mg kg^{-1}; total)	Ash (mg kg^{-1}; total)
K	258	1362	25611
Ca	1180	2412	286025
Mg	118	2568	17211
Na	16.3	180	6790
B	ND	3.911	218
Mn	43.5	156.5	8238
P	7.88	782.2	8427
S	–	836.1	14022
Al	952	18270	14767
Fe	129	16610	7647
Cd	ND	0.2342	10[a]
Cr	–	22.12	58
Cu	ND	24.68	58
Ni	–	8.311	60
Pb	–	34.04	57
Zn	10.2	45.09	2420

ND = not detected.
– = not analyzed.
[a]The Cd content of the spiked ash was 930 mg kg^{-1}.

the bacterial biomass ($PLFA_{bact}$) [12]. The amount of 18:2ω6 was used as an indicator of fungal biomass ($PLFA_{fung}$), since 18:2ω6 is suggested to be mainly of fungal origin in soil, and it is known to correlate with the amount of ergosterol [12].

Humus DNA extraction for PCR/DGGE (polymerase chain reaction/denaturing gradient gel electrophoresis) using general bacterial 16S [13] and fungal 18S rDNA primers [14] followed the protocol of Pennanen et al. [15] using 300 mg of fresh humus weighted into Bead Solution tubes (Ultraclean Soil DNA Isolation Kit, Mo Bio Laboratories Inc.). Consult Heuer et al. [13] for the PCR/DGGE conditions when using the bacterial F984+R1378 primer pair. In contrast to Heuer et al. [13] the denaturing gradient was from 35 to 60% of denaturant and the DGGE was performed in 1×TAE buffer at 58°C at a constant voltage of 150 V for 5 h. The fungal PCR/DGGE was performed with the primer pair FF390+FR1 [14], according to the descriptions given by Vainio and Hantula [14] and Pennanen et al. [15]. The wells of the DGGE gels were loaded with approximately the same amount of DNA. The DNA fragments were visualized by SYBRGreen I (FMC BioProducts) staining under UV light and photographed with a AlphaDigiDoc™ camera system.

The bioavailability of Cd in the humus samples after the treatments was determined by using the *Bacillus subtilis* strain BR151 [16] containing the Cd sensor plasmid pTOO24 [17] to control the expression of firefly luciferase. This biosensor emits light specifically in the presence of Cd. Air-dried humus samples of 2.5 g were used for the analyses. To obtain the relationship between bioluminescence and Cd concentration dilutions from a 10 mM $CdCl_2$ solution were prepared in Milli-Q (Millipore, Bedford, MA, USA) purified water to reach final Cd concentrations between 1 pM and 1 mM. The procedure was performed as described in detail by Fritze et al. [5]. Induction coefficients were calculated with different amounts of Cd as follows: induction coefficient $I = L_i/L_b$, where L_i is the light emitted by an induced sample (containing Cd) and L_b is the light emitted by the non-induced sample (containing Milli-Q water). The induction coefficient was then plotted against increasing Cd concentration, resulting in a typical standard curve for metal biosensors having a maximum I value (I_{max}) at a certain metal concentration where after the I value decreases again with increasing metal concentration. The same procedure was performed for the undiluted water extracts of the humus samples from the treatments. The light emission was converted to Cd concentrations using the linear part of the standard curve before reaching I_{max}.

2.4. Statistical analyses

The results were presented per d.m. of humus. The 38 identified individual PLFAs were expressed as mole percentage (mol% = area% of a single PLFA from the area sum of all identified PLFAs). The mol% values from the PLFA were standardized by dividing by the standard deviation (correlation matrix) before being subjected to principal component analysis (PCA). Two ACd treatments, one under water and one under SAR had to be removed from the PLFA data as outliers due to contamination in the laboratory. All the results, including the scores of the multivariate analysis, were subjected to analysis of variance (ANOVA) followed by the LSD (least significant difference) test for comparison of means. Two main effects and their interactions were tested in the ANOVA. The main effects were the treatments (C, A, ACd) and irrigation (water, SAR). If irrigation plot treatment replicates existed (respiration, biosensor), their mean was used and the final n for the statistical analyses was thus 30, except for the PLFA data where n was 28 (see above). In the case of the biosensor data a Kruskal–Wallis non-parametric ANOVA followed by a mean ranks test had to be performed due to the uneven distribution of positive results between treatments and irrigation (see Section 3). Pearson

Table 2
Treatment related humus physico-chemical and microbial variables (mean ± SE)

	Irrigation					
	Water			SAR		
Variable*	C**	A	ACd	C	A	ACd
pH	$5.0^{a} \pm 0.05$	$7.6^{b} \pm 0.08$	$7.6^{b} \pm 0.05$	$4.8^{a} \pm 0.03$	$7.5^{b} \pm 0.04$	$7.5^{b} \pm 0.05$
C (% of d.m.)	16.2 ± 0.43	15.1 ± 0.62	16.9 ± 0.51	16.9 ± 0.99	15.5 ± 0.37	16.2 ± 0.79
N (% of d.m.)	$0.87^{a} \pm 0.02$	$0.77^{b} \pm 0.03$	$0.86^{ab} \pm 0.03$	$0.89^{a} \pm 0.05$	$0.78^{b} \pm 0.02$	$0.81^{ab} \pm 0.03$
Ca (g kg^{-1})	$2.43^{a} \pm 0.06$	$18.8^{b} \pm 0.25$	$18.7^{b} \pm 0.45$	$2.40^{a} \pm 0.08$	$18.1^{b} \pm 0.41$	$18.5^{b} \pm 0.16$
Cd (mg kg^{-1})	$0.23^{a} \pm 0.02$	$0.53^{a} \pm 0.02$	$44.9^{b} \pm 9.05$	$0.24^{a} \pm 0.02$	$0.51^{a} \pm 0.03$	$34.1^{b} \pm 2.13$
CO_2–C (μg g^{-1} h^{-1})	$2.49^{a} \pm 0.13$	$3.24^{b} \pm 0.33$	$3.41^{b} \pm 0.16$	$2.42^{a} \pm 0.08$	$3.73^{b} \pm 0.16$	$4.03^{b} \pm 0.23$
$PLFA_{tot}$ (nmol g^{-1})	934 ± 38	857 ± 34	813 ± 92	966 ± 40	842 ± 44	913 ± 71
$PLFA_{bact}$ (nmol g^{-1})	395 ± 40	354 ± 17	333 ± 40	413 ± 16	351 ± 18	378 ± 32
$PLFA_{fung}$ (nmol g^{-1})	$33.9^{a} \pm 1.3$	$27.1^{b} \pm 1.3$	$27.5^{b} \pm 3.5$	$33.6^{a} \pm 1.9$	$26.8^{b} \pm 2.1$	$30^{b} \pm 2.5$

SAR = simulated acid rain.
*Means of bold variables are significantly different between the two irrigation treatments.
**Treatment means indexed by different letters are significantly different. Two-way-anova followed by LSD-test.

correlation tests were also performed between the PCA scores and the pH.

The bacterial and fungal DGGE gel photographs were analyzed as follows. All existing bands were first identified and named (A, B, C, ...). From the bacterial and fungal DGGE 19 and 34 separate bands, respectively, could be identified. Then all sample lanes ($n = 30$) were screened for the presence (1) or absence (0) of the respective bands, ending in a data matrix having only 1 and 0. This matrix was analyzed with a non-metric multidimensional scaling (MDS) method, which considers the rank order of distances. The principle on which MDS operates is to find a graphical representation of the data in a few dimensions, the distances in the ordination of the samples reflecting the (dis)similarities between the respective DGGE patterns as closely as possible. The pairwise (dis)similarities were computed using a Jaccard coefficient designed for this kind of data. Due to the nature of MDS, and unlike PCA, no further statistics can be made for the DGGE data.

3. Results

3.1. Effect of treatment

The treatment (C, A, ACd) means for the measured variables are presented separately for the water and SAR irrigation in Table 2. According to ANOVA no interactions occurred between treatments and irrigation (see Table 3 for an example). This meant that the treatments C, A, and ACd induced similar responses when irrigating with water or SAR. Therefore the overall mean treatment values, each treatment having $n = 10$, are presented here in the text. The ash treatments increased the humus pH significantly ($P < 0.001$) from the C treatment value 4.9 to 7.5 and 7.5 for the A and ACd treatments, respectively. The treatments had no effect on the C contents of the humus but the total N decreased from 0.88% (% of d.m.) to 0.78% due to the A treatment. The N content of the ACd treatment was 0.84% and did not differ from the control (Table 2). Both ash treatments increased the total humus Ca concentration from 2.4 g kg^{-1} to 18 g kg^{-1}. The humus total Cd concentration was 0.23 mg kg^{-1} in the C treatment and 0.52 and 39.5 mg kg^{-1} in the A and ACd treatments, respectively.

The basal respiration increased significantly ($P < 0.001$) due to the ash treatments. The mean CO_2–C production of the C treatments was 2.45 μg g^{-1} h^{-1} and increased due to the A treatment to a level of 3.48 μg CO_2–C g^{-1} h^{-1} and due to the ACd treatment to 3.73 μg CO_2–C g^{-1} h^{-1}. There were no significant differences between the A and ACd treatments. All the PLFA derived microbial biomass measures decreased due to the ash treatments (A and ACd). The total microbial biomass $PLFA_{tot}$ decreased from the mean C value of 950 nmol g^{-1} to 863 nmol g^{-1} and 850 nmol g^{-1} for the ACd and A treatments, respectively. This decrease was statistically insignificant and there were no differences between the two ash treatments. The respective values for the bacterial biomass $PLFA_{bact}$ were 404 nmol g^{-1}, 355 nmol g^{-1} and 353 nmol g^{-1} for the C, ACd and A treatments. This decrease was near statistical significance ($P = 0.051$) and no difference was detected between the A and ACd treatments. The fungal biomass $PLFA_{fung}$ decreased significantly ($P < 0.01$) from the mean C value of 33.7 nmol g^{-1} to the respective ACd and A values of 28.7 nmol g^{-1} and 26.9 nmol g^{-1}. No differences between the A and ACd treatments were detected.

The induction coefficient I of 21 samples was ≤ 1 and thus no bioavailable Cd could be detected with the biosensor in these samples. Bioavailable Cd was detected from nine samples. The treatment means for A ($n = 4$) and ACd ($n = 5$) were 0.27 ± 0.11 mg Cd kg^{-1} and 0.33 ± 0.09 mg Cd kg^{-1}, respectively, and did not differ

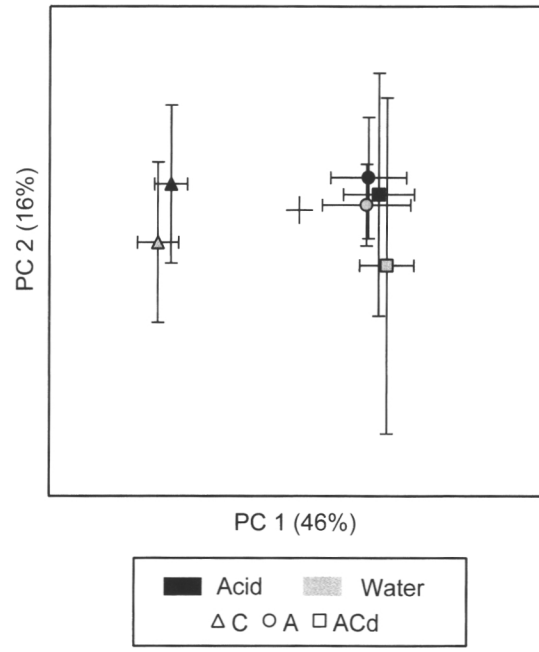

Fig. 1. PCA using the mol% of the PLFAs from irrigated humus samples. Abbreviations used: C = control, A = wood ash, ACd = cadmium spiked wood ash. Treatment means ± SD are presented.

Table 3
ANOVA for soil respiration

Source	*DF*	Mean square	*F*
Treatment (C, A, ACd)	2	9.13	23.03*
Irrigation (water, SAR)	1	0.901	4.55**
$T \times I$	2	0.338	0.203
Residual	24		

*$P < 0.001$.
**$P < 0.05$.

significantly from each other ($P = 0.5$; Kruskal–Wallis ANOVA).

The mol% of the individual PLFAs was subjected to PCA, which explained 62% of the data variation. The scores of PC1 were correlated to the humus pH ($r = 0.95$, $P < 0.001$) and separated the two ash treatments from the controls (Fig. 1). No further separation of the treatments could be detected. The loadings of the individual PLFAs on the first two PC axes are presented in Table 4. PLFAs with high positive or negative loading values on PC1 contributed most to the separation of the treatments along this axis (Fig. 1). MDS analyses of the DGGE patterns verified the result obtained with the PLFAs; both ash treatments had similar bacterial or fungal DGGE patterns, which were separated from the respective controls (Fig. 2). Respectively eight and seven bands were mainly responsible for the separation of the ash treatments in the bacterial and fungal DGGE.

Table 4
Loadings of the individual PLFAs on the first two PCAs

PLFA	PC1	PC2
i14	0.2262	−0.0067
14:0	0.1421	−0.1549
i15	−0.2036	0.0393
a15	−0.0734	0.0078
C15:1	0.0825	−0.3278
15:0	0.1681	−0.0421
i16:1	−0.2189	−0.0043
C16:0	−0.1964	−0.1679
i16:0	−0.1801	0.0132
16:1w9	−0.1437	0.0382
16:1w7c	0.2288	0.072
16:1w7t	0.0963	0.2674
16:1w5	−0.2168	0.0507
16:0	0.2221	0.0282
br17	−0.0351	−0.2251
10Me16	−0.2239	−0.0245
i17	−0.2187	0.0001
a17	−0.1758	−0.16
17:1w8	0.0696	−0.3701
cy17	0.2196	−0.001
C17:1	−0.2049	−0.0977
17:0	−0.0145	−0.0518
br18	−0.2293	−0.0346
10Me17	−0.1237	−0.0671
18:2a	0.0988	−0.1413
18:2w6	−0.1128	0.11
18:1w9	−0.2131	0.126
18:1w7	0.1797	0.13
18:1	−0.1046	−0.2455
18:0	−0.0643	−0.2082
19:1a	0.2202	−0.0095
18:2b	−0.0552	−0.0068
10Me18	−0.1645	−0.1193
19:1b	0.0097	−0.3857
cy19	−0.1658	−0.0106
20:5	0.1337	−0.291
20:4	0.0141	0.1401
20:0	0.1287	−0.2956

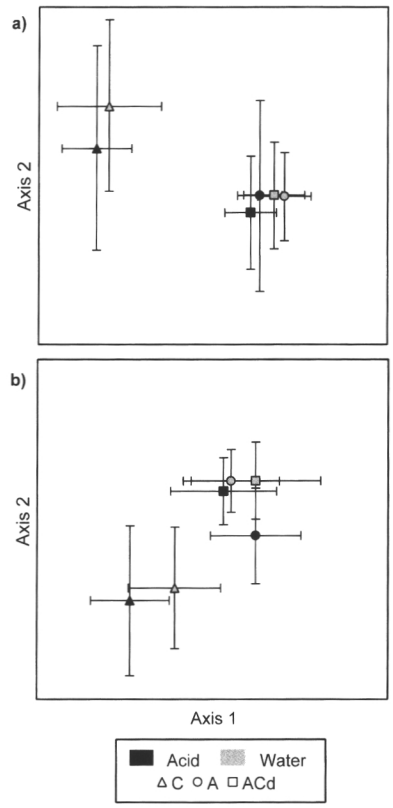

Fig. 2. Non-metric MDS ordination of the DGGE data matrix of (a) bacterial and (b) fungal rDNA fragments from irrigated humus samples. See legend of Fig. 1 for abbreviations. Treatment means ± SD are presented.

3.2. Effect of irrigation

The effect of SAR on the results was statistically verified by comparing the respective mean values of all watered ($n = 15$) to all acidified microcosms ($n = 15$). SAR decreased the humus pH of the microcosms significantly ($P < 0.05$) from a mean value of 6.7 of all watered plots to a mean pH value of 6.6. The difference between the two respective control treatments was 0.2 pH units (Table 2). Due to SAR the basal respiration increased significantly ($P < 0.05$, Table 2) from 3.05 μg CO_2–C g^{-1} h^{-1} to 3.4 μg CO_2–C g^{-1} h^{-1}. The irrigation had no effect on the humus C and N content, the total Ca and Cd concentration or the microbial biomass values $PLFA_{tot}$, $PLFA_{bact}$ and $PLFA_{fung}$. The scores of PC1 and PC2 were not significantly related to the irrigation treatment and thus no irrigation effect on the PLFA pattern could be obtained. Again the MDS analyses of the bacterial and fungal DGGE patterns verified this result. The positive biosensor data was unevenly distributed between the irrigation treatments. The SAR treatment had a mean ($n = 2$) bioavailable Cd of 0.14 ± 0.04 mg kg^{-1} and did not significantly differ

from the watering treatment mean ($n = 7$) of 0.35 ± 0.08 mg Cd kg^{-1} ($P = 0.15$; Kruskal–Wallis ANOVA).

4. Discussion

Application of wood ash onto the humus increased the pH and this was accompanied by an increase in basal respiration rate, a change in microbial PLFA pattern and a slight decrease in PLFA derived biomass values. These results thus confirm the published results [4,18–20]. In addition to the PLFA method, which is a measure of microbial community structure, also the PCR based DGGE method was used to test the treatment effects separately on the bacterial or fungal community. Both approaches showed that ash induces changes in the bacterial and fungal community. Again it could be shown that wood ash spiked with cadmium to a level of 1000 mg Cd kg^{-1} ash induced the same changes to the humus microflora as ash alone [4,5]. Without the ash this amount of Cd decreases soil respiration and changes the PLFA pattern [4].

Watering the microcosms with SAR in the field over the whole growing season slightly decreased the humus pH but did not change the interpretation of the results made above. Though there was probably a higher dissolution rate of the ash into the humus to be detected due to the SAR treatment the Cd of the wood ash was not liberated into bioavailable form and thus did not reach toxic levels. The higher dissolution of the ash induced a higher basal respiration rate. The ACd and A treatments would add 465 and 5 mg Cd m^{-2} onto the forest floor, respectively, which theoretically could leach into the environment when the ash induced pH effect stops. There are no investigations on the duration of how long the humus pH increasing wood ash effect lasts in the forest environment but it must be long, since ash induced effects on humus pH and microflora are still observed 18 years after fertilization [20]. Our results indicate that wood ash, containing Cd at levels between 1 to 30 mg Cd kg^{-1} ash, can be used in field trials to counteract soil acidification without the threat of Cd toxicity since much higher amounts of Cd spiked into the ash were not able to change any of the measured variables of this study.

The PLFA method is based on the extraction of PLFAs common in all bacteria. One of the 38 identified PLFAs is treated to be fungal. This makes the PLFA method mainly a bacterial community structure assay. It has been shown before that the humus PLFA pattern obtained from 40 environmental field plots correlated well to the bacterial PLFA pattern enriched on a nutrient rich agar media [21]. This implies that the PLFA method mainly measures changes in the dominant soil bacterial community, which can also be grown to pure culture. Using a general bacterial primer for PCR/DGGE resulted in the same resolution as the PLFA method. Therefore the use of general primers for PCR/DGGE in soil ecology pictures probably only the most dominant species of the investigated sample.

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Paper VII

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Boreal forest microbial community after long-term field exposure to acid and metal pollution and its potential remediation by using wood ash

Jonna Perkiömäki[a,*], Andreas Tom-Petersen[b], Ole Nybroe[b], Hannu Fritze[a]

[a]*Finnish Forest Research Institute, Vantaa Research Centre, P.O. Box 18, Vantaa 01301, Finland*
[b]*Department of Ecology, Royal Veterinary and Agricultural University, Frederiksberg C 1871, Denmark*

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Abstract

A 2^2-factorial design with sulphuric acid (pH 3.1) and Cu–Ni addition was used to assess the effects of moderate amounts of continuous acid (Acid and CuNi + Acid) and metal (CuNi and CuNi + Acid) deposition on humus microbial activity and community structure in the field after nine growing seasons. These 20 field experiment samples were also used to measure the suitability of wood ash for remediation. Microcosms were treated with wood ash at a fertilization rate of 5000 kg ha^{-1}, irrigated with water and incubated for 2 months in the dark at 20 °C and a constant relative humidity of 60%. Microcosms only irrigated with water served as a control. Microbial activity was measured as basal respiration. Microbial community structure was determined by phospholipid fatty acid analysis, which mainly targets bacteria. Fungal community structure was assessed by 18S rDNA-targeted polymerase chain reaction-denaturing gradient gel electrophoresis analysis. The bioavailability of Cu was tested with the *Pseudomonas fluorescens* DF57-Cu15 reporter strain, which bioluminescences in the presence of Cu. Our field study showed, that acid and metal treatments both changed the humus layer microbial community structure. Acid application decreased humus layer pH and base saturation (BS) and increased the amounts of both extractable and bioavailable Cu. Metal application increased the concentration of extractable Ni and changed the fungal community structure. In irrigated laboratory microcosms the above-mentioned treatment effects were still seen except for the acid and metal effects on microbial and fungal community structures. For ash-treated microcosms, neither acid nor metal effects were found for humus layer pH, BS, extractable Cu and Ni, or bioavailable Cu. Thus, wood ash can be used for remediation of acid and metal polluted humus.

Keywords: Bioluminescence; Boreal forest; Copper availability; DGGE; Metal pollution; Microbial activity; Microbial community structure; PLFA; Remediation

1. Introduction

Soil microbes play a key role during litter decomposition and nutrient release for plant growth. Hence changes in microbial activity and community structure could affect terrestrial ecosystems, especially those vulnerable such as boreal forests where litter degradation is slow. The deposition of heavy metals and acidifying substances change the chemical status of the soil (Tabatabai, 1985; Alloway, 1990) and affect structure, biomass and activity of the microbial community (Bååth, 1989; Pennanen, 2001). Furthermore, it is well known that soil acidity, in addition to the amount of soluble organic matter, is an important factor controlling metal mobility in soils (McBride et al., 1997; Temminghoff et al., 1998).

The industrial area of the Kola Peninsula in Northwest Russia has emitted S, Cu and Ni since the late 1930s (Tuovinen et al., 1993). At the end of the 1980s evidence began to accumulate that heavy metals and S from the Cu–Ni smelters in the Kola area were negatively affecting large forest areas in the region. Therefore a field experiment mimicking this deposition was established in 1991. Because in nature it is difficult to differentiate the effects caused by heavy metals and acid load this experiment was designed to analyse the individual as well as the combined effects of acid and Cu–Ni loads.

Experimental field plots have been irrigated with acidified water, water containing Cu and Ni or a combination of the two treatments. Pennanen et al. (1998) sampled these plots after 6 yr and by then the acid

* Corresponding author. Tel.: +358-10-211; fax: +358-211-2202.
E-mail address: jonna.perkiomaki@metla.fi (J. Perkiömäki).

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application resulted in acidification of the humus layer, as indicated by the decreased base cation concentrations and pH. The acid but not the metal treatment changed the microbial community structure and the bacterial community adapted to the changed humus layer pH (Pennanen et al., 1998).

The use of wood ash has been suggested for remediation of acidified and heavy metal polluted terrestrial environments, but seldom tested. Many investigators have reported that soil acidification results in nutrient cation release from soil and increased mobility of Al (van Breemen et al., 1984; de Vries and Breeuwsma, 1987; Koptsik and Mukhina, 1995). Wood ash amendment consistently leads to decreased concentrations of extractable Al, while values for pH and exchangeable base cations in the humus layer are increased (Unger and Fernandez, 1990; Bramryd and Fransman, 1995; Kahl et al., 1996). Wood ash fertilization might also reduce the toxic effects of heavy metals. Fritze et al. (1995) showed that an increase in soil pH after wood ash application reduced the toxicity of externally added Cd to microbes. Furthermore, Ca and Mg, which are major cations in wood ash, have been shown to reduce the inhibitory effects of heavy metals on microbes due to ion-antagonism, i.e. the protective effect of one cation on the toxicity of a second heavy metal cation (Gadd and Griffiths, 1978; Babich and Stotzky, 1980).

In addition to the effects on soil chemistry, wood ash fertilization has also resulted in higher microbial activity measured as soil respiration from acidic humus layer (Bååth and Arnebrant, 1994; Fritze et al., 2000), Cd-polluted humus layer (Fritze et al., 1995) and humus layer fertilized with Cd spiked wood ash (Fritze et al., 2000). Wood ash application also has the potential to change the humus layer microbial species composition as analysed by the phospholipid fatty acid (PLFA) technique (Bååth et al., 1995; Fritze et al., 2000).

We have assessed the effects of moderate amounts of continuous acid and Cu–Ni deposition on humus layer microbial community in the field after nine growing seasons. To determine whether these soils could be remediated, samples from the field were placed in laboratory microcosms and water irrigation combined with wood ash fertilization remediation treatment was evaluated. Microcosms that were only irrigated with water served as a control.

We measured microbial activity as basal respiration. Microbial community structure was determined by PLFA analysis, which mainly targets bacteria, while fungal community structure was assessed by 18S rDNA-targeted polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis. The bioavailability of Cu was tested using the *Pseudomonas fluorescens* DF57-Cu15 reporter strain (Tom-Petersen et al., 2001), which bioluminescences in the presence of Cu.

2. Materials and methods

2.1. Experimental area and sampling

The experimental area is situated near the Kevo Subartic Research Station (69°45′ N, 27°01′ E) in Finnish Lapland where the growing season is 110–125 d. The experimental area is dry, the soil nutrient content is poor, and has a mixed pine–mountain birch woodland cover (*Pinus sylvestris*, *Betula pubescens* spp. *cerepanovii*). The study area was divided into five adjacent blocks each consisting of four treatment plots totalling in 20 plots. Each plot (6×8 m^2) supported at least one pine and one mountain birch. The control plots (Control) were treated with spring water having a pH of 5.5; the acidified plots (Acid) were treated with simulated acid rain (spring water adjusted to pH 3.1 with sulphuric acid H_2SO_4); the heavy metal-treated plots (CuNi) were irrigated with water containing Cu and Ni sulphates (pH about 5.7); and the combined acid and heavy metal treatment plot (CuNi + Acid) contained the same amounts of Cu, Ni and H_2SO_4 as were added to the CuNi and Acid plots separately. The plots have been irrigated twice weekly between June and September since 1991. At each irrigation event, an amount of irrigation solution corresponding to 5 mm precipitation was spread over the plot. The cumulative S, Cu and Ni loads are shown in Table 1.

Humus layer samples were collected in September 2000 using a soil corer (40 mm diameter). The samples were sieved (2.8 mm mesh) and stored at +4 °C for 1–2 weeks before pH and basal respiration were measured, and PLFA and DNA were extracted. Chemical analyses and the assay for bioavailable Cu were performed on air-dried samples.

2.2. Remediation experiment in laboratory

Humus layer samples from the field were used for a laboratory remediation experiment. One week after sampling, soil from each field plot was used to establish two microcosms in pots (diameter at the surface 120 mm). The amount of soil weighed into the pots resulted in equal amounts of organic matter in each microcosm. One of the microcosms from each field plot was irrigated with water (= control treatment) while the other microcosm was watered and ash fertilized (= remediation treatment). To

Table 1
Cumulative sulfur and CuNi loads of the study plots

Treatment	Cumulative loads 1991–2000 (mg m^{-2})		
	S	Cu	Ni
Control	1080	–	–
Acid	18,730	–	–
CuNi	1220	160	100
CuNi + Acid	18,860	160	100

mimic a fertilization dose of 5000 kg ash ha^{-1}, 5.6 g of ash was added as top dressing. The microcosms were incubated for 2 months in the dark at 20 °C and at a constant relative humidity of 60%. Watering was made every 2- to 3-d to keep the moisture content of the humus at 30% of the water holding capacity (WHC). If water passed through the microcosm it was poured back onto the humus layer. After the termination of experiment the samples were stored at +4 °C for 1–2 weeks before pH, basal respiration, PLFA and DNA extractions, and determination of bioavailable Cu were performed in the same way as for the field experiment.

2.3. Chemical analyses

The chemistry of the humus layer sample was analysed as described by Tamminen and Starr (1990). Briefly, pH was measured in a water suspension (15 cm^3 humus + 25 ml water), dry weight determined after incubation at 105 °C overnight following organic matter (o.m.) determination at 550 °C for 4 h, and total organic C and N were determined by dry combustion (Leco CHN-600). For nutrient analyses, air-dried samples were prepared (48 h at 40 °C) and extracted with 0.1 M $BaCl_2$. Nutrient and metal concentrations in the suspension were determined with an inductively coupled plasma emission spectrometer (ICP-AES, ARL 3580). The chemical composition of the wood ash (Table 2) was determined after dry digestion (550 °C) and dissolution in concentrated HCl by ICP-AES. Base saturation (BS) and cation exchange capacity (CEC) were calculated.

2.4. Microbial analyses

Basal respiration rate was measured as the amount of CO_2–C evolved during 24–25 h as described by Pietikäinen and Fritze (1995). Fresh humus samples, equalling 2 g dry weight, were used in the analyses.

Bioavailable Cu was measured with a reporter bacterium responding specifically to Cu as described in Tom-Petersen et al. (2001). Briefly, the Cu-reporter *Pseudomonas fluorescens* DF57-Cu15 carries a promoterless Tn5 :: *luxAB* cassette inserted on the chromosome under the control of an indigenous Cu-induced promoter. Humus layer extracts were obtained from 1 g of sample (dry weight) shaken in polyethylene tubes for 2 h at 200 rev min^{-1} and room temperature with 5 ml of 50 mM $CaCl_2$ and the supernatant recovered by centrifugation (10,000*g*, 10 min, room temperature). Samples for the analysis were either 0.5 ml of the above supernatant, or 0.1 ml supernatant diluted in 0.4 ml 50 mM $CaCl_2$. The samples were mixed with 0.5 ml of a cell suspension of strain DF57-Cu15 (exponential-phase cells at a concentration of 2.5×10^8 cell ml^{-1}), and incubated for 1.5 h at room temperature as described by Tom-Petersen et al. (2001). Induction of bioluminescence from the *luxAB* reporter construct was measured using a luminometer (Bio Orbit 1253, Turku, Finland). A standard curve of bioluminescence was obtained by exposing DF57-Cu15 to increasing concentrations of $CuSO_4$ in 50 mM $CaCl_2$ and used to convert bioluminescence values into mg Cu kg^{-1} soil.

PLFAs were extracted as described by Frostegård et al. (1993b). Briefly, 0.5–1 g fresh weight of sample was extracted with a chloroform:methanol:citrate buffer mixture (1:2:0.8), and the lipids separated into neutral lipids, glycolipids, and phospholipids in a silicic acid column. The phospholipids were subjected to mild alkaline methanolysis, and the fatty acid methyl esters separated by a gas chromatograph (Hewlett Packard 5890) equipped with a flame ionization detector and a HP-5 (phenylmethyl silicone) capillary column (50 m in length) using He as a carrier gas. Peak areas were quantified by adding methyl nonadecanoate fatty acid (19:0) as an internal standard.

Fatty acids are designated in terms of their total number of C atoms:number of double bonds, followed by the position of the double bond from the methyl end of the molecule. The prefixes a and i indicate anteiso- and isobranching; br indicates unknown methyl branching position. The prefix cy indicates a cyclopropane fatty acid, and methyl branching (Me) is indicated as the position of the methyl group from the carboxyl end of the chain. The prefix C (C15:1) indicates that the PLFA has 15 C atoms and one double bond, but arrangement of the C atoms (e.g. branching position) is not known.

The total amount of PLFAs, $PLFA_{tot}$, was used to indicate the total 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 cy 19:0 was used as an index of the bacterial biomass ($PLFA_{bact}$) (Frostegård and Bååth, 1996). The quantity of 18:2ω6 was used as an indicator of fungal biomass ($PLFA_{fung}$), since it is suggested to be mainly of fungal origin in soil (Federle, 1986) and it is known to correlate well with the amount of ergosterol (Frostegård and Bååth, 1996). The $PLFA_{fung}$ to $PLFA_{bact}$ ratio was used to indicate the relative amounts of the fungal and bacterial biomasses.

Table 2
Elemental contents of the ash used in the experiment

Element	Wood ash (mg kg^{-1})
Ca	351,000
K	28,000
Mg	18,000
Fe	11,000
Mn	10,000
P	9000
Al	8000
Zn	2400
B	197
Cu	80
Ni	69
Cr	64
Cd	15

DNA extraction of the humus layer followed the procedure described by Pennanen et al. (2001). The extracted DNA was diluted 10-fold and subjected to PCR amplification of a 390 bp product using FR1 + FF390 primer pair, targeting fungal 18S (SSU) rDNA. The primer pair FR1 + FF390 amplifies a broad spectrum of fungal species without amplifying plant or bacterial DNA (Vainio and Hantula, 2000) or the DNA from soil animals common in coniferous forest humus layers (Pennanen et al., 2001). Fungal DNA isolated from pure cultures obtained from humus layer samples was used as positive controls. The resulting amplification products were checked for size, purity and product yields in 1% agarose gels. In order to obtain efficient separation in DGGE, a GC clamp was attached to the FR1 primer. The primer pair, the GC clamp, and the exact DGGE conditions were as described in Vainio and Hantula (2000). The wells of the DGGE gel were loaded with approximately the same amount of DNA as judged from the product yield in the agarose gel and stained using SYBRGreen I (FMC BioProducts).

2.5. Statistical analyses

The results from the three individual experimental designs (field, laboratory incubation plus irrigation and laboratory incubation plus irrigation with wood ash application), including the scores derived from a principal component analyses (PCA) of the PLFA measurements, were separately tested by analysis of variance (ANOVA) as a 2^2-factorial design. This design tested the acid (Acid and CuNi + Acid) and metal (CuNi and CuNi + Acid) effect and their interaction (see Table 3 as an example). PCA of PLFAs was performed separately for field, laboratory incubation plus irrigation and laboratory incubation plus irrigation with wood ash application samples although the result of a single PCA, including all samples is presented in Fig. 1. Treatment effects are discussed when $P < 0.10$.

When extractable Cu (Cu_{ext}) and Ni (Ni_{ext}) or bioavailable Cu were below the detection limit, values representing half the detection limit value were used for further calculations in order to avoid overestimation of differences between treatments and to ensure a full database for the statistics. Pearson correlation tests were used to evaluate the relationships between PCA scores and individual variables.

Table 3
Analysis of variance for bioavailable Cu in the field experiment

Source	df	MS	F	P
Acid effect (A)[a]	1	3.7701	36.97	0.000
Metal effect (B)[b]	1	0.4575	4.49	0.050
A × B	1	0.1592	1.56	0.230
Residual	16	0.1020		

[a] A = Acid and CuNi + Acid.
[b] B = CuNi and CuNi + Acid.

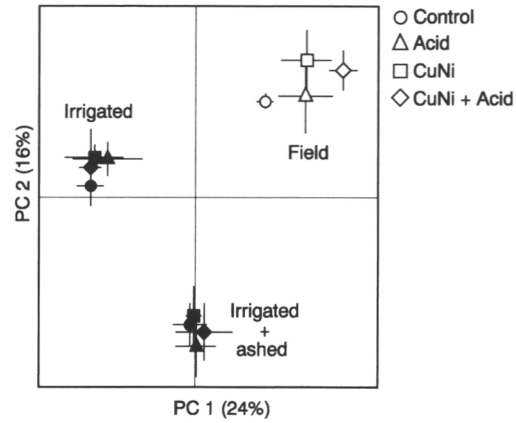

Fig. 1. Principal component analysis (PCA) using the mol% of the phospholipid fatty acids (PLFAs) from all the samples. Symbols indicate the mean of five replicate plots, and bars indicate the standard error. See Table 4 for treatment symbols.

All results are presented in the respective Tables and Figures but only those important to document potential remediation are presented in the text.

3. Results

3.1. Field experiment

In the field experiment the acid treatment (Acid and CuNi + Acid) lowered humus layer pH ($P = 0.005$) and base saturation (BS, $P = 0.002$), and increased the concentration of Cu_{ext} ($P = 0.018$) (Table 4). The metal treatment (CuNi and CuNi + Acid) increased the concentration of Ni_{ext} ($P = 0.0003$) (Table 4). Concerning the microbiological variables, the amounts of bioavailable Cu was increased due to acid treatment ($P = 0.001$) (Table 5). The remaining chemical (CEC, C-to-N ratio, organic matter) and microbiological variables (basal respiration, total microbial, bacterial and fungal biomasses) did not show any treatment effects, nor did the fungi-to-bacteria biomass ratio (Tables 4 and 5).

When PLFAs of the field experiment were subjected to PCA the first principal component (PC1) explained 24% and the second principal component (PC2) explained 15% of the variation. The individual PLFAs responsible for the changes along PC1 were: a15:0, 16:1ω7c, 16:1ω5, i16:0, 10Me16:0, 18:2ω6, 16:1ω9, i15:0, 18:1, i14:0 and i16:1. The PLFAs responsible for the separation along PC2 were: 16:1ω7t, br18:0, cy19:0, 10Me17:0, cy17:0, 18:2a, 17:1ω8, 16:0 and i16:0. Both acid ($P = 0.040$) and metal ($P = 0.022$) treatments separated from the control along PC1 (Fig. 1). The acid treatment decreased ($P < 0.10$) the amounts of PLFAs a15:0, 16:1ω5, 16:1ω9, cy17:0 and 18:1 and increased ($P < 0.10$) the amount of PLFA 18:2a (Table 6). The metal treatment decreased ($P < 0.10$)

Table 4
Chemical variables as means of five replicate plots

	Field				Irrigated				Irrigated + Ashed			
	Control	Acid	CuNi	CuNi + Acid	Control	Acid	CuNi	CuNi + Acid	Control	Acid	CuNi	CuNi + Acid
pH	4.07 (0.06)	3.99 (0.04)	4.08 (0.03)	3.89 (0.04)	4.26 (0.03)	4.18 (0.06)	4.31 (0.02)	4.21 (0.04)	7.32 (0.05)	7.31 (0.02)	7.26 (0.06)	7.32 (0.04)
CEC	49.2 (1.88)	39.1 (7.26)	45.6 (3.35)	51.2 (4.26)	55.9 (5.83)	58.0 (4.17)	45.8 (1.43)	58.7 (1.67)	158 (15.7)	170 (17.4)	127 (27.7)	110 (35.7)
BS	50.2 (2.90)	44.9 (1.44)	52.3 (1.87)	38.8 (3.20)	47.5 (2.95)	45.9 (3.26)	53.1 (2.72)	38.4 (2.74)	99.5 (0.04)	99.5 (0.01)	99.5 (0.01)	99.4 (0.04)
Cu_{ext}	bdl	2.93 (1.86)	bdl	3.89 (1.72)	bdl	4.50 (1.12)	bdl	4.63 (0.84)	bdl	bdl	bdl	bdl
Ni_{ext}	10.5 (0.68)	3.07 (1.88)	14.3 (2.91)	21.5 (3.06)	7.59 (3.25)	8.06 (2.45)	14.0 (2.27)	22.6 (2.29)	bdl	bdl	bdl	bdl
C-to-N ratio	35.7 (0.61)	35.3 (0.76)	36.7 (0.16)	35.1 (1.14)	34.3 (0.70)	34.5 (0.81)	35.4 (0.34)	33.6 (1.13)	34.6 (0.73)	35.1 (0.67)	35.9 (0.42)	34.5 (0.61)
o.m.	47.0 (5.30)	52.8 (5.31)	52.2 (2.35)	43.5 (2.08)	43.7 (3.62)	49.9 (5.67)	51.0 (2.02)	43.2 (2.06)	42.2 (4.47)	43.8 (4.58)	46.5 (1.99)	39.5 (1.67)

Treatments of the plots are coded as Control (water irrigation control), Acid (acid load), CuNi (Cu and Ni load) and CuNi + Acid (Cu, Ni and acid load). Field means situation in the field. Irrigated and Irrigated + Ashed means laboratory treatments, irrigation with water and water irrigation with wood ash amendment, respectively. Cation exchange capacity (CEC) is given in cmol kg^{-1} o.m. and base saturation (BS) as % of Ca, Mg, K and Na of CEC. The amount of extractable metals is expressed as $\mu g\ g^{-1}$ o.m. bdl = below detection limit. Standard error is in parentheses.

Table 5
Microbiological variables as means of five replicate plots

	Field				Irrigated				Irrigated + Ashed			
	Control	Acid	CuNi	CuNi + Acid	Control	Acid	CuNi	CuNi + Acid	Control	Acid	CuNi	CuNi + Acid
CO_2–C ($\mu g\ g^{-1}\ h^{-1}$)	16.1 (1.38)	14.9 (1.64)	16.3 (0.98)	17.9 (1.48)	7.3 (0.46)	6.5 (0.84)	5.8 (0.44)	6.9 (0.50)	20.1 (1.78)	22.9 (2.18)	22.8 (1.85)	24.0 (2.64)
Bioavailable (Cu mg kg^{-1})	bdl	0.79 (0.38)	0.10 (0.03)	1.61 (0.42)	0.02	0.63 (0.28)	0.10 (0.03)	0.76 (0.31)	0.02	bdl	bdl	bdl
$PLFA_{tot}$ (nmol g^{-1})	2206 (79)	1930 (228)	2137 (74)	2213 (57)	1723 (52)	1550 (98)	1578 (62)	1629 (135)	1523 (77)	1494 (63)	1622 (67)	1661 (81)
$PLFA_{bact}$ (nmol g^{-1})	635 (18)	569 (67)	611 (27)	603 (8.7)	582 (21)	507 (40)	533 (21)	528 (34)	524 (26)	508 (27)	546 (24)	542 (20)
$PLFA_{fung}$ (nmol g^{-1})	409 (46)	329 (47)	386 (21)	428 (28)	185 (12)	170 (8.1)	173 (7.8)	192 (22)	152 (10)	151 (5.7)	172 (6.9)	177 (14)
Fungal-to-bacterial ratio	0.64 (0.07)	0.58 (0.03)	0.63 (0.03)	0.71 (0.04)	0.32 (0.01)	0.34 (0.02)	0.33 (0.02)	0.36 (0.02)	0.29 (0.01)	0.30 (0.02)	0.32 (0.01)	0.33 (0.03)

All results are expressed as g^{-1} organic matter (o.m.). bdl = below detection limit. Standard error is in parentheses if not, then the other replicates were below detection limit. See Table 4 for treatment symbols.

Table 6
Treatment-induced changes in the humus PLFAs

PLFA	Field				Irrigated				Irrigated + Ashed			
	Control PLFA (mol%)	Ratio of control			Control PLFA (mol%)	Ratio of control			Control PLFA (mol%)	Ratio of control		
		Acid	CuNi	CuNi + Acid		Acid	CuNi	CuNi + Acid		Acid	CuNi	CuNi + Acid
14:0	0.27 (0.01)	0.95	0.90	0.77	0.36 (0.03)	0.90	0.99	0.95	0.32 (0.01)	1.10	1.23	1.14
14:0	1.17 (0.04)	0.96	0.93	0.93	1.04 (0.07)	0.99	1.01	0.96	1.25 (0.14)	1.01	1.08	0.91
i15:0	5.64 (0.12)	0.97	0.91	0.90	7.16 (0.22)	1.01	1.00	1.07	6.47 (0.17)	1.04	0.99	1.05
a15:0	2.15 (0.07)	0.92	0.92	0.76	3.39 (0.08)	0.90	0.99	0.94	2.98 (0.11)	0.99	0.95	0.89
C15:1	0.15 (0.02)	1.47	1.41	1.44	0.10 (0.01)	0.95	0.80	1.24	0.15 (0.03)	0.84	1.21	0.69
15:0	0.83 (0.03)	1.00	0.95	1.01	0.76 (0.03)	0.99	0.96	0.94	0.85 (0.03)	1.01	0.97	0.99
i16:1	0.56 (0.06)	0.94	0.69	0.81	0.51 (0.03)	0.98	0.97	0.86	0.46 (0.04)	1.02	1.06	0.81
C16:0	0.30 (0.03)	1.00	0.94	1.10	0.33 (0.02)	1.30	1.18	1.32	0.37 (0.06)	1.34	1.17	1.11
i16:0	2.22 (0.08)	1.05	0.96	0.93	2.77 (0.05)	1.01	0.96	0.92	3.24 (0.09)	1.03	1.04	0.97
16:1ω9	0.48 (0.03)	0.84	0.96	0.66	0.73 (0.06)	0.80	0.93	0.93	0.45 (0.04)	0.79	0.89	0.81
16:1ω7c	6.73 (0.07)	0.94	0.94	0.90	7.16 (0.18)	0.96	0.97	0.97	6.70 (0.09)	0.95	0.94	0.96
16:1ω7t	1.64 (0.07)	0.98	0.90	1.01	1.41 (0.07)	1.16	0.97	1.08	1.62 (0.07)	1.05	0.94	1.05
16:1ω5	2.09 (0.07)	0.88	0.99	0.75	3.49 (0.05)	0.85	0.96	0.85	1.85 (0.08)	0.94	0.99	0.92
16:0	13.3 (0.16)	0.94	0.97	1.02	11.9 (0.43)	1.03	1.02	0.98	12.5 (0.23)	0.99	1.01	1.03
br17:0	0.52 (0.09)	1.01	1.01	1.13	0.37 (0.03)	1.25	1.08	1.19	0.47 (0.04)	0.88	0.87	0.76
10Me16:0	4.16 (0.10)	1.03	0.95	0.96	4.52 (0.37)	0.96	0.82	0.98	4.93 (0.45)	1.01	1.02	0.99
i17:0	0.57 (0.02)	0.96	0.88	0.84	0.94 (0.02)	0.93	0.95	0.91	0.89 (0.04)	1.01	0.99	0.94
a17:0	0.75 (0.02)	0.99	0.83	1.08	0.88 (0.02)	0.95	1.00	0.91	0.93 (0.01)	1.01	0.97	1.01
17:1ω8	0.54 (0.01)	1.01	0.85	1.04	0.46 (0.10)	0.88	1.20	0.65	0.42 (0.12)	1.58	1.48	1.51
cy17:0	0.99 (0.03)	0.90	0.96	0.85	1.25 (0.05)	0.96	1.01	0.98	1.56 (0.10)	1.01	1.03	0.98
C17:1	0.17 (0.02)	1.10	1.43	1.02	0.24 (0.01)	0.95	1.02	1.10	0.20 (0.03)	0.99	0.87	1.00
17:0	0.56 (0.03)	1.14	1.18	1.14	0.61 (0.03)	1.04	1.03	1.01	0.64 (0.03)	1.04	0.96	0.98
br18:0	0.45 (0.06)	1.06	1.09	0.83	0.46 (0.06)	1.04	0.80	0.75	0.49 (0.07)	1.06	0.98	0.77
10Me17:0	0.75 (0.05)	1.15	1.01	1.00	0.80 (0.04)	1.16	0.98	0.97	0.99 (0.06)	1.09	1.03	0.93
18:2a	0.65 (0.08)	1.11	1.01	1.37	0.40 (0.05)	1.74	0.98	1.01	0.67 (0.10)	0.92	0.76	1.44
18:2ω6	17.1 (1.05)	0.99	1.05	1.14	10.7 (0.45)	1.03	1.03	1.09	9.99 (0.41)	1.01	1.06	1.06
18:1ω9	12.7 (0.73)	1.05	1.01	1.09	13.9 (0.41)	1.07	1.03	1.10	13.0 (0.38)	1.02	1.04	1.09
18:1ω7	8.44 (0.17)	0.88	0.98	0.88	7.18 (0.17)	0.89	0.99	0.86	8.42 (0.21)	0.89	0.92	0.85
18:1	0.70 (0.03)	0.88	1.02	0.75	0.88 (0.03)	0.86	0.95	0.73	0.34 (0.03)	0.92	1.04	0.84
18:0	2.11 (0.09)	1.07	1.03	1.05	2.29 (0.12)	1.01	1.01	0.94	2.05 (0.04)	1.08	0.98	1.03
19:1a	0.34 (0.01)	1.03	1.02	0.91	0.49 (0.02)	0.92	1.07	0.97	0.69 (0.05)	1.05	1.17	0.87
18:2b	0.15 (0.03)	1.03	1.04	1.01	0.09 (0.03)	1.31	1.08	1.64	0.15 (0.03)	0.57	0.70	0.64
10Me18:0	1.40 (0.14)	1.02	0.91	0.94	1.46 (0.11)	1.02	0.92	0.92	1.59 (0.10)	1.09	0.97	1.00
18:2c	0.22 (0.03)	1.19	1.27	1.08	0.24 (0.02)	0.67	1.09	1.18	0.29 (0.07)	0.88	0.75	0.69
19:1b	0.63 (0.05)	1.83	1.20	1.21	0.76 (0.03)	1.04	1.13	1.13	0.88 (0.12)	0.90	0.91	0.96
cy19:0	5.34 (0.18)	1.23	1.05	0.93	6.70 (0.14)	0.98	1.06	1.00	6.36 (0.11)	1.02	1.02	0.97
20:5	0.96 (0.07)	0.86	1.62	0.96	0.73 (0.07)	1.39	1.10	1.45	1.47 (0.38)	0.98	0.91	1.03
20:4	0.66 (0.05)	1.05	1.10	1.11	1.24 (0.12)	0.78	0.91	0.92	1.68 (0.12)	0.97	0.95	1.10
20:0	1.60 (0.10)	1.12	1.19	1.23	1.28 (0.14)	1.14	1.13	1.07	1.70 (0.24)	1.08	0.99	0.99

Mol% of PLFAs, expressed as means (± SE), from the controls are given. To show the effect of the treatments, the ratios of the means of individual PLFAs found in the treated humus samples to the means of the respective control treatment are presented. See Table 4 for treatment symbols.

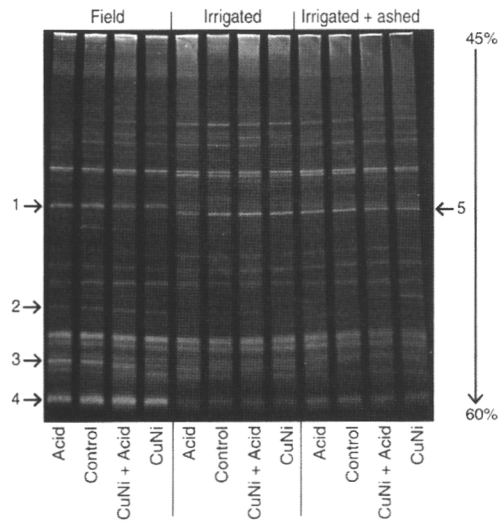

Fig. 2. Fungal DGGE of 390 bp 18S rDNA fragments from humus samples. The numbered bands indicated with arrows are discussed in the text. Each well is loaded with a mixture of PCR products of five replicate plots. See Table 4 for treatment symbols.

the amounts of PLFAs i14:0, i15:0, a15:0, i16:1, 16:1ω5 and 16:1ω9, and increased ($P < 0.10$) the amount of fungal fatty acid 18:2ω6. PC1 correlated with Cu_{ext} ($r = -0.70$), Ni_{ext} ($r = -0.51$), bioavailable Cu ($r = -0.78$) and fungi-to-bacteria ratio ($r = -0.80$).

The fungal community in the field experiment exhibited a metal effect since one band (no. 3) in the DGGE gel, which was obtained from the metal treated samples, had a weaker intensity (Fig. 2).

3.2. Irrigated microcosms

In the incubated and water irrigated microcosms the acid treated samples had still lower humus layer pH ($P = 0.046$) and BS ($P = 0.013$), and higher amounts of Cu_{ext} ($P = 0.0001$) and bioavailable Cu ($P = 0.011$) (Tables 4 and 5). Both acid ($P = 0.083$) and metal ($P = 0.001$) treatments increased the concentration of Ni_{ext} (Table 4). In addition both BS ($P = 0.040$) and Ni_{ext} ($P = 0.070$) had an acid and metal interaction, because their treatment effect was strongest in the CuNi + Acid treated microcosms, BS being lowest and Ni_{ext} highest due to this treatment.

There was no treatment effect for PCA scores of PLFAs in the irrigated microcosms (Fig. 1). The fungal community in the irrigated microcosms did not have the metal effect that was observed in the field samples. Three bands in the DGGE gel (no. 1, 2 and 4) faded due to laboratory incubation with irrigation and one band (no. 5) which did not occur in the field appeared in the gel (Fig. 2).

3.3. Irrigated and wood ash-treated microcosms

Following wood ash application no acid or metal treatment effect was detected on any measured variable. The ash-treated microcosms exhibited extractable metals (Cu_{ext} and Ni_{ext}) and bioavailable Cu amounts below the detection limit in general (Tables 4 and 5). Overall, ash-treated microcosms had higher humus layer pH, BS, CEC and basal respiration than irrigated microcosms had (Tables 4 and 5).

Wood ash-treated microcosms exhibited a different PLFA pattern than irrigation treatment alone had (Fig. 1). The proportion of 14:0, C15:1, 15:0, i16:0, 16:1ω7t, 16:0, 10Me16:0, a17:0, cy17:0, 10Me17:0, 18:1ω7, 19:1a, 10Me18:0, 20:5, 20:4 and 20:0 PLFAs were higher in the irrigation with ash treatment compared to irrigation alone (Table 6). Conversely, the proportion of i15:0, a15:0, 16:1ω9, 16:1ω7c, 16:1ω5, C17:1, 18:2ω6, 18:1ω9, 18:1, 18:0 and cy19:0 PLFAs were lower in the ash-treated microcosms.

Irrigation treatment with wood ash did not have any additional effect on fungal community structure, studied with 18S rDNA PCR-DGGE analysis, compared to irrigation alone (Fig. 2).

4. Discussion

4.1. Field experiment

After nine growing seasons the acid treatment resulted in decreased humus layer pH and BS and increased concentration of Cu_{ext}. In addition the Cu-sensor reacted to the acid effect indicating significantly more bioavailable Cu in the plots having received acid with or without the addition of Cu. The concentration of humus Ni_{ext} increased due to the metal treatment. The acid and metal treatments also changed the microbial PLFA pattern. The decrease in pH and increase in the amounts of Cu and Ni were not large enough to significantly decrease microbial activity (basal respiration) or biomass measures ($PLFA_{tot}$, $PLFA_{bact}$, $PLFA_{fung}$). Pennanen et al. (1998) obtained similar results from the same study site after the 6th treatment year. However, they did not observe a metal effect on the microbial PLFA pattern, and they did not include analyses for extractable Ni and bioavailable Cu.

With an 18S PCR-DGGE approach we observed a metal effect on the fungal community since one band (no. 3) had a lower intensity. None of the treatments decreased the number of DGGE bands, and therefore the fungal diversity was probably not affected. One must take into account that the DGGE analysis suffers from the limitation that although different band mobilities show non-identity, similar band mobilities do not confirm identity (Kowalchuk et al., 1997). For full interpretation of DGGE patterns, also DGGE band

excision and sequence determination would be necessary, but that was beyond the scope of our study.

4.2. Remediation experiment

Remediation of polluted soils aims to remove or immobilise the pollutants making them less mobile and less available for plants and microbiota. Wood ash, through increasing soil pH could counteract soil acidification and immobilize heavy metals since their solubility is pH dependent. Wood ash is also rich in mineral nutrients and so would improve the nutrient supply to plants. Our experimental design aimed in measuring the suitability of wood ash for remediation of acidified or metal polluted forest humus.

The irrigated microcosms displayed comparable, but less predominant acid and metal induced effects, as seen in the field experiment, except that no effects on PLFA and DGGE patterns could be observed. Ash-treatment removed all metal and acid treatment effects resulting in return of the acid and metal-induced disturbance towards respective control conditions.

The detoxifying effect of ash on Cu may be related to several possible causes. First, soil pH, CEC and BS are increased after wood ash application (Unger and Fernandez, 1990; Kahl et al., 1996), and these reduce metal solubility. This rise in soil pH, CEC and BS was also observed in the ash-treated microcosms compared to irrigated microcosms. Free metal (Cu^{2+}) activity decreases consistently with increasing pH (McBride et al., 1997). Thus, the reason for the decreased amount of bioavailable Cu after ash fertilization could be the decreased amount of Cu^{2+}. According to Dumestre et al. (1999) and Kiikkilä et al. (2002a,b) the activity of free Cu^{2+} in the soil solution is the best predictor of Cu toxicity to microbes.

Second, the incomplete combustion of fuel leaves carbonaceous substances in ash, which can bind heavy metals. Lin and Chang (2001) and Ricou-Hoeffer et al. (2001) demonstrated the removal of Cu^{2+} from aqueous solution by adsorption onto fly ash carbon. A comparable mechanism is probably not relevant in our study since after prolonged heating at 550 °C the organic matter content of the ash was as low as 4.7%. Thus, the amount of C (2.35%) in ash is so low that it does not adsorb significant amounts of Cu (Lin and Chang, 2001).

Third, Cu forms complexes with dissolved organic matter (Temminghoff et al., 1998), being mostly dissolved organic carbon (DOC). The toxicity of metal ions to bacteria decreases when they are complexed with organic compounds (Hughes and Poole, 1991; Menkissoglu and Lindow, 1991). Ash fertilization has been shown to increase DOC concentrations in soil (Weber et al., 1985; Ludwig et al., 2000) as has mulch (compost + woodchips) (Kiikkilä et al., 2001). After mulch application Kiikkilä et al. (2001) detected an increase in Cu complexation, which correlated to a decrease in exchangeable Cu and toxicity of soil solution to bacteria. It is not clear if the increase in soil DOC after ash fertilization is due to increased pH, increased microbial activity (Bååth and Arnebrant, 1994; Fritze et al., 2000) or both (Andersson et al., 2000).

Ash treatment increased the proportion of 14:0, C15:1, 15:0, i16:0, 16:1ω7t, 16:0, 10Me16:0, a17:0, cy17:0, 10Me17:0, 18:1ω7, 19:1a, 10Me18, 20:5, 20:4 and 20:0 PLFAs, while it decreased the proportion of i15:0, a15:0, 16:1ω9, 16:1ω7c, 16:1ω5, C17:1, 18:2ω6, 18:1ω9, 18:1, 18:0 and cy19:0 PLFAs compared to irrigated microcosms. The fatty acids 10Me16:0, 10Me17:0 and 10Me18:0 are produced by many actinomycete genera (Kroppenstedt and Kutzner, 1978; Kroppenstedt, 1985), and so can be used as actinomycete indicators. Thus, we detected an increased abundance of actinomycetes and lowered abundance of fungi following ash fertilization treatment. Similar changes after wood ash application on fungal (18:2ω6) and actinomycete PLFAs in a microcosms experiment were found by Fritze et al. (2000).

In the field studies of Frostegård et al. (1993a) and Bååth et al. (1995), the amounts of 16:1ω9, 16:1ω5 (2–3 times the control), cy17:0 and 10Me18:0 PLFAs increased after ash application, whereas i15:0 and 18:1ω9 decreased. The most striking difference between these field studies and our microcosm study was that the amount of the fatty acid 16:1ω5 decreased after ash amendment in our study. One reason for this difference could be the lack of plants in our microcosms. Herbs and grasses thrive after ash fertilization (Silfverberg and Hotanen, 1989; Ferm et al., 1992) and the amount of fatty acid 16:1ω5 increases when the amount of herbs and grasses increase (Pennanen et al., 1999; Saetre and Bååth, 2000).

Using DGGE, we did not detect the metal treatment effect on fungi obtained in the field after laboratory incubation. According to the number of bands in the DGGE profile, the amounts of fungal genotypes in the field and laboratory experiment were similar. In addition, the intensities of the bands were quite the same in the irrigated and irrigated plus wood ash-treated samples; showing they had the same amount of template DNA of these fungi. Fritze et al. (2001), in contrast, detected an ash effect on the fungal DGGE profile in their microcosm study. Bååth and Arnebrant (1993) also detected a clear difference in species composition of microfungi due to ash fertilization of coniferous forest soil analysed by an agar plate method. In our study, the intensities of three bands decreased, while that of one band increased during incubation of laboratory microcosms. These faded bands could be from mycorrhizas, since they depend on living plant for their C supply and are deprived of this at tree harvesting (Mahmood et al., 1999). Alternatively, the result is due to rhizosphere fungi, which suffer in the microcosms without plants.

4.3. Conclusions

Our field experiment showed that simulated acid rain and metal (Cu–Ni) application altered the microbial community structure of the humus layer. Acid application with or without metals decreased the humus layer pH, BS and increased the amounts of extractable and bioavailable Cu. Metal application with or without acid increased the concentration of extractable Ni and affected the fungal community. This is a situation found in the surroundings of industrial smelting plants. Incubating these samples for 2 months in laboratory conditions under water irrigation eliminated the detected acid and metal effects on the microbial and fungal community structure. All other above mentioned variables still exhibited the acid or metal effect. Addition of ash (5000 kg ha^{-1}) remediated the humus layer as indicated by a return of pH, BS, extractable Cu and Ni, and bacterial biosensor detecting bioavailable Cu to the respective control sample values. Thus, wood ash can be used for remediation of acid and metal polluted humus and should be tested in a field experiment.

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