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The effect of nickel contamination on the growth of litter-decomposing fungi, extracellular enzyme activities and toxicity in soil

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A multi-phased approach was used to assess microbial function and the growth of litter-decomposing fungi in the Ni-contaminated organic layer of a young *Pinus sylvestris* forest in southern Finland. Eight fungi tested (*Agaricus bisporus*, *Agrocybe praecox*, *Gymnopus peronatus*, *Gymnopilus sapineus*, *Mycena galericulata*, *Gymnopilus luteofolius*, *Stropharia aeruginosa* and *Stropharia rugosoannulata*) were able to grow in the Ni-contaminated soil (20 mg [kg soil]$^{-1}$). β-glucosidase, β-cellobiosidase, phosphomonoesterase, acetate-esterase and butyrate-esterase activities were mainly lower in the Ni-contaminated soil than in the non-contaminated soil. Low concentration of Ni (20 mg l^{-1}) inhibited the growth of all fungi on humus, RBBR and ABTS containing agar colour plates. EC_{50} for light inhibition in a test involving the yeast *Saccharomyces cerevisiae* was 294 mg Ni l^{-1}, which was quite high when compared with the inhibition of fungal growth on colour plates (20 mg Ni l^{-1}) and the EC_{50} for light inhibition of the bacterium *Vibrio fischeri* (85 mg Ni l^{-1}). Low Ni concentrations (20 mg [kg soil]$^{-1}$) in the present study inhibited microbial activity, the growth and enzyme production of litter-decomposing fungi.

Introduction

Nickel is a metal contaminating soils especially in the vicinity of industrial sites including smelters, mining sites and shooting ranges where concentrations can be as high as 40–4600 μg [g soil]$^{-1}$ (Paton *et al*. 2006, Robinson *et al*. 2008). Nickel has toxic effects on human (Riley *et al*. 2005), plants, soil microflora and -fauna (Baldrian 2003, Yuangen *et al*. 2006, Congeevaram *et al*. 2007, Gikas 2008) and it is regarded as belonging to the broad group of heavy metals (Duffus 2002). In addition, Ni^{2+} and other metal cations can replace nutrient cations (Ca^{2+}, Mg^{2+} and Mn^{2+}) from the cation exchange places of forest soil and thus decreasing the amount of available nutrients (Derome and Lindroos 1998). Still a few tools and applications are established to assess the environmental relevance of Ni contamination in the soil since the Ni concentration in soil is not directly correlated with its potential biological effects. The analysis of functioning of soil biota together with ecotoxicological tests are needed

to describe the impact of Ni contamination on forest soil ecology (Paton *et al*. 2006).

Microbial enzyme activities are essential in the cycling of organic carbon and nutrients in soil. Enzymes involved in the degradation of cellulose, hemicelluloses, starch and lignin — the most abundant biopolymers in forest soils — are essential to the nutrient cycling and releasing energy to the living organisms in soil. The main group of fungi decomposing poorly degrading lignin and lignocelluloses, the litter-decomposing fungi (LDF), produce oxidative enzymes, manganese peroxidase (MnP) and laccase, which are involved in the degradation of lignin (Hatakka 1994). Cellobiosidase and β-glucosidase produced by fungi and bacteria depolymerise cellulose to oligo- and monomers (Tomme *et al*. 1995, Warren 1996). Other hydrolytic enzymes like sulphatases and phosphatases hydrolyse ester bonds in sulphuric and phosphoric acids (Tabatabai and Fu 1992). Acetate- and butyrate-esterases are unspecific esterases, which are involved in the cycling of carbon (Tabatabai and Fu 1992).

Soil fungi are known to tolerate heavy metals like Pb (Baldrian 2003, Tuomela *et al*. 2005), but the sensitivity of fungi to heavy metals can differ between species and strains (Baldrian 2003, Fomina *et al*. 2004). To find out the effective concentrations of Ni on the growth and enzyme production on soil litter decomposing fungi and the overall enzyme production in forest soil and thus nutrient cycling, a multi-phased approach was used. Extracellular hydrolytic and oxidative enzyme activities produced mainly by fungi and bacteria (Warren 1996) were studied from soil and soil extract although the extracellular enzymes can be sorbed to particles in the soil (Tabatabai and Fu 1992, Kähkönen *et al*. 2008). Two light inhibition toxicity assays, based on the bacterium *Vibrio fischeri* and the yeast *Saccharomyces cerevisiae*, were used to measure the bioavailable Ni content and in order to allow comparisons of single species tests with our results of microbial functioning measured by growth and enzyme-activity tests. Non-sterile soil was used to better simulate the influence of Ni contamination in the natural forest soil. The hypothesis for this work was that Ni, even in low concentrations, has an effect on the growth and enzyme production of soil fungi and bacteria. This effect has to be studied closely to understand the mechanisms of Ni contamination in soil ecosystem, especially to the recycling on carbon and nutrients.

Materials and methods

Fungi and inocula

Litter decomposing fungi (LDF) were obtained from the Fungal Biotechnology Culture Collection (FBCC) at the Department of Food and Environmental Sciences, University of Helsinki, Finland, and the culture collection of the International Graduate Institute of Zittau, Germany (designated TM, X, 11372). The fungi, except for *Agaricus bisporus*, were selected for their ability to grow in heavy metal (Pb) contaminated soil as found in previous works (Tuomela *et al*. 2005, Kähkönen *et al*. 2008). The litter decomposing fungi were: *A. bisporus* ATCC 62459, *Agrocybe praecox* FBCC 476 (TM70.84), *Gymnopus peronatus* FBCC 635 (K220) (syn. *Collybia peronata*), *Gymnopilus sapineus* FBCC 1010 (HAM 1), *Mycena galericulata* FBCC 598 (K175), *Gymnopilus luteofolius* X9, *Stropharia aeruginosa* FBCC 521 (K47) and *Stropharia rugosoannulata* B FBCC 475 (DSM 11372) (Table 1). All fungi except for *S. rugosoannulata*, which is a straw-colonising fungus, can be found in boreal forests. The strains were maintained on 2% w/w malt extract agar. The inocula for the soil cultures were prepared from two-week-old malt agar (2% w/w, Biokar Diagnostics) plate cultures: 5 plugs (10 mm diameter) of the fungus growing on agar were removed and added to 200 ml sterilized (pH 5) basal liquid medium (Steffen *et al*. 2002) containing 2 g rye bran. The inoculum was homogenized by shaking with sterilised glass beads for 15 s.

Soil and soil plate cultures

The soil used in the study was from a young, about 20 years old, naturally generated *Pinus sylvestris* stand at Hälvälä in Hollola (67°67´N 34°18´E, southern Finland). The site is situated

on a moraine ridge, whose age is about 10 000 years. The forest site is *Vaccinium vitis-idea* type (Cajander 1925). The soil was well-stratified podsolic soil. The organic layer was sieved and was stone free having under 1% of stones with a diameter greater than 20 mm. The parent material was a glaciofluvial deposit with a texture from coarse to fine sand. Density of *Pinus sylvestris* was 0.15 trees m^{-2} and the production of total pine tree litter (needles, twigs, bark pieces, etc.) was 676 g m^{-2} $tree^{-1}$ in 5 months (from July to November) in 2004 (Rantalainen *et al*. 2006). Details of natural state site are described elsewhere (Rantalainen *et al*. 2006).

Soil of the organic layer of a podsolized forest soil was sampled with a shovel (0–6 cm) from the area being in natural state. Sampled soil was pooled and mixed. The carbon and nitrogen contents in the soil were measured with a Leco CHN analyzer (Leco Corporation, St. Joseph, MI) (Kähkönen *et al*. 1998). The soil pH was measured as described by Kähkönen *et al*. (2002). The carbon and nitrogen contents were 45.7% ± 1.0% per g_{dw} of soil and 2.1% ± 0.1% per g_{dw} of soil, respectively. pH of the soil was 4.5 ± 0.2. $NiSO_4$ solution (3 ml) was added to 9 g of soil in a Petri dish (10 cm diameter) to give a final Ni concentration of 20 mg per kg_{ww} soil (Ni-contaminated soil). Control soil cultures without added Ni were included. Sterile liquid medium (3 ml) was added to the non-contaminated control soils to achieve the same final moisture content as in the Ni-contaminated soil. Soil moisture was 47% of the final fresh weight. As an inoculum, 3 ml of suspension from a two-week-old liquid fungal culture was added to the Ni-contaminated and non-contaminated soils in the Petri dishes. Non-inoculated Ni-contaminated and non-contaminated soils served as controls for the fungal growth and enzyme activity determinations. Three replicate plates per treatment were incubated at 25 °C for three weeks and fungal growth was assessed visually and classified into the three categories: no growth (–), weak growth (+) and moderate growth (++). Samples for ligninolytic and hydrolytic enzyme activity assays were taken three weeks after inoculation.

Colour plate tests

The fungal growth and production of ligninolytic enzymes in the Ni-containing media were tested with colour plate tests. These tests were carried out on basal medium agar plates with glucose as a carbon source (Steffen *et al*. 2002) containing 1000 mg l^{-1} humic acid (Sigma Aldrich, U.S.A.), 250 mg l^{-1} 2,2´-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS; Sigma Aldrich, U.S.A.) (Steffen *et al*. 2000), or 400 mg l^{-1} Remazol Brilliant Blue R (RBBR; Serva, Germany) (Novotny *et al*. 1997) in 2% w/w agar. $NiSO_4$ was added to the media to give the final Ni concentrations of 0, 20, 50, 100, 500 and 1000 mg l^{-1}. Each plate of three replicates per concentration was inoculated with a 10 mm diameter agar plug from a 2–3-week-old malt-agar plate culture of fungi and incubated at 22 °C. After three weeks, the radial growth and color reaction zones were measured with a ruler. Laccase production by the fungi was evident when a dark-green color around the mycelium on

Table 1. The growth of selected litter decomposing fungi (n = 3) three weeks after inoculation into non-contaminated and Ni-contaminated soil (20 mg [kg_{ww} soil]$^{-1}$). The control soil was without fungal inoculum.

Fungus	Non-contaminated soil	Ni-contaminated soil
Agaricus bisporus ATCC62459	+	–
Agrocybe praecox TM70.84	++	++
Gymnopus peronatus K220	+	+
Gymnopilus sapineus HAM 1	++	++
Mycena galericulata K175	+	+
Gymnopilus luteofolius X9	+	+
Stropharia aeruginosa K47	+	+
Stropharia rugosoannulata DSM11372 B	+	+
Control soil without inoculum	–	–

ABTS plates appeared, indicating stable radical formation. On the RBBR plates, a decolorized zone around the fungal mycelium indicated the presence of extracellular laccases or manganese peroxidases. Clearing of the humic-acid plates was indicative of the action of depolymerizing ligninolytic enzymes produced by the fungi.

Hydrolytic enzyme activities from soil

All enzyme activities were measured in Ni-contaminated (20 mg [kg_{ww} soil]$^{-1}$) and non-contaminated soils inoculated with the fungi as shown in Table 1. Five hydrolytic enzyme activities were measured with 4-methyl-umbelliferyl (MUF) conjugated fluorogenic model compounds: MUF-β-cellobioside, MUF-β-glucoside, MUF-phosphate, MUF-acetate and MUF-butyrate (Sigma, St. Louis, Mo, USA). Two samples of soil (0.1 cm^3) were taken from each of the replicate soil plate cultures and transferred to wells in 96-well microtiter plates. Two wells with soil but without added substrate served as blanks. Deionized water (180 μl) was added to each well. The temperature was adjusted to 30.0 ± 0.5 °C 10 min before the enzyme activity measurements were made. A substrate solution (20 μl) was added to initiate the reaction. Enzyme activities were measured between 3 to 60 min depending on the activity at natural pH using an automated kinetic fluorometer (Fluoroskan Ascent Labsystems, Finland), as described by Wittmann *et al.* (2004). The excitation wavelength was 355 nm and the emission wavelength 460 nm. Calibration was performed using MUF.

Enzyme activities from soil extract

A soil sample (0.2 cm^3) from each replicate plate of fungal soil plate cultures was extracted for 30 min in 0.6 ml 500 mM sodium acetate buffer (pH 5.5) at 22 °C. The soil-buffer suspension was centrifuged for 5 min at 5000 *g*. The ligninolytic enzyme activities in the supernatant (soil extract) were measured spectrophotometrically (Shimadzu UV-1700). MnP activity was determined by following the formation of a Mn^{3+}-malonate complex at 270 nm (Wariishi *et al.* 1992). Laccase activity was measured by following the oxidation of ABTS at 420 nm (Eggert *et al.* 1996). To be able to compare the enzyme activities between soil samples and soil extract, we measured the hydrolytic enzyme activities also from the soil extract, which represent the free enzymes in the soil. Hydrolytic enzyme activities in the soil extract (180 μl) were measured with fluorogenic model substrates (20 μl) as described for soil samples.

Toxicity tests

To get test samples of varying Ni concentration (0–300 mg l^{-1}), first a 20% (w/w) slurry of soil was prepared from the soil containing 3000 mg Ni per kg. Nickel was added to soil in for of $NiSO_4$ solution. The 20% slurry was further diluted in water or in 2% NaCl (w/v) to get suitable concentrations for the *S. cerevisiae* test or for the *V. fischeri* test (ISO/DIS 21338*), respectively. The pH of soil samples was adjusted to 7. The final tested Ni concentrations of soil slurries were 0, 100, 200 and 300 mg l^{-1} (*S. cerevisiae* test) and 0, 12, 30, 38, 60, 75, 150 and 300 mg l^{-1} (*V. fischeri* test) to allow the calculation of EC_{50} value. $NiSO_4$ solution in water was used as a positive control in both tests.

Toxicity of Ni was measured using the yeast strain *S. cerevisiae* BMA64/luc (Leskinen *et al.* 2005). This strain constitutively expresses firefly luciferase from plasmid pRS316luc. Toxicity of Ni was assayed by comparing the light emission by the BMA64/luc strain in Ni-contaminated and non-contaminated soils. A 4-ml culture of *S. cerevisiae* was grown overnight at 30 °C with vigorous shaking in a synthetic minimal medium (SD; Difco yeast nitrogen base without amino acids, 6.7 g per 910 ml) supplemented with glucose (Amresco, Ohio, USA) and required amino acids (BD; Sigma-Aldrich Co., St. Louis, USA). The culture was diluted to an optical density of 0.4 at 600 nm (OD_{600}), and then grown at 30 °C until the OD_{600} reached 0.6–0.7. Soil slurry (50 μl) containing 0, 200, 400 or 600 mg Ni l^{-1} was added to three replicate wells of a 96-well plate, and 50 μl aliquots of *S. cerevisiae* culture were added to give a final volume of 100 μl. The plate was shaken for 20 s and then incubated at 30 °C

* ISO/DIS 21338 Water quality — Kinetic determination of the inhibitory effects of sediment, other solids and coloured samples on the light emission of *Vibrio fischeri* (kinetic luminescent bacteria test).

for 30 min, after which time it was shaken for another 20 s, and 100 µl of 1 mM D-luciferin (in 0.1 M sodium citrate buffer, pH 5; BioThema, Sweden) was added. The plate was again shaken briefly, and the luminescence was immediately measured using a Victor3 1420 multilabel counter (Perkin-Elmer Wallac, Turku, Finland) in luminescence mode, using a 1 s counting time. The EC_{50} value was calculated by fitting a linear regression to the results from the inhibition of light emission of *S. cerevisiae*.

The acute toxicity was tested with the luminescent bacterium *V. fischeri* using the BioTox™ Kit (Aboatox, Turku, Finland) as a Flash-test (ISO/DIS 21338*). Samples of 150 µl per well were dispensed in a 96-well microtiter plate. *Vibrio fischeri* suspension (150 µl) was added, and the maximum bioluminescence during 5 s was measured using the Victor3 1420 multilabel counter. The plate was incubated for 30 min at 15 °C, and the bioluminescence was measured from duplicate samples. The EC_{50} value was calculated from the inhibition of *V. fischeri* luminescence according to the instructions of the BioTox™ kit and the standard ISO/DIS 21338*.

Statistical analysis

To test significance of differences between enzyme activities (manganese peroxidase activity, β-glucosidase, β-cellobiosidase, phosphomonoesterase, acetate-esterase and butyrate-esterase) in the Ni-contaminated and non-contaminated soils with the same fungal inoculum; as well as between manganese peroxidase ($p < 0.05$, $n = 3$) β-glucosidase, β-cellobiosidase, phosphomonoesterase, acetate-esterase and butyrate-esterase activities ($p < 0.05$, $n = 6$) without and with fungal inoculum, a *t*-test (Sokal and Rolf 1969) was used. Calculations were performed using Microsoft® Excel®. Suitability of the data for the test was assessed with Shapiro-Wilk's test (Sokal and Rolf 1969).

Results

All eight fungi grew on the non-contaminated organic layer soil (Table 1). *Agrocybe praecox* and *G. sapineus* achieved the best growth in the non-contaminated and contaminated (20 mg Ni kg wet weight soil^{-1}) organic layer soils. All fungi except *A. bisporus* grew in the Ni-contaminated organic layer soil.

Nickel inhibited least the growth of *G. sapineus* and *S. rugosoannulata* [20 and 50 mg Ni l^{-1} on humic acid, and 20 and 50 mg Ni l^{-1} on ABTS, respectively, and 50 mg Ni l^{-1} on RBBR; (Fig. 1)]. More than 70% growth inhibition occurred in *A. bisporus*, *M. galericulata* and *S. aeruginosa* on humus, ABTS and RBBR colour plates containing 20 mg Ni l^{-1}. *Agrocybe praecox* and *G. sapineus* were the only fungi able to grow on ABTS and RBBR colour plates containing 100 mg Ni l^{-1}. The fungi did not grow in the presence of 500 and 1000 mg Ni l^{-1}. The MnP activity in the Ni-contaminated soil (20 mg kg of soil^{-1}) inoculated with *M. galericulata* or *S. aeruginosa* was lower (*t*-test: $p < 0.05$) than in the non-contaminated soil inoculated with these fungi (Fig. 2). *Agaricus bisporus*, *A. praecox*, *G. sapineus*, *G. luteofolius*, *S. aeruginosa* and *S. rugosoannulata* produced MnP in the Ni-contaminated soil (20 mg [kg soil]$^{-1}$), as the MnP activity in the soil inoculated with these fungi was higher than in the uninoculated soil (*t*-test: $p < 0.05$) (Fig. 2). The MnP activity in the non-contaminated soil inoculated with *A. praecox*, *M. galericulata* or *S. aeruginosa* was higher than in the uninoculated control soil (*t*-test: $p < 0.05$). The MnP activity in the Ni-contaminated soil (20 mg [kg soil]$^{-1}$) inoculated with *M. galericulata* or *S. aeruginosa* was lower (*t*-test: $p < 0.05$) than in the non-contaminated soil inoculated with these fungi. Nickel triggered MnP activity in *S. rugosoannulata*, as the MnP activity was higher in the inoculated Ni-contaminated soil than in the non-contaminated soil. No laccase activity was detected in the soil cultures, although the RBBR and ABTS basal medium plate cultures indicated production of laccase by all the fungi at the Ni concentration of 50 mg l^{-1}.

More than half (65%) of the tested hydrolytic enzyme activities were higher in the non-contaminated soil than in the Ni-contaminated soil inoculated with the same fungus (*t*-test: $p < 0.05$), indicating that these enzyme activities were decreased by Ni (Figs. 3 and 4). In case of the *G. penetrans* inoculated soil, all the hydro-

* ISO/DIS 21338 Water quality — Kinetic determination of the inhibitory effects of sediment, other solids and coloured samples on the light emission of *Vibrio fischeri* (kinetic luminescent bacteria test).

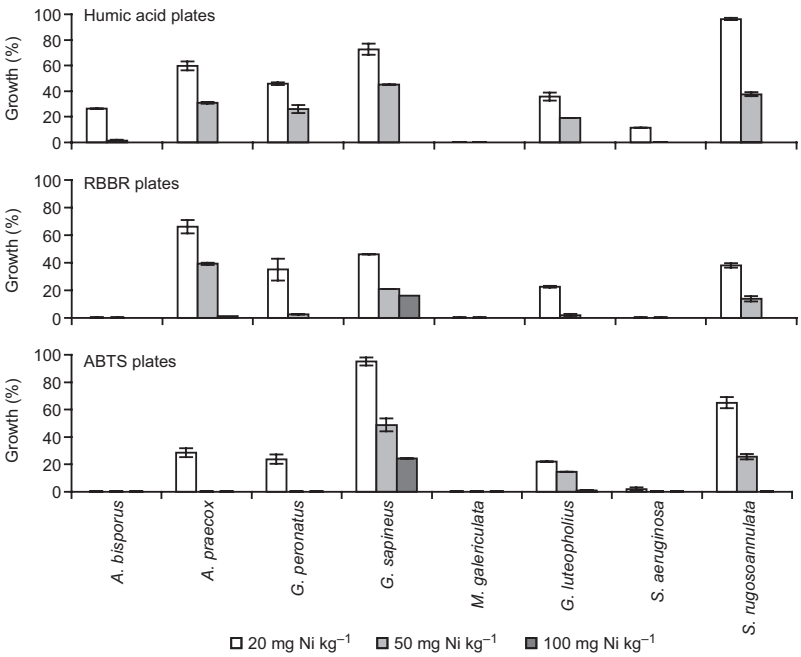

Fig. 1. The radial growth of eight selected litter-decomposing fungi after 3 weeks of growth on humic acid, RBBR (Remazol Brilliant Blue R) and ABTS (2,2´-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) colour culture plates (n = 2) containing 20, 50 and 100 mg Ni l^{-1}, relative to growth in control in the absence of Ni (assigned a value of 100%). The fungi did not grow in the presence of 500 and 1000 mg Ni l^{-1}.Bars are standard errors of the mean.

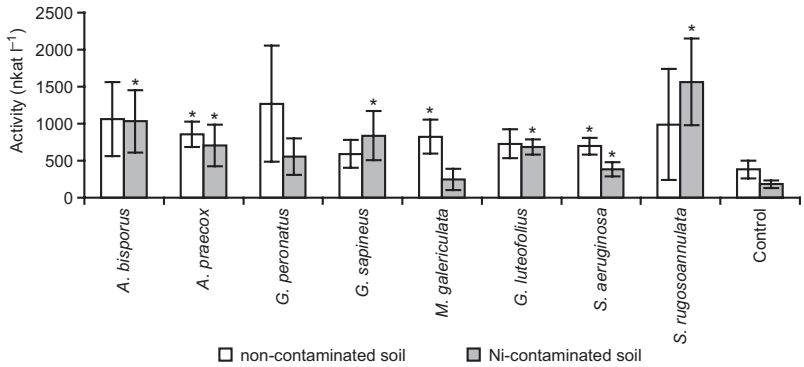

Fig. 2. Manganese peroxidase (MnP) activity in extracts of soil organic layer contaminated with Ni (20 mg [kg soil]$^{-1}$) and non-contaminated soil (n = 3). Control soil is without fungal inoculum. Bars are standard errors of the mean. Statistical differences (t-test: $p < 0.05$) between fungus-inoculated and non inoculated control soil are indicated with asterisks (*).

lytic enzyme activities (phosphomonoesterase, acetate-esterase, butyrate-esterase, β-glucosidase and β-cellobiosidase) were lower in the Ni contaminated than in the non-contaminated soils (t-test: $p < 0.05$).

Activity of β-cellobiosidase was higher (t-test: $p < 0.05$) in the non-contaminated and Ni-contaminated soils inoculated with *G. penetrans* than in the uninoculated control soil (Fig. 3), indicating that *G. penetrans* produced cellobiose hydrolyzing enzymes. Activity of phosphomonoesterase was higher in the Ni-contaminated and non-contaminated soils inoculated with *G. penetrans* or *M. galericulata* than in the uninoculated control soil (t-test: $p < 0.05$). From acetate- and butyrate-esterases only acetate-esterase activity was higher in the non-contaminated soil inoculated with *G. luteofolius* than in the uninoculated control soil (t-test: $p < 0.05$) (Fig. 4).

Phosphomonoesterase, acetate-esterase and butyrate-esterase were active in extracts of the non-contaminated and Ni-contaminated soils

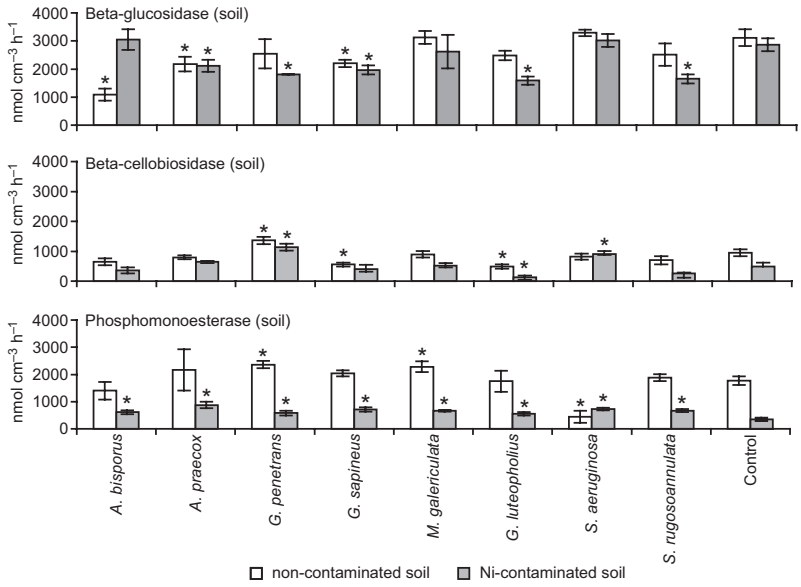

Fig. 3. β-Glucosidase, β-cellobiosidase and phosphomonoesterase activities in the organic layer of Ni-contaminated (20 mg [kg soil]$^{-1}$) and non-contaminated soil (n = 6). Bars are standard errors of the mean. Statistical differences (t-test: $p < 0.05$) between fungus-inoculated and non inoculated control soil are indicated with asterisks (*).

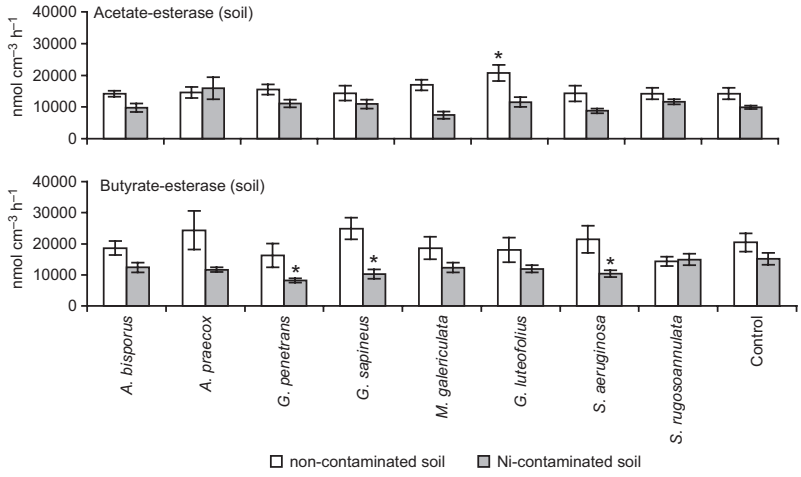

Fig. 4. Acetate-esterase and butyrate-esterase activities in the organic layer of Ni-contaminated (20 mg [kg soil]$^{-1}$) and non-contaminated soil (n = 6). Bars are standard errors of the mean. Statistical differences (t-test: $p < 0.05$) between fungus-inoculated and non inoculated control soil are indicated with asterisks (*).

with and without fungal inoculation (Fig. 5). β-glucosidase and β-cellobiosidase were not active in the acetate buffer extracts of the non-contaminated and Ni-contaminated soils, but active in the soil (native non-contaminated and Ni-contaminated; Fig. 3). Phosphomonoesterase, acetate-esterase, butyrate-esterase, β-glucosidase and β-cellobiosidase activities were higher in soil (Figs. 3 and 4) than in comparable soil extracts.

The EC_{50} values were calculated from the inhibition of light production by *S. cerevisiae* and *V. fischeri* in soil slurries containing varying Ni concentrations (0–300 mg l^{-1}). The EC_{50} values for the inhibition of light production were 294 mg Ni l^{-1} (*S. cerevisiae*) and 83 mg Ni l^{-1} (*V. fischeri*). The corresponding values for the $NiSO_4$ solution were 450 mg Ni l^{-1} (*S. cerevisiae*) and 280 mg Ni l^{-1} (*V. fischeri*). The EC_{50} value for Ni in the *S. cerevisiae* test was quite high relative to the concentrations causing toxicity in the fungal colour plate tests (20–100 mg Ni l^{-1}) However, the EC_{50} for inhibition of light production in the *V. fischeri* toxicity test was comparable to results obtained for the fungal plate toxicity and extracellular enzyme inhibition tests.

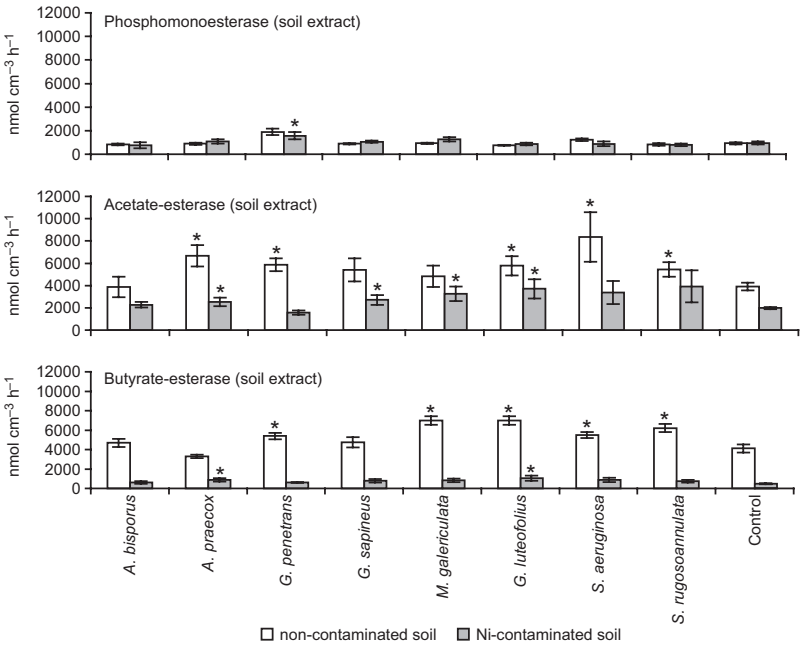

Fig. 5. Phosphomonoesterase, acetate-esterase and butyrate-esterase activities in extracts of the organic layer of Ni-contaminated (20 mg [kg soil]$^{-1}$) and non-contaminated soils ($n = 6$). Bars are standard errors of the mean. Statistical differences (*t*-test: $p < 0.05$) between fungus-inoculated and non inoculated control soil are indicated with asterisks (*).

Discussion

Impact of Ni on the growth of fungi and production of extracellular enzymes in the soil

The sensitivity of fungi to heavy metals can differ among species and strains (Baldrian 2003). The metal tolerance advances fungi in contaminated areas to continue carbon cycling: e.g. aquatic litter-degrading fungi are shown to tolerate Cu and Zn metal stress and continue degradation of litter in metal-contaminated streams (Azevedo *et al*. 2007). In this work, the basidiomycetous litter-degrading fungi *A. bisporus*, *A. praecox*, *G. peronatus*, *G. sapineus*, *M. galericulata*, *G. luteofolius*, *S. aeruginosa* and *S. rugosoannulata* were able to grow in the soil contaminated with 20 mg Ni kg^{-1}, with *A. praecox* and *G. sapineus* achieving the best growth. Kähkönen *et al*. (2008) and Tuomela *et al*. (2005) reported that *A. praecox* and *G. peronatus* grew also well in the soil heavily contaminated with Pb (40 g [kg soil]$^{-1}$). The growth of inoculated fungi in this work was clearly lower in the non-sterile soil than in experiments with the sterile soil (Tuomela *et al*. 2005) which is possibly at least partly due to soil microfauna consuming fungal mycelia from the soil plates. The growth of litter-degrading fungi in the presence of Ni is not much studied. Congeevaram *et al*. (2007) noticed that mitosporic (mold) fungi like *Aspergillus* species from Ni-contaminated soil were able to grow in the presence of Ni^{2+} concentration of 50–500 mg l^{-1} in liquid culture, but the growth decreased above the concentration of 100 mg l^{-1}. The findings of this work and those of Tuomela *et al*. (2005) and Kähkönen *et al*. (2008) show that *A. praecox* can tolerate both Ni and Pb which both are divalent (+2) heavy metals.

Heavy metals (Mn, Cu, Co, Cd) in low concentrations can increase the ligninolytic activity (MnP and laccase) of fungi (Baldrian and Gabriel 2002, Baldrian 2003). Mn is an essential part of the catalytic cycle in MnP and Cu is a co-factor of laccase. Nickel can act as a co-factor for several microbial enzymes (Dosanjh and Michel 2006, Gikas 2008), and Ni and other heavy metal stress may cause elevated peroxidase levels in plants (Schickler and Caspi 1999, Ahonen-Jonnarth *et al*. 2004, Lin and Kao 2005), but it is not known to have that role in fungal oxidoreductive enzymes. Mycorrhizal fungi are shown to mitigate the stress caused by soil metal (Cu, Cd, Pb and Zn) contamination

in plants (Jentschke and Godbold 2000, Colpaert 2008) but the mechanisms of mitigation is not clear. In our work, only *S. rugosoannulata* increased MnP production in the Ni-contaminated soil (20 mg [kg soil]$^{-1}$) as compared with that in the non-contaminated soil. Divalent Pb^{2+} also appears to slightly increase the production of MnP by *G. peronatus* and *G. sapineus* in soil cultures (Kähkönen *et al*. 2008). We suggest that the production of MnP by *S. rugosoannulata* in the presence of divalent Ni^{2+} may reflect fungal detoxification processes under stress, as shown for fungal laccases (Baldrian and Gabriel 2002), or plant peroxidases

Microbial toxicity of Ni

At a low concentration (20 mg [kg soil]$^{-1}$) Ni was toxic to fungi and bacteria, and affected microbial functioning in the soil. Major part of the tested hydrolytic enzyme activities were higher in the non-contaminated than in the Ni-contaminated soil indicating that Ni reduced activities of these enzymes. Particularly *G. penetrans* seemed to be sensitive to Ni, since all the hydrolytic enzyme activities (phosphomonoesterase, acetate-esterase, butyrate-esterase, β-glucosidase and β-cellobiosidase) in the *G. penetrans* inoculated soil were lower in the Ni contaminated than in the non-contaminated soil ($p < 0.05$).

A Ni concentration of 20 mg [kg soil]$^{-1}$ is well below the limit values for contaminated soil (100–150 mg Ni [kg_{dw} soil]$^{-1}$) set by the Finnish administration (Valtioneuvoston asetus 2007) and those reported for the most Ni-contaminated areas (up to 5000 mg Ni [kg soil]$^{-1}$, Derome and Lindroos 1998, Paton *et al*. 2006). Bååth *et al*. (1998) reported that Ni at a concentration of 69 mg [kg soil]$^{-1}$ negatively influenced soil microbial communities. The natural background for Ni is about 1–5 mg [kg soil]$^{-1}$ in rural areas in southern Scandinavia (Hovmand *et al*. 2008). Thus sensitive parts of the soil ecosystem, such as bacteria involved in production of hydrolyzing enzymes and litter-degrading fungi tested in this work, are vulnerable to low Ni concentrations studied in this work (20–100 mg kg^{-1}).

The present study is the first to report Ni toxicity in the soil using a luminescent yeast assay. Luminescent yeast cells have previously been used to test Ni toxicity in solution (Soares *et al*. 2003, Knight *et al*. 2004). The yeast luciferase assay directly reflects the condition of the cell, as the light-producing reaction uses ATP (Hollis *et al*. 1999). The EC_{50} (294 mg Ni l^{-1}) for light inhibition in the yeast test was higher than that concentration (100 mg Ni l^{-1}), which almost completely inhibited fungal growth in the colour plate tests. Knight *et al*. (2004) calculated an EC_{50} value of 21 mg Ni l^{-1} for the genotoxicity of fluorescent *S. cerevisiae*. On the other hand, an EC_{50} of 6100 mg Ni l^{-1} has been reported for *S. cerevisiae* in solution (Codina *et al*. 1993). Pearce *et al*. (1999) suggested that the detoxification process of Ni by the ascomycetous yeast *S. cerevisiae* may be due to accumulation of the heavy metal with histidine in the vacuole in the cell, and this may explain the high tolerance of yeast to Ni. In the present study, the higher EC_{50} value for light inhibition in both the *V. fischeri* and *S. cerevisiae* tests, relative to growth inhibition of the fungi and production of extracellular enzymes, indicates that the tolerant members of microbial communities may be able to survive in Ni-contaminated soil ecosystems; however, the ability of the basidiomycetous fungi to degrade plant biopolymers (lignocelluloses) may be reduced in these environments. This could have a deleterious impact on carbon cycling in the Ni-contaminated soil.

Environmental fate of extracellular enzymes in the soil

The phosphomonoesterase, acetate-esterase and butyrate-esterase activities were higher in the soil than in the soil extracts. β-glucosidase and β-cellobiosidase activities were not detected in the soil extracts, although they were detected in the soil. Enzymes form soil–enzyme complexes (Tabatabai and Fu 1992), which can preserve the enzyme activity. Kähkönen *et al*. (2008) showed that sulphatase, α-glucosidase, β-xylosidase, β-glucosidase and β-cellobiosidase activities were higher in *Pinus sylvestris* forest soil than in acetate extracts of that soil. Our results suggest that phosphomonoesterase, acetate-esterase, butyrate-esterase, β-glucosidase, β-cellobiosidase,

α-glucosidase and β-xylosidase enzymes may be bound to soil particles in the organic layer in the podsolized coniferous forest soils.

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