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Influence of lead on organisms within the detritus food web of a contaminated pine forest soil

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The shooting range site at Hälvälä in southern Finland is heavily contaminated by lead pellets. Still, the appearance of the forest is not visibly changed. Lead contamination strongly decreased the amount of enchytraeid worms, while reporter bacteria showed no or very minor decrease in viability. The bioavailability of lead in the soil was measured to be low, which was verified by the very low water extractability of lead. Nevertheless, the frequency of lead resistant cultivable bacteria was elevated, and the bacterial community composition in lead contaminated soil was altered. Some enzymes of litter decomposing fungi isolated from Hälvälä also showed an elevated lead tolerance, though generally their activity was highly variable and in some cases lead enhanced enzyme production of a fungus. In conclusion, our results point to a low acute toxicity of lead, but to a risk of deleterious long term effects in a pine forest environment.

Introduction

Healthy looking, well growing trees are normally interpreted as a sign of a clean, unpolluted environment. Also contaminated sites may, however, appear healthy. Good examples of this are the forests at shooting ranges. The Hälvälä shooting range, located in Hollola, southern Finland has been in use since 1964 (Turpeinen *et al*. 2000). Although this site is heavily contaminated by lead pellets the visual appearance of the forest at the shooting range is indistinguishable from the nearby clean forest and at least pine growth and ground cover vegetation are not visually disturbed (Rantalainen *et al*. 2006). The Hälvälä shooting range, the model ecosystem used in this study, thus appears normal from a distance, although the ground in the most heavily polluted sections is virtually covered with lead pellets from many years of shotgun activity. Even after

removal of the pellets the total concentration of lead in soil is high and the ecosystem can be considered as saturated (Turpeinen *et al*. 2000, Rantalainen *et al*. 2006).

Lead is considered to be highly toxic to living organisms, and it has been reported to decrease microbial biomass and alter their community structure in contaminated soil (Kuperman and Carreiro 1997, Rantalainen *et al*. 2006). Earlier we have shown that various representatives of the soil fauna are affected (Salminen *et al*. 2002). The hazard posed by lead is, however, a function of the mobility and bioavailability, which in turn are dependent on soil characteristics such as pH, mineralogy, organic matter content and the biota of the soil (Tuomela *et al*. 2005). In bacteria, resistance to heavy metals is often a plasmid- or transposon-borne trait (Busenlehner *et al*. 2003). An increase in the bacterial community tolerance to lead does therefore not necessarily require a reshaped bacterial community structure, if instead the resident bacteria are equipped with mobile genes or operons to deal with lead related stress. Using community fatty-acid analysis (PLFA) Bååth *et al*. (2005) found, however, a shift towards dominance of Gram positive bacteria in the soil naturally containing high concentrations of lead, whereas no decrease in bacterial cell counts was reported by these authors.

Lead tolerance of fungi is generally thought to be higher than that of bacteria, and the fungal proportion in lead contaminated soil has in some cases been found to increase (Rajapaksha *et al*. 2004). In earlier studies performed in the soil from the Hälvälä site, growth of certain fungal species showed remarkable tolerance to lead, although lignin mineralization by these fungi was slightly retarded (Tuomela *et al*. 2005). Thus, it appears likely that such species are enriched at lead contaminated sites. Although fungi in general tolerate higher concentrations of heavy metals than bacteria, also fungal population may be affected by high heavy metal contamination (Chander *et al*. 2001). The disturbed fungal activity may slow down the carbon cycle, as litter-decomposing fungi are the most important degraders of organic matter in boreal forests. In addition to growth, heavy metals usually inhibit the enzymatic activity and often have an influence on morphology of fungi (Baldrian 2003, Fomina *et al*. 2003). However, sometimes the activity of laccase — one of the ligninolytic enzymes — increase in the presence of heavy metals (Baldrian and Gabriel 2002).

The "healthy" appearance of the Hälvälä forest in combination with prior knowledge of clear differences in response to lead by different groups of organisms caught our attention and desire to compare the influence of lead on different biota, and thereby get a preliminary view of how these groups deal with lead contamination. We hypothesize that the response by each member of the soil biota is linked to the specific bioavailability of lead to this organism. Bioavailability is here defined as the fraction of lead that causes an effect on the organisms that are used as bioreporters. According to our hypothesis there is thus no universal method of measuring bioavailability, and although bioavailability correlates with the water solubility, the bioavailable fraction of a heavy metal may be larger than the water soluble fraction (Petänen and Romantschuk 2003). Only the bioavailable portion of lead or other heavy metals is toxic, and toxicity (lethal effects, inhibition of metabolism, etc.) can thus be used as a measure of bioavailability (Petänen and Romantschuk 2003, Petänen *et al*. 2003). A very sensitive method of determining bioavailability of a toxic compound is to use inducible bacterial bioreporters (Petänen *et al*. 2001) that respond, in a measurable fashion (e.g. by producing bioluminescence), to the toxicant at concentrations that are far below the acutely toxic level. Such bioreporters can detect subtoxic concentrations of heavy metals, which are expected to have long term effects on the soil biota.

We hypothesize that (1) the site history (duration of contamination) is reflected in the distribution (stratification) of lead in the upper layers of the soil, (2) that the effect of lead on different soil biota correlates with, and only with, the bioavailability of lead to each group of organisms, and (3) that the microbial diversity and species composition, but not the density, i.e. colony forming units, are affected by the concentration and distribution of bioavailable lead. Testing of these hypotheses is mainly based on data presented in this article, but we also seek explanations from observations published earlier.

Materials and methods

Study area

An established shooting range, located at a pine forest site in Hälvälä in southern Finland (67°37´N, 34°18´E) has been studied since 2003 as our model ecosystem. The forest mainly consists of Scots pine (*Pinus sylvestris*) with some sporadic silver birch (*Betula pendula*). The field layer consists of dwarf shrubs (*Vaccinium myrtillus*, *V. vitis-idaea*), and grasses (mainly *Deschampsia flexuosa*), while the ground layer comprises various mosses (mainly *Pleurozium schreberi* and *Dicranum* spp.; Rantalainen *et al*. 2006). One sector of the site was used as a shooting range during the years 1964–1987, called hereafter the 'old contaminated area' (OC), and after that this part of the range was allowed to reforest naturally. The shooting sector was then moved to an adjacent area, and this sector is still in use (referred to as the 'new contaminated area', NC). The contaminated soil from the old sector has been described earlier (Turpeinen *et al*. 2000, Salminen *et al*. 2002, Rantalainen *et al*. 2006). In the OC area, the soil surface and litter layer are free from lead shotgun pellets, but the humus layer is heavily contaminated by partly eroded pellets, and lead levels are very high even after their removal. In the NC area, shotgun pellets and lead contamination is high both in the surface litter and the humus layers. A nearby uncontaminated area was chosen as a control (clean control, C). This area was to the left of the shooting sectors ca. 500 m from the old contaminated area, and was assessed as 'clean' based on the previous analyses of the soil lead concentrations together with information on the pellet fall-out pattern of the shooting range (*see* Rantalainen *et al*. 2006). The vegetation, terrain, and visual appearance of the control site were indistinguishable from the contaminated areas.

Field studies

Studies aiming at exploring the impacts of lead contamination on litter degradation rate, plant growth and enchytraeid worm numbers at the Hälvälä site were conducted in 2003 for getting a general view about the functioning of the contaminated forest ecosystem. For soil sampling, ten permanent plots of 1.5 × 1.5 m were thus established on C and OC areas (Rantalainen *et al*. 2006), thus contrasting to the rest of the studies, where also NC area was included.

Enchytraeid worms

Numbers and biomasses of enchytraeid worms (Enchytraeidae) were estimated at each study plot (n = 10) in the C and OC areas in October 2003. Two soil cores (∅ 5 cm) per plot were taken from the entire humus layer and the worms were extracted using the wet funnel method (Sohlenius 1979), after which the enchytraeid worms from two soil cores were pooled. The number of individuals was counted under a binocular microscope, and classified in length categories for biomass determination (Abrahamsen 1973).

Degradation of litter

A reciprocal litter transplantation approach was applied to investigate the influence of litter quality on its degradation rate. Senescent litter material (predominantly pine needles and birch leaves) was collected from the C and OC areas in June 2003. At both areas, one nylon mesh bag containing a known amount of litter mixture from the same area and another one containing litter from the adjacent area were placed at ten locations (study plots). The litter bags (altogether 40 bags) were collected 17 months after the initiation of the study. All the roots growing into the litter bags were carefully removed, after which the remaining bag content was dried in an oven at 60 °C for 24 hours for the determination of dry mass loss during the field incubation.

Plant growth

The influence of lead contamination on seed germination and biomass production of a mixture of grass species was explored by establishing 30 cm × 30 cm subplots in the C (n = 5) and OC (n

= 5) areas in June 2003. In these subplots, the moss and litter layers, as well as the uppermost humus layer, were carefully removed and a mixture of *Festuca* seeds (ca. 2300 seeds) was sown. The seed mixture composed of *F. rubra* (80%), *F. ovina* (10%) and *F. arvensis* (10%). After sowing the seeds, the uppermost litter layer was placed back on the seeds and the soil was gently irrigated with tap water. Since the beginning, the subplots were watered frequently and the experiment terminated after three months. The grasses were cut from the soil surface, dried at 60 °C for 48 hours, and weighed for dry mass.

Statistical analyses for the field data

The response of enchytraeid worms to lead concentration was tested using regression analysis with soil lead concentration in each sampling plot serving as the independent variable and enchytraeids as the dependent variable. Due to many zero values in the worm data, a log transformation $x' = \log(x + 1)$ was performed to normalize the data. To analyze the influence of litter quality on its decomposition rate a two-way ANOVA was conducted with the area (C *vs*. OC) and litter type (litter from the C *vs*. OC area) as factors. Difference in grass growth between the C and OC areas was tested with Student's *t*-test. All statistical analyses were performed using SPSS-statistical package (SPSS 11.5) for Microsoft Windows.

Analysis of lead and its bioavailability

To test our hypothesis 1, the soil samples were divided into two layers. The upper (litter and fermentation, L + F) layer will hereafter be called the litter layer, and the lower layer (humus, H) will be called the humus layer. Soil samples were collected from ten plots at C, OC and NC areas. Concentrations of total and water extractable lead were analyzed after removing the lead pellets from one gram of fresh soil by ICP-MS by standard methods (Hartikainen and Kerko 2009). The solid-solution partitioning of lead was compared to predictions (Sauvé *et al*. 2000) at pH values measured at the site. pH (0.01 M $CaCl_2$) and soil organic matter contents were measured with standard methods (Rantalainen *et al*. 2006).

Bioavailability of lead was estimated with three methods using both bacteria (tests 1 and 2) and enchytraeid worms (test 3) as the indicator species. In the bacterial tests two bacterial strains were used. For test 1 (inhibition "INH"-test) we used *P. fluorescens* equipped with the plasmid pDN*lux* (Leedjärv *et al*. 2006), which constitutively expresses the bacterial *lux* gene cluster. This strain required no luminescence substrate. Inhibition of bioluminescence produced by a constitutively bioluminescent bacterial strain is commonly used in measurements of toxicity of environmental samples (e.g. Petänen *et al*. 2003). This method is comparable to the commercially available Microtox® toxicity test. Here we use a soil compatible luminescent bacterium to determine the level of lead that inhibits bacterial metabolism and thereby light production. The standard curve obtained is then used when determining the toxicity of the soil samples.

For test 2 (induction "IND"-test) we used *P. fluorescens* OS81 (Sarand *et al*. 2000, Petänen *et al*. 2001) equipped with the plasmid pJH01, which carries a Pb inducible promoter (Ivask *et al*. 2004) expressing the *luc* luciferase gene. In pJH01 the *mer* promoter in pTPT11 (Petänen *et al*. 2001) was replaced by a Pb inducible promoter. In the presence of sub-toxic levels of lead the bacterium is induced to produce luciferase, which in the presence of added luciferin as a substrate gives rise to light emission. A standard curve of increasing light production is plotted against a rising concentration of lead. The standard curve has a rising trend until toxic levels of lead are reached. Lead concentrations above the toxic threshold are measured by test 1.

For both types of bacterial bioavailability measurements the strains were inoculated in 5 ml HMM (La Rossa *et al*. 1995) liquid amended with tetracycline (15 μg ml^{-1}) in the absence of lead. The cultures were incubated overnight with shaking (260 rpm) at 30 °C. If necessary the cultures were diluted and grown to early exponential growth phase (OD_{600} 0.5; OD) before use.

For the inhibition test (test 1) 1 g of fresh soil, collected from the litter (L + F) sample and the humus (H) sample were mixed with 1 ml of ultra pure water in 15 ml Falcon tubes and vortexed

for one minute. In the case of OC and NC areas, the samples were taken from the most heavily contaminated sections of each area. Directly after vortexing, 50 μl of the resulting soil slurry (soil suspended in water) was used in tests where the reporter bacteria were mixed with the slurry. In order to measure the water soluble fraction of the samples, one ml of the slurry was centrifuged at 13 000 rpm for three minutes, and 50 μl of the supernatant (named hereafter water extract) was used in the tests using the same reporter bacteria. The luminescence measurements were carried out as earlier described by Petänen *et al*. (2001) and Ivask *et al*. (2002).

A 50 μl aliquot of bacterial suspension was mixed with 50 μl of water extract or soil slurry in 96 well micro-titer plates. The most repeatable results were obtained by performing the luminescence measurement approximately 30 min. after mixing the bacteria with the lead samples. Bioluminescence was measured using the multi-reader Wallac 1420 Victor^{2TM}. The light emission without addition of lead was given the value 1. Light emission remaining in the samples was calculated as relative light units (RLU) L_{inh}/L_{max} where L_{inh} is the luminescence in a lead-spiked sample or an actual sample to be measured, and L_{max} is the luminescence from the lead free control. In order to account for light quenching caused by the sample (e.g. soil slurry and dissolved humic substances in the water extract), separate lead-spiked standard curves were made for each sample type.

In test 2 the sample treatment and luminescence measurements were identical to those in test 1, but the incubation times were different. The inducible strain *P. fluorescens* OS81 (pJH01) reached maximum of light production 5 hours after incubation with lead standards at 30 °C with shaking. After such a time, 100 μl of D-luciferin solution (1 mM D-luciferin in 0.1 M Na-citrate buffer, pH 5.0) was added as the substrate for the luciferase. Bioluminescence was measured approximately 20 min. after addition of substrate. In test 2 the induction coefficient (relative light units, RLU) was calculated using the formula: L_{ind}/L_{bg} (Ivask *et al*. 2002) where L_i is the luminescence value in the metal-containing sample, and L_{bg} is the background luminescence value in pure water, lead free soil-water slurry, or water extract of lead free soil.

In the third bioavailability test (test 3), enchytraeid worms collected from the C area were used as toxicity reporters. Lead contaminated soil from the heavily contaminated sector of the OC area was mixed with clean C soil in different proportions, and 10 g portions were placed in small beakers. Enchytraeid worms were added to the defauned soil mixes at an initial density of 25 individuals per gram of soil, and the beakers were incubated at 18 °C with a 16/8 hour light/dark period for 33 days. The number of viable worms was counted after extraction (*see* above).

Isolation and cultivation of soil bacteria

For determination of frequency of lead resistant (Pb^R) and lead sensitive (Pb^S) bacterial strains and for isolation of bacterial strains, the litter (L + F) and the humus (H) soil layer samples were collected from three locations per study area (C, OC, NC). Lead pellets were removed manually and the samples were homogenized. Dilutions were made in sterilized water, and plating was carried out on lead-free 1/5 TGY-plates (Hernesmaa *et al*. 2005) and heavy metal medium (HMM)-plates (La Rossa *et al*. 1995) containing 0.35 mM or 1.8 mM Pb.

After five days of incubation in room temperature, the number of CFU's per gram of fresh soil were calculated. For characterization of cultivable bacteria, the vertical layers of the soil samples were not separated. The samples thus represent areas but not soil layers. Randomly chosen bacterial colonies growing on lead containing and lead-free plates were picked and pure-cultured by restreaking single colonies twice on TGY plates. The pure-cultures were restreaked on HMM plates with and without lead to confirm the original $Pb^{R/S}$ phenotype, and the identity of each pure-culture was then determined by DNA-sequencing (*see* below).

Molecular methods for analysis of soil bacterial community

Soil sampling from the C, OC and NC areas were performed as described above, but the

litter and humus layers were not separated. DNA was extracted with FastDNA SPIN Kit for Soil (Qbiogene Inc., USA) according to the manufacturer's instructions. For analysis, the bacterial 16S ribosomal DNA universal primers pA and pH´ (Edwards *et al*. 1989) were used in PCR to amplify the whole length of 16S ribosomal RNA gene. A mixture of Taq polymerase and Pfu polymerase to increase fidelity was used for the PCR reaction. The amplified DNA fragment was cloned using a commercial T/A cloning kit according to the manufacturer's instructions (QIAGEN PCR CloningPlus, QIAGEN, Hilden, Germany), and sequenced using capillary sequencing techniques (ABI) as described by Hultman *et al*. (2008). The sequences were assembled with the Staden Package Program (Staden 1996) and annotation was achieved using the EMBL Fasta3 database (http://www.ebi.ac.uk/fasta33/).

Identification of bacterial pure-cultures was achieved by sequencing the PCR fragment of the 16S ribosomal RNA gene amplified with the promoters MR907 (5´-CCG TCA ATT CMT TTG AGT TT-3´) and MF394 (5´-CTA CGG GAG GCA GCA G-3´). Sequences were analyzed as above.

Fungal enzyme activities

Basidiomycetous litter-decomposing fungal species used in this study were typical boreal forest species. Vouchered specimens are deposited in the culture collection of the Department of Applied Chemistry and Microbiology, University of Helsinki (K), or in the culture collection of the International Graduate School of Zittau, Germany (TM or plain number): *Agrocybe praecox* TM70.84, *Gymnopilus penetrans* K244, *Gymnopus dryophilus* (*Collybia dryophila*) K209, *Mycena amicta* K91, and *Mycena epipterygia* K72, and a German edible fungus *Stropharia rugosoannulata* B 11372. The species *G. penetrans* was isolated from the Hälvälä shooting range. All strains were maintained on 2% malt extract agar slants.

Lead sensitivity and tolerance of litter-decomposing fungal strains were evaluated by the ABTS plate test (Steffen *et al*. 2002). Fungi were inoculated on agar plates containing ABTS (2,2´-azino-*bis*-(3-ethyl)-benzthiazoline-6-sulfonate), a dye oxidised by ligninolytic enzymes of litter-decomposing fungi. The positive colour change is from colourless to green. Lead was added to agar plates at two concentrations, 0.5 or 1.5 mmol Pb l^{-1}. Control plates remained lead free. The growth and activity of fungi were evaluated during the incubation period of 30 days.

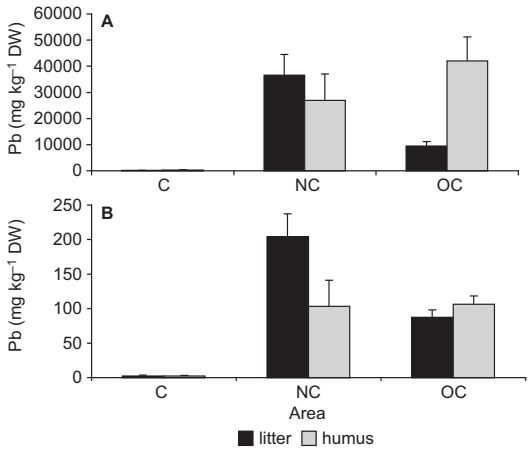

Fig. 1. Concentrations of (**A**) total and (**B**) water soluble lead, in the Hälvälä shooting range soils. Clean control, C; new contaminated area, NC; old contaminated area, OC. The litter (L + F) and humus (H) layers of soil were analyzed separately. Standard error (SE) is indicated by error bars (n = 3).

Results

Lead concentration in soil

While soil from the C area contained only low concentrations of lead, comparable to background values elsewhere, both OC and NC contained very high concentrations of lead (Fig. 1) even though lead pellets were removed before analysis. As reported earlier (Rantalainen *et al*. 2006), the samples contained high numbers of shotgun pellets at various stages of weathering. The concentration of lead in the litter layer (L + F) from the OC area was clearly lower than that in the humus layer (H). The concentrations of the water-extractable portion of lead followed the same trends as the total lead (Fig. 1), how-

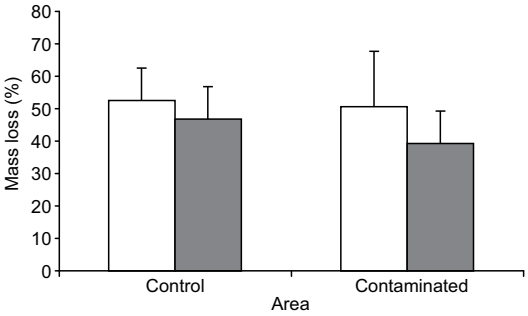

Fig. 2. Decomposition rate (percentage of initial mass decomposed; mean ± S.D., n = 10) of a mixture of pine and birch litter at the control (C) and at the old contaminated (OC) areas. Open bars represent litter collected from the control area, hatched bars represent litter from the contaminated area.

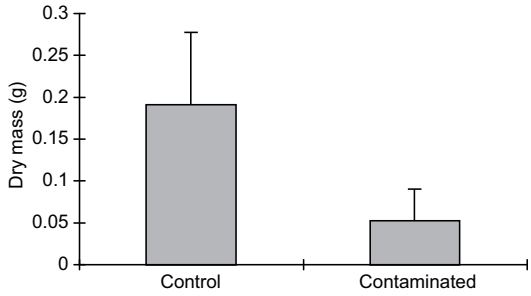

Fig. 3. Biomass (mean + S.E., n = 5) of the grass sward grown at the control area (C) and at the old contaminated area (OC) four months after sowing in June 2003.

ever, this water-extractable share represented only 0.2%–1% of the total lead, giving a solid-solution partitioning value of K_d = 100–500 l kg^{-1} (Sauvé *et al*. 2000). This relatively low K_d value apparently reflecs the low pH of the soil which was between 3.2 and 4.1, the avarge being pH 3.52. This is typical for the humus soil in the region (Rantalainen *et al*. 2006). The soil organic matter (SOM) contents of the humus soil at the site as determined for 24 separately collected samples, was 54.9% (SE 2.2%). The small variability in recorded pH and organic matter contents allows us to compare lead bioavailability data and effect on organisms within this study.

Enchytraeid worms

Sub-optimal soil moisture conditions for naturally occurring enchytraeids were created as a result of an exceptionally dry season in 2003. Our results showed a clear negative influence of the drought on the numbers of enchytraeid worms in both the C and OC areas (0.30 ± 0.32 and 0.18 ± 0.38 individuals g^{-1} dry weight of soil, respectively). Lead concentration in the soil had no influence on either individual number (R^2 = 0.010, $p > 0.05$) or biomass (R^2 = 0.024, $p > 0.05$) of the worms. However, there was a visual indication that the worms collected from the OC area were more threadlike than those collected from the C area.

Litter degradation

The litter derived from the OC area decomposed at a slower rate than the litter from the C area (two-way ANOVA: $F_{1,36}$ = 4.6, p = 0.04). The area where decomposition took place exerted no statistically significant influence on litter degradation rate (two-way ANOVA: F_8 = 1.196, p = 0.282), although there appeared to be a tendency towards slower degradation rate for the OC derived litter in OC than in C (Fig 2).

Grass biomass production

Lead contamination exerted a significant influence on the growth of grass during three months' experiment in the field. The grass biomass from the OC area was significantly lower (t-test: t_8 = 0.754, p = 0.025) than that from the C area (Fig. 3).

Bioavailability of lead based on bacterial biosensor measurements

Lead bioavailability: test 1

Standard curves using the constitutively luminescent soil bacterium *P. fluorescens* (pDN*lux*) were made by spiking pure water, water extract of soil, and soil slurries with known concentrations of lead. In water, the reduction in luminescence started to show at 30 μM Pb, and the luminescence (RLU) was two orders of magnitude lower at 300 μM (data not shown). Using

the water extracts (Fig. 4A) a reduction in luminescence was clearly observed at 100 μM Pb and a 100-fold decrease in RLU was seen at 1 mM Pb. Since the measurement was normalized by giving luminescence in a lead-free solution the value 1, the higher toxicity threshold in the water extract was not a result of quenching from components in the extract. Rather, lead may have precipitated in contact with components in the extract. In soil slurry standard curve (Fig. 4B), luminescence reduction was barely evident at 100 μM Pb but was clearly visible at 1 mM. At 10 mM Pb, light production was completely suppressed. This indicates that a large portion of the added lead adsorbed to soil particles, which to some degree decreases the reliability and repeatability of bioavailability measurements performed in soil slurries.

Water extracts and soil slurries from the sample areas C, NC, and OC were tested for their effect on light production by the bioluminescent reporter bacterium along with the standard curve samples. In the case of water extracts, only the water extract of the NC litter layer (L + F) reduced the bioluminescence produced by the reporter bacterium (Fig. 4A) to a level that equaled 40 μM on the standard curve. In the C and OC sample extracts, lead concentration was indistinguishable from the lead free sample in the standard curve (< 30 μM). Slurries made from the litter layer of C and OC samples produced no significant reduction in luminescence, while reduction caused by the NC litter layer slurry was equivalent to a 0.8 mM lead concentration as read from the standard curve (Fig. 4B). The humus layer (H) samples were subjected to similar measurements using appropriate standard curves (for results *see* Table 1).

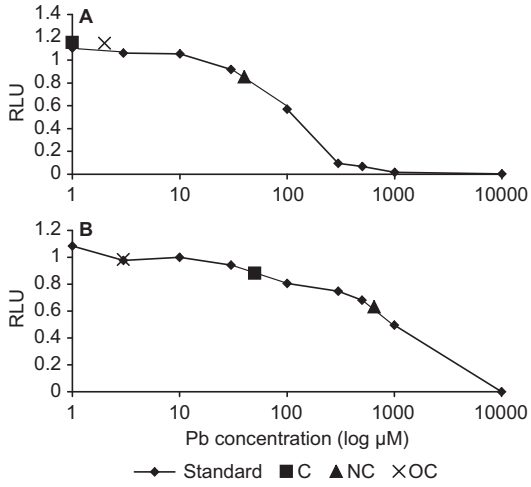

Fig. 4. Light (relative light units, RLU) produced by the constitutively bioluminescent reporter bacterium *P. fluorescens* (pDN*lux*) when exposed to increasing concentrations of lead in (**A**) water extract and (**B**) soil slurry. Luminescence produced by the reporter when exposed to extract or slurry of the sampled soils (C, NC, OC) is indicated in the figures.

Lead bioavailability: test 2

Bioavailable lead concentration in samples was measured using the lead inducible stain *P. fluorescens* OS81 (JH01), where a lead inducible promoter expresses the *luc* gene. As above, the lead concentration was read by comparing to standard curves. Standard curves were made by spiking samples of the same type (water extract, soil slurry) as the actual samples. Measurement based on test 2 of litter layer extract and slurry samples of C, NC and OC (Fig. 5) are in approximate agreement with the readings of test 1 (Fig. 4). Based on observed reduction in constitutive luminescence (test 1, Fig. 4), only the NC samples were slightly toxic to the reporter bacterium, which is in agreement with the test 2 result that both NC samples are close to maximum of the induction standard curve (Fig. 5). Thus, for NC samples both types of analyses gave the same result, and in both cases the bioavailable fraction of lead was higher in the soil slurry than in the water extract. (For results *see* Table 1.)

The concentration of bioavailable lead determined by inhibition of constitutive luminescence (test 1) and induction of luminescence (test 2), respectively, were in most cases in agreement with each other (Table 1), the exceptions being litter layer slurry and humus layer extract of the OC sample. Measurements of induction and inhibition complemented each other in this study. The induction measurements were designed to reveal low, nontoxic levels of lead, while inhibition measured the high, toxic levels. The inhibition test also verifies that a low induction reading is not a result of toxic levels of lead or any other toxicant. Using the luminescent bacterial strains

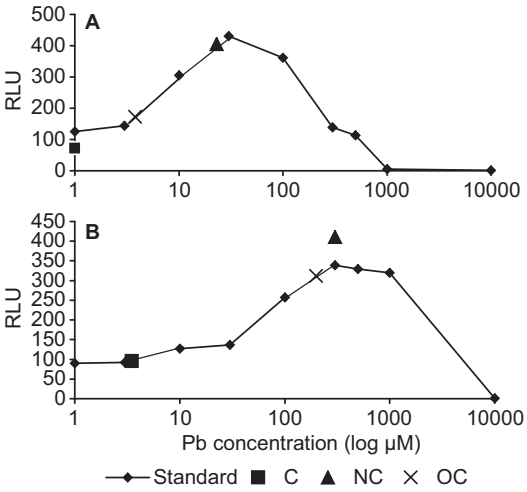

Fig. 5. Light (relative light units, RLU) produced by the inducible bioluminescent reporter bacterium *P. fluorescens* OS81 (pJH01) when exposed to increasing concentrations of lead in (**A**) water extract and (**B**) soil slurry. Luminescence produced by the inducible reporter when exposed to extract or slurry of the sampled soils (C, NC, OC) is indicated in the figures.

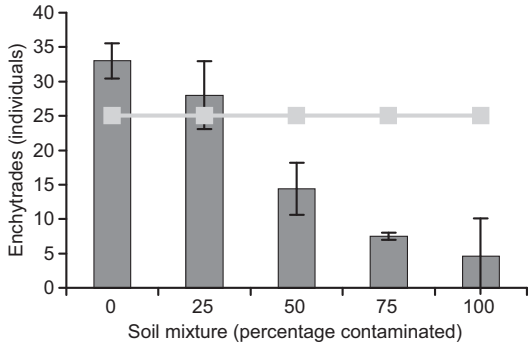

Fig. 6. Effect of lead contaminated soil on enchytraeid viability. Lead contaminated soil (OC) was mixed with clean soil (C) in proportions indicated on the *x*-axis. Live worms (*y*-axis) were mixed into the soil and survival was evaluated after a 33 days incubation period (mean ± SE, n = 5). The horizontal line denotes the number of worms added at the start of incubation.

in tests 1 and 2 the measurable lead concentration range was extended from approximately 3 μM to 1000 μM.

Influence of lead on enchytraeid worm in pot exposure experiments

The influence of lead contaminated soil collected from the OC area on the viability of enchytraeids was tested by exposing the worms to mixtures of lead contaminated and clean soil. The OC soil used had a total lead concentration of 21.9 ± 0.7 g Pb kg^{-1} soil of which 0.67 ± 0.02 g kg^{-1} was water soluble, while the C soil had a total background concentration of 0.16 g Pb kg^{-1} soil, with a water soluble fraction below detection. Already a 50% portion of OC soil killed 50% of the worms, while even undiluted OC soil did not kill all of them (Fig. 6). The results show that the enchytraeid worms are sensitive to lead despite low bioavailability based on bacterial measurements.

Lead sensitive and resistant cultivable bacteria in the soil

The number of colony forming units (CFU's) on

Table 1. Bioavailability of lead measured by bioluminescense inhibition and induction in litter and humus extractions and slurries. C, OC and NC refer to the study areas. INH denotes inhibition of luminescence as presented in Fig. 4 and IND denotes induction of luminescence as presented in Fig. 5. Values indicate concentrations (μM) calculated from standard curves as those presented in Figs. 4 and 5. The numbers in brackets should be interpreted with caution since the humus slurry standard curve did not extend to the level of the highest measured sample value.

	C		OC		NC	
	INH	IND	INH	IND	INH	IND
Litter extr	≤ 30	≤ 3	≤ 30	4	40	30–50
Litter slurry	≤ 100	≤ 10	≤ 100	250	800	500–1000
Humus extr	≤ 30	≤ 3	≤ 30	300	100	100
Humus slurry	≤ 300	(3)	≤ 300	(100)	≤ 300	(200)

the nonselective media 1/5 TGY was similar in each of the soils (Fig. 7), whereas there was a clear difference in levels of CFU's when plating on lead containing media. Nevertheless, the number of CFU's using nonselective media was considerably larger in samples from all types of soils, indicating that the majority of bacteria, also in the lead contaminated soil, were lead sensitive. When a lead concentration of 1.8 mM was used in the selection plates, the portion of lead resistant bacteria isolated from the C area was below detection (1×10^{-4} CFU g^{-1}) while in samples from OC and NC the portion was up to 0.5% of total cultivable CFU's.

In both OC and NC samples, there were increased numbers of lead-resistant bacteria in the litter layer. In addition, high numbers of resistant bacteria were found in the humus layer in the OC area (Fig. 7). Plates containing bacteria from the C area and from the humus layer of the NC area showed low Pb^R frequencies.

The plating of bacteria from the C, OC and NC areas on both lead containing and lead-free plates has been performed totally five times at different times of the year and under different conditions with similar results. When a concentration of 0.35 mM Pb was used in the lead containing plates, the fraction of Pb^R was higher, but also the difference between the lead containing and clean samples was smaller (data not shown). The lower lead concentration reflects more closely the true field conditions as portrayed by the bioavailability measurements.

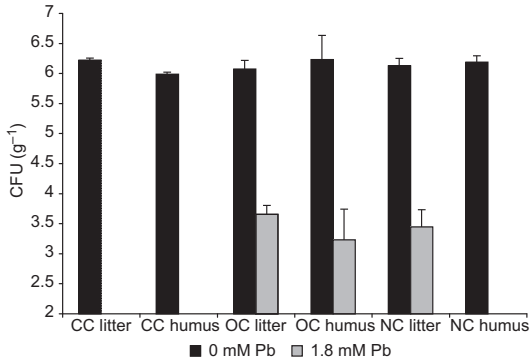

Fig. 7. Colony forming units (CFU g^{-1} fresh weight) of total (plates with 0 mM Pb) and lead resistant bacteria (plates with 1.8 mM Pb) in three Hälvälä study areas (C, OC, NC). The soil samples were divided into litter (L + F) and humus (H) soil layers. CFU's and their standard errors are presented in logarithmic form. The *y*-axis lower cutoff is set at log 2 which is the detection limit in the measurement used.

Partial assessment of bacterial diversity in lead contaminated and clean soil

Cultivation-based analysis

The bacteria that were pure cultures and identified from the soil samples are listed in Table 2. None of the bacteria originally isolated from the C area as being Pb^R (0.35 mM) could be restreaked and pure-cultured on lead containing plates. Thus, we do not have evidence for lead resistant bacteria in the clean soil. In contrast, the Pb^R bacteria isolated from lead contaminated soil samples were confirmed to be lead resistant. Conversely, the lead sensitive strains isolated

Table 2. Cultivated and identified soil bacteria from the study areas C, OC and NC. Bacteria were pure cultured on HMM plates containing 0 or 0.35 mM lead. Twenty colonies originating from plates with no lead (0 mM) and ten colonies from plates containing 0.35 mM were picked randomly, tested and identified. The number following the identity of the isolate denotes how many times this strain was picked.

		Pb 0 mM	Pb 0.35 mM
C	*Burkholderia phenazinium*	2	
	Burkholderia sp.	18	
OC	*Arthrobacter*	5	
	Bradyrhizobium japonicum		1
	Burkholderia phenazinium	11	
	Burkholderia cepacia	1	
	Burkholderia sp.		3
	Peanibacillus		1
	Pseudomonas fluorescens	3	1
	Pseudomonas sp.	2	
	Rhodococcus		1
	Bacterium BTH 16S gene		1
NC	*Arthrobacter*	9	
	Beta proteobacterium MZT1A	1	
	Brevibacterium	7	
	Burkholderia sp.	2	
	Pseudomonas fluorescens	4	
	Pseudomonas sp.	4	
	Pseudomonas sp.	2	
	Rhizobacterium loti	2	
	Bacterium BTH 16S gene	1	

from all three areas (OC, NC, and C) were confirmed to be lead sensitive.

In all three soils, bacteria from the genus *Burkholderia* were present, and in the C and OC samples, this was the most common genus. Since a clear majority of the CFU's in all samples were lead sensitive (Fig. 7), *Burkholderia* is clearly a common bacterium in this type of forest soil. No *Burkholderia* spp. were detected among the Pb^R strains, and in the NC sample, where the lead contamination was high also in the litter layer, only two *Burkholderia* species were found among the 20 characterized isolates. In the OC sample, where the total lead concentration in the litter layer had already decreased (Fig. 1), 15 out of 20 strains were *Burkholderia*. Other genera found in the contaminated soil sites include *Pseudomonas*, *Brevibacterium* and *Rhizobacterium*. Among the lead resistant strains, *Arthrobacter* was clearly predominant, with 14 out of 20 strains identified.

DNA-based analysis

Analysis of the bacterial community composition using DNA-sequencing of amplified 16S ribosomal RNA-genes resulted in a considerably higher number of detected genera (Table 3) than the cultivation based method. As often is the case, this method generated a high number of sequences with no close matches in the database. Of the total 264 sequences analyzed, 59% were unidentifiable/unknown. The percentage of unknown sequences was particularly high in the NC soil. The most common cultivable bacteria *Burkholderia*, was represented among the sequences by three hits each in the C and OC samples respectively, but was absent in the NC sample. Of the other cultivable bacteria, only a *Paenibacillus* sequence was identified. Acidobacteria and *Rhodoplanes* species were the only bacteria detected in all soils by DNA analysis. The phylum Acidobacteria is among the most common soil bacteria (Janssen 2006) and has only been recently cultivated, but many subphyla of Acidobacteria still remain uncultured. The two other dominant soil phyla, Proteobacteria and Actinobacteria, made up the majority of the remaining bacteria, both among the cultivated and those found with direct sequencing. A substantial portion of both cultivated and sequenced bacteria were Proteobacteria (*Bradyrhizobium*, *Rhizobiales*, *Afipia*, *Burkholderia*) that are associated with nitrogen fixing (Sawada *et al*. 2003). The low number of Actinomycetes among the cultivated bacteria isolated, compared to the sequences, probably stems from the slow growth of many of the bacteria of this phylum.

One can conclude that the cultivation based and sequence based analysis of the bacterial biodiversity generate comparable results on a phylum level, whereas the diversity is too large for a genus or species based inspection.

Analysis of litter decomposing fungi — the ABTS plate test

The results showed that lead strongly inhibited the growth of *Agrocybe praecox* and *Mycena* species, but stimulated the growth of *Stropharia rugosoannulata* on ABTS plates (Fig. 8). However, *Mycena* species did not grow well even on control plates and lead inhibited the growth of *M. epipterygia* totally. The growth of other fungi was significantly affected by lead. All screened fungi gave positive colour reaction in ABTS plates (Fig. 8). In addition to ligninolytic activity, colour reaction on ABTS plates may also reflect a stress reaction of the fungus. Thus, also the existing mycelium of *M. epipterygia*, which did not grow on lead containing plates, was able to turn the ABTS plate green. No colour change was observed before day 20 on plates cultivating *Gymnopilus penetrans,* after which time complete colour change occurred all at once. Therefore, *G. penetrans* was omitted from the figure.

Discussion

Lead contamination was found to retard plant biomass production. As earlier suggested (Rantalainen *et al*. 2006), this may be due to altered soil physical and chemical characteristics, including an increase in soil alkalinity by pH 0.2 and reduced concentration of NH_4^+ in contaminated as compared to the control area (Rantalainen *et al*. 2006). Retarded plant growth in the con-

Table 3. Bacterial 16S sequences in soil samples. Sequences were grouped into the same genus/phylum when the similarity to database entries was at least 94%. Sequences with a similarity below 94% to database entries were placed in a single group.

C		OC		NC	
Genus/Phylum	No. of seq.	Genus/Phylum	No. of seq.	Genus/Phylum	No.of seq.
Acidobacteria/Acidobacteriaceae	11	*Acetobacteraceae*	2	Proteobacterium	1
Actinomadura	1	*Acidobacteria*	15	*Acidobacteria*	1
Bradyrhizobium	2	*Acidocella*	2	*Rhodoplanes*	2
Burkholderia/Burkholderiaceae	3	*Acidosphaera*	1	*Rhizobiales*	1
Frateuria	1	*Actinobacterium/Actinomycetes*	8	Similarity below 94%	41
Janthinobacterium	1	*Afipia*	14		
Methylocapsa	1	*Bradyrhizobium*	1		
Methylocystis	1	*Burkholderia*	3		
Mycobacterium	5	*Caulobacter*	2		
Paenibacillus	1	*Conexibacter*	1		
Rhodoplanes	1	*Duganella*	1		
Solibacter	2	*Frateuria*	1		
Similarity below 94%	35	*Gemmata*	1		
		Leifsonia	1		
		Methylocapsa	1		
		Methylocella	1		
		Methylocystis	6		
		Methylosinus	1		
		Mycobacterium	4		
		Pseudonocardia	1		
		Rhodoplanes	2		
		Sinorhizobium	1		
		Sphingobacterium	1		
		Sphingomonas	2		
		Similarity below 94%	80		
Total	65		153		46

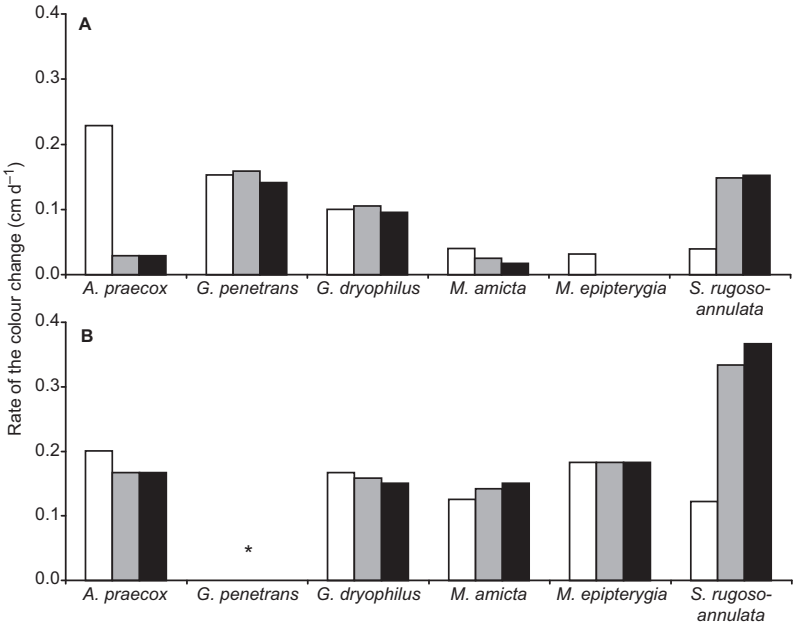

Fig. 8. Effect of Pb on ABTS agar plates on (**A**) the growth rate of fungus and (**B**) on the rate of colour production. The plates were incubated for 30 days. Rate of fungal growth or colour production on ABTS plate is expressed as a function of the time (cm d^{-1}). White column = control, grey column = 0.5 mM Pb, black column = 1.5 mM Pb, * = *G. penetrans* changed the colour of the plates at once on day twenty of the cultivation.

taminated area may also be derived from a direct toxic effect on the grasses, and the effect may be combined with possible direct toxic effects of lead on the decomposer microflora and the whole soil food web. Although in the very dry season in 2003 the biomass of enchytraeid worms was not affected by lead, their biomass was significantly decreased by lead by September 2004 (Rantalainen *et al*. 2006).

The litter originating from the old contaminated area degraded at a slower rate than the litter from the control area, which may point to lower nutrient concentrations of the litter at the former area, possibly due to retarded nutrient mineralization. This hypothesis is supported by our earlier observations according to which soil ammonium concentrations are lower in lead contaminated than in the control soils (Rantalainen *et al*. 2006, S. Selonen unpubl. data).

The occurrence and density of lead resistant bacteria coincided in most cases with the amount of lead found in the soil samples. Most of the lead pellets found in the new contaminated area were still near the surface of the soil while they were dispersed into deeper layers in the old contaminated area. With the analysis techniques used here, lead resistant bacteria were not detected in the control area, and the level of these bacteria was below the detection limit also in the humus layer of the new contaminated area, possibly reflecting the relatively short time of NC humus exposure to lead.

No decrease in the number of bacterial CFU's was observed in lead contaminated soils. This contrasts with the earlier findings (Rantalainen *et al*. 2006) that the bacterial biomass measured by PLFA is inversely correlated with lead concentration. A possible explanation is found in the study by Bååth *et al*. (2005), where a higher proportion of the total number of bacterial cells formed colonies when lead containing samples were plated than when clean soils were used. Thus, a lower bacterial biomass in lead contaminated soil may still give rise to the same number of colony forming units per gram of soil.

An interesting observation was that even in the most heavily lead contaminated soils the vast majority of the bacteria were found to be lead sensitive. This may be explained by either lead-free micro-niches where lead sensitive bacteria can grow, or alternatively, a high proportion of immigrating bacteria that merely survive in the presence of lead, without being capable of multiplying in the contaminated soil. In principle, the latter explanation is possible only if lead is biostatic. The CFU's would then represent

viable bacteria arrested in growth. An argument against this hypothesis is that such arrested bacteria should not be as numerous as the bacteria present in the clean control soil. Furthermore, one would expect that the humus layer would not contain high concentrations of bacterial immigrants arrested in growth.

An additional possible explanation for the high proportion of lead sensitive bacteria is that despite extremely high total concentrations, the bioavailability of lead is very low. The results presented here partly support this notion. The toxicity of water extracts and soils slurries, as shown by bioavailability test 1, was very low shown by the fact that the constitutively luminescent reporter bacterium exhibited reductions in light output only when exposed to the litter samples of the new contaminated area. The luminescence induction measurements (bioavailability test 2), however, showed that the local environment is not totally devoid of bioavailable lead. In all samples shown to be highly contaminated (litter and humus layers of the NC area and the humus soil layer of the OC area), the induction level was close to the maximum of the standard curve. This was true for water extracts as well as for soil slurries. In the latter case the bacterial exposure to soil particles is direct, and therefore also lead potentially mobilized by the bacterial cells is measured. The tests performed in litter slurries indicated that lead may in some cases be mobilizable by bacteria, a phenomenon that previously was reported in the case of mercury (Petänen and Romantschuk 2003). In agreement with the previously published mercury results, the reporter bacterium was not able to mobilize lead bound to humus.

Despite the very low acute toxicity on bacteria used in the bioavailability tests, lead did have an effect on the bacterial community composition in the studied soils. This limited effect might be comparable to the limited but significant effect seen on the growth of grass, the degradation of litter, and the slight influence on the appearance of the enchytraeid worms in the field. To these organisms the exposure to lead was sublethal, but it was still apparently a stress factor with an effect on growth and well-being. As shown by Rantalainen *et al*. (2006) and by the lead-soil exposure test presented here (Fig. 6), enchytraeids are strongly influenced by lead contaminated Hälvälä soil if the conditions are in other respects optimal for their growth. Unlike the conditions during the dry summer of 2003, soil water content typical for an average summer (Rantalainen *et al*. 2006), or typical for optimized laboratory conditions, appears to expose the enchytraeids to the acute toxic effects of lead. A possible explanation for this can be attributed to a more active ingestion of lead contaminated microbes and organic material in conditions that promote growth.

Lead resistant bacteria were readily isolated only from the lead contaminated soils, and the selection of cultivable bacteria was somewhat changed. The low number of isolates did not, however, allow for a statistical analysis. The analysis based on DNA sequences supports the notion of a shift of total bacterial biodiversity in the presence of lead. The proportion of Actinomycetes appeared to be elevated in the lead contaminated soil. Statistical proof is, however, still lacking. A drawback with DNA analysis is that the microbes observed could not be tested for $Pb^{R/S}$ or any other traits. Taken together, both the cultivation and the DNA-analysis results suggest that lead had an effect on the diversity of bacteria in the coniferous forest soil at the Hälvälä shooting range.

Lead clearly inhibited the growth of the most litter-decomposing fungi, but the inhibition seems to be very variable among fungal species, a finding, which has also been confirmed with other heavy metals (Baldrian 2003). We also observed the stimulation of growth in one fungal species. However, the activity of fungi in lead contaminated growth media is not governed only by growth, which has been earlier observed by Chander *et al*. (2001). Thus, the impact of lead contamination to saprophytic fungal populations in the soil is very hard to predict.

In conclusion, organisms representing different trophic groups adapt to the presence of lead in different ways. While plants may respond by growth retardation, the bacterial microflora responds and adapts with a partial change in diversity. Furthermore, fungal adaption is in some cases evidenced by increased lead tolerance of a secreted enzyme, while enchytraeid worms fluctuate in population density and physical appearance of the individuals.

The results presented here point to a need for a versatile approach when assessing the environmental impact of lead contamination in forest soil. Each single type of test, type of organism, and testing condition only tells a small part of the whole story.

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