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Phospholipid Fatty Acid Composition and Heavy Metal Tolerance of Soil Microbial Communities along Two Heavy Metal-Polluted Gradients in Coniferous Forests

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The effects of long-term heavy metal deposition on microbial community structure and the level of bacterial community tolerance were studied along two different gradients in Scandinavian coniferous forest soils. One was near the Harjavalta smelter in Finland, and one was at Rönnskär in Sweden. Phospholipid fatty acid (PLFA) analysis revealed a gradual change in soil microbial communities along both pollution gradients, and most of the individual PLFAs changed similarly to metal pollution at both sites. The relative quantities of the PLFAs br18:0, br17:0, i16:0, and i16:1 increased with increasing heavy metal concentration, while those of 20:4 and 18:2ω6, which is a predominant PLFA in many fungi, decreased. The fungal part of the microbial biomass was found to be more sensitive to heavy metals. This resulted in a decreased fungal/bacterial biomass ratio along the pollution gradient towards the smelters. The thymidine incorporation technique was used to study the heavy metal tolerance of the bacteria. The bacterial community at the Harjavalta smelter, exposed mainly to Cu deposition, exhibited an increased tolerance to Cu but not to Cd, Ni, and Zn. At the Rönnskär smelter the deposition consisting of a mixture of metals increased the bacterial community tolerance to all tested metals. Both the PLFA pattern and the bacterial community tolerance were affected at lower soil metal concentrations than were bacterial counts and bacterial activities. At Harjavalta the increased Cu tolerance of the bacteria and the change in the PLFA pattern of the microbial community were found at the same soil Cu concentrations. This indicated that the altered PLFA pattern was at least partly due to an altered, more metal-tolerant bacterial community. At Rönnskär, where the PLFA data varied more, a correlation between bacterial community tolerance and an altered PLFA pattern was found up to 10 to 15 km from the smelter. Farther away changes in the PLFA pattern could not be explained by an increased community tolerance to metals.

Disturbances caused by heavy metals to microbial biomass and activity are known to be reflected in decreased litter decomposition and subsequently less-efficient soil nutrient cycling (see reviews in references 3, 13, 17, and 44). Several studies have also demonstrated heavy metal-induced changes in specific parts of the soil microbial community. Traditional ways of studying the soil microbial community are time-consuming and dependent on the success of culturing the organisms. In the case of soil bacteria the culturable portion is known to represent only a limited part of the total bacteria (37). An approach to detect possible changes in the soil microbial community, in a nonselective way, is analysis of the phospholipid fatty acid (PLFA) composition in the soil (42). PLFAs are located in membranes of the cells, which implies a rather fast turnover of these compounds (42). Different subsets of microorganisms have different PLFA patterns, and it is possible to characterize the features of the microbial community directly in a natural habitat, without an initial isolation step. The PLFA profile does not give an actual species composition but instead gives an overall picture of the community structure. However, in some cases, changes in the concentrations of certain PLFAs may be correlated to changes in more specific groups of organisms. On the basis of PLFA analysis, the effects of alkaline pollution and different management practices have been detected (7, 8, 25).

Often the effect of a toxic substance on the soil microbial community is measured by standard techniques, such as measurements of activity and biomass. However, such measurements have the disadvantage that they will not be specific for the toxic substance studied. For example, although soil respiration has often been found to be a sensitive measurement to reveal heavy metal toxicity under natural conditions (3), soil respiration will be affected by other toxic substances as well as environmental factors such as soil moisture content, temperature, carbon availability, and pH. In laboratory studies, where such environmental factors can be standardized, this is usually a lesser problem. In a more complex field situation this will increase the variability in the data, thus decreasing the possibilities to detect toxic effects. This criticism would hold for the PLFA analysis also. However, the enhanced tolerance of a community to a toxic compound suggests that a selective pressure has been exerted by the compound in question (9, 10). The measurement of community tolerance would therefore be a more direct technique to indicate toxicity in nature. Such measurements would also be less affected by the environmental factors mentioned above and would therefore be a more sensitive technique for use in field studies.

Increases in the numbers of metal-tolerant bacteria (14, 15, 18, 30, 38), actinomycetes (32), and fungi (2, 32, 46) have been seen as the results of heavy metal pollution by the traditional

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plate count technique. Recently, the radioactive thymidine incorporation technique, which estimates the growth rate of bacteria by the rate of thymidine incorporation into bacterial cells, was modified to allow measurements on bacteria extracted from soil by homogenization-extraction (4). It has successfully been adapted to estimate the community heavy metal tolerance of bacteria exposed to artificial pollution (5, 12). PLFA analysis and the thymidine incorporation technique (to measure community tolerance) have previously been shown to be effective methods to reveal the changes in the microbial community in soils artificially polluted with heavy metals (12, 26). They have not, however, been applied to a more complex field situation. In the present study we analyzed two different heavy metal pollution gradients, one around a smelter in Finland with a rather simple pollution pattern (mainly Cu emitted) and one around a primary smelter in Sweden, where a mixture of metals is emitted. In addition to studying to what extent the heavy metals affected the microbial community, we wanted to compare the changes in PLFA patterns at the two sites, to see if changes in the community structure (as revealed by the PLFA analyses) were correlated to changes in community tolerance. We also wanted to see if increased bacterial community tolerance to different heavy metals could be found.

MATERIALS AND METHODS

Study areas and sampling. Two heavy metal-contaminated gradients were sampled, one in southwest Finland at the Harjavalta smelter and one in northern Sweden around the Rönnskär smelter. Humus (the F/H-layer) samples from 12 plots were collected from Scots pine (*Pinus sylvestris* L.) forest stands in Harjavalta. Each sample consisted of 20 soil cores (diameter, 7.2 cm). The plots were situated in groups of three at four sampling locations 0.5, 2, 4, and 8 km southeast of the smelter and represented the controls of a larger fertilization experiment along the pollution gradient. The samples were sieved (4-mm mesh) and stored at 4°C for 3 to 5 weeks before analysis. The soil organic matter content varied between 64 and 98% of the dry matter (d.m.), and the H_2O pH was about 4.2 along the gradient. For a more detailed description of the humus chemistry, see the work of Fritze et al. (22).

The sampling sites around Rönnskär were also coniferous forest stands, consisting mainly of Norway spruce (*Picea abies* L.). Thirty-nine samples were collected from the humus (F/H) layer. The sites were situated from 2 to 55 km both south and north of the smelter. The soil samples were frozen for 1 year, and they were then thawed and stored at 4°C for 4 weeks before further processing. Soil characteristics of Rönnskär were similar to those of Harjavalta with respect to their organic matter content (about 87%) and pH (around 4). The area has been previously described by Nordgren et al. (35).

For the last 50 years Cu has been the dominant source of pollution in the Harjavalta area. Only small quantities of Ni, Zn, Pb, and Cd have been deposited (22). The Rönnskär area has been exposed since the 1930s to a mixture of heavy metal deposition consisting mainly of Pb, Cu, Zn, As, Cd, and Hg. The Cu concentration of the humus layer was chosen to represent the pollution gradient for both areas. Variation in humus Cu concentrations (determined after acid digestion) with distance from the smelters is shown in Fig. 1. The concentrations of other heavy metals in Harjavalta and Rönnskär are given by Fritze et al. (22) and Nordgren et al. (35), respectively.

PLFA analysis. The phospholipid extraction and analysis of PLFAs was as previously described by Frostegård et al. (26). Briefly, 0.5 g (fresh weight) of humus was extracted with a chloroform-methanol-citrate buffer mixture (1:2:0.8), and the lipids were separated into neutral lipids, glycolipids, and phospholipids on a silicic acid column. The phospholipids were subjected to mild alkaline methanolysis, and the fatty acid methyl esters were separated by gas chromatography (flame ionization detector) using a 50-m HP-5 (phenylmethyl silicone) capillary column. Hydrogen was used as a carrier gas. Peak areas were quantified by adding methyl nonadecanoate fatty acid (19:0) as an internal standard. PLFA determinations were carried out twice on the Harjavalta samples, while the Rönnskär samples were analyzed only once.

Fatty acids are designated in terms of 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 a and i indicate anteiso and iso branching, br indicates unknown branching, and cy indicates a cyclopropane fatty acid. Methyl branching (Me) is indicated as the position of the methyl group from the carboxyl end of the chain.

The sum of PLFAs considered to be predominantly of bacterial origin (i15:0, a15:0, 15:0, i16:0, 16:1ω9, 16:ω7t, i17:0, a17:0, 17:0, cy17:0, 18:1ω7, and cy19:0) was chosen to represent bacterial biomass (bacterial PLFA) (24). The quantity of 18:2ω6 was used as an indicator of fungal biomass (fungal PLFA), since 18:2ω6 is suggested to be of fungal origin (20) and is known to correlate with the amount of ergosterol (24), a sterol found only in fungi. The ratio fungal PLFA/bacterial PLFA was used as an index of the ratio of fungal/bacterial biomass in soil.

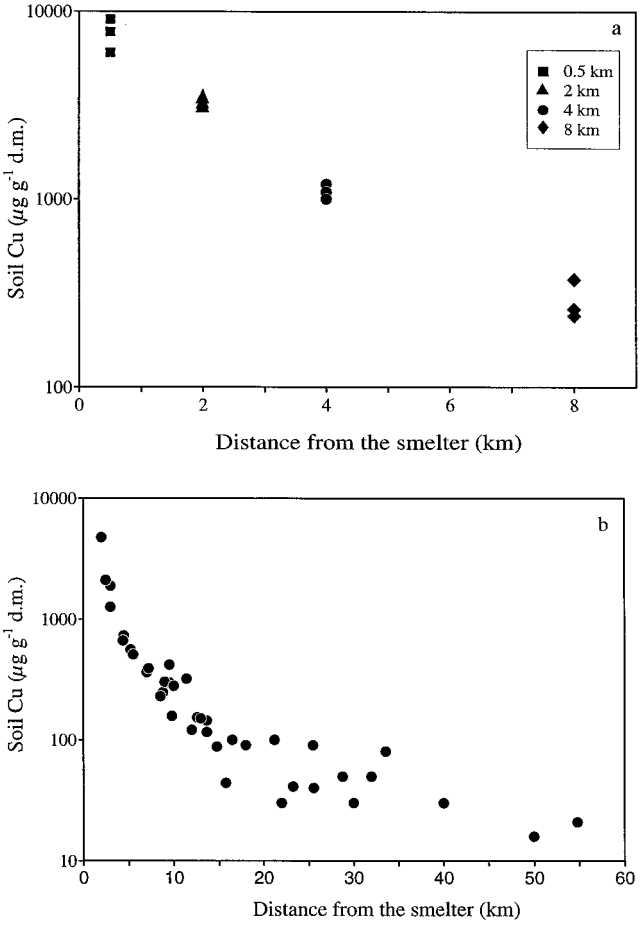

FIG. 1. Soil humus Cu concentrations at different distances from the smelters at Harjavalta (a) and Rönnskär (b).

Bacterial growth rates and community tolerance to metals. Bacterial growth rates were estimated by the thymidine incorporation technique using the bacterial community extracted by homogenization-centrifugation as described by Bååth (4). Briefly, soil (2.5 g [fresh weight]) was homogenized with 100 ml of distilled water and centrifuged for 10 min at 750 × *g*. The supernatant, containing about 20% of the bacteria in the soil, was subsequently used. Two-milliliter portions of this bacterial suspension were incubated for 2 h with 100 nM [*methyl*-^{3}H]thymidine (925 GBq mmol^{-1}; Amersham) at 20°C. Thymidine incorporation was then measured in cold acid-insoluble material after filtration and washing (4).

Heavy metal tolerance of the bacterial community was measured by the thymidine incorporation technique (5, 12). The following metal salts were used: $CuSO_4$, $ZnSO_4$, $CdSO_4$, and $Ni(NO_3)_2$. Each metal was added at 10 concentrations, ranging between 10^{-2} and 10^{-7} M (final concentration), to the bacterial solution before thymidine was added. A control without any added metals was always included. Thymidine incorporation was then measured as described above and expressed as a percentage of this control value. The heavy metal tolerance of the different bacterial communities was then estimated by calculating the concentration of added metals which results in 50% incorporation into the bacterial suspension compared with the control (no metal added) (IC_{50}). Percent inhibition was plotted against log added metal concentration, and the IC_{50}s were determined from the slope of the decreasing linear part of the curve. The changes in the level of tolerance to different metals were expressed as ΔIC_{50}s, which were calculated by subtracting the IC_{50}s of unpolluted control plots from those of the three most polluted ones.

In Rönnskär the tolerance of the bacterial community to Cu was measured in soil from 24 plots. Tolerance to the three other metals was measured in only six plots, three of the most polluted (2, 2.5, and 3 km from the smelter) and three control plots (55, 50, and 40 km from the smelter). From the Harjavalta area the

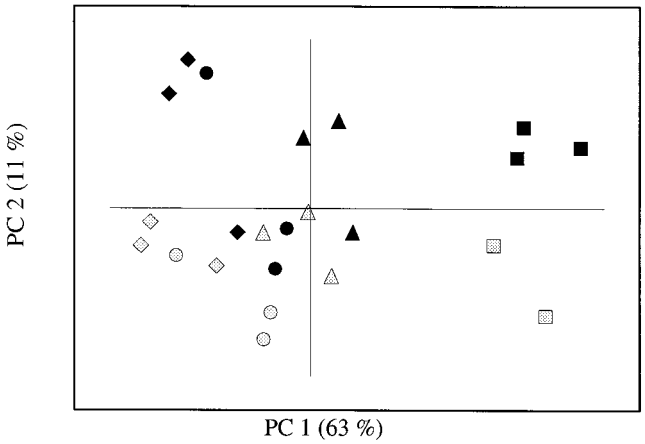

FIG. 2. Score plot of PCA showing the separation of the Harjavalta plots along the first two principal components (PC 1 and PC 2) using PLFA data. Black and grey symbols indicate two different laboratory measurement occasions. Distances from the smelter are indicated as follows: squares, 0.5 km; triangles, 2 km; circles, 4 km; and diamonds, 8 km.

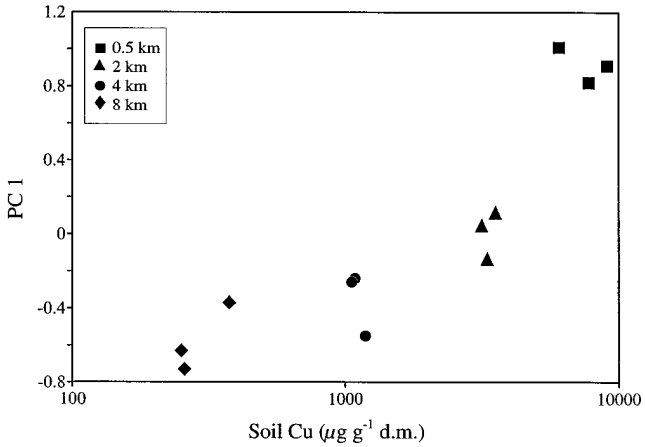

FIG. 3. Scores of the first principal component (PC 1) from the analysis in Fig. 2 in relation to the soil Cu concentration showing the pollution effect on the PLFA pattern.

tolerance of the bacterial community to Cu, Zn, Ni, and Cd was analyzed with all the 12 study plots.

The number of total bacterial cells in the suspension was determined by counting acridine orange-stained cells in the same bacterial solution used for thymidine incorporation measurements (6).

Statistical analyses. The moles percents of the PLFA values from Harjavalta and Rönnskär were $\log_{10}$ transformed before being subjected to separate principal component analyses (PCA) using the Sirius program (33). The scores of the first principal component were then used to compare changes in the PLFA pattern with the changes in bacterial community tolerance to Cu, while the loadings from the two sites for the individual PLFAs were regressed against each other to detect if the changes in the PLFA pattern were similar for the two areas studied.

RESULTS

PLFA patterns. PCA of all PLFAs at Harjavalta resulted in a separation of the different plots along the first axis, which explained 63% of the variation in the data (Fig. 2). The most polluted plots having a high score were found to the right, and the least polluted sites having a low score were found to the left in the graph. The two measurements of the same soil samples separated along the second axis (explaining 11% of the total variation). The results from the different measurement occasions were reproducible in relation to the first axis. This indicated that the scores for the different plots along the first axis showed the effect of the pollution. Plotting the scores for the first axis against the Cu concentrations of the humus (Fig. 3) stressed this further. The pollution effect levelled off with decreasing soil Cu content and thus with increasing distance from the smelter. The plots 4 km away did not differ much from the remotest plots (8 km), indicating that a Cu concentration around 1,000 μg g of d.m.$^{-1}$ was a threshold value for a pollution effect on the PLFA pattern at Harjavalta.

The PCA performed on all PLFAs at Rönnskär extracted two components, explaining totally 52% of the variation (data not shown). As with the Harjavalta data, the scores of the first component (explaining 38% of the total variation) appeared to reflect the pollution level of the humus. However, the variation between plots with similar Cu concentrations was greater than at Harjavalta, and thus a less good relationship between the scores for the different plots along the first axis and the Cu content of the soil was found at Rönnskär (Fig. 4). Still, the highest scores were found in the most polluted plots, and the lowest scores were usually found in less polluted plots.

To investigate if the metal pollution at the two areas induced the same changes in the PLFA patterns, the loadings for the individual PLFAs for the first PCA axis (indicating the pollution) were plotted against each other (Fig. 5). The metal pollution caused a similar change in most of the PLFAs in both study areas, and thus, a good correlation between loadings was found ($r = 0.76$; $P < 0.01$). The relative moles percent of several branched PLFAs, especially br18:0, br17:0, i16:0, and i16:1, increased in both areas because of pollution (indicated by a high positive loading value for these PLFAs). On the other hand, the PLFAs 18:2ω6 and 20:4 decreased the most because of the pollution in both areas (Fig. 5). To give an indication of how the proportions of PLFAs changed because of the pollution, the mean moles percents of some PLFAs of the most polluted (0.5 km) and the remotest (8 km) plots at Harjavalta can be compared. The values for the most polluted plots were 6.15, 0.60, 1.55, 3.28, and 0.16 mol% for the PLFAs i16:0, br17:0, br18:0, 18:2ω6, and 20:4, respectively, while the corresponding values in the least polluted plots were 3.70, 0.21, 0.44, 14.7, and 2.39 mol%.

At both sites increasing humus Cu concentrations resulted in

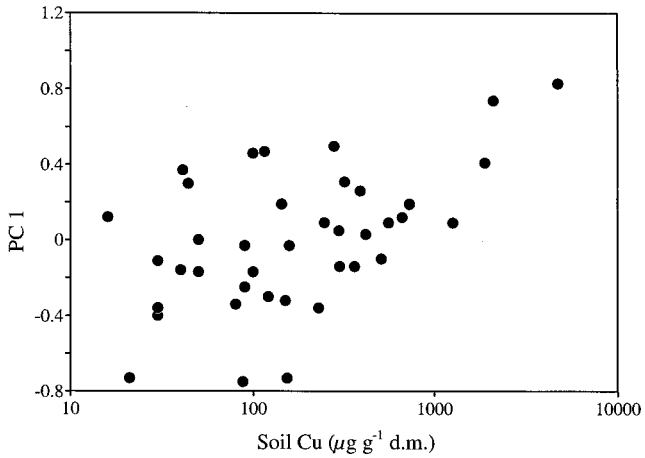

FIG. 4. Scores of the first component (PC 1) from a PCA of PLFA data from Rönnskär in relation to the soil Cu concentration showing the pollution effect on the PLFA pattern.

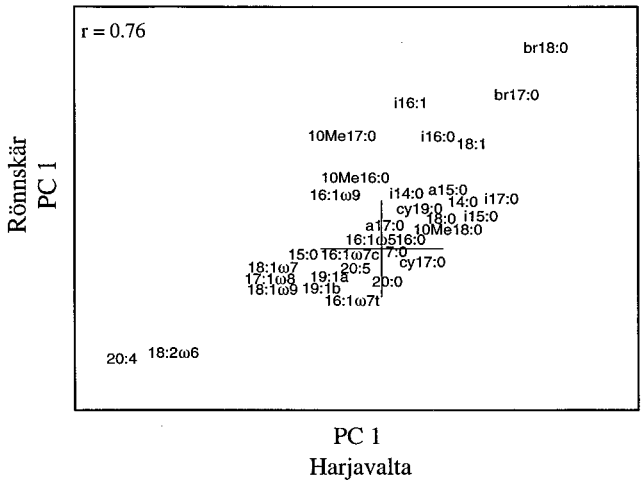

FIG. 5. Loading values for the individual PLFAs of the first principal component (PC 1) from Harjavalta plotted against those from Rönnskär. PLFAs that decreased in proportional abundance at higher pollution levels are found in the lower left corner of the plot, while those that increased are found at the upper right corner.

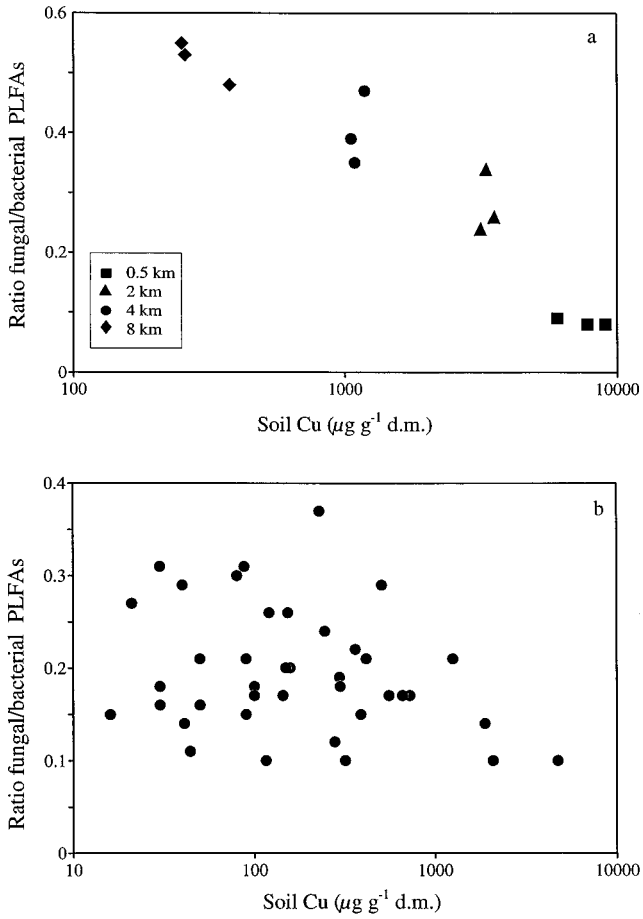

FIG. 6. The effect of the soil Cu concentration on fungal/bacterial biomass ratio estimated from PLFAs at Harjavalta (a) and Rönnskär (b).

a reduced fungal/bacterial biomass ratio as estimated from the fungal PLFA 18:2ω6 and the sum of bacterial PLFAs. The reduction of the ratio due to increasing metal load was clearly seen in Harjavalta (Fig. 6a). At Rönnskär low ratios were always found for the most polluted plots, while in the control plots both high and low values were found (Fig. 6b).

Bacterial community tolerance to heavy metals. The bacterial community tolerance to Cu was estimated by comparing the measured IC_{50}s (log metal concentration) by the thymidine incorporation method. Plotting the IC_{50}s from Harjavalta against the Cu concentration of the humus (Fig. 7a) showed a higher tolerance of the bacterial community to Cu with increasing humus Cu concentrations. Cu tolerance of the bacterial communities clearly increased in the plots situated 2 and 0.5 km from the smelter. At a distance of 4 km from the smelter, where the Cu concentration of the humus was around 1,000 μg of Cu g of d.m.$^{-1}$, IC_{50}s were similar to those at the remote plots (8 km). No or little increased tolerance to Zn, Ni, or Cd was observed, even in the most polluted plots. This is shown in Table 1, where the increases in bacterial community tolerance (ΔIC_{50} [the difference between IC_{50}s for the three most and three least polluted plots]) to all of the tested metals are presented.

A strong increase in the IC_{50}s of Cu with increasing metal concentrations of the humus was found at Rönnskär (Fig. 7b), indicating an increased bacterial community tolerance to Cu. There appeared to be an exponential relationship between IC_{50}s and log Cu content of the soil, where the IC_{50}s levelled off at Cu concentrations between 100 and 200 μg of Cu g of d.m.$^{-1}$, corresponding to distances between 10 and 15 km from the pollution source. Tolerance of the bacterial communities to other metals was also found, but the increases in level of tolerance, measured as ΔIC_{50}, were smaller than with Cu (Table 1). This could also be indicated by estimating threshold pollution levels for changes in IC_{50}s for the different metals by a linear extrapolation. Thus, the toxicities of different metals to the bacterial communities at Rönnskär were estimated by calculating the crossing points of the slopes of the IC_{50} curves for the different heavy metals from the three most polluted plots and three control plots. Cu appeared to be the most toxic metal, since an increase in tolerance was found at a lower pollution level than tolerance to the other metals (Table 1). It must be emphasized that the threshold levels indicated by the linear extrapolation will be higher than that found by using the exponential relationship, but since data for Cd, Ni, and Zn were from only the three least and three most polluted plots, only linear approximations were possible. However, even if the actual threshold level was too high, it was still possible to compare levels for tolerance to different metals.

Relation between bacterial community tolerance to copper and the PLFA pattern. The relationship between the bacterial community heavy metal tolerance and the metal pollution-induced change in the microbial community structure is shown in Fig. 8 as a correlation between the tolerance of bacterial communities to Cu (IC_{50}s) and the scores for the first axis of the PCA of the PLFA data. In Harjavalta the tolerance of the bacterial community and the PCA scores appeared to have a strong linear correlation ($r = 0.93$; $P < 0.01$) (Fig. 8a). However, in Rönnskär only the study plots with concentrations of more than 200 μg of Cu g of d.m.$^{-1}$, which were situated no more than 10 km from the smelter, showed a correlation between tolerance of the bacterial communities to Cu and the PCA scores ($r = 0.86$; $P < 0.01$) (Fig. 8b). In the more remote plots changes in the PLFA pattern were not reflected in a concomitant change in the bacterial community tolerance to Cu.

Microbial biomass, bacterial number, and activity. The specific thymidine incorporation (expressed per bacterial cell) de-

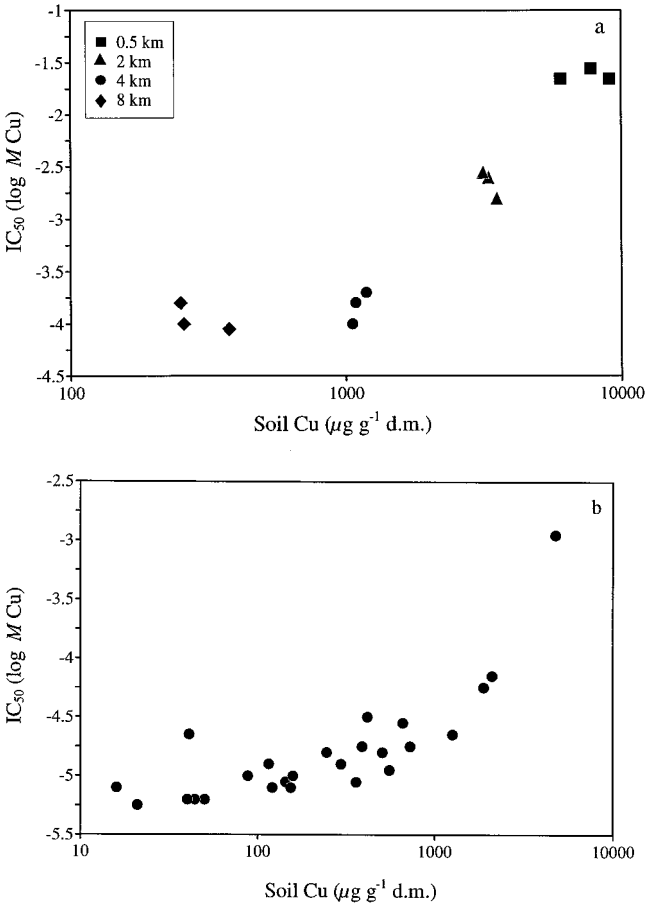

FIG. 7. Bacterial community tolerance to Cu (expressed as IC_{50}s) along the pollution gradients at Harjavalta (a) and Rönnskär (b).

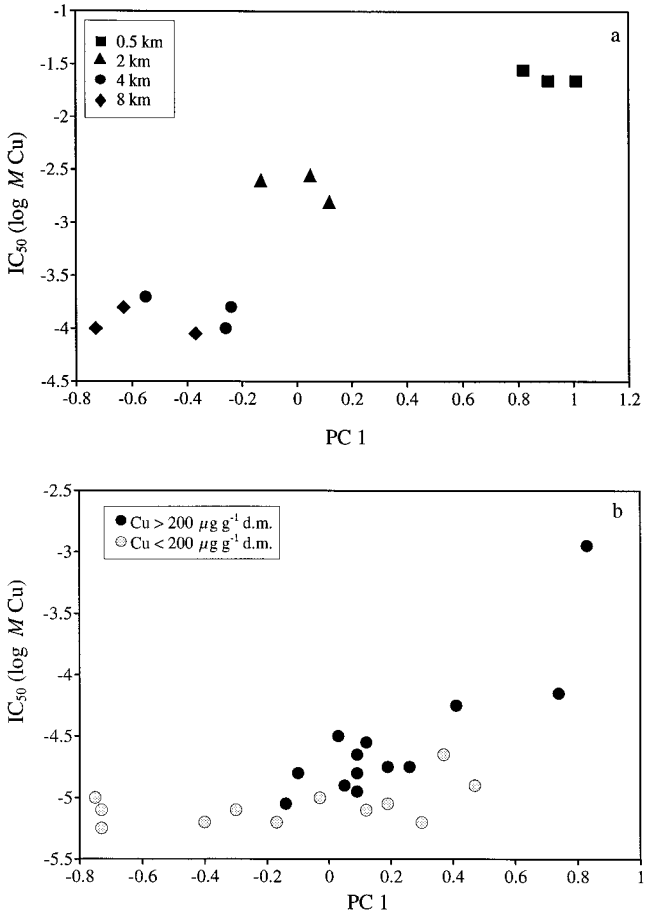

FIG. 8. The relationship between the scores of the first component (PC 1) from the PCA (indicating pollution effects on the microbial community structure) and bacterial community tolerance to Cu (expressed as IC_{50}s) at Harjavalta (a) and Rönnskär (b).

creased in Harjavalta with increasing Cu concentration (Fig. 9a), indicating a decreased growth rate of bacteria in the vicinity of the smelter. A reduction in specific thymidine incorporation was found only in the most polluted plots in Rönnskär (between 2 and 3 km from the smelter), but in most of the plots the growth rate seemed to be unaffected by the pollution (Fig. 9b).

The total number of acridine orange-stainable bacteria extracted from the humus of Harjavalta by the homogenization-centrifugation method was not affected by the increasing copper concentration of the humus, except at the most polluted plots, 0.5 km from the smelter (Fig. 10a). In Rönnskär bacterial numbers were affected only in the two plots with the highest Cu concentrations (Fig. 10b).

The total amount of bacterial PLFAs that were chosen to represent the bacterial part of the biomass was also little affected by the metal pollution. The amount of the bacterial PLFAs was 390 nmol g of $d.m.^{-1}$ in the least polluted plots of Harjavalta, which decreased to 360 nmol g of $d.m.^{-1}$ in the plots nearest to the smelter. Corresponding values for the Rönnskär area were 530 and 470 nmol g of $d.m.^{-1}$.

The total microbial biomass, measured as the total amount of PLFAs, showed a reduction in both study areas because of metal pollution. The total amount of PLFAs was 1,380 nmol g of $d.m.^{-1}$ in the least polluted plots of Harjavalta, which decreased to 910 nmol g of $d.m.^{-1}$ in the plots situated nearest to the smelter. Corresponding values for the Rönnskär area were 1,550 and 1,250 nmol g of $d.m.^{-1}$.

TABLE 1. Increase in bacterial community tolerance (ΔIC_{50}) between the three most polluted plots and the three least polluted plots at Harjavalta and Rönnskär[a]

Pollutant	Harjavalta ΔIC_{50}	Rönnskär result	
		ΔIC_{50}	Threshold pollution level for tolerance (mg of Cu g of $d.m.^{-1}$)
Cu	2.33	2.52	0.9
Cd	0.18	1.62	1.3
Ni	0.18	1.63	1.2
Zn	0.04	1.32	1.3

[a] By linear extrapolation the pollution level (expressed as soil Cu concentrations) eliciting no tolerance to the different metals was estimated at Rönnskär.

DISCUSSION

The effect of heavy metal pollution on the PLFA pattern was most clearly seen at the Harjavalta smelter, where 63% of the variation in the PLFA data was explained by the first principal component. The first principal component for the Rönnskär data explained only 38% of the variation, indicating that much

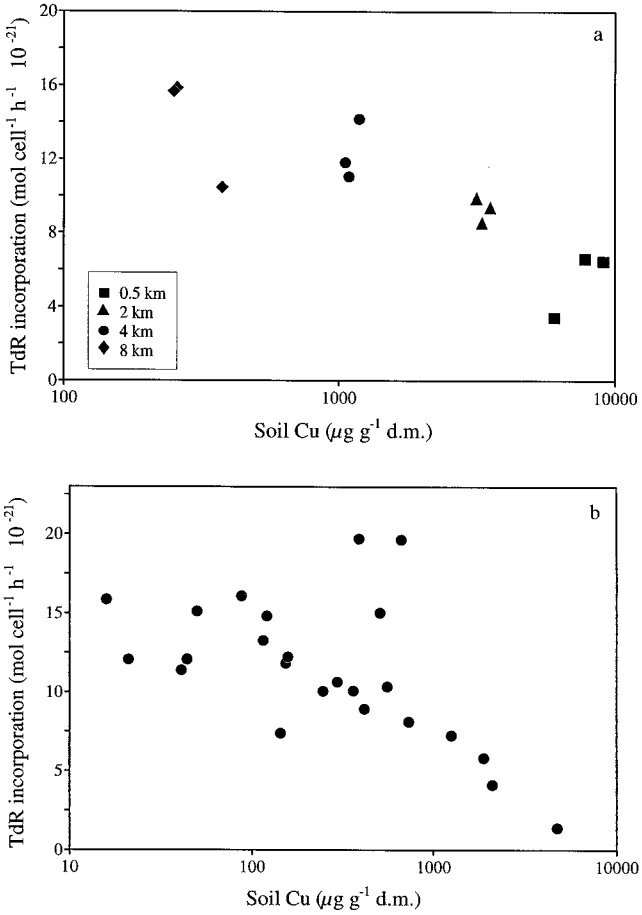

FIG. 9. The specific thymidine (TdR) incorporation of the extracted bacterial community along the pollution gradients at Harjavalta (a) and Rönnskär (b).

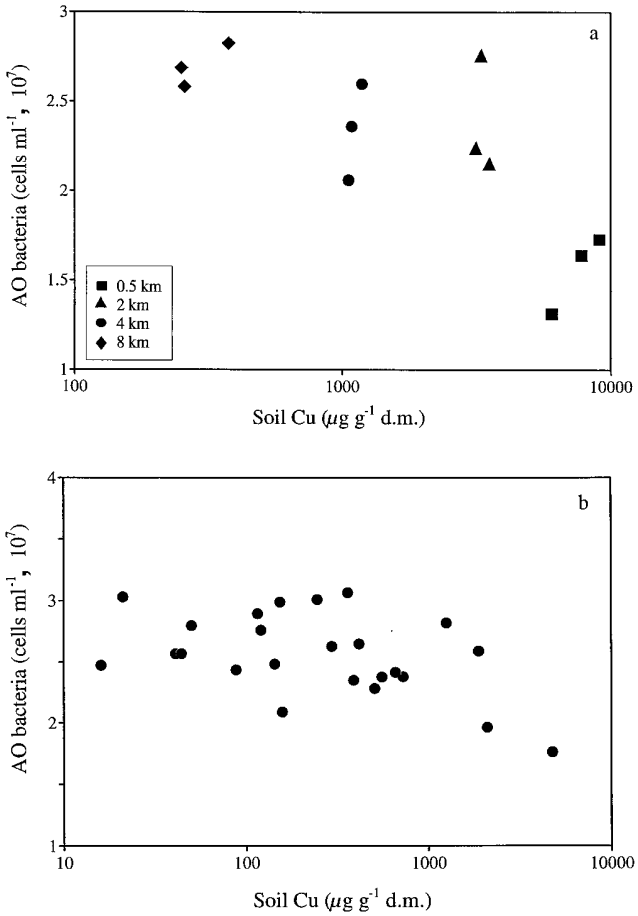

FIG. 10. The total number of the acridine orange (AO)-stainable bacteria extracted from the soil by the homogenization-centrifugation technique along the pollution gradients at Harjavalta (a) and Rönnskär (b).

of the variation in the data was due to factors other than the heavy metal pollution at this site. This was expected, since more control samples and few polluted samples were taken at Rönnskär compared with the Harjavalta site. Still, the scores for the first component were correlated with the pollution level at both sites (Fig. 3 and 4). This could therefore be considered a pollution component, and the changes in the PLFA loadings along this axis could be considered to be due to metal pollution.

The majority of the fatty acids which exhibited changes in relative concentrations in relation to heavy metal pollution reacted in the same way, either increasing or decreasing, at both sites. This was seen as a correlation between PLFA loadings along the first principal component of the different sites (Fig. 5). The changes in the PLFA patterns of the Harjavalta and Rönnskär coniferous forest soils were therefore similar, suggesting that the heavy metal pollution had resulted in similar changes in the microbial community structure at both sites, despite the fact that different pollution situations (different mixtures of heavy metals) were present. This is thus an indication that the microbial community structure will change in similar ways because of the heavy metal pollution in different coniferous forest soils. If similar changes in the PLFA pattern are found at other sites, this could thus be taken as an indication of a heavy metal effect. A different pattern would indicate that other environmental factors are of greater importance.

According to previous investigations, fungi appear to be more tolerant to heavy metals than bacteria and actinomycetes (29, 32). In agreement with this, a laboratory study showed increased proportions of the fungal 18:2ω6 in metal-polluted soil, at least in an arable soil (26). However, in the present field study, a decrease in 18:2ω6 could clearly be seen in Harjavalta, and a similar tendency was also found for the Rönnskär site (Fig. 5). One explanation could be that the main part of 18:2ω6 in the soils studied here originated from ectomycorrhizal fungi, since these are known to constitute a great part of the fungal biomass in forest soils (21). The decrease in the amount of 18:2ω6 would then be due to a decrease in ectomycorrhizal fungi, which in turn could be due to damage to the fine roots of trees because of pollution, as found in Harjavalta by Helmisaari et al. (28). The trees were also visually affected near the Rönnskär smelter. In the present study the strong reduction in the PLFA 18:2ω6 was responsible for the decrease in the ratio of fungal to bacterial biomass (Fig. 6). There was only a slight decrease in the amount of bacterial PLFAs and number of the acridine orange-stained bacteria in the gradient (Fig. 10). The assumption that the reduction in 18:2ω6 was due to fungi was supported by microscopic measurements of fungal lengths (22) and ergosterol (23) in Harjavalta showing a decrease in total length of hyphae near the smelter. The decrease in fungal/bacterial biomass ratio was not as clearly seen in Rönnskär as in Harjavalta (Fig. 6). This might explain why Nordgren et al. (35) did not find changes in the length of fungal hyphae in the Rönnskär area.

Many branched PLFAs, like br17:0 and br18:0 (branching site unknown), or iso- and anteiso-branched PLFAs, like i15:0, i16:0, i16:1, and i17:0, increased in the high-metal-content soils (Fig. 5). They are commonly found in gram-positive bacteria (36). It is usually thought that gram-negative bacteria dominate in metal-contaminated soils compared with gram-positive bacteria (13, 14, 18, 29). Reports indicating a dominance of gram-positive bacteria in metal-polluted soils are rare (41). One explanation of these contradictory results could be that in soil, gram-negative bacteria are typically found in the rhizosphere environment (27, 40). A reduction of rhizosphere microhabitats could therefore lead to a decrease in the abundance of gram-negative bacteria. There was a reduction in the forest floor vegetation due to the metal pollution in the vicinity of the Harjavalta smelter (22, 28), and a subsequent reduction in plant root biomass would be expected.

The PLFA 16:1ω5 is an example of a fatty acid which was not affected by metal contamination. Recently, Olsson et al. (39) demonstrated that 16:1ω5 can be used as a signature fatty acid for arbuscular mycorrhizal fungi, but it is also found in bacteria (31, 34, 45). In the present study the latter is more probable, since the forest floor vegetation consisted of only a few species of dwarf shrubs up to 4 km from the Harjavalta smelter (22) and the presence of arbuscular mycorrhizal plants was unlikely.

In a laboratory study in which a forest soil was contaminated artificially with different metals and then incubated for 6 months, the PLFA pattern was drastically affected by the metal addition (26). The changes in the PLFA pattern in the present field study were different from those results. Although several PLFAs, e.g., 20:4, br17:0, i16:0, and i16:1, reacted to the heavy metals similarly in both studies, several exceptions were seen. This discrepancy between field and laboratory studies could be due to roots, since in the laboratory experiments with incubated soils no plants were present. Although laboratory studies can give indications of the direct effects of metals and may be better to indicate the sensitivity of a new technique because of reduced variation of other factors, they can never simulate field conditions. However, PLFAs that were similarly affected both in the field and in the laboratory studies should be indicative of organisms that are good indicators of metal pollution. This was the case with 20:4, which was the PLFA most sensitive to heavy metals in both studies. This PLFA is typical for eucaryotic organisms and has, for example, been found in special fungi (*Mortierella* subgenus *Mortierella* [1]) which are common in forest soils (16), and it is known to be sensitive to heavy metals (2).

Heavy metal pollution increased the Cu tolerance of the bacterial communities in both Harjavalta and Rönnskär (Fig. 7), and bacterial community tolerance was the most sensitive technique to detect heavy metal pollution of the methods used in the present study when both areas were considered. Our results showed that it was possible to use the new thymidine incorporation technique to measure community tolerance for field samples with high organic matter content and not only in laboratory studies with soils low in organic matter (12). In the case of Rönnskär it was also possible to use soil samples that had been frozen for a year with good results. This is a further advantage, as it is not always possible to process samples immediately.

The increased heavy metal tolerance of the bacterial community could be due to either an acquired tolerance by adaptation, a genetically altered tolerance, or a shift in species composition in which organisms already tolerant become more competitive and thus more numerous (3). The latter explanation was suggested to be the major reason for the high level of tolerant fungi in metal-polluted soils (2), and it is probable that this, at least partly, was the reason for the increased community tolerance of bacteria in the present study. Consequently, the heavy metal tolerance of the bacterial community should be reflected in a change in microbial community structure, which would be detected as a shift in the PLFA pattern. This appeared to be the case, since the bacterial community tolerance to Cu and PLFA scores of the first component were correlated linearly in the Harjavalta samples and in the most polluted plots of Rönnskär (Fig. 8). Thus, both the Cu tolerance and the shift in community structure due to metals were found at the same level of pollution. This was also found in laboratory studies using these two techniques (12, 26). The changes in PLFAs of the remote plots of Rönnskär were not correlated with the bacterial community tolerance to Cu (Fig. 8b). This indicated variation in the microbial community structure (as revealed by the PLFA analyses), which was not directly related to the heavy metal pollution. The measurement of bacterial community tolerance is a more direct way of detecting toxic effects in soil than less specific measurements like the PLFA analysis. The same would probably also be true for biomass and activity measurements.

The tolerance of the bacterial community to Cu was higher with increasing amounts of heavy metals in the humus layer (Fig. 7). The Rönnskär data indicated that bacterial tolerance was unaltered up to a humus Cu concentration of between 100 and 200 μg g of d.m.$^{-1}$ (equivalent to a distance of 10 to 15 km from the smelter). Closer to the smelter, the community tolerance increased exponentially with the pollution level (Fig. 7b). Díaz-Raviña et al. (12) used only three different metal levels in their laboratory study. This provided too few datum points to detect an exponential function. Instead, they used a linear approximation, although they stated that this linearity between IC_{50}s and added metals did not necessarily hold at low pollution levels. This was also shown later, when lower pollution levels were studied in the laboratory system and a clear exponential relationship between IC_{50}s and added amount of metals was found (11). Any exponential relationship between IC_{50}s and soil Cu content was more difficult to detect in the Harjavalta area because of too few datum points (Fig. 7a). An increased bacterial community tolerance was found around a humus Cu concentration of 1,000 μg g of d.m.$^{-1}$. It was, however, impossible to determine the lowest level of deposition to induce increased Cu tolerance in Harjavalta, since the average of the Cu concentrations in the remotest plots (8 km from the smelter) was 294 μg g of d.m.$^{-1}$.

The heavy metal deposition in Harjavalta induced a bacterial community tolerance only to Cu, while at the Rönnskär smelter, tolerance to all the metals tested in this study (Cu, Zn, Cd, and Ni; Table 1) was found. Even though the concentrations of Zn and Ni near the Harjavalta smelter were about 10-fold higher than those in the control plots (22), no or little tolerance to Zn or Ni was found at this site (Table 1). The time of exposure to metals could not be the limiting factor for the tolerance development in this field study, since in laboratory studies bacterial community tolerance to Cd, Cu, Ni, and Zn was found after 6 to 8 months (12).

An increased tolerance to metals other than the principal contaminant (multiple tolerance) is a phenomenon reported several times (reviewed by Doelman [13]). Using artificially polluted arable soil, Díaz-Raviña et al. (12) revealed tolerance couplings for the bacterial community between Zn and Cd, Cd and Pb, and Zn and Pb. In contrast, they did not find that Cu pollution, inducing Cu tolerance, automatically led to community tolerance to other metals. This was also found at Harjavalta, where only Cu tolerance was found, with no evidence of multiple tolerance to Cd, Ni, and Zn. The interpretations of

multiple tolerance are difficult in field experiments, since several pollutants are present at the same time, as was the case in Rönnskär. However, since the Cu pollution resulted in almost the same increase in bacterial community tolerance at both sites (Table 1), the increased tolerance to Cd and/or Zn at Rönnskär probably was due to these metals exerting a toxic influence and not to multiple tolerance due to Cu. However, some indications of multiple tolerance in Rönnskär were found. The bacterial community was tolerant to Ni, even though Ni emissions were minor and the amount of Ni in the humus was similar to that at Harjavalta.

It is difficult to elucidate in a complex pollution situation, as in Rönnskär, which metal has the most toxic influence. Stepwise multiple regression analysis (e.g., see references 19 and 43) is not possible, as there will be a strong correlation between the different metals emitted. The use of bacterial community tolerance measurements will give some indications, although multiple tolerance will complicate the interpretation. The increased community tolerance to Cu was larger than tolerance to the other metals at Rönnskär (Table 1), indicating that of these four metals Cu was the most important one. However, we do not know if we can compare ΔIC_{50}s for different metals directly. Díaz-Raviña et al. (12) compared threshold levels for bacterial tolerance of different metals using a linear extrapolation and found that often the lowest threshold level was found for the metal exerting the toxic influence. In Rönnskär, using the same linear extrapolation, Cu gave the lowest threshold level compared with the other three metals (Table 1), indicating that Cu was the most toxic of these metals in the Rönnskär area.

Decreased microbial activity, measured as the ratio of microbial respiration to microbial biomass, and microbial biomass in the vicinity of the Harjavalta smelter were found by Fritze et al. (22, 23). In our study the continuous presence of heavy metals also resulted in decreased activity and biomass of bacteria, since a general reduction in the growth rate of bacteria (indicated by the specific thymidine incorporation) was observed in the vicinity of both smelters (Fig. 9), and the number of bacteria was reduced in the most polluted plots in Harjavalta and in the two sites nearest the smelter at Rönnskär (Fig. 10). Changes in the microbial community structure (estimated as both the PLFA pattern and bacterial community tolerance) were found at lower soil metal concentrations than the changes in bacterial biomass and activity. This suggested that the altered microbial community with increased tolerance to metals had at least partly counteracted the toxic effects of the metals, as seen by biomass and activity.

The present study has shown the potential for using PLFA analysis and bacterial community tolerance to study heavy metal pollution in field sites. Both techniques appeared to be well suited to detect effects of heavy metals, although the use of community tolerance was more directly related to the heavy metal pollution. Thus, measurements of community tolerance can be used to deduce which changes in the PLFA pattern are due to the metal toxicity in field studies, while changes in the PLFA pattern can be used to some extent to elucidate which groups of organisms are affected.

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