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Multiple Heavy Metal Tolerance of Soil Bacterial Communities and Its Measurement by a Thymidine Incorporation Technique

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A thymidine incorporation technique was used to determine the tolerance of a soil bacterial community to Cu, Cd, Zn, Ni, and Pb. An agricultural soil was artificially contaminated in our laboratory with individual metals at three different concentrations, and the results were compared with the results obtained by using the plate count technique. Thymidine incorporation was found to be a simple and rapid method for measuring tolerance. Data obtained by this technique were very reproducible. A linear relationship was found between changes in community tolerance levels obtained by the thymidine incorporation and plate count techniques ($r = 0.732, P < 0.001$). An increase in tolerance to the metal added to soil was observed for the bacterial community obtained from each polluted soil compared with the community obtained from unpolluted soil. The only exception was when Pb was added; no indication of Pb tolerance was found. An increase in the tolerance to metals other than the metal originally added to soil was also observed, indicating that there was multiple heavy metal tolerance at the community level. Thus, Cu pollution, in addition to increasing tolerance to Cu, also induced tolerance to Zn, Cd, and Ni. Zn and Cd pollution increased community tolerance to all five metals. Ni amendment increased tolerance to Ni the most but also increased community tolerance to Zn and, to lesser degrees, increased community tolerance to Pb and Cd. In soils polluted with Pb increased tolerance to other metals was found in the following order: Ni > Cd > Zn > Cu. We found significant positive relationships between changes in Cd, Zn, and Pb tolerance and, to a lesser degree, between changes in Pb and Ni tolerance when all metals and amendment levels were compared. The magnitude of the increase in heavy metal tolerance was found to be linearly related to the logarithm of the metal concentration added to the soil. Threshold tolerance concentrations were estimated from these linear relationships, and changes in tolerance could be detected at levels of soil contamination similar to those reported previously to result in changes in the phospholipid fatty acid pattern (Å. Frostegård, A. Tunlid, and E. Bååth, Appl. Environ. Microbiol. 59: 3605–3617, 1993).

Numerous reports of the effects of heavy metals and other toxic substances on bacteria obtained from soils, sediments, and aquatic habitats have been published. In most of these studies bacteria were isolated from their natural habitats, and then the susceptibility of isolated strains to the toxic substance was studied in pure culture (14, 21, 36, 37). Such studies have often revealed that bacteria isolated from environments with elevated levels of heavy metals exhibit greater metal tolerance than bacteria isolated from unpolluted habitats. However, metal-tolerant bacteria are also found in environments that are not polluted by heavy metals (18, 28). Thus, the existence of bacterial species that are tolerant to a pollutant in an environment does not provide information concerning the selection pressure due to that pollutant. This has to be studied at the community level.

Studies at the bacterial community level are, however, not as common, and little is known about the sensitivity of whole soil bacterial communities to heavy metals. Jordan and Lechevalier (28) investigated the influence of Zn on bacteria in soils close to a zinc smelter. These authors found an increased proportion of tolerant bacteria in polluted soils, but, as mentioned above, zinc-tolerant bacteria were also readily isolated from soils containing low Zn concentrations. Using soils artificially contaminated in a laboratory, Doelman and Haanstra (14) observed that Pb-polluted soils contained a greater proportion of bacteria that were able to grow in medium containing high concentrations of Pb. In a field study the metal resistance of soil bacteria was related to the total concentrations of Cd, Zn, and Pb in soils (33). Similar results have also been found in different terrestrial and aquatic environments (5, 6, 11, 39). However, in some cases no changes in tolerance have been found (10, 18, 24, 29).

In all of the studies mentioned above the authors used plate count techniques and media containing different metal concentrations in order to quantify the levels of tolerance of soil bacteria. Plate count techniques, however, are selective, and only up to 5% of the soil bacteria, depending on the medium used, are able to grow on agar plates (32). Therefore, there is no information available concerning the metal tolerance of the majority of soil bacteria.

Recently, a new technique for studying the tolerance of soil bacteria to heavy metals was described (2). In this procedure the bacteria are first extracted from soil by homogenization and centrifugation. Then the level of tolerance is estimated by measuring the incorporation rate of labelled thymidine into total bacterial macromolecules following the addition of different amounts of metals to bacterial suspensions. The thymidine incorporation technique has an advantage over the techniques based on plate counts in that it provides information about a larger proportion of the bacterial community without the selectivity of the plate count technique. Furthermore, Bååth (2) also reported that this new approach could be successfully used with a Cu-polluted soil to determine multiple tolerance to different heavy metals (that is, the bacterial community became tolerant to metals other than the metals added to the soil). No information about soils contaminated

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TABLE 1. Soil treatments studied

Symbol	Treatment		pH
	Metal added	Dose (mmol kg^{-1} [dry wt])	
Cu_8	Cu	8	7.86
Cu_{16}	Cu	16	7.27
Cu_{32}	Cu	32	7.04
Cd_4	Cd	4	7.73
Cd_8	Cd	8	6.93
Cd_{16}	Cd	16	6.58
Zn_8	Zn	8	7.53
Zn_{16}	Zn	16	7.00
Zn_{32}	Zn	32	6.71
Ni_{16}	Ni	16	7.86
Ni_{32}	Ni	32	7.60
Ni_{64}	Ni	64	7.41
Pb_{16}	Pb	16	7.54
Pb_{32}	Pb	32	6.88
Pb_{64}	Pb	64	6.22

with metals other than Cu is available, and thus we know little about the pattern of multiple heavy metal tolerance at the community level.

The aim of this work was to use the thymidine incorporation technique to study multiple tolerance of a soil bacterial community to different metals in soil artificially contaminated with Cu, Cd, Zn, Ni, or Pb. In order to determine the degree to which heavy metal contamination affected the level of tolerance, the metals were added to the soil at different rates. Furthermore, to evaluate the validity of this new method, the results obtained were compared with results obtained by the traditional plate count technique.

MATERIALS AND METHODS

Experimental design. This study was performed by using an agricultural sandy loam soil from southern Sweden; this soil contained 4.4% organic matter, and its pH was 7.8. Soil samples (100 g) that had been previously sieved (mesh size, <2 mm) and thoroughly mixed by hand were contaminated in our laboratory with five different metals. Cd, Cu, Zn, Ni, and Pb were added to the soil as $CdSO_4$, $CuSO_4$, $ZnSO_4$, $Ni(NO_3)_2$, and $Pb(NO_3)_2$ solutions at the concentrations indicated in Table 1. Metal concentrations which resulted in reductions in soil ATP content of approximately 50% (20) were selected as the medium levels of application. The low and high doses of each metal corresponded to concentrations that were two times lower and two times higher than the medium level. Soil samples that received no metal addition were used as controls. All samples were stored in plastic beakers at room temperature (approximately 22°C). Distilled water was added occasionally to maintain the soil moisture at a constant level.

Two experiments were performed. The first experiment was performed by using soils contaminated only with the low level of each metal, and levels of tolerance were measured after 9 months of incubation by using the thymidine incorporation technique. The second experiment was performed with soils that were polluted at the medium and high levels, and both thymidine incorporation and plate count techniques were used to determine levels of tolerance. Tolerance was measured after 5, 6, 7, 7.5, and 8 months of storage for soils polluted with Cu, Pb, Ni, Zn, and Cd, respectively. Unpolluted soil samples were included in each experiment.

Extraction of bacteria. Bacteria were extracted from soil by the homogenization-centrifugation method of Faegri et al. (19), as modified by Bååth (3). A 10-g (wet weight) portion of soil was homogenized in 200 ml of distilled water with a Sorvall Omnimixer operated at 80% of the top speed for 1 min. The soil suspension was then centrifuged at 750 × *g* for 10 min at 5°C, and the supernatant was poured through glass wool. This resulted in a bacterial suspension containing approximately 10 to 20% of the total bacteria in the soil (as determined by acridine orange staining).

Tolerance measured by the thymidine incorporation technique. Bacterial growth rates were determined by measuring the incorporation of [^{3}H]thymidine into the cold trichloroacetic acid-insoluble material (total macromolecules) in each bacterial suspension, using the procedure described by Bååth (2). Samples (1.8 ml) of each bacterial suspension were placed into plastic vials, and 0.2-ml portions of distilled water (control) or the different metal solutions were added. Heavy metal solutions whose concentrations ranged from a concentration that resulted in no inhibition to a concentration that resulted in total inhibition of thymidine incorporation were prepared by using the metal salts mentioned above. For each metal between 13 and 15 concentrations in the range from 1×10^{-9} to 2×10^{-3} M (final concentrations) were prepared in the second experiment, whereas only 6 concentrations were used in the first experiment. One replicate of each concentration was used. The suspensions were incubated at 20°C in the presence of 100 nM [*methyl*-^{3}H]thymidine (925 GBq $mmol^{-1}$; Amersham). After 2 h incorporation was stopped by adding 1 ml of 5% formalin; for zero-time controls the formalin was added at the same time as thymidine. The suspensions were then filtered through glass fiber filters (Whatman GF/F filters soaked in 1% Calgon) and washed three times with 5 ml of ice-cold 80% ethanol and three times with 5 ml of ice-cold 5% trichloroacetic acid. The rims of the filters were then cut away. The filters were placed in scintillation vials containing 1 ml of 0.1 M NaOH and kept at 90°C for 2 h to solubilize the macromolecules. After cooling, 10 ml of Ultima Gold scintillation liquid (Packard) was added. The radioactivity was measured with a Beckman model LS 1801 liquid scintillation spectrometer by using an external standard method (Beckman method H#) to correct for quenching.

Tolerance measurements as determined by the plate count method. The numbers of CFU were determined only for soils polluted with Cu, Cd, and Zn at the highest levels tested (Cu_{32}, Cd_{16}, and Zn_{32}) (Table 1) and soils polluted with Ni and Pb at medium levels (Ni_{32} and Pb_{32}). The bacterial suspension that was used for the thymidine incorporation technique was also used for plate counting. The bacterial suspension was diluted with 0.1% peptone. The bacterial counts were determined by spreading 50-μl portions of appropriate dilutions on plates containing TSB agar (0.2% tryptic soy broth, 1% agar, 50 μg of cycloheximide per ml) that was either not supplemented (control) or supplemented with metals at different concentrations. Each heavy metal solution and cycloheximide were filter sterilized and added to the autoclaved agar medium at 50°C before the plates were filled. For each metal between six and eight concentrations in the range from 3×10^{-3} to 1×10^{-7} M (final concentrations) were prepared, and three or four plates were used for each dilution. The final pH of the medium varied between 6.0 and 6.5 depending on the metal and concentration added. The plates were incubated at 22°C for 6 weeks and were counted at 2-week intervals. Only dilutions that produced between 20 and 80 colonies per plate were considered.

Statistical analysis. All tolerance measurements determined by the thymidine incorporation and plate count techniques were expressed as percentages of the control values. To

increase the precision of the control values, the mean of the data obtained at low metal concentrations when there was no inhibition effect was used to calculate the control value. The logarithms of the concentrations that resulted in 50% inhibition (IC_{50}) (i.e., a 50% decrease in the number of CFU or the thymidine incorporation rate) were then calculated. The IC_{50} values for plate counts and thymidine data obtained in the first experiment were determined from the slope of the decreasing linear part of the plot of percentage of inhibition versus log metal concentration. Since more datum points were available in the second experiment, incorporation data could be analyzed by using a logistic model (23, 41) having the general form $Y = c/[1 + e^{b(X - a)}]$, where Y is the observed level of thymidine incorporation, X is the logarithm of the metal concentration, a is the logarithm of the concentration at which the growth rate was one-half of the control value (IC_{50}), c is the growth rate in the control with distilled water, and b is a slope parameter indicating the inhibition rate. As the logarithm of the heavy metal concentration in the control was not known, it was replaced by the logarithm of a very low heavy metal concentration (10^{-9} M). Nonlinear regression by using the statistical program STATGRAPHICS was used to fit the data to the model.

We observed no differences between IC_{50} values calculated by using the logistic model and IC_{50} values calculated by using the linear part of the plot. Therefore, the results obtained in the first and second experiments could be compared. IC_{10} values (concentrations which resulted in 10% inhibition) were also calculated from the logistic equation. A linear-logistic model (41) was also applied to the data that produced a dose-response curve which indicated that there was stimulation at low doses. This model included a new parameter in order to model this stimulation. Similar IC_{50} values were obtained with the logistic model and the linear-logistic model. This indicated that the stimulation effect, which modified the shape of the curve, was not critical for determining IC_{50} values.

The effect of heavy metal amendment on the level of tolerance (ΔIC_{50} or ΔIC_{10}) was estimated by determining the difference between IC_{50} or IC_{10} values in contaminated and uncontaminated soils (ΔIC_{50} = IC_{50} in polluted soil − IC_{50} in unpolluted soil). To do this, we used the means of several different measurements of IC_{50} in unpolluted soil in the second experiment, while only one measurement was used in the first experiment.

RESULTS

Thymidine incorporation. The rates of thymidine incorporation into cold trichloroacetic acid-insoluble material for soil bacteria extracted from unpolluted soil after different storage times (5 to 8 months) varied between 9.9×10^{-14} and 21.7×10^{-14} mol h^{-1} ml^{-1} (mean ± standard error, $14.7 \times 10^{-14} \pm 0.9 \times 10^{-14}$ mol h^{-1} ml^{-1}) (Table 2). A marked, but not consistent, effect of heavy metals on the thymidine incorporation rate was observed. For some metals the thymidine incorporation rate for bacteria obtained from polluted soils was greater than the rate for bacteria obtained from unpolluted soil, but for other metals the rate was lower (Table 2). Addition of Ni and Pb at the highest levels reduced the growth rate to undetectable values, and therefore tolerance levels could not be measured.

On the basis of the IC_{50} values (log metal concentration), Cu was the most toxic metal for soil bacteria extracted from unpolluted soil (IC_{50}, −6.48), followed by Cd and Zn (IC_{50}, −5.32 and −5.01, respectively), while Pb and Ni, with IC_{50} values of −4.61 and −4.47, respectively, were the least toxic (Table 3). Differences in the slopes of the dose-response curves were also found. Pb, Cu, and Zn produced dose-response curves with steep slopes (with values ranging from 3 to 3.5), whereas the slopes of the curves for Cd and Ni were less steep (the slopes obtained with Cd and Ni were 2.3 and 1.0, respectively).

TABLE 2. Bacterial colony counts (CFU) after 6 weeks of incubation of agar plates and thymidine incorporation into total macromolecules of bacteria extracted from unpolluted and polluted soils by using homogenization and centrifugation

Treatment[a]	Colony count		Thymidine incorporation rate (10^{-14} mol ml^{-1} h^{-1})
	CFU (10^5 ml^{-1})	% of CFU after 2 weeks[b]	
Control	1.4 ± 0.4[c]	84 ± 1[c]	14.7 ± 0.9[c]
Cu_{16}	ND[d]	ND	10.8
Cu_{32}	14.5	58	6.5
Cd_8	ND	ND	10.6
Cd_{16}	2.8	67	19.2
Zn_{16}	ND	ND	11.8
Zn_{32}	2.8	40	31.9
Ni_{32}	2.6	76	38.5
Ni_{64}	ND	ND	BDL[e]
Pb_{32}	4.8	78	132.9
Pb_{64}	ND	ND	BDL

[a] See Table 1.
[b] CFU after 2 weeks of incubation, expressed as a percentage of the CFU obtained after 6 weeks of incubation.
[c] Mean ± standard error of the values obtained at different sampling times (n = 4 for colony counts; n = 5 for thymidine incorporation).
[d] ND, not determined.
[e] BDL, below detectable levels.

Since IC_{50} and slope values were obtained at different times during storage of the unpolluted soils in the second experiment, it was possible to compare how reproducible the measurements were (Table 3). The standard deviations were lower for the IC_{50} estimates than for the slope estimates, except for Ni. Ni also exhibited the greatest variation in IC_{50} values, while Cu and Zn exhibited the least variation. This was also evident when the IC_{50} values for the unpolluted soil were compared for the two different experiments (Tables 3 and 4). Only the values for Ni deviated substantially from each other in the two experiments.

Both differences in the IC_{50} values and differences in the slopes of the dose-response curves were observed when we compared bacteria extracted from unpolluted and polluted soils, showing that soil metal amendment resulted in changes in the sensitivities of microorganisms to heavy metals (Tables 3 and 4). This was shown by the inhibition curves for Cd obtained for bacteria extracted from unpolluted and Cd-polluted soils (Fig. 1). The increases in IC_{50} values compared with the unpolluted soil value were 0.14, 1.00, and 1.65 logarithmic units for the 4-, 8-, and 16-mmol kg^{-1} (dry weight) treatments, respectively.

The metal concentrations in the bacterial suspensions were not measured. Thus, the metal concentrations might have differed for bacteria extracted from unpolluted and heavily polluted soils. However, this apparently did not affect the results. In an additional trial, the bacterial suspensions from unpolluted and Zn-polluted (16 mmol kg^{-1} [dry weight]) soils were washed once by centrifugation at 12,000 × g for 30 min, and the resulting bacteria were resuspended in new distilled water. The IC_{50} values and thus the ΔIC_{50} for Zn tolerance were the same for both the original and the washed bacterial suspensions (data not shown).

TABLE 3. Levels of bacterial community tolerance to different heavy metals measured by the thymidine incorporation and plate count techniques in the second experiment

Treatment[a]	Method[b]	Parameter[c]	Cu	Cd	Zn	Ni	Pb
Control[d]	TdR	IC_{50}	−6.48 ± 0.02	−5.32 ± 0.03	−5.01 ± 0.02	−4.47 ± 0.05	−4.61 ± 0.03
		Slope	3.03 ± 0.04	2.35 ± 0.04	3.25 ± 0.06	1.04 ± 0.04	3.53 ± 0.08
	PC	IC_{50}	−3.84 ± 0.05	−5.29 ± 0.09	−4.36 ± 0.08	−3.83 ± 0.06	−3.05 ± 0.01
Cu_{16}	TdR	IC_{50}	−5.55	−5.03	−4.66	−4.65	−4.50
		Slope	4.23	2.98	2.51	1.21	4.94
Cu_{32}	TdR	IC_{50}	−5.25	−4.83	−4.27	−4.04	−4.52
		Slope	4.76	2.31	1.97	1.13	4.40
	PC	IC_{50}	−3.44	−4.48	−4.06	−3.56	−3.40
Cd_8	TdR	IC_{50}	−6.18	−4.32	−3.27	−4.54	−4.18
		Slope	3.04	2.24	3.15	1.00	5.93
Cd_{16}	TdR	IC_{50}	−6.10	−3.67	>−2.70	−2.83	−3.89
		Slope	3.64	1.59	ND[e]	1.13	5.87
	PC	IC_{50}	−3.20	−4.10	>−3.30	−3.84	>−2.90
Zn_{16}	TdR	IC_{50}	−6.12	−4.49	−3.68	−4.91	−4.28
		Slope	4.08	3.66	4.78	1.08	8.63
Zn_{32}	TdR	IC_{50}	−6.23	−3.76	−2.69	−4.10	−4.09
		Slope	5.08	4.22	9.71	0.71	27.19
	PC	IC_{50}	−3.40	−4.42	>3.30	−3.86	−2.90
Ni_{32}	TdR	IC_{50}	−6.43	−5.13	−4.31	−3.28	−4.20
		Slope	3.51	1.54	2.03	0.98	3.07
	PC	IC_{50}	−3.72	−4.92	−4.48	−3.18	−3.38
Pb_{32}	TdR	IC_{50}	−6.03	−4.35	−4.22	−3.40	−4.56
		Slope	3.35	2.63	1.82	1.30	10.68
	PC	IC_{50}	−3.56	−4.82	−3.88	−3.58	−2.90

[a] See Table 1.
[b] TdR, thymidine incorporation method; PC, plate count method.
[c] IC_{50} values are expressed as log metal concentration. IC_{50} and slope values were calculated by using the logistic equation for the thymidine incorporation data. For plate counts, IC_{50} values were calculated from regression of the linear parts of the dose-response curves.
[d] For the control soil, means ± standard errors are given (n = 4 for the plate count technique; n = 5 for the thymidine incorporation technique).
[e] ND, not determined.

Two different inhibition curve types were obtained as a consequence of the short-term metal effects on the thymidine incorporation rate used for tolerance measurements (Fig. 2). In most cases, the data fitted the logistic model well since gradual decreases in the growth rate in response to increases in the metal concentrations in the bacterial suspensions were observed (Fig. 2, unpolluted soil). In some cases, however, a significant stimulatory effect at low metal concentrations was found (Fig. 2, polluted soil). This stimulatory effect was especially prominent for the bacterial communities extracted from soils that received the highest concentrations of Zn and Cd.

In the first experiment, in which only the lowest soil metal concentration was studied (Table 4), generally only a small increase, or even a decrease, in metal tolerance was found in the metal-contaminated soils compared with the unpolluted soil. Nevertheless, increased tolerance to Zn and Cd was

TABLE 4. Levels of bacterial community tolerance to different heavy metals in unpolluted and polluted soils measured by using the thymidine incorporation technique in the first experiment

Treatment[a]	IC_{50} (log metal concn)[b]				
	Cu	Cd	Zn	Ni	Pb
Control	−6.28	−5.28	−5.04	−3.78	−4.74
Cu_8	−6.18	−5.56	−5.20	−4.16	−5.06
Cd_4	−6.18	−5.14	−4.00	−4.18	−4.64
Zn_8	−6.40	−5.00	−4.16	ND[c]	−4.94
Ni_{16}	−6.36	−5.38	−4.62	−2.82	−4.66
Pb_{16}	−6.62	−5.28	−4.82	−4.32	−4.88

[a] See Table 1.
[b] IC_{50} values were calculated from regression of the linear parts of the dose-response curves.
[c] ND, not determined.

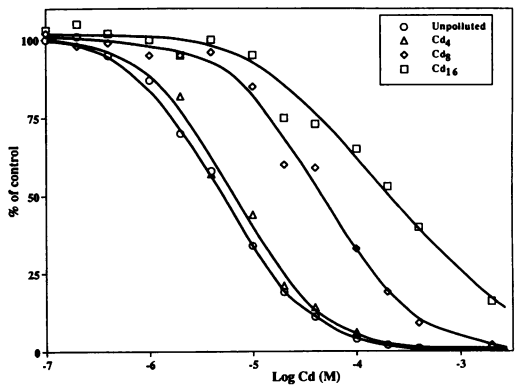

FIG. 1. Dose-response curves showing the effect of added Cd on thymidine incorporation into macromolecules of bacteria extracted from unpolluted soil and soil polluted with 4 (Cd_4), 8 (Cd_8), and 16 (Cd_{16}) mmol of Cd kg^{-1} (dry weight).

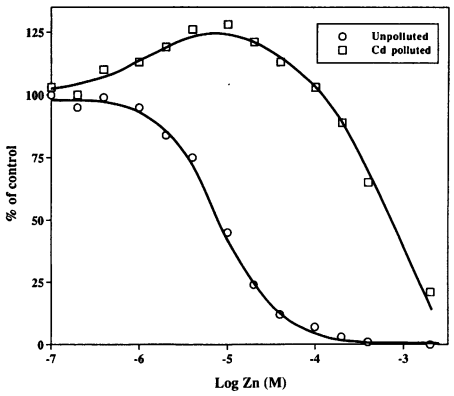

FIG. 2. Dose-response curves showing the effect of added Zn on thymidine incorporation into macromolecules of bacteria extracted from unpolluted soil and Cd soil polluted with Cd (16 mmol of Cd kg^{-1} [dry weight]).

observed with the bacteria in the soils that received Zn and Cd at concentrations of 8 and 4 mmol kg^{-1} (dry weight), respectively (ΔIC_{50}, 0.28 and 0.88 for Cd and Zn, respectively, for soil that received 8 mmol of Zn per kg and 0.14 and 1.04, respectively, for soil that received 4 mmol of Cd per kg). Increased tolerance to Ni and Zn in soil that received 16 mmol of Ni per kg was also observed (ΔIC_{50}, 0.96 and 0.42 for Ni and Zn, respectively). In the soils that received 8 mmol of Cu per kg and 16 mmol of Pb per kg no increase in tolerance to Cu, Pb, or any of the other metals studied was found.

When the results of the two experiments were combined, higher levels of tolerance were found with higher concentrations of soil metals (Tables 3 and 4 and Fig. 3). Generally, contamination with a specific metal increased the level of tolerance of the bacterial community to that metal. However, increases in the levels of tolerance to metals other than the metal added also occurred, especially at higher levels of contamination. Thus, addition of Cu led to a bacterial community that was more tolerant to Cu and, to a lesser extent, to Zn, Cd, and Ni. No tolerance to Pb was detected. After contamination of soil with Zn and Cd, higher levels of tolerance to all of the metals studied were observed. These two metals produced similar effects; i.e., the levels of tolerance to Zn and Cd were increased considerably, tolerance to Ni was also enhanced, particularly in Cd-polluted soil, and tolerance to Pb and Cu increased to lesser extents. It should be pointed out that tolerance to Zn was actually increased more by addition of Cd than by addition of Zn. In the case of soil contaminated with Cd at the highest level, IC_{50} values for Zn could not be properly calculated since even the highest metal concentration did not result in 50% inhibition. Addition of Ni increased tolerance to Ni the most, followed by Zn and, to lesser degrees, Pb and Cd, whereas no increase in tolerance to Cu was detected. Finally, in soils that received Pb, increases in the levels of tolerance of bacteria to all metals except Pb were observed, in the following order: Ni > Cd > Zn > Cu.

Multiple heavy metal tolerance of the soil bacterial community was also observed when the correlation coefficients for the ΔIC_{50} values obtained for all amendment levels of the different metals were calculated (Table 5). The results indicated that there was a close relationship between ΔIC_{50} values for Cd, Zn, and Pb tolerance ($r > 0.8$); changes in tolerance to Cd and Zn were particularly highly correlated ($r = 0.92$). A positive correlation between changes in Pb tolerance and changes in Ni tolerance, although significant at a lower level, was also found.

A positive linear correlation between changes in IC_{50} and changes in IC_{10} ($r = 0.909$, $P < 0.001$, $n = 39$) was found when the values for all of the metals were combined. In general, the same conclusions concerning the levels of tolerance were reached by using ΔIC_{50} or ΔIC_{10} values for Cu, Cd, Zn, and Ni (data not shown). In contrast, the increases in levels of Pb tolerance based on IC_{10} values were about two times greater than the increases in Pb tolerance levels observed when IC_{50} values were used. Changes in the slopes of the dose-response curves explained this finding (Table 3). Differences in the slopes of the inhibition curves for polluted and unpolluted soils were observed for all metals except Ni, indicating that the rates of inhibition obtained with increasing metal concentrations in short-term measurements varied as a consequence of soil metal additions. However, the changes in the slopes of the inhibition curves for Cu, Cd, and Zn were only minor. Thus, similar results for the levels of tolerance were obtained by using ΔIC_{50} and ΔIC_{10} values. In contrast, changes in the slopes were particularly evident in the case of Pb when polluted soils were compared with unpolluted control soil. Thus, whereas small or even no detectable changes in IC_{50} values were observed, greater changes were observed in the corresponding IC_{10} values.

Although data for only three soil metal concentrations were available for Cd, Cu, and Zn, the results indicated that the level of tolerance increase (ΔIC_{50}) was proportional to the amount of heavy metal added to the soil (Fig. 3). Using linear regression and assuming that the linear relationship could be extended to soil metal concentrations lower than the concentrations used in our experiments, we estimated the threshold concentration at which changes in the tolerance of the bacterial community became evident (Table 6). Estimates were also obtained for Ni and Pb tolerance, although the estimated threshold soil concentrations were very imprecise since only two soil amendment levels were used. The lowest threshold concentrations were generally found in the Cd-polluted soils (range, 1 to 6 mmol kg^{-1} [dry weight] of soil for the five different metals), while the highest threshold concentrations were found for the Pb-polluted soils (range, 13 mmol kg^{-1} [dry weight] of soil to no tolerance detected). The lowest threshold concentration for altered bacterial tolerance was found for the same metals used for soil contamination in the Cu-, Zn-, and Ni-polluted soils. In the Cd-polluted soils, the threshold concentrations for altered tolerance were similar for Cd, Cu, Pb, and Zn. In the Pb-polluted soils no tolerance to Pb was detected. Instead, the lowest threshold concentration was found for Cd and Zn. If the lowest threshold value irrespective of metal was used, the data indicated that tolerance of the bacterial community should not exist with metal concentrations less than 6, 1, 4, 1, and 13 mmol kg^{-1} (dry weight) of soil for Cu, Cd, Zn, Ni, and Pb, respectively.

Plate counts. The numbers of CFU obtained for unpolluted soils after different storage times varied from 0.5×10^5 to 3.6×10^5 CFU ml^{-1} (mean ± standard error, $1.4 \times 10^5 \pm 0.4 \times 10^5$ CFU ml^{-1}) (Table 2). Between 76 and 88% (mean ± standard error, 84% ± 1%) of the colonies were formed during the first 2 weeks of incubation. After the addition of heavy metals, three- to sixfold increases in the number of CFUs were observed in bacterial suspensions extracted from polluted soils compared with suspensions extracted from unpolluted soils (Table 2). A delay in the appearance of colonies was also detected. Only 40 to 78% of the colonies (depending on the metal added to the plates) appeared during the first 2 weeks. Increases in the pH of the agar medium of between 1 and 2 pH units were detected with both polluted and unpolluted soils after 6 weeks of incubation as a result of bacterial growth.

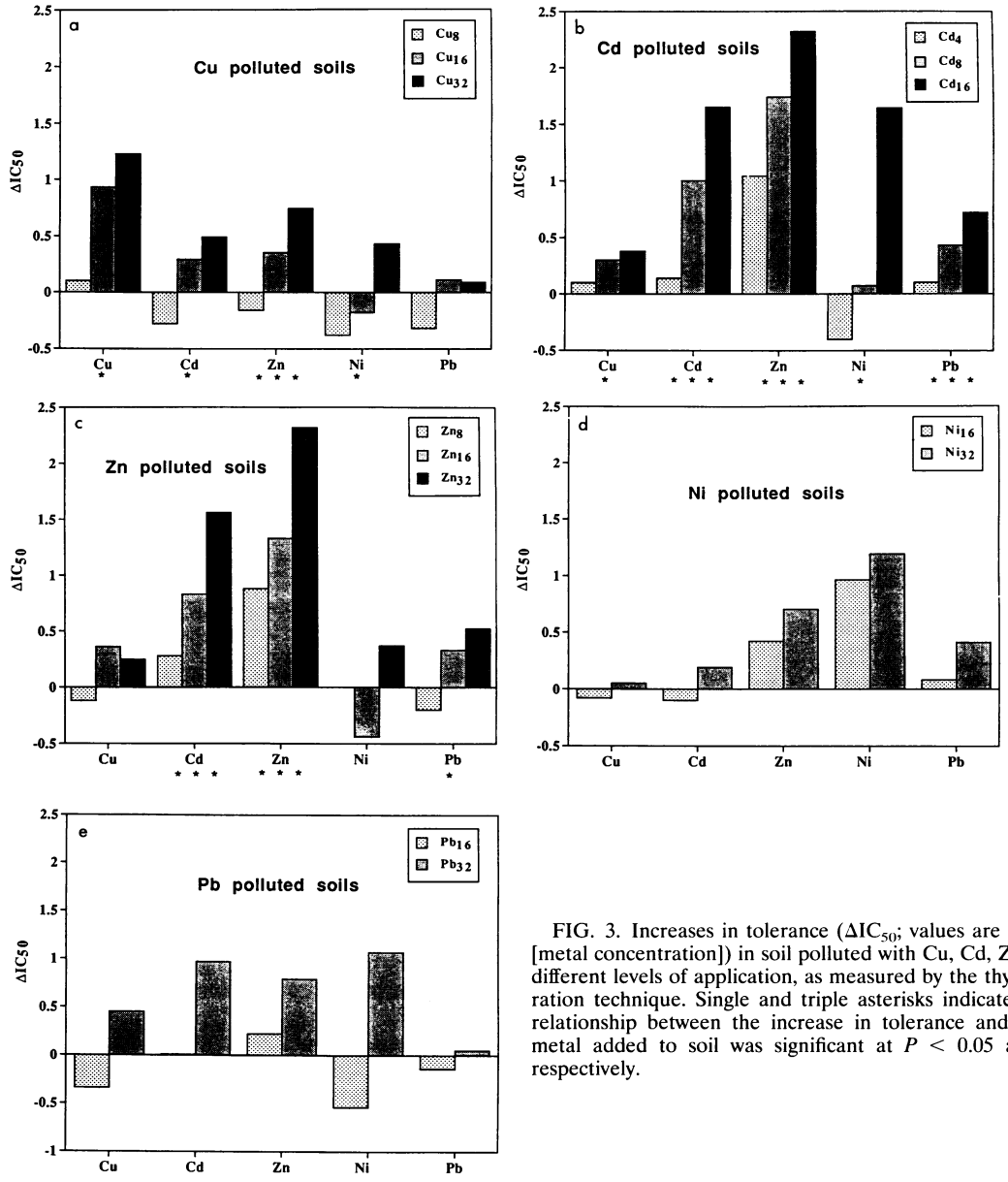

FIG. 3. Increases in tolerance (ΔIC_{50}; values are expressed as log [metal concentration]) in soil polluted with Cu, Cd, Zn, Ni, and Pb at different levels of application, as measured by the thymidine incorporation technique. Single and triple asterisks indicate that the linear relationship between the increase in tolerance and the amount of metal added to soil was significant at $P < 0.05$ and $P < 0.001$, respectively.

IC_{50} values (log metal concentration) showed that Cd (mean IC_{50}, -5.29; $n = 4$) was the most toxic metal for bacteria extracted from unpolluted soils, followed by Zn (IC_{50}, -4.36). Cu and Ni had similar values (IC_{50}, -3.83), while Pb was the least toxic metal (IC_{50}, -3.05) (Table 3). The greatest variations in IC_{50} values were found with Zn and Cd, and the smallest variations were found with Pb. The data also indicated that the variability of different measurements, as judged from the standard deviation data, was larger for IC_{50} values calculated by plate counts than for values obtained by the thymidine incorporation technique.

A change in the tolerance of bacteria to heavy metals was detected in polluted soils when they were compared with unpolluted soils by using plate counts (Table 3). Addition of Cu at the highest level (32 mmol kg^{-1}) increased the tolerance to all of the metals studied except Pb. Addition of Ni at the medium level (32 mmol kg^{-1}) increased tolerance to Ni and also, to lesser extents, tolerance to Cd and Cu. Contamination of soil with Cd and Zn at the highest levels (16 and 32 mmol kg^{-1}, respectively) produced similar effects (namely, considerable enhancement in the tolerance of bacteria to Cd and Zn and also an increase in tolerance to Cu and Pb). No change in tolerance to Ni was observed. Addition of Pb at the medium level (32 mmol kg^{-1}) increased the levels of tolerance to all of the metals studied; the smallest increase was for tolerance to Pb.

A comparison of the plate count results with the results obtained by using the thymidine incorporation technique showed that smaller increases in tolerance for the different polluted soils were often obtained by the plate count technique than by the thymidine incorporation technique. However, a positive relationship between the two measurements ($r = 0.732$, $P < 0.001$, $n = 24$) was found (Fig. 4). This indicated that the two methods were equivalent in terms of suitability for measuring tolerance.

TABLE 5. Correlation coefficients for changes in levels of bacterial tolerance to different heavy metals (ΔIC_{50} values) obtained for different polluted soils (n = 13) by using the thymidine incorporation technique

Heavy metal	Correlation coefficient				
	Cu	Cd	Zn	Ni	Pb
Cu	1.000				
Cd	0.284	1.000			
Zn	0.111	0.920[a]	1.000		
Ni	0.100	0.428	0.355	1.000	
Pb	0.225	0.807[a]	0.847[a]	0.544[b]	1.000

[a] Significant at $P < 0.001$.
[b] Significant at $P < 0.05$.

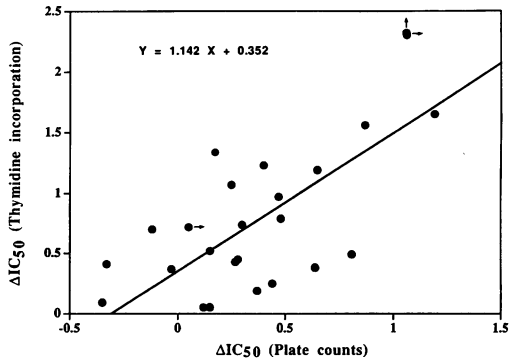

FIG. 4. Relationship between changes in bacterial community tolerance to different heavy metals (ΔIC_{50}; values are expressed as log [metal concentration]) as measured by the thymidine incorporation and plate count techniques. An arrow indicates that a value was underestimated, since the highest metal concentration used in the tolerance measurements did not result in 50% inhibition.

DISCUSSION

Increases in the levels of tolerance to metals added to soil were observed in bacterial communities obtained from Cu-, Cd-, Ni-, and Zn-polluted soils when these communities were compared with communities obtained from unpolluted soil (Fig. 3). This indicated that these metals provided selection pressure. In contrast, in soils contaminated with Pb no significant increase in the level of tolerance of bacteria to this metal was observed. Doelman and Haanstra (14) demonstrated that higher proportions of Pb-tolerant bacteria were found in Pb-containing soils than in soils containing low concentrations of Pb. However, these authors also noted that the selection of Pb-tolerant strains in a sandy soil containing 1,500 μg of Pb g^{-1} occurred only after 2 years. Thus, the 6-month incubation time which we used might be too short to detect Pb tolerance in Pb-polluted soils. Furthermore, tolerance to different metal may evolve at different rates. Other explanations for the results observed with Pb might involve problems that make it impossible to detect Pb tolerance by the thymidine incorporation technique. However, this seems improbable since Pb tolerance was detected in soils amended with Cd, Zn, and Ni (Fig. 3).

Increases in levels of tolerance to metals other than the metals originally added to the soil were found for the bacterial communities from polluted soils when these communities were compared with communities obtained from unpolluted soil, indicating that multiple metal tolerance had developed at the community level (Fig. 3). It is well known that bacteria can develop tolerance to two or several metals after exposure to only one metal (see, for example, the review by Doelman [13]). Enhanced tolerance of a community to a toxic compound suggests that selective pressure has been exerted by the compound in question (7, 8). However, if multiple tolerance is common, as shown in this study for metal tolerance, interpretation of field data will be difficult, since several pollutants are usually present simultaneously. Blanck and Wängberg (9) studied patterns of tolerance in marine periphyton communities established under arsenate stress with a technique similar to ours, which they called pollution-induced community tolerance. These authors found that multiple tolerance occurred but that the tolerance was only to toxic compounds that had very similar mechanisms underlying their toxicity.

We found correlations between the increases in levels of tolerance (ΔIC_{50}) to Cd and Zn (r = 0.920), Cd and Pb (r = 0.807), and Zn and Pb (r = 0.847) (Table 5). A positive correlation, which was significant at a lower level, for the ΔIC_{50} values of Ni and Pb (r = 0.544) was also observed. This may indicate either that there are similar tolerance mechanisms or that there is a common site of action for these metals, as discussed by Doelman (13). Our results support previous indications of resistance coupling between Zn and Cd, as well as between Zn and Pb, for bacteria living in contaminated sediments (22, 30). Bacteria that exhibit resistance to both Zn and Cd have also been isolated from severely Zn-contaminated soils (31). Resistance to both metals was found in the same plasmid and was lost by incorporation of a transposon at one site, indicating that there is a common resistance mechanism for the two metals. In contrast, our results showed that Cu tolerance was not significantly related to tolerance to any other metal tested. Thus, for example, contamination with Cu increased tolerance to Cu and Ni but not tolerance to Pb, while tolerance to Cu was enhanced by adding Pb but not by adding Ni (Fig. 3). This indicated that although pollution with different metals could select for Cu tolerance, tolerance to Cu did not automatically lead to resistance to the other metals.

Frostegård et al. (20) studied the effect of heavy metal pollution on the microbial community in the soil used in this study by performing a phospholipid fatty acid (PLFA) analysis. These authors found that addition of Cd, addition of Pb, addition of Zn, and addition of Ni induced similar changes in the PLFA pattern, while the effect of Cu addition on the PLFA pattern differed from the effects of the other metals. Likewise, they observed greater similarity in the changes induced by Cd, Pb, and Zn than in the changes induced by Ni. The bacterial communities in polluted soils appeared to be correlated in the same way according to their patterns of tolerance (Table 5). Changes in Cd, Pb, and Zn exhibited the highest correlation values, Ni tolerance was less correlated with these three

TABLE 6. Threshold soil metal concentrations for changes in levels of bacterial community tolerance estimated by using the thymidine incorporation technique

Soil pollutant	Threshold concn for tolerance (mmol kg^{-1} [dry wt])[a]				
	Cu	Cd	Zn	Ni	Pb
Cu	6	12	10	17	NT[b]
Cd	2	3	1	6	3
Zn	9	6	4	23	10
Ni	NT	20	6	1	13
Pb	24	16	13	19	NT

[a] The data were calculated by assuming that there was a linear relationship between ΔIC_{50} values (on a log scale) and log metal addition.
[b] NT, no tolerance detected.

metals, and no correlation between changes in Cu tolerance and tolerance to the other metals was found. Thus, there are indications that there is a relationship between changes in the community tolerance pattern and changes in the community species composition (as judged from the PLFA patterns) after metal contamination of soil.

Strong positive correlations between the amounts of Cu, Cd, and Zn added to soil and the increases in the levels of tolerance to the different metals tested were found (Fig. 3). This finding is consistent with the results of a study performed by Olson and Thornton (33), who showed that the metal resistance patterns of bacterial populations were related to the total concentrations of Cd, Zn, and Pb in soils from mine areas. Positive correlations between metal contents and the percentages of bacteria tolerant to heavy metals have also been observed with sediments and in aquatic environments (26, 39). The opposite result was observed by Dean-Ross and Mills (12) in a study of the responses of planktonic, sediment, and epilithic bacterial communities to heavy metal pollution. However, these authors suggested that the lack of correlation between metal concentration and resistance in the sediment bacterial community was due in part to the high pH of the river water, which reduced the toxic effect of the heavy metals present.

There is little information available concerning the metal concentrations required to cause changes in soil microbial community tolerance. As we observed a positive correlation between the increase in tolerance (ΔIC_{50}) and soil metal amendment, the threshold concentrations at which changes in tolerance occurred could be estimated (Table 6). These threshold concentrations, however, must be interpreted with caution, especially the concentrations of Ni and Pb, for which only two pollution levels were studied. Furthermore, the linear correlation between ΔIC_{50} and soil metal concentration does not necessarily hold at low pollution levels. Blanck and Wängberg (7) indicated this in their pollution-induced community tolerance study of aquatic systems. This means that the actual threshold values might be lower than the values calculated in this study. The lowest threshold concentrations were observed with Cd-polluted soils, and the highest values were observed with Pb-polluted soils, which is consistent with the finding that Cd is more toxic than Pb for microorganisms, as reported previously in numerous studies (1, 40).

Our data indicated that the bacterial community could withstand single metal additions of 6, 1, 4, 1, and 13 mmol of Cu, Cd, Zn, Ni, and Pb, respectively, per kg without showing a change in metal tolerance (Table 6). Frostegård et al. (20) found that the lower limits of heavy metal concentrations at which changes in PLFA patterns could be statistically detected in the same soil were 2, 1, 8, 2, and 8 mmol kg^{-1} (dry weight) for Cu, Cd, Zn, Ni, and Pb, respectively. These values are very similar to those calculated for tolerance thresholds, indicating that the two methods exhibit similar sensitivities for detecting the effects of heavy metal pollution. Moreover, since the PLFA pattern analysis was performed with a whole soil sample, while tolerance was measured by using a bacterial suspension that contained only 10 to 20% of the total bacteria in the soil, this similarity suggests that the bacteria extracted from soil are representative of the whole bacterial community. This supports the validity of the results of the thymidine incorporation technique, in which bacteria extracted from soil by homogenization and centrifugation are used in studies at a community level.

In general, the increases in tolerance observed by the plate count technique were lower than the increases measured by the thymidine incorporation technique (Fig. 4), indicating that the latter technique was more sensitive. These results are partially consistent with the results obtained by Jonas et al. (27), who showed that with water samples thymidine incorporation was a much more sensitive indicator of metal toxicity than bacterial viability was. However, since there was a linear relationship between the ΔIC_{50} values obtained by the two methods, the results also indicate that the susceptibility of the whole soil bacterial community (thymidine incorporation values) to heavy metals is similar to the susceptibility observed with culturable bacteria (plate count values). This makes it possible to compare results obtained by the two methods.

There were more problems associated with the plate count method than with the thymidine incorporation method. Since use of a large number of agar plates containing different amounts of heavy metals was necessary in order to enhance the sensitivity and reproducibility of the measurements, the plate count technique was the most time-consuming method. The variability in IC_{50} values was also larger (Table 3). This is not surprising considering that in total no more than 300 colonies were counted per dilution by the plate count technique, while the 1.8 ml of bacterial suspension used for the thymidine incorporation method contained more than 10^7 bacteria. Problems associated with the choice of growth media were also observed. The pH of the agar medium differed from the soil pH. Furthermore, and more importantly, variations in pH during incubation occurred, with increases of up to 2 pH units occurring as a consequence of bacterial growth during the 6-week incubation. It is well known that pH affects metal toxicity (4). Thus, the plate count data were more prone to the influence of a variable pH than the thymidine incorporation data were, since the latter involved only 2 h of incubation.

Another disadvantage of the plate count method was the long incubation time needed to obtain maximum counts. In polluted soils, particularly soils contaminated with Cu, Zn, and Cd, the appearance of colonies was delayed compared with unpolluted soil (Table 2). Thus, our data indicated that special care has to be taken when bacterial counts for polluted and unpolluted soils are compared, since the plates have to be incubated for a relatively long time. The differences observed in the appearance of colonies when polluted and unpolluted soils are used might reflect differences in the values of the parameters λ and t_r in the first-order reaction model of Hattori (25). The results also suggest that the kinetics of colony formation might be used to detect the effects of heavy metal pollution by using plate counts.

The thymidine incorporation method was simple and less laborious than the plate count method. Another advantage of this technique was the fact that measurements were made at the field pH, which remained constant during the analysis since the addition of metals to the bacterial suspension did not significantly change the original pH of the bacterial suspension. Furthermore, the thymidine incorporation method provides information about the response to heavy metals of a larger portion of the soil bacterial community than the culturable bacteria. Thus, the thymidine incorporation technique appears to be a more suitable method to detect changes in tolerance than the plate count technique. Therefore, we suggest that the thymidine technique should be used as a routine technique to study tolerance of soil bacterial communities.

Despite the fact that the thymidine incorporation rates and CFU values obtained for unpolluted soils differed at different sampling times (Table 2), similar IC_{50} values were observed for all metals tested (Tables 3 and 4). This indicated that no change in the sensitivity of the bacterial community extracted from unpolluted soil to heavy metals could be detected during the storage period by using either the thymidine incorporation

technique or the plate count technique. However, IC_{50} values do differ with different soil types (34) because of, for example, different soil pH values, organic matter contents, or bacterial community compositions. Thus, comparisons of community tolerance measurements by the thymidine incorporation technique have to be made by using the same soil type.

Differences in both IC_{50} values and the slopes of the dose-response curves were observed between polluted and unpolluted soils. The biological meaning of the differences in slopes is unclear. The data support the previous conclusions of Doelman and Haanstra (15, 16), however, who found that the slopes of the dose-response curves for Cd, Cr, Cu, Ni, and Zn for urease and phosphatase activities obtained in soils polluted with these metals differed from the slopes obtained in unpolluted soils. To overcome this problem, Doelman and Haanstra suggested the use of an ecological dose range (defined as the dose range in which activity decreases from 90 to 10% of the undisturbed activity) instead of the IC_{50} values used in ecotoxicological studies. In our study IC_{50} and IC_{10} values were closely related ($r = 0.909$, $P < 0.001$). Considering that there are fewer errors associated with the determination of IC_{50} values than with the determination of IC_{10} and IC_{90} values, tolerance changes based on IC_{50} values are probably preferable.

The levels of tolerance of the bacterial community to the different metals in soils polluted with low doses were in some cases even lower than the levels of tolerance in the unpolluted control (Fig. 3). One reason for this could be inaccuracies inherent in the method, such as overestimation and underestimation of the rate of thymidine incorporation in unpolluted and polluted soils, respectively. This is indicated by the fact that a decrease was found more often for Ni tolerance, for which the variation in the measurements was greatest (Table 3 and Fig. 3). However, it is also possible that addition of one metal increased the sensitivity to another metal. The presence of plasmids conferring increased tolerance to Cu and Ag and increased sensitivity to Zn isolated from an *Escherichia coli* strain obtained from the feces of pigs fed copper sulfate (38) indicates that this is possible at the species level. There is also evidence that increased resistance of *Klebsiella aerogenes* to Cd decreases resistance to Zn (35).

Qualitative as well as quantitative shifts in bacterial community structure after exposure to heavy metals have often been observed (1, 13, 17, 40). It is generally assumed that such changes lead to the establishment of a tolerant population. The results obtained in this study and those reported previously by Frostegård et al. (20) seem to support this assumption since (i) an increase in the tolerance of the bacterial community and changes in the PLFA pattern were found at similar metal concentrations and (ii) similar effects of heavy metal additions were deduced after the bacterial communities were grouped on the basis of the results of a PLFA pattern analysis and tolerance pattern measurements. However, such observations have been made in only one soil type so far. Further research should be carried out to determine whether these assumptions can be verified in a wider range of soils. We also know very little about the development of tolerance in soil ecosystems, and thymidine incorporation appears to be a useful method for assessing the effect of heavy metals on soil bacterial communities over time.

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