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Development of Metal Tolerance in Soil Bacterial Communities Exposed to Experimentally Increased Metal Levels

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The development of metal tolerance in soil bacterial communities exposed to different heavy metals was examined under laboratory conditions. An agricultural soil amended with different Zn concentrations was studied most intensively, and measurements were made over a 28-month incubation period by means of the thymidine incorporation technique. Tolerance levels were not affected by metal concentrations lower than 2 mmol of Zn kg (dry weight) of soil^{-1}, but above this value, the level of Zn tolerance increased exponentially with the logarithm of the soil Zn concentration. An increased metal tolerance was detected after only 2 days of Zn exposure. Thereafter, stable tolerance values were observed at different sampling times for bacterial communities exposed to up to 8 mmol of Zn kg (dry weight)$^{-1}$, indicating no changes in tolerance with time. The tolerance of bacterial communities exposed to 32 mmol of Zn kg (dry weight)$^{-1}$ increased rapidly within the second week of incubation, but then the values remained unchanged until the end of the experiment. Bacterial communities from soil contaminated with 16 mmol of Zn kg (dry weight)$^{-1}$ showed an increase of the same magnitude, but the increase started later, after 4 months of incubation, and took place for a much longer period (more than 1 year). Cd, Cu, and Ni addition also resulted in metal-tolerant communities, and the level of tolerance increased with prolonged incubations of the soils. The bacterial community at the end of the incubation period also exhibited a lower pH optimum and an increased tolerance to low osmotic potential. The results suggest that the increase in metal tolerance of the community after adding metals can be attributed to an immediate effect due to the death of sensitive species and a later effect due to different competitive abilities and adaptation of surviving bacteria.

The effects of metal pollution on soil microorganisms have been studied extensively under field and laboratory conditions (3, 12, 21). Usually, measurements are made only once in such ecotoxicological studies. However, several studies have shown that when repeated measurements are made, different results concerning the toxicity can be found (13, 18, 19, 31, 33, 36, 37). Although an influence of time on the effect of metal exposure can thus be expected, it has seldom been taken into account.

Most of the investigations cited above have focused on the estimation of numbers, biomass, or activity of soil organisms. It is well known that these variables can be influenced by different environmental factors, such as temperature and water content, and not only by heavy metals. In addition, altered activity in polluted habitats can be caused by factors altered by the presence of metals, for example, changes in pH or nutrient availability. Information obtained from metal impact studies can therefore be difficult to evaluate because of the problems of separating the effect of metal toxicity from that of other environmental factors.

A more direct method of studying the effect of heavy metal pollution on microorganisms might be to estimate the number of tolerant microorganisms. It has been shown that bacteria and fungi isolated from polluted environments are frequently tolerant of higher levels of metals than those isolated from unpolluted areas and that tolerant microorganisms are found at high frequencies in polluted habitats (1, 3, 17, 20, 28, 32). However, information about the time scale of development of a tolerant community is scarce. Yamamoto et al. (39) investigated the sensitivity to Cu of the soil fungal flora, showing that selection for Cu tolerance may proceed rapidly. The same observation was made for forest soil bacteria in response to Zn stress (29). In contrast, Doelman and Haanstra (18) studied the effects of Pb on soil bacteria and found that selection for Pb tolerance took 2 years.

In aquatic habitats, increased community tolerance levels of periphyton has been used as a simple way of assessing the ecological impact of several pollutants (10, 11, 34). In soil, the ratio of metal-tolerant to metal-sensitive bacteria (determined by plate counts) has been suggested as a sensitive and relevant measure of community tolerance to metals (20, 28). Recently, the thymidine and leucine incorporation techniques, originally used for estimating growth rates of bacterial cells, were modified to study tolerance of soil bacterial communities (5, 14, 16). With this technique, bacteria are extracted from soil by homogenization-centrifugation and challenged with different metal concentrations before thymidine or leucine incorporation is measured. The degree of inhibition can then be used as an estimate of the bacterial community tolerance. This technique was shown to be faster and less variable than plate count techniques. By using thymidine incorporation, soil bacterial communities artificially exposed to Cu, Cd, Zn, or Ni during a 5- to 8-month incubation period were shown to have an increased metal tolerance compared with those in the corresponding unpolluted soil (16). This technique has also been used to study the impact of heavy metals in field sites (35).

Three different hypotheses have been put forward to explain the effect of time on heavy metal toxicity and the microbial community tolerance. A decreased toxicity, and presumable metal tolerance of the community, over time was reported for

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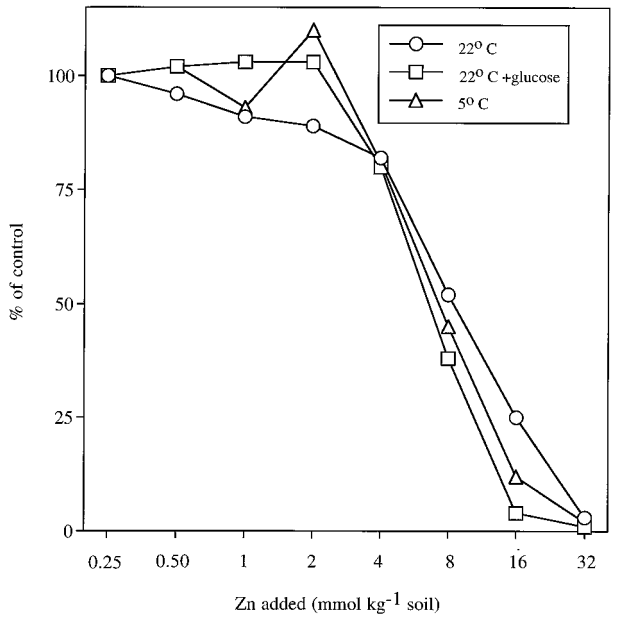

FIG. 1. Thymidine incorporation into cold-TCA-insoluble material of bacteria extracted from an agricultural soil after 1 day of exposure to 0.25 to 32 mmol of Zn kg (dry weight) of soil^{-1}. For each soil treatment, the values were expressed as percentages of the uncontaminated control (with no metal added).

respiration measurements in soils contaminated with different metals (19). This could be due to the added metals becoming less available over time as a result of binding to organic matter. On the other hand, Frostegård et al. (24) studied changes in the microbial community structure in two different soils following Zn addition as revealed by the analysis of the phospholipid fatty acid patterns. They found that during the incubation of the soils under laboratory conditions, gradual changes over time occurred in the microbial community structure of the polluted soil as compared with that of the unpolluted soil, suggesting that the microbial population had become more tolerant over time. The third hypothesis is that the addition of the metals would immediately kill sensitive organisms, thus leaving tolerant ones. This would result in an immediate increase in the tolerance of the microbial community, which would be correlated to the number of surviving organisms.

In the present investigation, the validity of these different hypotheses concerning the development of metal toxicity was examined. By using experimentally polluted soil, the development of metal tolerance in soil bacterial communities in the presence of Zn was monitored by means of the thymidine incorporation technique. Four other metals were also studied, but with less frequent measurements. Furthermore, by use of a range of metal concentrations, the relationship between the level of bacterial community tolerance and the level of metal addition was determined.

MATERIALS AND METHODS

Experimental design. An agricultural sandy loam soil containing 4.4% organic matter and with a pH of 7.8 was used. The soil, previously sieved (<2-mm pore size), was artificially contaminated in the laboratory with solutions of five different metals, Zn as $ZnSO_4$ [0.25, 0.5, 1, 2, 4, 8, 16, and 32 mmol kg (dry weight) of soil^{-1}, Cd as $CdSO_4$ (8 and 16 mmol kg^{-1}), Cu as $CuSO_4$ (16 and 32 mmol kg^{-1}), Ni as $Ni(NO_3)_2$ (32 and 64 mmol kg^{-1}), and Pb as $Pb(NO_3)_2$ (32 and 64 mmol kg^{-1}). The metal solutions were thoroughly mixed into the soil to ensure homogeneous distribution of the metals. Soil samples without metal additions were used as controls. The contaminated soil samples and the control (100 g [dry weight] per replicate) were kept in plastic beakers at room temperature (approximately 22°C). Distilled water was added regularly to maintain the soil moisture at a constant level (about 75% of water-holding capacity). Between one and three replicates of each treatment were used.

Three experiments with different incubation periods (short-, medium-, and long-term incubations) were conducted. In the long-term experiment, soils were contaminated with two doses of the five different metals (16 and 32 mmol kg^{-1} for Zn, Cu, Ni, and Pb and 8 and 16 mmol kg^{-1} for Cd). Measurements of tolerance to the metals present in the soils were made after 7, 14, and 28 months of incubation (only Zn and Cd were measured at 28 months). In addition, bacterial communities exposed to 32 mmol of Zn kg^{-1} for 28 months were also examined for tolerance of different pH values and NaCl concentrations (see below).

In the short- and medium-term experiments, the development of metal tolerance was studied in soil bacterial communities exposed to eight to nine different concentrations in the range of 0 to 32 mmol of Zn kg (dry weight)$^{-1}$. In the medium-term experiment, tolerance measurements were made for all soil treatments at different times between 8 and 128 days. In the short-term incubation experiment, the effects of metals were studied for up to 16 days. Tolerance was measured only in soils contaminated with 8, 16, and 32 mmol of Zn kg (dry weight)$^{-1}$, but bacterial activity (thymidine incorporation) following the application of different Zn concentrations (0 to 32 mmol of Zn kg^{-1}) was also examined. The growth rate of the bacterial community was altered in three ways in this experiment. A low-temperature incubation (5°C) and the addition of a labile substrate (glucose) were used to obtain bacterial communities with low and high growth rates, respectively. In the glucose treatments, glucose (1 mg of C g [dry weight]$^{-1}$) was added initially and after 8 days to each soil metal treatment (0 to 32 mmol of Zn kg^{-1}).

Extraction of bacteria and tolerance measurements. Bacteria were extracted from soil by a homogenization-centrifugation method (4) resulting in a bacterial solution with approximately 10 to 20% of the total number of bacteria counted in situ. It was assumed that the metal tolerance of the extracted bacteria was representative of the metal tolerance of the whole bacterial community. Metal tolerance of soil bacterial communities to different metals present in the soil was determined by measuring metal toxicity by means of the thymidine incorporation technique (5, 16). The bacterial solution was poured in 1.8-ml quantities into plastic vials and mixed with 0.2 ml of distilled water (control) or different metal solutions with a range of concentrations (1×10^{-8} to 2×10^{-3} M final concentration) giving no inhibition to total inhibition of the incorporation rate. Heavy metal solutions were prepared with the metal salts mentioned above. Bacterial solutions with metals were incubated with 100 nM [*methyl*-^{3}H]thymidine (925 GBq/mmol; Amersham) at the temperature of the incubated soils (5 or 22°C for 10 and 2 h, respectively) and then the incorporation was stopped by adding 1 ml of 5% formalin. The length of incubation was selected to give a similar incorporation rate per milliliter of bacterial solution at both temperatures. Filtration on glass fiber filters, washing of the filters (with ice-cold 80% ethanol and 5% trichloroacetic acid [TCA]), solubilization of macromolecules (0.1 M NaOH at 90°C for 1.5 h), and scintillation counting were as described previously (4).

The responses of soil bacterial communities to pH and NaCl were determined in a similar way to the metal tolerance measurements. The pH of the bacterial suspension was adjusted with potassium phosphate buffer (6.6 mM final concentration) to values of 5.5, 6.2, 7.2, and 8.2. In the NaCl tolerance test, six different NaCl concentrations ranging from 0.25 to 3.5% (final concentration) were added to the bacterial suspension. For each test, two controls with distilled water were prepared. The suspensions thus obtained were then incubated at room temperature with the labelled thymidine for 2 h. Incorporation of thymidine into cold-TCA-insoluble material was measured by the same procedure as that described above.

Statistical analysis. Nonlinear regression analysis was performed with the statistical program STATGRAPHICS to fit the incorporation data obtained for the heavy metal toxicity assays to the logistic model (38) defined by the equation $Y = c/[1 + e^{b(X - a)}]$, where Y is the observed level of thymidine incorporation, c is the growth rate in the control with distilled water, b is a slope parameter indicating the inhibition rate, and X is the logarithm of the metal concentration in the bacterial solution at which the thymidine incorporation was one-half of the control value (50% inhibitory concentration [IC_{50}]). The IC_{50} values were used as an estimate of the bacterial community tolerance. Changes in the bacterial community tolerance level were estimated from the difference between IC_{50} values in polluted and unpolluted soils ($\Delta IC_{50} = IC_{50}$ polluted $- IC_{50}$ unpolluted). For the pH response and NaCl toxicity assays, the percentage activity was calculated in reference to the distilled water control.

RESULTS

A clear dose-response relationship between the level of Zn addition and bacterial activity measured as thymidine incorporation was detected 1 day after adding the metal (Fig. 1). The thymidine incorporation was similar to that of the control in the soils contaminated with metal concentrations of up to 2 mmol of Zn kg (dry weight) of soil^{-1}, but a decrease was found at higher metal levels. This initial reduction was followed by a

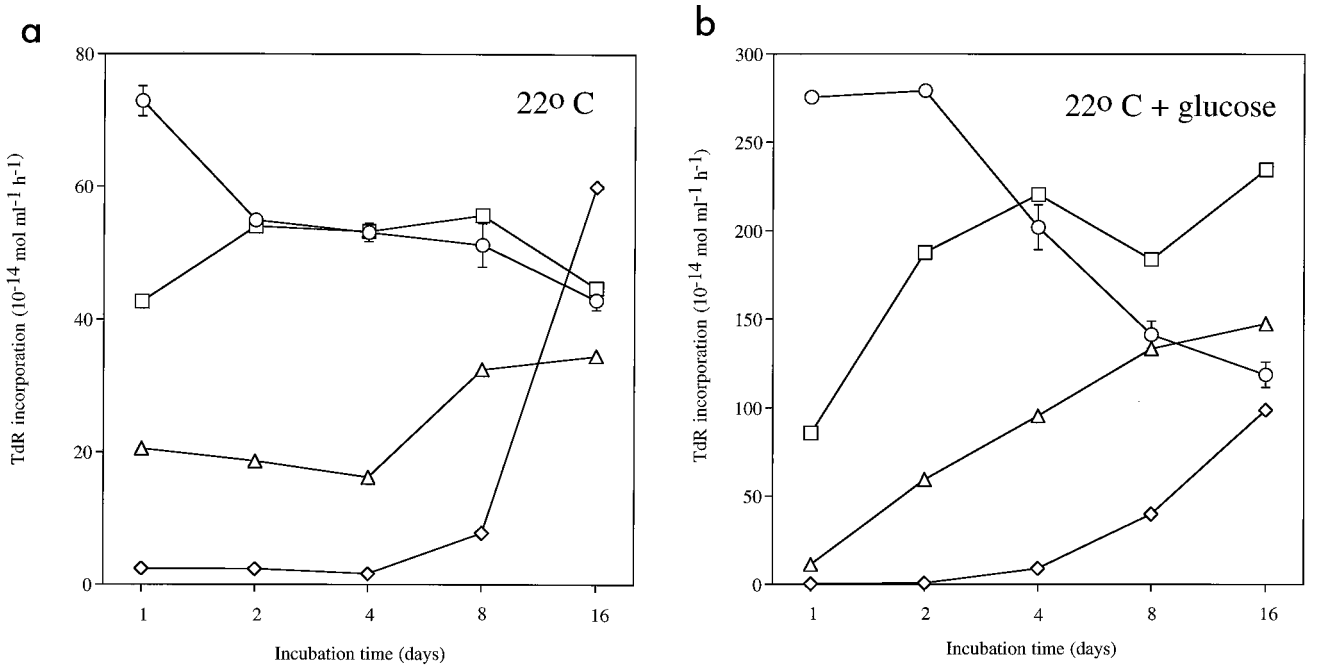

FIG. 2. Thymidine (TdR) incorporation into cold-TCA-insoluble material of bacteria extracted from an agricultural soil contaminated with 0 to 32 mmol of Zn kg (dry weight) of soil^{-1} over a 16-day incubation period. (a) Normal growth rate with soil incubated at room temperature (22°C); (b) high growth rate with glucose-amended soil incubated at room temperature; (c) low growth rate with soil incubated at 5°C. Symbols: ○, unpolluted soil and soil polluted with 0.5, 1, 2, and 4 mmol of Zn kg^{-1} (mean ± SE [n = 5]; bars indicating SE are shown only when larger than the symbol); □, soil polluted with 8 mmol of Zn kg^{-1}; △, soil polluted with 16 mmol of Zn kg^{-1}; ◇, soil polluted with 32 mmol of Zn kg^{-1}. For the three latter treatments, only single replicates were measured.

recovery of bacterial activity levels in all soil metal treatments, reaching values similar to that in the unpolluted control soil 16 days after the addition of metals (Fig. 2a). Thus, at this time, no dose-response effect of metal pollution could be detected on the basis of the thymidine incorporation values, since they were not correlated with the soil metal amendments. The highest value was actually found in the most polluted soil.

Zn tolerance of the bacterial community was not measured the first day after metal application, but an increased tolerance to Zn was observed 2 days after the metal addition (Fig. 3). Stable ΔIC_{50} values were then obtained at the different sampling times in soils that received 8 and 16 mmol of Zn kg (dry weight) of soil^{-1}, indicating no changes in community tolerance of Zn over time during the 16 days of incubation. In contrast, for bacteria in soils that received 32 mmol of Zn kg (dry weight)$^{-1}$, an increase in tolerance over time was observed (Fig. 3).

Glucose addition and the low-temperature treatments, respectively, increased and decreased the thymidine incorporation rate of the soil bacterial communities by a factor of about 3 (Fig. 2b and c). Despite this difference in activity, the initial inhibition of thymidine incorporation by the Zn amendments was the same as that observed for the unamended soil incubated at room temperature (Fig. 1). The recovery, over time, of the thymidine incorporation in the glucose-treated soils with metals was similar to that of the corresponding unamended soil incubated at room temperature (Fig. 2a and b). In contrast, bacterial activity in the metal-treated soils incubated at low temperature recovered slowly, and reductions of 22, 78, and 96% (with respect to that of the corresponding unamended control) were still detected after 16 days of incubation in soils contaminated with 8, 16, and 32 mmol of Zn kg^{-1}, respectively (Fig. 2c).

The development of tolerance over time for bacteria in soils that received 8 and 16 mmol of Zn kg^{-1} did not differ between the treatments with different growth rates (Fig. 3). At the highest Zn concentration (32 mmol of Zn kg [dry weight]$^{-1}$), however, the development of the bacterial Zn tolerance between 2 and 16 days was slower for the low-temperature treatment than for the other two treatments. Tolerance measurements were also made after 64 and 128 days of incubation. No differences in tolerance between bacteria showing different

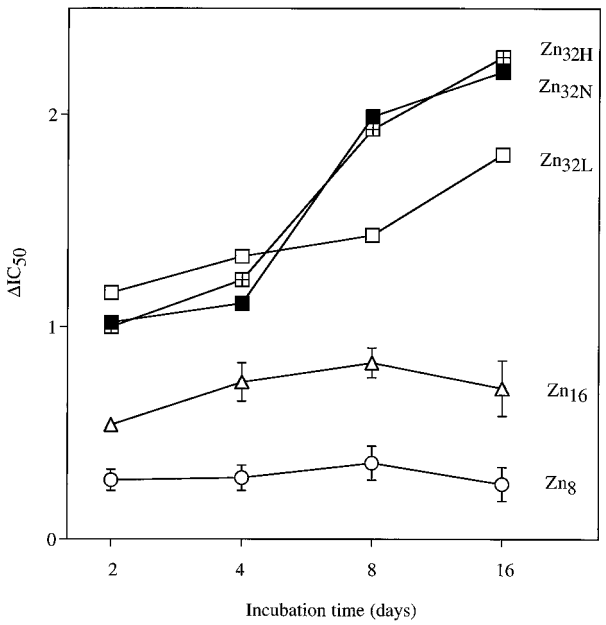

FIG. 3. Development of tolerance over time for bacterial communities from soil contaminated with 8, 16, and 32 mmol of Zn kg (dry weight) of soil^{-1} (Zn_8, Zn_{16}, and Zn_{32}, respectively). N, normal growth rate with soil incubated at room temperature (22°C); H, high growth rate with glucose-amended soil incubated at room temperature; L, low growth rate with soil incubated at 5°C. Data for Zn_8 and Zn_{16} are means of the three different treatments (N, H, and L) ± SE, since they did not differ significantly. Bars indicating SE are shown only when larger than the symbol.

growth rates were observed for the metal treatments tested (8, 16, and 32 mmol of Zn kg [dry weight]$^{-1}$) (data not shown).

When combining the results of the three laboratory experiments (short-, medium-, and long-term incubations), the observed time course of development of metal tolerance was different for bacterial communities exposed to 4, 8, 16, and 32 mmol of Zn kg^{-1} (Fig. 4). Initial tolerance increases were already detected after 2 days of incubation for bacteria in all soil treatments (ΔIC_{50} = 0.20, 0.5, and 1.0 for soils that received 8, 16, and 32 mmol of Zn kg^{-1}, respectively). After this time, the tolerance stabilized at a constant value in soils amended with 4 and 8 mmol of Zn kg^{-1}. In contrast, with the two highest metal concentrations, bacterial tolerance increased during the incubation. For bacteria in the soil that received 16 mmol of Zn kg (dry weight)$^{-1}$, the tolerance remained unchanged for 4 months and then a gradual increase in the IC_{50} value of 1 logarithmic unit took place between 4 and 28 months of incubation. In the case of bacterial communities in soil that received 32 mmol of Zn kg (dry weight)$^{-1}$, a rapid increase in tolerance of around 1 logarithmic unit occurred within the second week of incubation and then the values remained unchanged until the end of the experiment.

Measurements with incubation times longer than 4 months were not replicated. However, there were no changes over the total incubation period in the parameters describing the dose-response curve for the unamended soils. Average values (means ± standard errors [SE]) for IC_{50} and the slope were −4.83 ± 0.02 and 3.71 ± 0.21, respectively (n = 12). This showed that the measurements were reproducible and that the increase in ΔIC_{50} values after 4 months of incubation with 16 mmol of Zn kg^{-1} was much larger than the normal variation.

An increased bacterial tolerance over time was also indicated for other metals, although they were not studied as extensively as Zn. Table 1 shows the results of tolerance mea-

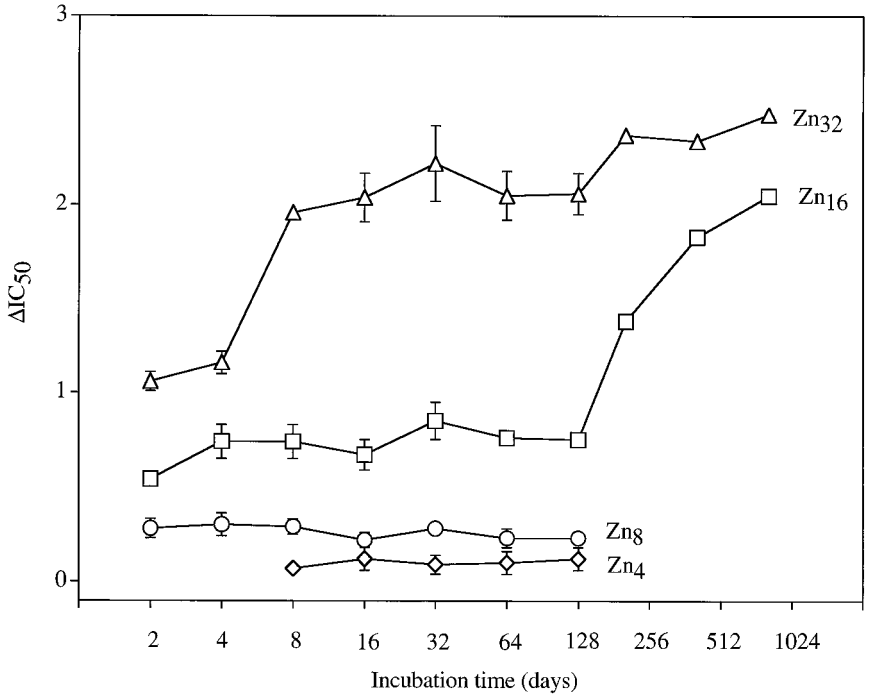

FIG. 4. Development of tolerance over time for bacterial communities from soil contaminated with 4, 8, 16, and 32 mmol of Zn kg (dry weight) of soil^{-1} (Zn_4, Zn_8, Zn_{16}, and Zn_{32}, respectively) (mean ± SE; n = 2 to 5) and incubated at 22°C. Bars indicating SE are shown only when larger than the symbol. For the last three sampling times, only single measurements were made.

TABLE 1. Heavy metal tolerance of soil bacterial communities extracted from unpolluted and metal-polluted soils

Soil treatment[a]	Log metal concn after incubation for:			
	7 mo		14 mo	
	IC_{50}	ΔIC_{50}	IC_{50}	ΔIC_{50}
Unpolluted	−6.42	0	−6.65	0
Cu_{16}	−5.55	0.87	−5.33	1.32
Cu_{32}	−5.25	1.17	−5.13	1.52
Unpolluted	−5.28	0	−5.51	0
Cd_{8}	−4.32	0.96	−4.38	1.13
Cd_{16}	−3.67	1.65	−3.37	2.14
Unpolluted	−4.13	0	−4.62	0
Ni_{16}	−3.27	0.85	−2.43	2.19
Ni_{32}	BDL[b]		−2.56	2.06
Unpolluted	−4.86	0	−4.67	0
Pb_{16}	−4.56	0.30	−4.46	0.21
Pb_{32}	BDL		−3.84	0.83

[a] Subscripts after metal symbols indicate the level of metal addition in millimoles per kilogram (dry weight) of soil.

[b] BDL, below detection limit (thymidine incorporation was below the detection limit, and thus IC_{50} values could not be estimated).

surements of bacterial communities obtained in soils contaminated with Cu, Cd, Ni, or Pb at two different concentrations and incubated for 7 and 14 months. An increased tolerance was found after 14 months of incubation compared with 7 months for all metals except Pb. In the case of the treatment with 16 mmol of Cd kg^{-1}, this tolerance continued to develop, and after 28 months, the ΔIC_{50} was 2.93. For the highest level of Cd and Zn addition, the extent of multiple tolerance was also estimated after 28 months by use of a single replicate. The ΔIC_{50} for Zn tolerance in the Cd-treated soil was 2.49, and that for Cd tolerance in the Zn-treated soil was 1.59. For the Pb and Ni amendment, an increased tolerance could be detected only at the low level of metal addition since no thymidine incorporation could be measured after 7 months of incubation at the high amendment rate (Table 1).

No significant differences were observed between IC_{50} values for bacterial communities exposed to the different Zn concentrations between 8 and 128 days. The mean tolerance changes with respect to the unamended control soil (ΔIC_{50}) were therefore calculated for each soil metal treatment and plotted against the logarithm of the added metal concentration (Fig. 5). Increases in the bacterial community tolerance level were observed only at concentrations above 1 mmol of Zn kg^{-1}. Increasing the level of metal amendment increased the bacterial community tolerance (ΔIC_{50}) exponentially (Fig. 5). The slope variable from the logistic equation used to model the inhibition curves for the heavy metal toxicity assays was affected little by the soil metal amendment (data not shown).

The bacterial community tolerance to increased ionic strength of the soil solution was tested with NaCl as the osmolyte. An inhibitory effect of high levels of NaCl was observed on thymidine incorporation in bacterial communities of both soils (Fig. 6). However, the inhibition was higher for the unamended soil for all NaCl concentrations compared with that of the Zn-treated soil. This indicated that the bacterial community extracted from the metal-amended soil was more tolerant of high ionic strength than that in the corresponding unamended soil.

Differences between bacterial communities from these soils were also observed on the basis of their pH response (Fig. 7). Higher incorporation values at pH 5.5 and 6.2 and lower values at pH 7.2 and 8.2 were observed in Zn-amended soil than in the unamended one, indicating that the optimum pH value for thymidine incorporation was lower in the former treatment.

DISCUSSION

An increased metal tolerance was detected in the soil bacteria community 2 days after the addition of Zn, and the increase was greater at the highest concentrations of metal application (Fig. 4 and 5). An initial reduction in thymidine incorporation rate (indicating bacterial activity) was also observed after the addition of different Zn concentrations to soil (Fig. 1). The extent of the reduction was correlated with the metal level and was not affected by altering the bacterial activities (by changing the incubation temperature or by adding glucose). Assuming that the initial decrease in thymidine incorporation was due to metal toxicity, the initial tolerance increase could thus be attributed to the inhibition and death of sensitive species and survival of Zn-tolerant bacteria already present in the soil. It should be noted that even a small percentage of surviving bacteria, as occurred in the case of the high metal amendments, was sufficient to allow measurements of the increase in the bacterial tolerance levels.

After the initial tolerance increase, no further changes in the levels of tolerance of soil bacteria to Zn were detected during the incubation of the soil that received 8 mmol of Zn kg^{-1} or less, while an increase in tolerance took place within the first 2 weeks in bacteria in soils contaminated with 32 mmol of Zn kg^{-1} (Fig. 3). This tolerance increase could be due to different competitive abilities of the surviving bacteria, the most tolerant ones outcompeting the less tolerant ones. If this development of an increased tolerance was due to competition, one would expect the development to be slower at lower turnover rates (activities) of the bacterial community, unlike the effect of direct toxicity. The fact that bacteria showing a high growth rate appeared to became tolerant more rapidly than those showing a low growth rate (due to low temperature) (Fig. 3) therefore appears to support the hypothesis about competi-

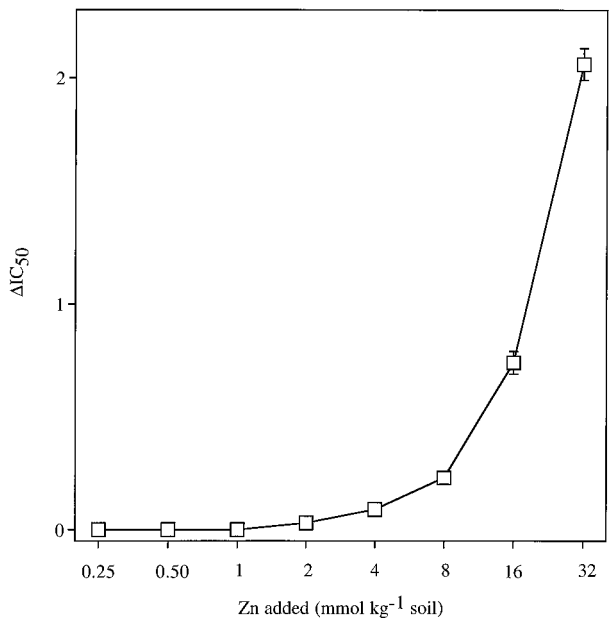

FIG. 5. Relationship between Zn added to soil and changes in tolerance levels (ΔIC_{50}). ΔIC_{50} values are expressed as log metal concentrations and are means ± SE. Bars indicating SE are shown only when larger than the symbol.

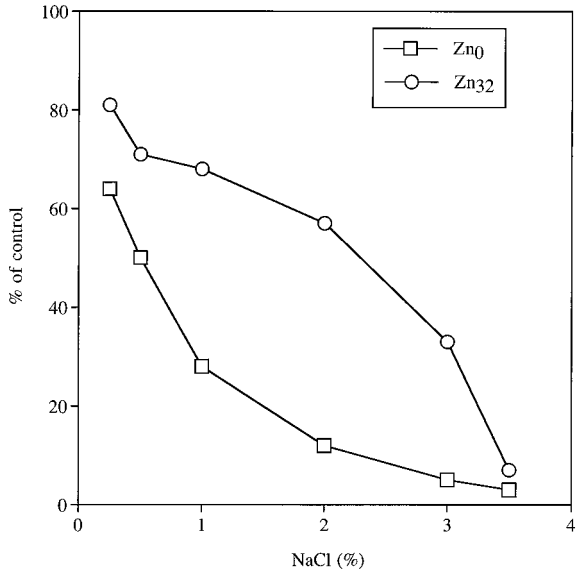

FIG. 6. Effect of different NaCl concentrations on thymidine incorporation into cold-TCA-insoluble material of bacteria extracted from uncontaminated soil (Zn_0) and soil contaminated with 32 mmol of Zn kg (dry weight) of $soil^{-1}$ (Zn_{32}). Values are expressed as percentages of the control (distilled water added).

tion. There were no differences between the glucose-amended and nonamended soils with respect to development of tolerance, although the former treatment was supposed to have a higher turnover rate. However, since the metal addition killed such large numbers of microorganisms, resulting in an input of carbon to the surviving bacteria in both glucose-amended and nonamended treatments, there was not as much difference in activity between these two treatments at the highest metal level as there was in the nonamended control.

A gradual increase in the levels of tolerance of soil bacteria occurred much later, between 4 and 14 months of incubation, in the soil that received 16 mmol of Zn kg^{-1} (Fig. 4). Although only single measurements from one replicate were made, the large increase in tolerance (more than 1 logarithmic unit) was much greater than the normal variation in IC_{50} measurements, indicating that the increase in tolerance was real. Since it took 4 months before this increase in tolerance started, it is unlikely that this was due to competition between phenotypes existing in the soil directly after the metal amendment. This late, gradual tolerance increase could instead be due to physiological or genetic adaptation in this treatment, where the adapted microorganisms then outcompeted the formerly dominant microorganisms. This development of tolerance was a slow process, which could be due to the activity and thus the turnover rate of the microbial community being very low after 4 months of incubation. A similar increase in tolerance (more than 1 logarithmic unit) was also found for the soil polluted with 16 mmol of Cd kg^{-1} between 7 and 28 months of incubation.

Three different mechanisms are therefore suggested as causes of the increased metal tolerance observed for soil bacterial communities in Zn-polluted soils: (i) an immediate, toxic effect killing sensitive species; (ii) a selection for Zn tolerance due to the different competitive abilities of surviving bacteria; and (iii) adaptation of bacteria developing in these polluted soils due to physiological and/or genetic changes. The predominance of one or another mechanism might depend on the level of pollution. In general, the initial killing effect was found to be the dominant factor in increasing the metal tolerance, particularly with bacteria in soils that received low metal concentrations. At higher soil metal concentrations, competition and adaptation also became important later. This was also suggested by Duxbury and Bicknell (22), who suggested that at low levels of pollution, phenotypic selection probably accounts for a greater proportion of tolerant bacteria in soils, whereas genetic selection could become more important in heavily polluted soils.

The metal concentrations in the bacterial suspensions were not measured. Thus, one possible confounding factor from use of the thymidine incorporation technique to measure metal tolerance could be that metals were extracted from the soil by the bacterial extraction method and that this affected the tolerance measurements. For example, if the amount of extractable metals would decrease over time of incubation, this could result in an apparent increase in metal tolerance of the bacterial community. However, washing of the extracted bacteria to remove extracted metals did not alter the measured IC_{50} values (16), indicating that extracted metals did not affect the tolerance measurements. Furthermore, if the amount of extractable metals would change over time, it is likely that this change would be similar in all metal treatments. Such a change could therefore not explain that the development of tolerance differed between different treatments (Fig. 4).

The presence of one metal not only increases bacterial community tolerance of that specific metal but also can affect tolerance of other metals (16). The development of such multiple tolerance was not specifically studied here. However, for the highest metal amendment levels of Zn and Cd, the tolerance to the other (nonamended) metal was also measured after 28 months of incubation. The results can be compared with earlier results with the same soil type incubated for around 7 months (16). In the case of the Zn-polluted soil, only small differences in ΔIC_{50} values were found between the two incubation times, irrespective of whether Cd or Zn tolerance was considered. However, in the case of the Cd-polluted soil, Cd tolerance had increased by more than 1 logarithmic unit, while Zn tolerance was only affected to a minor degree. Thus, after

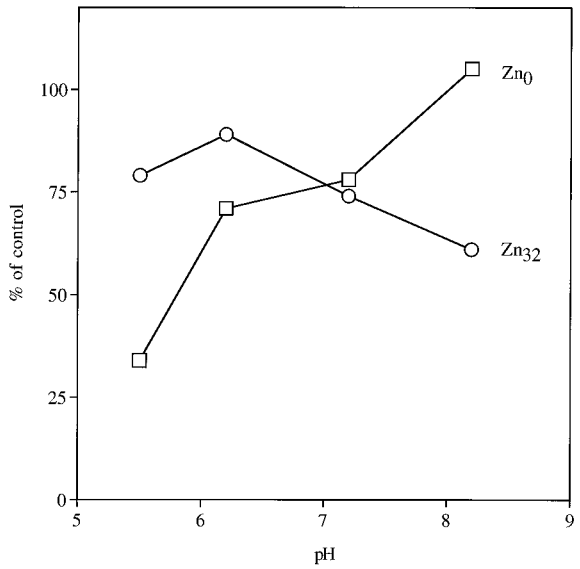

FIG. 7. Effect of different pH values on thymidine incorporation into cold-TCA-insoluble material of bacteria extracted from the uncontaminated soil (Zn_0) and soil contaminated with 32 mmol of Zn kg (dry weight) of $soil^{-1}$ (Zn_{32}). Values are expressed as percentages of the control (distilled water added).

7 months of incubation, it was not possible from the tolerance measurements to deduce whether the Cd-polluted soil was actually polluted with Zn or Cd, since tolerance of both metals had increased to the same degrees (16). However, it was clear after 28 months of incubation that Cd was actually the metal exerting the selective pressure. It therefore appears as if the development of tolerance for the toxic metal might be faster than the development of multiple tolerance of metals not present, at least in the case of Cd. If this is true for other metals, the presence of multiple tolerance might be less of a problem when interpreting field data than previously thought.

Two different effects of the metal addition on the bacterial activity could be detected, an initial inhibitory effect probably caused by the death of the Zn-sensitive bacteria due to metal toxicity and a stimulatory effect on the growth rate of the surviving, tolerant bacteria due to an increased substrate availability, where the new substrate was derived from dead cells (Fig. 2a). This means that the effect of the metal addition differed with the time of incubation. Initially, the thymidine incorporation data fitted well with a logistic dose-effect curve (Fig. 1), suggesting that activity measurements shortly after metal addition could be useful in identifying pollution effects. Because of the carbon input from dead cells, this adverse effect disappeared, and bacterial activity levels in polluted treatments approached that in the unpolluted soil after 16 days of incubation (Fig. 2a). At this time, the bacterial activity could thus not be used to indicate metal toxicity because of the confounding effect of nutrient levels. Similar findings have been reported for the same soil in a detailed study of bacterial activities following the addition of Zn, Cu, Cd, Ni, or Pb in two doses during an incubation time of 14 months and measured by the thymidine and leucine incorporation techniques (15). The overall activity of soil bacteria in polluted soils will therefore depend on the relative importance of these two opposite effects, which can confound attempts to estimate the effects of heavy metal pollution. This can also be true for other general measurements of activity and biomass of soil microorganisms and may explain the contradictory results observed by different authors concerning the medium-term effects of heavy metal pollution on soil microorganisms (2, 26, 27, 31). However, an increased bacterial tolerance to the metal present in the soil could be detected throughout the entire incubation period (Fig. 4), suggesting that the level of tolerance of the bacterial community could be successfully used for detecting metal pollution independently of the time exposure. This agrees well with recent studies using plate counts showing that the fraction of metal-tolerant bacteria can be used as a sensitive measure of the metal impact (20, 28).

Doelman and Haanstra (19) reported that the toxic effects of different metals on the microbial respiration rate in different soils generally appeared to decrease during long-term storage of the soil. The same observation was reported by Frostegård et al. (24) in a study concerning the effect of Zn on both ATP and respiration rate. Haanstra et al. (25) interpreted a similar result found during storage of a Ni-polluted soil as the soil providing increasing protection against the toxic effects of metal. However, our tolerance measurements indicated the opposite long-term effect, since little change or increased tolerance was observed over time, suggesting no change or even an increase in metal toxicity during incubation of the soils (Fig. 3 and 4). We believe that the earlier interpretation of a decreasing metal toxicity with length of time of incubation is due to the problems of separating different factors affecting the measurements and not necessarily due to the fact that metals become unavailable. Traditional measurements of biomass and activity (e.g., ATP and respiration) are affected not only by the metals but also by the carbon availability in the soil. During long-term incubation of soils, easily decomposable material will be used up, resulting in a decreased microbial biomass and activity over time. This effect is likely to develop more quickly in the uncontaminated soils than in polluted soils where microbial activity is depressed. Thus, the microbial biomass and activity will decrease more quickly in unpolluted soils than in metal-polluted soils since the former soils will become more deprived of easily available carbon. The difference in activity between uncontaminated and contaminated treatments will therefore decrease over time, which will look like a decreasing metal toxicity over time. Kirchbaum (30) discussed a similar problem when measuring the effect of temperature on soil respiration rate. Exhaustion of easily decomposable material would proceed at higher rates at higher temperatures than at lower temperatures, resulting in an underestimation of the temperature sensitivity of the respiration rate during prolonged storage of the soil.

A significant correlation was found between changes in IC_{50} and the logarithm of the soil metal concentration in a previous study with a soil artificially contaminated with Cu, Cd, Zn, Ni, or Pb at relatively high doses of application (16). This correlation led us to assume a linear relationship between these variables, which was used to calculate the threshold metal concentrations at which metal effects could be detected. Studies by Huysman et al. (28) also indicated a linear relationship between bioavailable soil Cu and the proportion of Cu-tolerant bacteria in manured soils. The results of the present work show, however, that when a wide range of soil metal treatments was considered, an exponential instead of a linear relationship was observed between community tolerance and log metal concentration (Fig. 5). This means that the previously reported threshold concentrations (16) were too high. For example, the actual threshold metal concentration for Zn tolerance was 2 mmol kg^{-1} instead of the earlier reported value of 4 mmol kg^{-1}. The results from the present laboratory study agree well with a recent field study (35), where an exponential relationship was also suggested.

Studies carried out under both laboratory and field conditions have shown that the phospholipid fatty acid (PLFA) compositions of metal-contaminated and uncontaminated soils differ (23, 35), indicating differences in the microbial species composition. Recently, Frostegård et al. (24), in a study concerning the changes in microbial community structure in two contrasting contaminated soils during long-term incubation, observed that the differences in PLFA patterns due to Zn pollution increased gradually over time. They attributed these changes in PLFA patterns to either the gradual development of a microbial community which became increasingly tolerant over time or to the successive breakdown of PLFAs of dead cells and the subsequent proliferation of the organisms feeding on them. The tolerance measurement data reported here indicate that both of these explanations might be true. Usually, the metal tolerance increased over time, at least for higher amendment levels (Fig. 4; Table 1), and there was also increased bacterial growth on killed, sensitive bacteria as discussed above (Fig. 2a).

An increased tolerance of NaCl (Fig. 6) and a lower pH optimum (Fig. 7) were observed for bacterial communities from the soil amended with 32 mmol of Zn kg^{-1} as compared with that in the corresponding unamended control. The lower pH optimum was expected, since as a consequence of Zn addition, the bulk soil pH decreased from 7.8 to 6.7. Changes in the pH response of the bacterial community have earlier been shown to correlate to soil pH after different treatments modifying soil pH such as alkaline dust deposition, ash fertil-

ization, liming, or burning (7–9), and the bacterial community response was well correlated to soil pH in a range of soils with pH values between 4 and 8 (6). The increased tolerance to NaCl was also expected since high metal doses will increase the ionic strength of the soil solution, thus selecting for microorganisms tolerating a lower osmotic potential. This demonstrates that the thymidine technique to measure tolerance could easily be adapted to differentiate between different side effects causing an altered community, in the present case, the effects of metal addition, which causes changes in pH and ionic strength of the soil solution in addition to the direct toxic metal effect. This would not be possible if general measurements of activity and biomass were used.

The results obtained in the present study are likely to be extended to the development of bacterial tolerance after exposure to metals other than Zn. However, the results with different metals (compare, for example, Cd and Pb in Table 1) indicated that the development of tolerance might proceed with different speeds depending on the metal causing the selection pressure. A different time scale might also be operative if metals are added to the soil in small amounts on several occasions instead of as a single large dose and if combinations of metals are used. This would more accurately reflect pollution conditions occurring in the field, where the actual metal concentrations usually are the result of accumulating small inputs of several heavy metals. By use of the techniques of the present laboratory study, it will, however, be possible to monitor the development of tolerant bacterial communities in such a field situation.

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