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Association of Microbial Community Composition and Activity with Lead, Chromium, and Hydrocarbon Contamination

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Microbial community composition and activity were characterized in soil contaminated with lead (Pb), chromium (Cr), and hydrocarbons. Contaminant levels were very heterogeneous and ranged from 50 to 16,700 mg of total petroleum hydrocarbons (TPH) kg of soil^{-1}, 3 to 3,300 mg of total Cr kg of soil^{-1}, and 1 to 17,100 mg of Pb kg of soil^{-1}. Microbial community compositions were estimated from the patterns of phospholipid fatty acids (PLFA); these were considerably different among the 14 soil samples. Statistical analyses suggested that the variation in PLFA was more correlated with soil hydrocarbons than with the levels of Cr and Pb. The metal sensitivity of the microbial community was determined by extracting bacteria from soil and measuring [^{3}H]leucine incorporation as a function of metal concentration. Six soil samples collected in the spring of 1999 had IC_{50} values (the heavy metal concentrations giving 50% reduction of microbial activity) of approximately 2.5 mM for CrO_4^{2-} and 0.01 mM for Pb^{2+}. Much higher levels of Pb were required to inhibit [^{14}C]glucose mineralization directly in soils. In microcosm experiments with these samples, microbial biomass and the ratio of microbial biomass to soil organic C were not correlated with the concentrations of hydrocarbons and heavy metals. However, microbial C respiration in samples with a higher level of hydrocarbons differed from the other soils no matter whether complex organic C (alfalfa) was added or not. The ratios of microbial C respiration to microbial biomass differed significantly among the soil samples ($P < 0.05$) and were relatively high in soils contaminated with hydrocarbons or heavy metals. Our results suggest that the soil microbial community was predominantly affected by hydrocarbons.

Heavy metals at excessive concentrations may have great impacts on soil microbial community structure, biomass, and activities. The adverse effect of heavy metals has often been observed as a reduction in microbial biomass and activity (1, 7, 22). Low microbial biomass and activity may limit the decomposition of soil organic matter and lead to the accumulation of organic materials in metal-contaminated soils (2, 8). Reduced microbial activity may originate from the change of microbial community structure after long-term exposure to a heavy metal. Doelman et al. (14) observed that metal-contaminated soil contained more metal-resistant microbes, but these microbes had a restricted ability to degrade organic pollutants. Most of our knowledge about the ecological effects of heavy metals on soil microorganisms comes from data collected for a few metals such as Cu and Zn or from data collected for sewage sludge that contains a broad mixture of heavy metals at relatively low concentrations (<1,000 mg kg^{-1}) for a relatively short period of time (several years). Cr and Pb are important pollutants in hazardous waste sites (21), and little is known regarding the effects of high concentrations of Cr and Pb on microbial community structure and activity.

Heavy metals are often mixed with organic pollutants in contaminated sites. The presence of multiple contaminants may present extreme challenges to the maintenance of a phylogenetically and functionally diverse microbial community. In soils contaminated with both heavy metals and hydrocarbons, for example, only microbes that tolerate both heavy metals and toxic levels of hydrocarbons may survive. Of course, some microbes may also utilize hydrocarbons as their energy source. The changes in community structure may not simply equal the microbial responses to the individual pollutants. Moreover, the restricted ecological niche may simultaneously affect microbial function and activity. In the present study, our objective was to examine microbial community composition and activity after long-term exposure to Pb, Cr, and hydrocarbons. We expected a significant association between the levels of contaminants and alterations in the microbial community.

MATERIALS AND METHODS

Soil sampling. We collected soils from a site in Seymour, Indiana, where the Indiana Department of Transportation had maintained a service location that had been contaminated in the late 1960s with Pb, Cr, and hydrocarbons (primarily toluene). The level of contaminants varied to a great extent within the site due to the heterogeneous nature in which contamination was introduced, thereby leading to different concentrations of Pb, Cr, and hydrocarbons both horizontally and vertically (19). Based on a previous survey, we collected soil samples not only from soil patches with high contamination but also from those patches with slight or no contamination. We sampled six soils from 0 to 30 cm depth in early spring 1999 and 10 soils from 30 to 60 cm in late summer 1999, and those samples were collected within approximately a 20- by 3-m area. Soil samples were stored on ice and transported to the laboratory, wet sieved through a 10-mm screen, and then stored at 4°C until use. Air-dried soils were used for chemical analyses, and freeze-dried soils were used for phospholipid analyses. The soil matrix was comprised of 12% clay, 10% silt, and 78% sand. Selected soil properties are given in Table 1.

Experimental design. The relationships between microbial community composition and Pb, Cr, and hydrocarbons were assessed by the analysis of phospholipid fatty acid (PLFA) patterns with six soils collected in early spring 1999

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TABLE 1. Selected properties of soil samples

Sample[a]	Organic C (g kg^{-1})	CEC[b] ($cmol_c$ kg^{-1})	pH	TPH[c] (mg kg^{-1})	Pb (mg kg^{-1})	Cr (mg kg^{-1})
S1	6.3	4.0	8.2	46	235	64
S2	6.1	4.5	8.2	61	526	105
S3	11.3	11.9	7.1	188	3,885	962
S4	15.3	2.4	7.5	1,435	552	76
S5	9.7	9.9	8.1	95	2,612	608
S6	24.5	7.5	8.1	163	9,198	1,949
U1	13.2	4.1	7.5	224	1	4
U2	32.8	8.4	7.6	66	30	8
U3	10.9	4.0	7.9	66	4	3
U4	94.7	3.8	7.6	16,692	5,054	365
U5	114.0	1.2	7.2	8,984	1,504	240
U6	19.1	5.4	7.7	66	1,259	238
U7	100.5	2.5	5.7	13,477	17,083	3,260
U8	95.3	2.2	5.8	6,709	16,636	2,956
U9	78.3	2.5	7.4	713	8,649	1,042
U10	8.0	ND[d]	ND	ND	6	4

[a] Soil samples labeled with S were collected in March 1999, and soil samples labeled with U were collected in August 1999. U1 to U10 correspond to HN4, HN8, HN11, HS1, HN3, HN5, HN2, HS3, HS2, and HS8, respectively (20).
[b] CEC, cation exchange capacity.
[c] TPH represents total petroleum hydrocarbons.
[d] ND, not determined. However, U10 was collected from the soil patch without contamination, as for soil samples U1, U2, and U3. Thus, the values of CEC, pH, and TPH might be similar to those in U1, U2, and U3.

(S1 to S6) and eight soils collected in later summer 1999 (U1 to U8) (Table 1). The 14 samples covered broad levels of Pb, Cr, and hydrocarbons. We used two methods to compare microbial responses to heavy metals in metal-contaminated soils with those in slightly or noncontaminated soils. One method measured microbial [^{3}H]leucine incorporation by soil bacterial suspensions of the six soils collected in early spring 1999 (S1 to S6). The other method measured microbial mineralization of [^{14}C]glucose in intact soils. We selected U9 versus U10 to assess the microbial activity under high levels of heavy metals as well as organic C. To determine whether a representative sample of bacteria was being extracted from the soil matrix, as in the microbial [^{3}H]leucine experiment, bacterial community structure was compared in sample U10 between intact soil and a soil suspension by using denaturing gradient gel electrophoresis (DGGE) analysis. Microcosm experiments were conducted to evaluate the effects of hydrocarbons and heavy metals on microbial biomass and activities following addition of organic substrates with soils S1 to S6.

Phospholipid analysis. Three replicates of each soil sample (5 g [dry weight]) were extracted with a one-phase chloroform-methanol-phosphate buffer mixture (1:2:0.8 [vol/vol/vol]), mixed with a vortex for approximately 30 s, and then left standing for 24 h. The extraction mixtures were adjusted to 1:1:0.9 (vol/vol/vol) by adding the appropriate amounts of chloroform and phosphate buffer and incubated overnight for phase separation. The chloroform phase was filtered, and the phospholipids were separated from other lipids with silicic acid columns. A 100-μl portion was used to measure phospholipid phosphate (PL-P) (3), while the remainder was subjected to mild alkaline methanolysis. Fatty acid methyl esters were then analyzed using a Hewlett Packard 5890 gas chromatograph as described in reference 3, and the PLFA pattern was analyzed.

Microbial [^{3}H]leucine incorporation. Dose-response analyses of microbial activities to CrO_4^{2-} and Pb^{2+} were performed by a modified method of microbial [^{3}H]leucine incorporation (6). Briefly, soil bacteria were extracted with distilled water (1:20 soil [wt/vol]) using a homogenization-centrifugation technique (4). The pH of the bacterial extracts was adjusted to 6.5 with 10 mM morpholineethanesulfonic acid buffer. The bacterial extracts (2 ml) were transferred into 20-ml glass vials. A series of 0.2-ml K_2CrO_4 or 0.4-ml $Pb(NO_3)_2$ solutions were added to give final concentrations of 0, 0.001, 0.01, 0.1, 1, 5, and 10 mM. After preincubation for about 30 min, we added 3 μl of (4,5-L) [^{3}H]leucine (1 mCi ml^{-1}, 58 mCi mmol^{-1}; Amersham Life Science, Piscataway, N.J.) to each glass vial. Then, the bacterial extracts were incubated at 25°C for 2 h with continuous shaking at 125 rpm. The incubation was terminated with the addition of 1 ml of ice-cold 15% trichloroacetic acid (TCA; 5% final concentration). Zero-time controls were also included by adding 3 μl of [^{3}H]leucine and 1 ml of 15% TCA simultaneously. After 45 min in an ice-cold water bath to precipitate macromolecules, the suspensions were filtered through 0.45-μm Gelman membrane filters (prerinsed with 5% ice-cold TCA) and washed with three 2-ml portions of ice-cold 5% TCA and three 2-ml portions of ice-cold 80% ethanol. The filters with the precipitated macromolecules were placed into scintillation vials containing 1 ml of 0.1 M NaOH and were heated at 90°C for 1.5 h. After cooling to the room temperature, 15 ml of CytoScint scintillation cocktail (ICN Biomedicals, Costa Mesa, Calif.) was added. Radioactivity was measured in a liquid scintillation spectrometer (Tri-Carb model 1600 TR; Packard Instrument Company, Meriden, Conn.), using an external standard of ^{3}H to determine counting efficiency in each vial.

Microbial glucose mineralization. Uniformly ^{14}C-labeled glucose (200 mCi mmol^{-1}; ICN Biomedicals) was used. One gram (dry weight equivalent) of soil was added to each of a series of 25-ml serum vials. The ^{14}C-labeled glucose (0.27 μCi), varying amounts of $Pb(NO_3)_2$, and water were added to the soils to achieve a moisture content of 118% water-holding capacity. Soils were mixed with a spatula to ensure uniform distribution of the isotope. The vials were sealed with a rubber serum stopper that positioned a small plastic cup above the soil. The cup contained 250 μl of 1 M KOH as an alkaline trap for CO_2. Each treatment was prepared in triplicate. All vials were incubated at 20°C for 8 h. The KOH in the trap was placed in scintillation vials containing 7 ml of CytoScint scintillation cocktail (ICN Biomedicals). Radioactivity was quantified in a scintillation counter (Tri-Carb model 1600 TR; Packard Instrument Company). An external standard was used to determine counting efficiency in each vial. Soil bacterial extracts were also subjected to the addition of [^{14}C]glucose, and these data were compared with those from intact soils. The cell suspensions were derived as described above for the experiment employing [^{3}H]leucine.

Characterization of the microbial community in soil extracts. The community structures of bacteria from intact soil and liquid soil suspension were assessed by DGGE analysis. Total bacterial genomic DNA was extracted either from intact soil with a sample size of 0.5 g or from the liquid soil suspension with a sample size of 1 ml. Extraction was performed using a Q-BIOgene FastDNA spin kit (Q-BIOgene, Vista, Calif.) with a modification of the manufacturer's method. Then, PCR was carried out on the DNA total extract and the products were separated by DGGE (23). Band positions in each lane were scored visually; community similarity was determined using the Dice similarity coefficient (12).

Microcosm experiment. An aerobic incubation experiment was conducted either without any addition of organic C or after the addition of complex organic matter (alfalfa) at 10 mg of C g of soil^{-1}. Three replicate microcosms were run for each soil sample. For each replicate, two 20-ml vials containing 5.5 g (equivalent to 5 g of oven dry weight) of soil were placed into a 1-liter Mason jar along with a 20-ml vial containing 1 ml of 0.5 M NaOH. The jar was then capped tightly. Each week, the Mason jar was flushed with air to maintain aerobic conditions. On days 1, 4, 7, 14, 21, and 28, the alkaline trap was replaced and capped tightly for later analysis of CO_2 trapped in the base. Mason jars containing only the alkaline trap were used as blanks. The rate of CO_2 production was determined by titration with standardized 0.1 M HCl (27). On days 7 and 28, one vial containing soil was removed from the Mason jar and microbial biomass was measured as PL-P. At the beginning of the incubation, soil water content was adjusted to 45% water-holding capacity (as determined by the method of Forster [16]). Thereafter, soil water content was maintained at that level.

Data analysis. We used principal component analysis (PCA) (26) to compare the PLFA profiles in the 14 soil samples. Fatty acids with carbon lengths of 14 to 20 were used to analyze microbial community structure. Fatty acids were transformed to their mole fractions, and CANOCO software (Microcomputer Power, Inc., Ithaca, N.Y.) was used to perform PCA. In addition, the first principal component was regressed with the environmental variables of hydrocarbons, Pb, and Cr.

Dose responses of microbial [^{3}H]leucine incorporation to heavy metals were fit to a logistic model (11, 13) with the following equation: $A = 100/[1 + e^{b(S-a)}]$, where A is the measured microbial activity expressed as the percentage of microbial activity in the sample with no metal addition; b is a slope parameter indicating the inhibition rate; S is the logarithm of heavy metal concentrations; and a is the logarithm of the IC_{50}, i.e., the heavy metal concentration giving 50% reduction of microbial activity relative to the sample to which no metal was added. The IC_{50} and b values were derived using nonlinear regression (SigmaPlot 5.0; SPSS Inc.).

We calculated the microbial specific activity (microbial C respiration per unit of microbial PL-P) by dividing CO_2 released during a given period by the average of microbial biomass at the beginning and end of that period (10). Effects of soil contamination on microbial C respiration, microbial biomass, and microbial specific activity with the incubation time were analyzed using a completely randomized design with two-way analysis of variance (Data Desk 6.0; Data Description, Inc. Ithaca, N.Y.).

RESULTS AND DISCUSSION

Soil characteristics. The samples collected from the Seymour site exhibited substantial variation in the level of contamination (Table 1). Total petroleum hydrocarbon (TPH) varied by more than 2 orders of magnitude, Cr varied by 3 orders of magnitude, and Pb varied by 4 orders of magnitude. Samples with high total organic C were those that appeared to have been exposed to hydrocarbons. Subsequent analyses of some samples indicated that the petroleum hydrocarbon assays were detecting primarily toluene, xylene, and ethylbenzene. Changes in cation exchange capacity (CEC) and pH among samples were much smaller in magnitude, but the CEC values were consistently lower in highly TPH-contaminated soils than in slightly or noncontaminated soils.

Microbial community structure. PLFA have been used as biomarkers for evaluating the changes of microbial major groups, for example, with gram-positive and gram-negative bacteria, actinomycetes, and fungi (17, 20, 24). Seventeen PLFA were detected in all 14 soil samples, including five branched-chain, iso-, and anteiso-PLFA (i15:0, a15:0, i16:0, i17:0, and a17:0), five saturated PLFA (14:0, 15:0, 16:0, 17:0, and 18:0), four monounsaturated PLFA (16:1ω9c, 16:1ω9t, 18:1ω9c, 18:1ω11c/9t), one polyunsaturated PLFA (18:2ω9,12), and two cyclopropane PLFA (cy17:0 and cy19:0c11,12) (Table 2). Generally, the mole fractions of branched-chain PLFA, which represent gram-positive bacteria (24), were considerably higher in sites with lower TPH, Pb, and Cr than in the more contaminated sites. In addition, the mole fractions of cyclopropane PLFA, which are the biomarkers for gram-negative bacteria (24), were also considerably higher in less contaminated sites. However, the PLFA of fungal origin, i.e., polyunsaturated PLFA (24), were much higher in sites with higher TPH, Pb, and Cr than in sites with less contamination. The ω-9 monounsaturated PLFA, which usually represent the fungi (24), were also higher in highly contaminated sites. These results suggested that microbial community compositions were associated with soil contaminants.

Community composition was further analyzed by PCA (Fig. 1A). The first two principal components explained 92.0% of the variance. Five samples (S4, U4, U5, U7, and U8) that contained high levels of hydrocarbons formed a distinct cluster along the first principal component. The loading plots (Fig. 1B) indicate that high levels of two PLFA found in microeukaryotes (18:2ω9,12 and 18:1ω9c) were responsible for shifting these samples along the *x* axis. PLFA of 18:2ω9,12, 18:1ω9c, 16:1ω9c, i15:0, i16:0, and i17:0 were critical to separate microbial communities on the first principal component, whereas PLFA of 18:0, 18:1ω9c, 16:0, and cy17:0 had the greatest impact on the second principal component. The clustering of samples along the second principal component appeared related to the time of sampling (Fig. 1A). The log (soil hydrocarbon level) was significantly correlated ($r^2 = 0.85$; $P < 0.001$) to the value of the first principal component (Fig. 2), whereas the correlation of this component to the Pb and Cr levels was poor (data not shown). Contaminated soils had significantly different microbial community compositions than slightly or noncontaminated soils, and a major community effect was a shift from bacteria (both gram positive and gram negative) to fungi.

Microbial responses to heavy metals. The PLFA analyses suggested that the microbial communities had shifted as a consequence of the contaminants present in the habitat for the past several decades. One expected shift would be to populations with a higher level of tolerance for the metals Pb and Cr. [^{3}H]leucine incorporation into protein was used as an assay of microbial activity. In order to minimize physical and chemical binding of added metals within the soil matrix in these experiments, bacteria were extracted from soil particles and then challenged with a series of Pb or Cr concentrations. The extraction techniques are not 100% efficient, but they do produce a representative microbial community. Profiles of community structure (determined by DGGE of a portion of the 16S rRNA gene) were similar for DNA extracted from intact soil and the bacterial extracts (Fig. 3). The Dice similarity coefficient for the band patterns obtained from whole soil and bacteria extracted from soil particles was >0.9.

We had hypothesized that soil samples that differed in metal contamination would show different dose responses of microbial activity across a series of Cr or Pb concentrations. However, there were no differences among the six soil samples collected in early spring 1999 (Fig. 4). The IC_{50} values were about 2.5 mM for Cr and 0.01 mM for Pb regardless of exposure history. The lower IC_{50} value for Pb than for Cr suggests that Pb was more toxic than Cr, or that bacteria were more resistant to Cr than to Pb. Our analyses of bacterial isolates from these soils have shown that the maximum resistance level to Pb does not exceed 150 μM, whereas bacteria capable of growth at 10 mM Cr have been obtained (unpublished observations).

The shape of the sigmoid response suggests that there are subpopulations with different tolerance levels to metal. With pure laboratory cultures, a reduction in activity from 80 to 20% of the maximum occurred over a change in metal concentration of 1.5- to 3-fold for Pb and Cr (unpublished observations). In all the natural communities, this level of activity reduction occurred over a much broader concentration range. However, the ranges differed among samples. For Pb, the concentration ratios that decreased activity from 80 to 20% in samples S3 and S4 were only slightly greater (3 and 5, respectively) than what was observed in pure cultures. Samples S2 and S5 had the highest ratios (approximately 15). In the case of Cr, the community from S1 had a rather sharp response (ratio of 10), and samples S2, S4, and S5 had ratios of about 25. These heterogeneities were not related to the physical or chemical characteristics of the soil samples in any obvious way. The important point that they do illustrate is that all of the contaminated soils contained complex microbial communities that consisted of both metal-sensitive and metal-resistant types. This raises an issue regarding the heterogeneity of metal bioavailability within these soils.

The measurement of macromolecular synthesis has been shown to be a sensitive method to detect changes in microbial metal resistance (5, 11). Soils experimentally contaminated with heavy metals develop microbial communities more resistant to heavy metals, and soils contaminated with higher amounts of heavy metals show a relatively larger population of resistant bacteria (11). Unexpectedly, we did not detect these differences when using the most toxic forms of these metals [Pb(II) and Cr(VI)]. Perhaps the bioavailable levels of toxic Cr

TABLE 2. PLFA from soils with various levels of hydrocarbons and heavy metals[a]

PLFA	PLFA level (mol%)												
	S1	S2	S3	S4	S5	S6	U1	U2	U3	U4	U5	U6	U7	U8
14:0	1.35 ± 0.17	1.35 ± 0.10	1.73 ± 0.49	1.47 ± 0.20	0.88 ± 0.05	0.72 ± 0.10	1.85 ± 0.20	3.04 ± 0.04	3.00 ± 0.52	2.62 ± 0.35	1.09 ± 0.16	2.98 ± 0.22	1.01 ± 0.32	0.68 ± 0.09
i15:0	5.87 ± 0.58	3.30 ± 0.16	1.77 ± 0.50	0.35 ± 0.05	2.08 ± 0.03	0.76 ± 0.02	2.94 ± 0.53	3.47 ± 0.04	4.16 ± 0.61	0.00 ± 0.00	0.54 ± 0.09	4.68 ± 0.43	0.20 ± 0.07	0.06 ± 0.03
a15:0	4.36 ± 0.52	2.65 ± 0.14	4.05 ± 1.14	5.15 ± 0.86	1.72 ± 0.05	0.63 ± 0.02	4.63 ± 0.89	4.56 ± 0.06	6.30 ± 1.00	0.27 ± 0.14	4.18 ± 0.48	5.75 ± 0.55	2.09 ± 0.61	0.84 ± 0.11
15:0	0.74 ± 0.02	0.67 ± 0.04	1.45 ± 0.39	0.36 ± 0.04	0.45 ± 0.06	0.27 ± 0.04	1.45 ± 0.26	1.91 ± 0.07	2.47 ± 0.46	0.15 ± 0.15	0.38 ± 0.04	2.29 ± 0.13	0.26 ± 0.08	0.12 ± 0.02
i16:0	1.68 ± 0.14	1.01 ± 0.04	1.63 ± 0.46	0.89 ± 0.13	0.81 ± 0.03	0.35 ± 0.00	1.21 ± 0.29	1.66 ± 0.04	2.22 ± 0.23	0.00 ± 0.00	0.41 ± 0.06	2.35 ± 0.26	0.26 ± 0.07	0.03 ± 0.03
16:1ω9c	14.94 ± 1.65	9.72 ± 0.42	5.55 ± 1.43	0.42 ± 0.05	7.18 ± 0.11	5.39 ± 0.22	16.48 ± 3.57	11.07 ± 0.25	12.67 ± 1.34	0.57 ± 0.32	1.04 ± 0.26	15.05 ± 1.44	1.28 ± 0.36	0.57 ± 0.11
16:1ω9t	0.00 ± 0.00	0.00 ± 0.00	1.45 ± 0.45	0.24 ± 0.04	0.00 ± 0.00	0.30 ± 0.15	0.00 ± 0.00	1.90 ± 0.07	2.76 ± 0.31	0.00 ± 0.00	0.16 ± 0.02	2.64 ± 0.47	0.15 ± 0.03	0.16 ± 0.04
16:0	18.80 ± 2.09	26.00 ± 2.83	26.01 ± 5.74	13.88 ± 3.12	20.79 ± 2.31	22.98 ± 6.27	20.63 ± 4.52	18.94 ± 0.16	20.02 ± 2.15	16.06 ± 1.69	16.46 ± 2.33	17.77 ± 2.17	15.73 ± 3.17	13.06 ± 1.44
i17:0	1.18 ± 0.14	0.71 ± 0.03	0.42 ± 0.10	0.04 ± 0.01	0.43 ± 0.02	0.19 ± 0.00	0.60 ± 0.07	0.94 ± 0.01	1.29 ± 0.17	0.10 ± 0.10	0.07 ± 0.03	1.16 ± 0.12	0.04 ± 0.02	0.04 ± 0.02
a17:0	1.42 ± 0.13	0.86 ± 0.04	0.93 ± 0.24	0.79 ± 0.20	0.64 ± 0.02	0.23 ± 0.01	0.88 ± 0.12	1.13 ± 0.10	1.56 ± 0.16	0.56 ± 0.19	0.40 ± 0.04	1.42 ± 0.13	0.37 ± 0.07	0.23 ± 0.05
cy17:0	2.57 ± 0.17	1.74 ± 0.10	2.19 ± 0.55	0.23 ± 0.04	0.88 ± 0.02	0.48 ± 0.05	14.27 ± 3.16	9.74 ± 0.36	9.29 ± 4.24	0.00 ± 0.00	0.58 ± 0.12	7.90 ± 0.32	0.06 ± 0.03	0.08 ± 0.05
17:0	0.93 ± 0.16	1.02 ± 0.05	1.03 ± 0.26	0.32 ± 0.10	0.63 ± 0.06	0.61 ± 0.07	1.22 ± 0.19	1.65 ± 0.35	1.31 ± 0.69	0.38 ± 0.19	0.41 ± 0.02	1.28 ± 0.15	0.37 ± 0.06	0.33 ± 0.07
18:2ω9,12	5.76 ± 0.62	6.54 ± 1.38	5.04 ± 0.84	36.13 ± 9.95	21.04 ± 4.58	20.28 ± 6.99	8.68 ± 1.35	14.91 ± 2.49	6.99 ± 3.59	52.41 ± 6.08	47.85 ± 8.18	11.46 ± 2.80	49.94 ± 9.85	59.49 ± 3.15
18:1ω9c	14.00 ± 1.75	18.46 ± 2.49	17.71 ± 3.48	34.01 ± 8.78	25.09 ± 4.42	30.47 ± 9.53	4.81 ± 0.94	9.98 ± 0.42	6.53 ± 0.77	21.51 ± 2.32	20.78 ± 3.22	7.86 ± 1.40	11.24 ± 5.71	19.71 ± 1.53
18:1ω11c/9t	17.21 ± 1.73	12.41 ± 0.82	12.50 ± 2.57	0.04 ± 0.02	9.23 ± 0.43	6.68 ± 0.79	12.52 ± 2.85	9.67 ± 0.57	10.06 ± 1.39	0.00 ± 0.00	0.00 ± 0.00	8.64 ± 0.92	11.41 ± 11.41	0.00 ± 0.00
18:0	4.73 ± 0.56	8.11 ± 0.92	8.17 ± 1.74	5.24 ± 1.24	6.99 ± 1.00	8.66 ± 2.56	3.16 ± 0.50	4.18 ± 0.27	4.10 ± 0.32	5.22 ± 0.50	5.57 ± 1.01	3.02 ± 0.51	5.36 ± 1.13	4.53 ± 0.59
cy19:0c11,12	4.45 ± 0.26	5.44 ± 0.33	8.37 ± 1.46	0.43 ± 0.02	1.15 ± 0.07	1.00 ± 0.05	4.66 ± 2.36	1.26 ± 1.26	5.28 ± 2.65	0.14 ± 0.14	0.08 ± 0.08	3.74 ± 0.32	0.20 ± 0.03	0.06 ± 0.03

[a] All values are means ± standard errors ($n = 3$).

FIG. 1. Principal component ordination of PLFA analyses from contaminated soils. (A) Discrimination of soil samples along the first two principal components. (B) Loading factors for individual fatty acids along the first two principal components. Soils labeled with S were sampled in the early spring, and soils labeled with U were sampled in the summer.

and Pb were similar in all the soil samples. Several factors, including organic matter, pH, and redox potential might influence the solubility of heavy metals (9, 18, 25). In the case of Cr, the oxidation state of the metal impacts mobility and toxicity; Cr(VI) is several orders of magnitude more toxic than Cr(III). Our analytical method did not distinguish these forms in soil. In addition, there may be spatial heterogeneities in metal distribution on smaller scales than we assayed.

The capacity of soil physical and chemical properties to protect microbes from metal toxicity was assessed by measuring the activity of microbes directly in the soil matrix. It was not feasible to use the same activity assay as with the bacterial suspensions because of the difficulty in quantitatively separating all microbes from the soil matrix after incubation. Therefore, [^{14}C]glucose was added, and the production of $^{14}CO_2$ was assayed.

Two samples were chosen for analysis. U9 contained high levels of Pb contamination (as well as Cr and organic pollutants), whereas low levels of Pb and other contaminants were found in U10 (Table 1). When Pb was added to soil sample

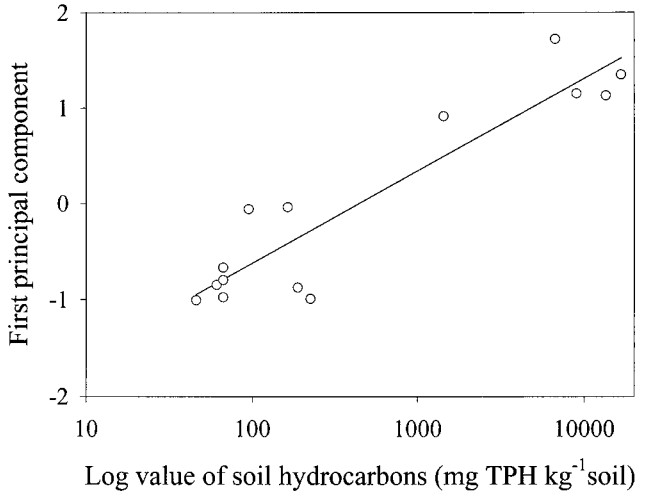

FIG. 2. Linear regression of the first principal component from PLFA analysis with the logarithm of soil TPH.

U10, there was a progressive decrease in $^{14}CO_2$ production from [U-^{14}C]glucose (Fig. 5) as increasing amounts of Pb were added. However, much larger inputs of Pb were required to inhibit microbial activity in soil than that found when microbes were removed from soil particles. Approximately 10,000 mg of Pb kg of soil^{-1} was required to reduce $^{14}CO_2$ production by a factor of 2 in soil, whereas only 0.1 mM Pb (about 400 mg of Pb kg of soil^{-1}, using a 1:20 ratio of soil weight/H_2O volume

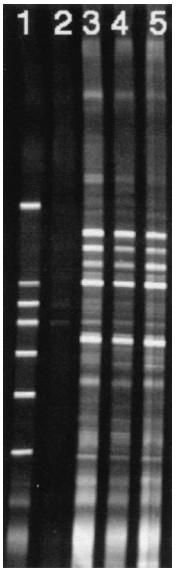

FIG. 3. DGGE fingerprints of microbial community composition in soil. The soil used for this experiment was U10. Lane 1, markers (see Materials and Methods); lane 2, negative control with no DNA in PCR; lane 3, DNA extracted from bulk soil; lane 4, DNA obtained from the cell suspensions extracted from soil particles; lane 5, DNA extracted from the soil residue after extraction of bacteria. The actual gel contained 15 lanes of samples; the other samples contained different banding patterns and, thus, serve as positive control. As these samples were not relevant to the issue at hand, they were not included in the figure.

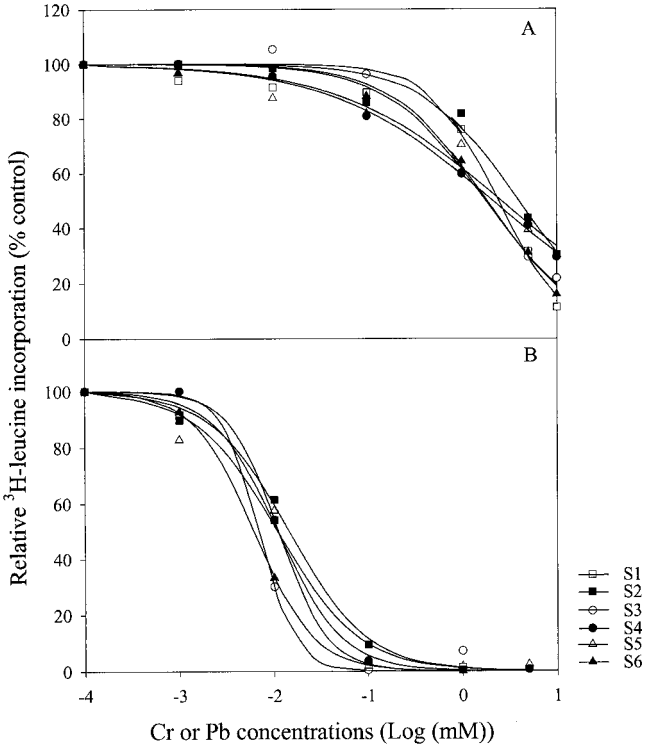

FIG. 4. Responses of microbial [^{3}H]leucine incorporation to a series of CrO_4^{2-} and Pb^{2+} concentrations in various Pb- and Cr-contaminated soils. Data points are expressed as the percentage of control (no metal added) values. Lines are fitted from the logistic model.

for conversion) could reduce microbial activity by 50% in a suspension of bacteria.

When experiments were run with the metal-contaminated soil sample U9, a different response was found than with U10

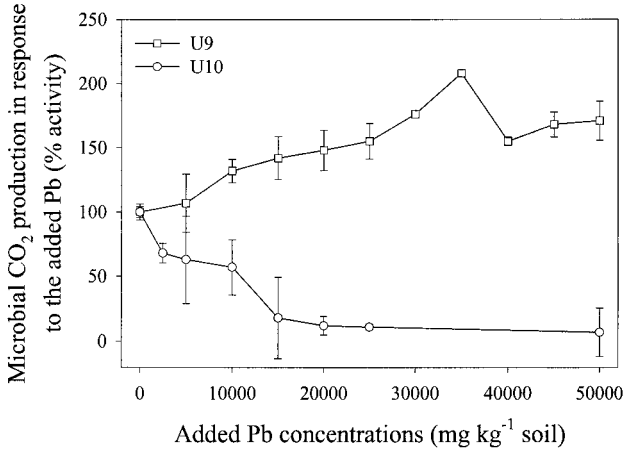

FIG. 5. Effect of lead nitrate additions upon microbial activity (mineralization of [^{14}C]glucose) in soils U9 and U10. Soils were amended with lead nitrate and glucose and incubated for 8 h, and the amount of $^{14}CO_2$ was determined. Activity values are normalized to that found when no lead nitrate was added. Values are the means of three replicates. Error bars represent the standard error; when error bars are not visible, the standard error was smaller than the size of the symbol.

TABLE 3. Soil microbial biomass in microcosms

Sample	Biomass (nmol of PL-P/g of soil)[a] at:				
	Day 0	Day 7		Day 28	
		No C	Added C	No C	Added C
S1	7.34 (0.80)	3.39 (0.44)	17.60 (0.49)	3.92 (0.07)	13.96 (0.27)
S2	3.67 (0.40)	2.13 (0.16)	18.43 (2.03)	3.82 (0.61)	10.17 (2.10)
S3	5.78 (0.70)	6.25 (0.90)	20.05 (4.44)	9.50 (1.11)	17.20 (5.47)
S4	3.08 (0.80)	2.76 (0.52)	13.57 (1.15)	3.97 (1.44)	17.08 (1.73)
S5	4.24 (0.40)	3.19 (0.33)	14.90 (2.35)	6.68 (0.66)	16.55 (1.75)
S6	3.10 (0.30)	2.90 (0.25)	16.94 (2.74)	7.78 (2.04)	17.05 (0.39)

[a] Results for microcosms to which either no organic amendments were made (No C) or 10 mg of alfalfa C g of soil^{-1} was added (Added C). Samples were analyzed before microcosms were constructed (Day 0) and after 7 or 28 days of incubation. All data are means plus standard errors ($n = 3$) (in parentheses).

(Fig. 5). Activity did not decrease as $Pb(NO_3)_2$ was added, but in fact it increased up to twofold. This general trend was observed in four separate experiments. The physiological basis for the phenomenon remains unknown. However, the addition of KNO_3 rather than $Pb(NO_3)_2$ did not stimulate activity (data not shown). Therefore, we conclude the effect was not due to N limitation in soil. Although $Pb(NO_3)_2$ is acidic, the experiment were run under buffered conditions where its addition did not alter the pH. The response appeared to be due to properties of the soil matrix. When bacteria were removed from U9 soil particles and their activity was tested with [^{14}C]glucose at a series of Pb concentrations in suspension, a sigmoidal response was noted in which activity could be fully inhibited by high Pb additions. A 50% inhibition was observed at a metal concentration of 0.06 mM Pb (250 mg of Pb kg of soil^{-1}, using a 1:20 ratio of soil weight/H_2O volume for conversion).

The comparison of effects observed when metals were added directly to soil versus when metals were added to suspensions of bacteria extracted from soil illustrates two points. The first is well known: physical and chemical interactions of metals with soil reduce the bioavailability of metals. Therefore, the mass of metal required to inhibit microbial activity was several orders of magnitude higher in soil than in suspensions. These interactions were the reason our first experiments were conducted with [^{3}H]leucine on extracted bacteria. However, the unexpected similarities of tolerance levels found in bacteria extracted from soils with high and low metal contents prompted a direct examination of effects in soil. The inability of high Pb concentrations to inhibit [^{14}C]glucose mineralization in the highly metal-contaminated soil suggests that these soils contain a biotic (or biotically produced) factor that reduces metal bioavailability that was not present in soil sample U10, which had low metal contamination. The difference in results between experiments with extracted bacteria and with whole soil was not due to the different activity assay methods used. When [^{14}C]glucose mineralization was used to assay activity in extracted bacteria, the levels of Pb tolerance from the two soils were within a factor of 2.

Microbial responses to organic C input. The levels of microbial biomass (assayed as PL-P) were similar in contaminated and noncontaminated soils; they ranged from 3.0 to 7.3 nmol of PL-P g of soil^{-1} (Table 3). The levels were not correlated to the levels of heavy metals or to hydrocarbons (r^2_{Cr} = 0.081, $P = 0.58$; $r^2_{Pb} = 0.111$, $P = 0.52$; $r^2_{TPH} = 0.195$, $P = 0.38$). The ratio of microbial biomass to organic C was not correlated with heavy metals or hydrocarbons, either ($r^2_{Cr} = 0.299$, $P = 0.26$; $r^2_{Pb} = 0.326$, $P = 0.24$; $r2_{TPH} = 0.219$, $P = 0.35$) (Tables 1 and 3). These results are in contrast to other field studies in which contaminated soils had reduced microbial biomass (1, 7, 8, 22). However, it is important to note that none of our sample sites were vegetated. Therefore, the inputs of degradable organic C from primary production were low. To determine if there were differences in the potential for organic mineralization and microbial growth in the soils contaminated with not only heavy metals but also organic pollutants, microcosms were set up in which soils either received no organic C or a complex source of organic C (alfalfa). The amount of organic C added was similar to the total soil organic C. The six soil samples used (S1 toS6) varied about 30- to 40-fold in Pb, Cr, and TPH concentrations (Table 1). When no organic C was added, the level of microbial biomass was relatively stable over a 28-day incubation period, although the alterations of microbial biomass were statistically significant in some soils ($P < 0.05$) (Table 3). In particular, microbial biomass in soils contaminated with heavy metals (S3, S5, and S6) was unchanged from day 0 to day 7 and then significantly increased from day 7 to day 28. The addition of organic C stimulated microbial growth in all samples (Table 3). Microbial biomass increased two- to fourfold after the addition of alfalfa (Table 3), which suggests that all soils had a similar capacity to utilize degradable organic C.

The kinetics of CO_2 production were similar in all samples except S4, which had a high level of TPH contamination (Fig. 6). In the absence of added organic C, there was a release of 0.3 to 0.5 mg of CO_2-C g of soil^{-1}. This occurred during the first 3 days of incubation. Sample S4 released slightly more CO_2 (reaching 0.8 mg of C g of soil^{-1} after 28 days). When 10 mg of organic C g of soil^{-1} was added, there was detectable CO_2 production in all samples except S4 after 1 day. Carbon dioxide evolved at a linear rate for 5 days in those five samples and then reached a plateau at values of 40 to 50% of the amount of organic C added to the soils. Although the initial rates of CO_2 evolution were indistinguishable regardless of the level of metal contamination, sample S6, which had the highest metal concentrations, produced 18% more CO_2 than samples S1 and S2. Sample S4, which contained high TPH concentrations, exhibited different dynamics of CO_2 evolution. There was a lag of at least 1 day before CO_2 production was detected. Secondly, production did not plateau after 2 weeks of incubation. When the experiment was terminated after 28 days, a mass of CO_2 equivalent to 60% of that added had been mineralized, and there was little indication that CO_2 production was declining. Our assay method is not specific for the source of organic C that mineralized. Therefore, we cannot exclude that the addition of organic C stimulated enhanced catabolism of hydrocarbons.

The relationship between microbial C mineralization and population size was evaluated by calculating the specific rate of respiration for the day 0 to 7 period (when most CO_2 was produced) and the day 7 to 28 period (after biomass accumulation had peaked). During the first 7 days of incubation for unamended soils, CO_2 production was stimulated by physical disturbances such as soil rewetting and soil mixing (Fig. 6), and

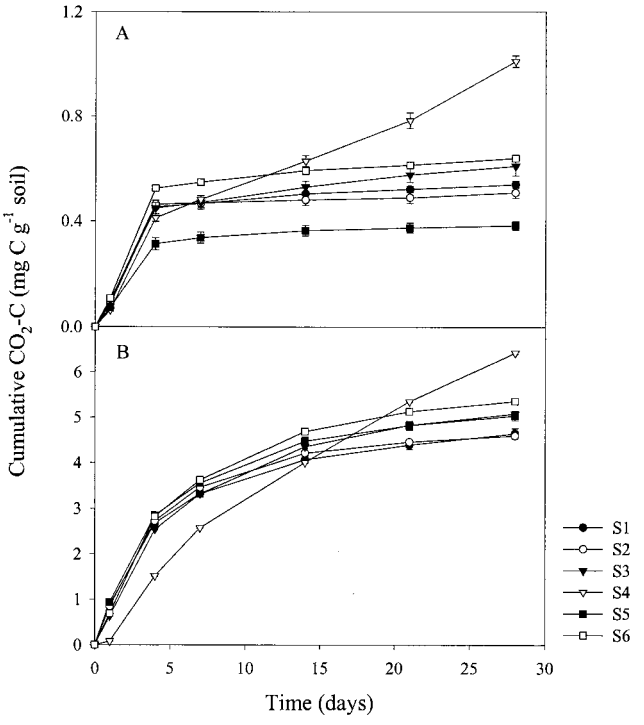

FIG. 6. Cumulative production of CO_2-C in soil samples with no organic addition (A) or with the addition of alfalfa at 10 mg of C g of $soil^{-1}$ (B). Values are means ($n = 3$); standard errors were calculated but the error bars are smaller than the data points. See Table 1 for chemical characteristics of soil samples.

specific respiration rates were high (Table 4). These rates declined 10- to 30-fold during the day 7 to 28 incubation period, with a significantly ($P < 0.01$) higher rate for S4, which had a high level of TPH. For soils amended with organic C, both microbial growth (Table 3) and CO_2 production (Fig. 6) were stimulated during the first 7 days of incubation and specific respiration rates were high (Table 4). Due to the confounding effects of organic amendments and physical disturbance, microbial specific respiration rates among soils were not statistically different during the first 7 days (Table 4); the rates appeared highest for the samples (S5 and S6) with the highest Pb and Cr contamination. During the day 7 to 28 incubation period, specific respiration rates declined, and as in unamended soils S4 had the highest specific respiration rate (Table 4).

TABLE 4. Specific respiration rates

Sample	Respiration rate (mg of CO_2-C/μmol of PL-P/day)[a]			
	No C		Added C	
	Days 0–7	Days 7–28	Days 0–7	Days 7–28
S1	12.6 (1.0)	1.0 (0.2)	38.1 (1.2)	4.0 (0.1)
S2	24.4 (0.6)	0.7 (0.1)	46.3 (4.4)	3.8 (0.1)
S3	11.6 (1.6)	0.9 (0.1)	40.0 (8.1)	4.6 (0.6)
S4	23.9 (1.9)	7.6 (1.0)	44.6 (3.0)	11.8 (0.5)
S5	13.0 (1.2)	0.5 (0.1)	54.5 (6.2)	4.7 (0.6)
S6	26.2 (0.9)	0.9 (0.1)	54.5 (9.3)	5.1 (0.7)

[a] Results are specific respiration rates in soils to which either no organic amendments were made (No C) or 10 mg of alfalfa C g of $soil^{-1}$ was added (Added C). The rates were calculated over the first 7 days of incubation or over the period of 7 to 28 days. All data are means plus standard errors ($n = 3$) (shown in parentheses).

Specific activity has been used to estimate the effects of environmental variables on soil microorganisms. Under the stress of heavy metals, activities are generally thought to increase due to increased maintenance energy or increased substrate utilization efficiency (10, 15). However, we did not observe high specific activities in metal-contaminated soils (Table 4). Instead, the highest specific activity was found in hydrocarbon-contaminated soils (Table 4). The results suggest that the soil microbial activity was mainly associated with hydrocarbons.

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