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Biomass Carbon Measurements and Substrate Utilization Patterns of Microbial Populations from Soils Amended with Cadmium, Copper, or Zinc

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Samples of a sandy loam soil taken from a long-term liming experiment in southeast England were amended with solutions of metal sulfate salts. Soils with a range of pHs were amended to contain Cu, Cd, or Zn at concentrations around the maximum permissible values for these metals in agricultural land receiving sewage sludge. After a 3-year equilibration period, the microbial biomass was determined by the fumigation-extraction technique. These results were compared with data from substrate utilization patterns of microbial populations extracted by using a weak salt solution. There was no reduction in microbial biomass due to pH or metal treatment in any of the soils except the Cu treatment. Principal-component analysis of the respiration patterns in Biolog plates demonstrated effects of both pH and metal treatment on the extracted microbial population which were independent of gross biomass size. pH and soil amendments with Cu and Zn were found to reduce the metabolic potential of the extracted soil microbial population.

Measurements of soil microbial biomass have demonstrated reduction in the size of the overall microbial community in soils polluted with heavy metals. This reduction only becomes apparent at metal concentrations above current accepted metal loading limits (5). One criticism of gross biomass measurements is that the structure of the soil microbial community could change without any measurable change in the total biomass. Hence, the degree of metal pollution of soils permitted under current legislation may not cause measurable changes in the total amount of biomass, but a small chronic metal stress may change the community structure.

One method to study changes in the structure of a soil microbial community is to assess the range of phenotypes present. This has been approached in a number of ways, including the number of similar biotypes grouped by the Biomerieux "api" identification system (11) and the qualitative assessment of phospholipids present in a sample of the microbial community (6). A third way in which the phenotypic characterization of microbial communities has been attempted is by determination of the range of carbon substrates which the microorganisms are capable of using. The Biolog microtiter plate system for identifying microorganisms offers a simple and fast method of testing for the ability to use 95 sole carbon sources. Several authors have demonstrated that substrate utilization patterns for whole communities generated by Biolog microtiter plates differ between diverse habitats and even within the different size fractions of particles of the same soil (7, 13). This difference in carbon source utilization pattern offers a method of characterizing the functional diversity (15) of the microbial community. However, Haack et al. (8) expressed concern that perceived differences in substrate utilization patterns may be related to inoculum densities and not simply diversity.

This study compares the results from measurements of soil microbial biomass on whole soil by fumigation-extraction and the incubation of soil extracts in Biolog microtiter plates. The soils used were amended with Cu, Cd, or Zn to contain these metals at close to the current limits in the United Kingdom for soils receiving sewage sludge (3). The aim was to determine whether the Biolog assessment of the range of carbon sources which microorganisms extracted from soils can utilize could be used to screen soils to show heavy-metal stress.

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MATERIALS AND METHODS

Soils. Soils were sampled from a long-term liming experiment at the Woburn experimental farm in southeast England. A range of soil pH conditions have been maintained in this experiment (sandy loam, Typic Udipsamment) since 1967 (2). Soils from four pH treatments (4.5, 5.1, 6.3, 7.0), with similar fertilizer additions, were sampled, and the soils were sieved moist (<3-mm pore size) with steel sieves to give 40 kg (dry weight) of soil at each pH. Each 40-kg batch was separated into 1-kg pots. Sets of 10 pots were amended with solutions of metal sulfates to raise the soil content of either Cu, Cd, or Zn to approximately the maximum concentration permitted in soils receiving sewage sludge (3). The contents of these 1-kg pots were mixed individually with a food processor to ensure thorough mixing. The contents of similar 1-kg pots were then bulked to give a 10-kg batch of soil amended with either Cd, Cu, or Zn and a control soil receiving no heavy metals. Total heavy-metal concentrations in soil were determined by aqua regia digestion (9) of bulked pH batches and of the samples receiving individual treatments after the metal additions (Table 1). Copper and Zn concentrations were determined by inductively coupled plasma atomic emission spectrometry (ARL34000), and Cd concentrations were determined with a graphite furnace (Perkin-Elmer 4100ZL). The heavy-metal-amended 10-kg pots of soil were sown with ryegrass. The ryegrass was removed after 1 year, and the remaining soil was incubated in an open-air cage for 2 years. The soils were watered once a month by the addition of deionized water to individual saucers used for each pot. After 2 years without plant cover, five cores were taken from each pot, bulked, and thoroughly mixed.

A subsample of each soil was taken for determination of moisture content, and the remaining soil was placed in plastic bags with the necks loosely tied. These samples were then kept in a cold room at 4°C (soils were kept for no more than 1 month) until they were used in the experiments described below.

Soil microbial biomass measurements. The fumigation-extraction method described by Vance et al. (12) was used, and the total carbon contents of the 0.5 M K_2SO_4 extracts were measured with a Dohrman DC80 carbon analyzer. Wu et al. (14) describe the technique in detail and suggest a factor of 2.22 for the extraction efficiency of the method. This value was adopted and used to convert all the total carbon results for the extracts to biomass carbon in the soil samples.

Substrate utilization patterns of microbial populations. Biolog gram-negative microtiter plates contain 95 separate, sole carbon sources and a blank well with no substrate. Each well contains the redox dye tetrazolium, which is reduced by NADH produced by respiration pathways. The rate and extent of color formation indicate the rate and extent to which respiration occurs with the substrate

TABLE 1. Analysis of Woburn soil (Typic Udipsamment)

Soil pH[a]	Metal concn (mg kg^{-1} [dry weight][b]) in soil					
	Zn		Cd		Cu	
	Control	Amended	Control	Amended	Control	Amended
4.5	53.1	307	0.45	4.3	13.4	143
5.1	58.0	330	0.37	3.1	19.5	154
6.3	52.9	354	0.36	3.6	14.3	153
7.0	54.0	345	0.31	3.7	14.2	157

[a] pH measurements are the means of three replicates with standard deviations of 0.1 U.

[b] All metal concentrations shown are the average of four replicates, with the SE not more than ±9%.

present in that well. All of the plate readings were carried out with a Multiscan MCC plate reader driven by Titretek II software.

The equivalent of 10 g (dry weight) of soil was added to 90 ml of sterile one-quarter-strength Ringer's solution. This slurry was shaken with a reciprocal shaker set at 200 oscillations min^{-1}. After shaking for 2 h, the flasks were allowed to stand for 3 min before a 10-ml sample of the suspension was removed and added to 90 ml of sterile one-quarter-strength Ringer's solution. Ringer's solution was used since this was found to maintain the pH of the extract at the pH of the soil. This was done so that both the effect of soil pH and the effect of Cu, Cd, or Zn at each pH value could be assessed. This extraction procedure removed suspended soil particles and reduced the amount of organic material which was carried over into the microtiter plate. The soil extract was then placed in a sterile tray with a magnetic stirrer. This kept the suspension homogeneous during the inoculation of the microtiter plates. In each case, three replicate plates were used. After inoculation, the plates were wrapped in plastic wrap and incubated at 20°C. Periodically, the plates were inspected for color development, and once purple dye formation was noted, the plates were scanned every 12 h.

Statistical methods. Measurements of soil microbial biomass and the average well color development (AWCD) are shown as the mean of three replicate measurements with standard error (SE) values in each case. Comparison of mean determinations to show significant differences was carried out by the Tukey method. Two-way analysis of variance was carried out to determine if there was a significant effect of pH and metal treatment on both microbial biomass and AWCD. Absorbance readings for the 95 separate substrates from each of the 48 plates used were analyzed by principal-component analysis with the statistical software Genstat, and analysis of variance was used to show the significance of soil pH and heavy metal treatment on the data set.

RESULTS

Soil microbial biomass. The measured biomass carbon values ranged from 24 to 90 μg of C (g of soil)$^{-1}$ (Fig. 1). Soil pH had no significant (Tukey honest significant difference, $P < 0.05$) negative effect on biomass values except in the soils amended with Cu. Soil amendment with Cd or Zn had no negative effect on biomass size. Biomass decreased with Cu as pH decreased but only resulted in biomass values significantly less than that of the control soils at the lowest soil pH of 4.5 (Fig. 1).

Substrate utilization patterns of microbial populations. Three replicate plates of each soil were used to check the reproducibility of the Biolog readings. A time point of 60 h of incubation was chosen since this gave the greatest differences in the response of the microbial extracts to the substrates. The choice of a single time point for analysis has been made in previous studies (15) and was used here to compare all of the treatments by principal component analysis and AWCD.

The first principal component accounted for 80% of the variation, with the second component accounting for 4.5%. The subsequent components accounted for <2.2% of the variation. Replicate microtiter plates gave similar patterns of substrate utilization (Fig. 2). Analysis of variance confirmed that the soil pH exerted more influence on the first principal component axis, whereas heavy-metal treatment had the most influence on the second principal component axis (Table 2).

The AWCD is the mean absorbance value for the plate at a given time point and showed gross differences in the response of the inocula to the array of substrates presented in the Biolog plate (Fig. 3). After 60 h of incubation, reduced substrate utilization was found in the plates inoculated with microorganisms taken from the lower-pH soils. Copper reduced microbial respiration, measured by AWCD, at all soil pHs. The Zn-amended soil extracts gave a reduction in the AWCD only at pHs of 4.5 and 5.1, and the Cd-amended soil extracts gave reduced AWCD only in the pH 5.1 soil (Fig. 3). Analysis of variance showed highly significant ($P < 0.001$) effects of pH and metal treatment and also a highly significant ($P < 0.001$) interaction between pH and metal treatment.

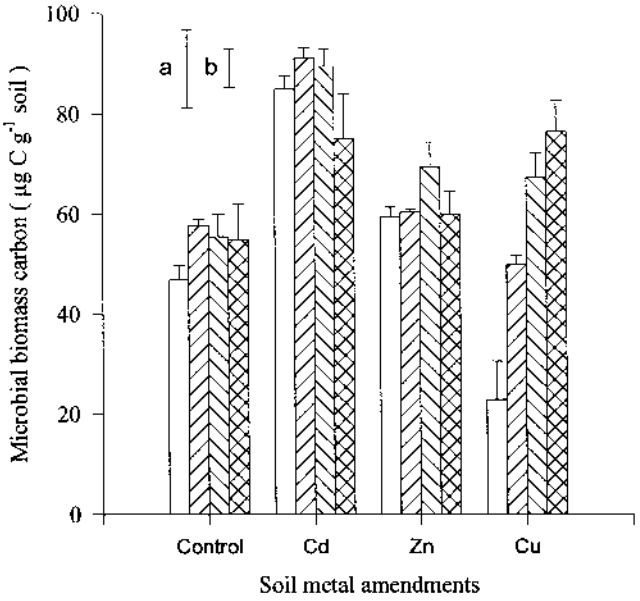

FIG. 1. Microbial biomass estimated by fumigation-extraction in the metal-amended soils. Individual error bars indicate SEs of the means. Vertical lines show significant difference (Tukey's honest significant difference, $P < 0.05$) between mean values across all the data (a) or within the same pH or metal amendment (b). Symbols: ▭, pH 4.5; ▨, pH 5.1; ▨, pH 6.3; ▩, pH 7.0.

The response of soil extracts to the substrates, taken as an average over the whole plate, was consistent (standard deviation in AWCD, <0.015 absorbance unit). However, replicate extracts did not give consistent profiles of the extent of use of individual substrates. For example, replicates of the pH 5.1 control soil gave a consistent picture of the types of substrate used, but the absorbance values recorded for the same substrates varied greatly (Fig. 4). Extracts from the pH 5.1 Cu-amended soil showed a large reduction in the number of substrates used (Fig. 5) compared with that of the control soil (Fig. 4). The variation in substrate utilization seemed to increase in the metal-amended soil with much less consistency in the type of substrates used in the replicate plates (Fig. 5).

DISCUSSION

The low values for biomass C were expected because of the 2-year incubation of the soils with no plant inputs. Copper at pH 4.5 was the only metal treatment to give a notable reduction in biomass. Copper is known to be very toxic to microorganisms in the free ionic form (16), and it has been demonstrated (10) that the proportion of the total soil Cu content found in the soil solution as free Cu^{2+} decreases as pH increases.

The apparent stimulation of biomass due to Cd is harder to

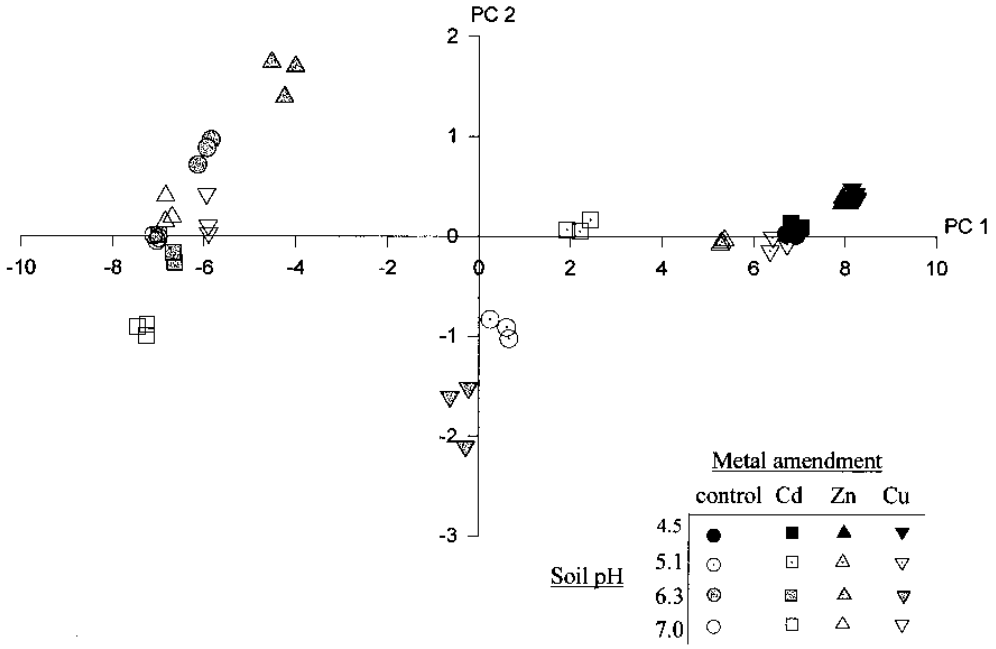

FIG. 2. Two-dimensional principal-component diagram of absorbance data from the Biolog plates inoculated with soil extracts after 60 h of incubation.

explain. Cadmium is not an essential element and so cannot have a direct positive influence on the soil microbes. There may be an indirect effect of Cd either on the availability of other essential micronutrients or as a legacy of some effect of Cd on the ryegrass crop which was grown on the metal-amended soils for 1 year after the metals were added. The yield of the ryegrass grown on the metal-amended soils was not significantly different from that of the control plots, but it was smaller in the pH 4.5 and 5.1 Cu-amended soils.

The use of Biolog microtiter plates relies on dehydrogenase activity as a measure of microbial activity with a single carbon source. Copper is known to interfere with measurements of dehydrogenase activity performed directly on soil (4). The extraction and dilution of the extract used here resulted in maximum possible Cu concentrations present in the Biolog wells which were below that found to cause reduction in dehydrogenase activity (4). Therefore, the differences in cellular activity noted by this method cannot be an artifact of Cu interference.

Haack et al. (8) reported that changes in the substrate use patterns for model bacterial communities were related to differences in inoculum density as well as differences in the microbial composition of the inoculum. In the Cu-treated soils, decreased activity, shown by less substrate utilization in the Biolog plates, was found in soils with smaller microbial biomass (Fig. 1 and 4). However, the Biolog results did not correspond to the biomass determinations made for the control or Zn- or Cd-amended soils. The AWCD (Fig. 3) and principal-component analyses (Table 2) both indicated that lower soil pH and metal amendment significantly reduced microbial activity and changed the pattern of substrates used. This effect of

TABLE 2. Analysis of variance of principal components against soil pH and metal treatment

Principal component	% Variation accounted for by axis	Variance ratio[a]		
		pH	Metal treatment	Interaction (pH × metal)
1	80.1	4,229*	1,041*	199*
2	4.5	32*	190*	64*

[a] Variance ratio of >5.0 means significant effect, $P < 0.001$ (*).

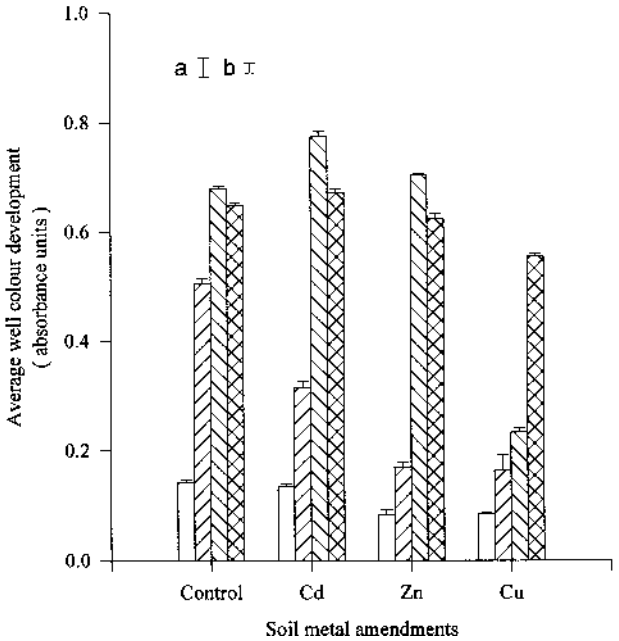

FIG. 3. Average well absorbance after 60 h of incubation of Biolog plates inoculated with extracts from soils with a range of pHs amended with Cd, Cu, or Zn. Individual error bars indicate SEs of the means. Vertical lines show significant difference (Tukey's honest significant difference, $P < 0.05$) between mean values across all data (a) or within the same pH or metal amendment (b). Symbols: ☐, pH 4.5; ▨, pH 5.1; ▧, pH 6.3; ▩, pH 7.0.

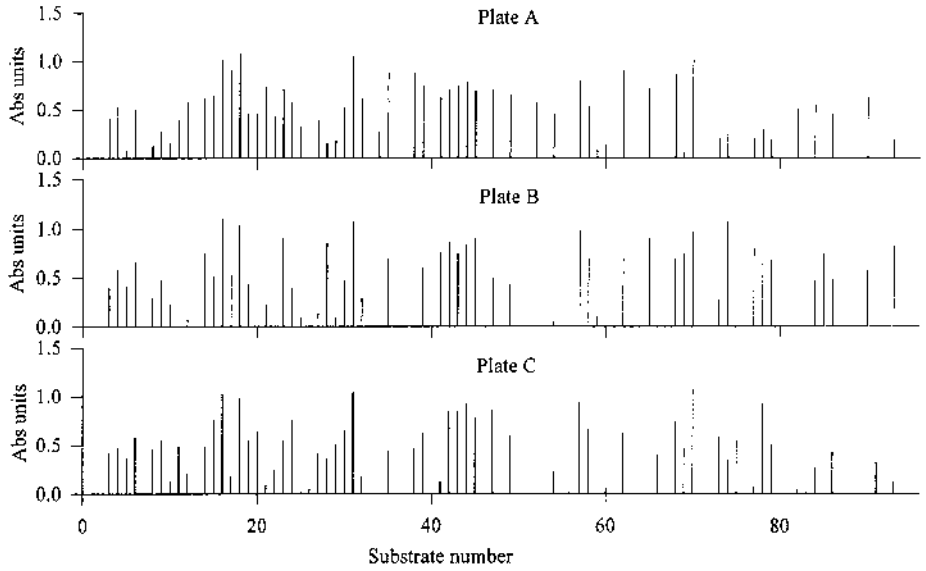

FIG. 4. Type and extent of substrates used in Biolog plates after 60 h of incubation with extracts from pH 5.1 unamended soil. Abs, absorbance.

soil pH and metal treatment was not seen in the measurements of microbial biomass except for the Cu-amended soil (Fig. 1).

Throughout the whole data set, soil pH had the dominant effect on the change in substrate utilization. However, there was a highly significant effect of metal treatment ($P < 0.001$) (Table 2). The strong interaction between pH and metal effect was expected since the solubility of heavy metals, and hence their effect on soil microorganisms, increases at lower pH conditions.

The composition and activity of a soil microbial community are known to be changed by environmental stress, such as low soil pH (1, 6). The results presented in this study indicate that both soil pH and heavy-metal amendment significantly change the pattern and extent of substrate use.

Previous workers have attributed reduced substrate use to reduced microbial diversity. However, extraction of a representative subset of the soil microflora is difficult to prove. In addition, techniques such as diversity indices and evenness scores rely on precise and reproducible measurements of color formation. In this study, the types of substrate used were consistent for replicates with high metabolic rates, but the extent of dye formation was variable (Fig. 4 and 5). In extracts with low metabolic rates, there was little or no reproducibility in terms of type or extent of substrate use (Fig. 5). It is equally obvious from Fig. 4 and 5 that there is a difference between the abilities of the soil extracts from two treatments to utilize the range of sole carbon sources.

The technique described here demonstrates that the activity of the microbial community has changed and that this reduction in carbon source utilization is most clear with decreased soil pH. However, there was also a significant reduction in substrate utilization in soils amended with Zn or Cu sulfate

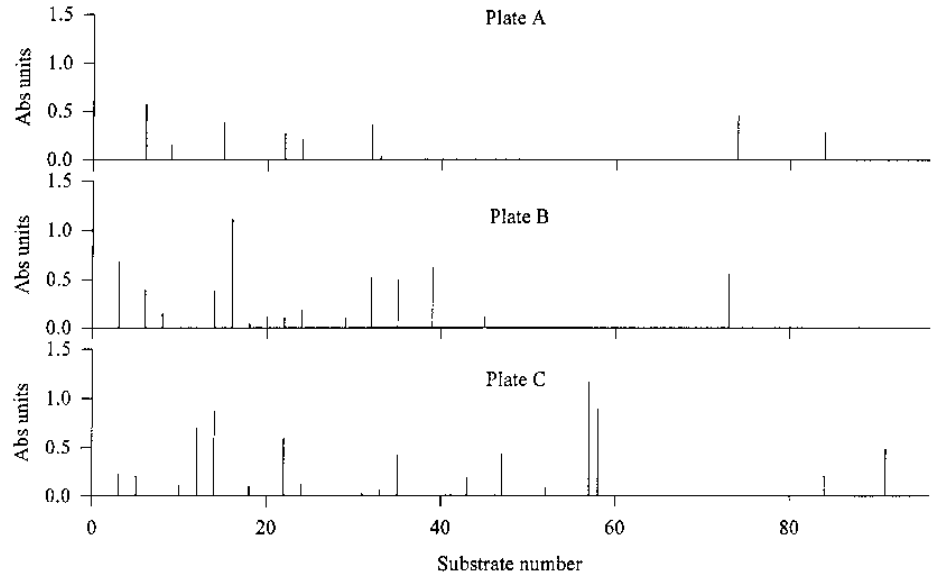

FIG. 5. Type and extent of substrates used in Biolog plates after 60 h of incubation with extracts from pH 5.1 soil amended with Cu. Abs, absorbance.

compared with that of control soils at pHs of 6.3 and 5.1. By comparison, the measurement of soil microbial biomass showed no significant reduction due to pH, and only the Cu treatment combined with the lowest soil pH resulted in a significantly smaller microbial biomass. The use of Biolog microtiter plates is rapid and could offer a fast screening technique to detect stressed populations in situations where an appropriate control soil is available.

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