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Impact of Carbon and Flooding on the Metabolic Diversity of Microbial Communities in Soils

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The assumption that carbon and soil water content are major determinants of microbial community structure and function is rarely questioned because of substantial evidence of the impacts of these variables on specific populations and functions. The significance of carbon and water for metabolic diversity at the microbial community level was tested on the field scale in agricultural plots varying in carbon inputs and in whether they were flooded. Surface soils in which rice straw was incorporated or burned and which were flooded or unflooded were sampled at monthly intervals three times during the flooded winter period (January to March) and again 1 month postdraining. Biomass carbon and nitrogen were not affected by treatments, active bacterial counts showed slight increases, and respiration rates were increased by carbon inputs and flooding. Biolog microplates were inoculated with soil extracts to quantify the metabolic diversity of the soil microbial community. Canonical correspondence analysis and the Monte Carlo permutation testing showed that differences in substrate utilization patterns were significantly related ($P < 0.001$) to carbon and flooding treatments. Biolog substrates whose metabolism was altered by the treatments were consistent across dates and tended to be positively related (utilization enhancement) to carbon inputs and negatively related to winter flooding. The importance of carbon as an environmental variable increased over time after straw treatment, whereas the importance of water became evident after flooding and decreased after drainage. The effect of long-term rice straw incorporation on substrate utilization patterns at another field site was consistent with these results despite the dissimilarities of the two soils.

Soil microorganisms live in complex communities and are responsible for the principal mineralization reactions that recycle important nutrients and degrade environmental pollutants. Changes in microbial communities resulting from agricultural practices, ecosystem management, and global change can have profound impacts on ecosystem dynamics. Interest in quantifying impacts on the biotic component of the soil ecosystem has increased with concern for the sustainability of our agricultural systems and as agricultural and industrial pollutants have become more prevalent in the environment.

In ecological studies, analyses of biological communities may consider community structure, e.g., species diversity, and/or function, e.g., the presence or rates of specific processes. The past decade has witnessed a virtual revolution in microbial ecology with the development of metabolism-based, biochemical, and molecular techniques for directly characterizing the structure and function of microbial communities without requiring isolation and culturing of individual species. Biochemical methods can help characterize microbial community structure on the basis of the cell constituents of its members, such as phospholipid fatty acids (3, 19, 24); however, these methods are highly complex and currently limited to only a few laboratories. Molecular approaches can be used to explore both community structure through analysis of rRNA and function through hybridization to specific catabolic probes. In order to relate community patterns back to known organisms or processes, both phospholipid fatty acid analysis and most molecular approaches still require culture-based information to develop the data bases needed for these interpretations. Whole community DNA hybridization can provide information about the relative similarity and complexity in the genetic diversity of microbial communities (8); however, this method is complex and may generate information that is difficult to interpret when applied to soils (10). An alternative and relatively simple method that characterizes community function based on net sole-carbon source utilization patterns is Biolog analysis.

Because sole-carbon-source utilization is so important in classifying isolates of bacteria, Garland and Mills (4) proposed that the same approach could be used as a functionally based measure for classifying heterotrophic microbial communities. Garland found that microplates containing 95 different carbon sources, which were prepared by Biolog, Inc., for isolate identification, were capable of separating the microbial communities of a variety of soil and water samples on the basis of substrate utilization patterns. Zak et al. (23) used these microplates to assess the functional diversity of microbial populations associated with six plant communities. More recently, Haack et al. (6) investigated the factors affecting substrate utilization patterns and found the patterns to be highly reproducible for model bacterial communities. An important advantage of the Biolog method is its ease of use and thus feasibility for use in large-scale field studies. However, this method requires that organisms are metabolically active in solution culture under conditions that are quite different from those in the environment and, therefore, cannot be expected to reflect the metabolic capabilities of the entire soil community.

The availability of carbon and water strongly governs the activities of specific microbial populations and functions. A unique opportunity to test the importance of these factors for microbial community diversity at the field scale was provided by a study of rice straw decomposition in northern California. This multidisciplinary, replicated field experiment addresses the concerns of farmers who are required to phase out the

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TABLE 1. Soil water content over time

Date	Soil water content (%)[a]	
	No flood	Flood
Jan. 3	44 (2.8)	58 (4.0)
Feb. 2	44 (3.6)	54 (3.4)
Mar. 7	42 (3.6)	54 (3.8)
Mar. 30	31 (2.8)	33 (2.0)

[a] Values are means of burned and incorporated plots (with standard deviations in parentheses).

traditional practice of burning rice straw because of the negative impacts of combustion products on air quality. Interest in providing habitat for migrating birds by flooding rice fields during winter months has also been integrated into the field study. Various management practices, differing in their treatment of rice straw and flooding, on a commercial rice farm in Maxwell, Calif., are being compared. Field plots have gradients of carbon availability and water content and so provide an ideal experiment for testing the impacts of these variables on microbial communities.

The objective of this study was to quantify seasonal differences in microbial communities, through the use of Biolog microplates, in soils receiving different inputs of carbon and water. Additional data on microbial population densities and process rates in the same soils were collected. Previous studies employing Biolog analyses of soil communities in microcosms or on nonreplicated field samples have been performed. No other statistically based field study by this method exists. Additionally, this is the first study to apply canonical correspondence analysis (CCA) (15, 16) to Biolog substrate utilization data. This technique, which has been developed for vegetation community ecology, is a multivariate method that reveals relationships among species abundances and environmental variables.

MATERIALS AND METHODS

Field study and sampling. A 75-acre field study of rice straw incorporation methods and winter flooding effects was initiated in October 1993 on a commercial farm near Maxwell in Colusa County, Calif. It was a randomized complete block experiment with split plots; two water treatments were used as the main plots (winter flood or no flood), and two rice straw management methods were used as split plots (burned or incorporated). All treatments were replicated four times. Rice was harvested on 3 to 7 October 1993, and straw was either incorporated (6 to 8 November) or burned (15 November). Flood water was applied on 22 November and was maintained at a depth of 5 to 15 cm until it was drained on 7 March 1994. The soil at the Maxwell site is Willows clay with a pH of 6.8. Representative surface soil samples (0 to 15 cm) were taken by pooling 20 cores from a 100-m transect in each plot in early winter (3 January), midwinter (2 February), predraining (7 March), and 1 month postdraining (30 March). Incorporated plots received an average of 3,278 kg of carbon per ha in the form of mature, dry rice straw, while burned plots received an average of 762 kg of carbon per ha from rice straw which remained as stubble after burning. Soil moisture contents in flooded and unflooded plots were, on average, 55 and 43% (equivalent to matric potentials of 0 and 0.03 MPa), respectively, during the flooded period (Table 1). One month after draining, moisture contents were 33% in previously flooded plots and 31% in unflooded plots, or approximately 0.10 MPa for both treatments.

A long-term experiment on a commercial farm near Pleasant Grove in Sutter County, Calif., also measured the effects of rice straw incorporation on the rice production system. The soil type, a loam with a pH of 4.5, was quite different from that at the Maxwell site. In addition, there were no flooded treatments. Burned and incorporated rice straw treatments were started in October 1988 and were applied each fall to the same plots through 1993. The soils were sampled on 22 March. Twenty cores taken from throughout the 0.25-ha basins were pooled to obtain a representative surface soil (0- to 15-cm) sample.

Microbial analysis. All samples were kept on ice after collection and were stored without disturbance at 4°C until analysis. Storage time was less than 7 days for all analyses except fumigation extraction, which was completed within 14 days. Soil pH was measured in a 2:1 dilution of 0.01 M $CaCl_2$-soil. Substrate-induced respiration (with glucose as the substrate) and basal respiration were measured by determining CO_2 production within 2 h in 2:1 water-soil slurries (20) of three 5-g subsamples from each plot. Biomass carbon and nitrogen values were determined by fumigation extraction (1, 18) of three 20-g subsamples. Carbon and nitrogen were analyzed by a Shimadzu carbon analyzer (22) and by ninhydrin-reactive nitrogen (2), respectively. Three 10-fold dilution series were made for samples from each plot, starting with 1 g of moist soil in 9 ml of 0.05 M phosphate buffer as the lowest dilution. The 10^{-1} dilution was shaken for 5 min in a 120° arc at 60 rpm. Counts of fluorescein diacetate (FDA)-active bacteria and fungal hypha length were made with the 10^{-1} dilution according to the method described by Ingham and Klein (7).

Biolog analysis. The metabolic diversity of the soil communities was analyzed with gram-negative microplates (Biolog, Inc.). A preliminary study of the effects of dilution on Biolog gram-negative microplate readings showed that with Willows clay soil, a dilution of 10^{-3} gave the most desirable results. Lower dilutions contained too much clay that interfered with readings, and higher dilutions resulted in increased variability in substrate utilization among replicate microplates. To avoid potential losses of cells attached to clay particles, soil material was not removed by centrifugation prior to inoculating Biolog plates. The plates were incubated at 25°C. Inoculum density varied little across treatments (Table 2) and therefore had little, if any, impact on overall well color development. This assumption was tested by including inoculum density as an environmental variable in CCA of all Biolog data; in all cases, inoculum density was not significant in explaining observed changes in substrate utilization patterns.

A_{595} levels in each well were read with a microplate reader (Molecular Devices, Inc.) equipped with SOFTmax software (version 2.01). Biolog plates from the 2 February sampling date were read at frequent intervals in order to generate a relationship between average well color development and time (data not shown). On the basis of this relationship, 50 h was chosen as an optimum time to compare treatments, because average well color development was increasing rapidly (linear phase). Analysis of substrate utilization at different time point readings produced the same results with respect to significance of carbon inputs and flooding as environmental variables, although only 25 to 30% of the specific substrates involved in separating treatment plots remained the same throughout a 33- to 69-h incubation time period.

Statistical analysis. Biolog results were analyzed with CANOCO software from Microcomputer Power, Inc. (Ithaca, N.Y.). Actual absorbance values after correction of the absorbance from the control well, rather than positive-negative binary responses, were used in the analysis. Each substrate value for a plot was the average for three replicate plates prepared from dilutions of separate subsamples of soil. For analysis by CANOCO, the 95 Biolog substrates were considered individual species. All negative absorbance values were set to zero. To avoid excessive influence of rarely utilized substrates, any substrates that exhibited positive absorbances in fewer than five field plots were not included in the analysis.

CCA was used to analyze the data. CCA differs from other ordination techniques, such as principal-component analysis, correspondence analysis, or discriminant analysis, in that it allows direct analysis of the effect of specific environmental variables of interest because ordination axes are constrained to be linear combinations of environmental variables (15). All measured soil parameters (Tables 1 through 6) were tested as environmental variables for significant contribution to observed changes in substrate utilization patterns with the forward selection subroutine available in CANOCO. The statistical validity of the association between environmental variables and variance in Biolog substrate data was tested by the Monte Carlo permutation test (14). The influence of spatial position in the field on results was accounted for in CCA by including field blocks as a covariable in all analyses.

Biolog data from the three sample dates falling within the time of winter flooding (3 January, 2 February, and 7 March) were analyzed as a composite datum set, and Biolog data for all treatments were also analyzed by date to investigate changes in treatment effects over time. All replications and all sam-

TABLE 2. Biolog gram-negative microplate average inoculum densities for each sample date at both sites

Site and date	Inoculum density (FDA-active cells/ml [10^5])[a]
Maxwell	
Jan. 3	1.03 (0.11)
Feb. 2	0.75 (0.09)
Mar. 7	0.46 (0.10)
Mar. 30	1.09 (0.15)
Pleasant grove	
Mar. 22	1.02 (0.14)

[a] Values are means of 16 treatment plots for Maxwell samples and 10 treatment plots for Pleasant Grove samples (standard deviations in parentheses).

TABLE 3. FDA-active bacteria over time

Date	No. of FDA-active bacteria (cells [10^8]/g of soil)[a]			
	No flooding	Flooding	Burning	Incorporation
Jan. 3	1.44 (0.15)	1.67[b] (0.17)	1.47 (0.14)	1.65[c] (0.20)
Feb. 2	1.08 (0.14)	1.17 (0.14)	1.07 (0.15)	1.17 (0.13)
Mar. 7	0.61 (0.14)	0.76 (0.17)	0.62 (0.10)	0.75 (0.15)
Mar. 30	1.33 (0.08)	1.56 (0.26)	1.36 (0.11)	1.53[c] (0.27)

[a] Values are means of eight treatment plots (standard deviations in parentheses). Grams of soil on an oven-dryed-weight basis.
[b] Significant difference between flooded and nonflooded treatments ($P < 0.05$).
[c] Significant difference between burned and incorporated treatments ($P < 0.05$).

ples were included in the analysis, with the exception of one, a burned, unflooded sample, which was dropped from the 30 March sampling date because of high standard deviations among substrate absorbance readings on replicate Biolog plates. Biolog results from late March at the two different field sites were also compared.

CCA results are displayed on biplots in which relationships among environmental variables and either treatment plots or Biolog substrates are displayed. In CCA, as in other ordination techniques, the positions of the treatment plots on the diagram are based on the weighted mean substrate utilization scores for each plot. The distribution of the treatment plots reveals patterns of association between plots and the environmental variables. On CCA biplots, the influence of environmental variables is indicated by arrows whose directions describe the directions of the environmental gradients and whose lengths are proportional to their importance. Environmental variables with long arrows are more strongly correlated with the ordination axes than those with short arrows and, thus, are more closely related to the pattern of community variation shown in the ordination diagram (16). Polygons surrounding like treatment plots were drawn on biplots to facilitate interpretation.

To interpret the substrate-environment biplot, ter Braak (17) recommends that each arrow representing an environmental variable be considered an axis in the diagram. The position of each substrate point projected onto an environmental gradient axis is based on the weighted average of the substrate score with respect to that environmental variable and, therefore, shows its approximate correlation to that environmental axis (9, 13). The positions of individual substrates on environmental axes are given directly by CANOCO. Axis positions of $\geq|0.20|$ for the composite analysis or $\geq|0.25|$ on individual dates were used to identify substrates most affected by each environmental variable. These threshold values allowed us to identify between 20 and 34 substrates (21 to 36% of the total substrates) which were most important in distinguishing microbial communities.

RESULTS

Soil parameters and microbial analysis. Rice straw incorporation of approximately 3,300 kg of C per ha was associated with FDA-active bacterial counts that were higher than those with burned plots, with differences being significant on 3 January and 30 March (Table 3). Respiration rates were even more sensitive to treatment effects (Table 4), showing significant increases due to straw incorporation on 3 January, 2 February, and 7 March. There was a dramatic drop in the respiration rates in all plots on the 30 March sample date,

TABLE 4. Respiration rates over time

Date	Respiration rate (μg of CO_2/g of soil/h)[a]			
	No flooding	Flooding	Burning	Incorporation
Jan. 3	5.19 (1.39)	10.74[b] (4.12)	6.34 (2.72)	9.59[b] (4.80)
Feb. 2	5.56 (1.41)	21.16[c] (9.67)	10.48 (6.12)	16.23[c] (13.44)
Mar. 7	7.80 (5.10)	25.71[b] (13.59)	11.00 (6.40)	22.51[c] (17.14)
Mar. 30	0.29 (0.29)	1.23 (1.93)	0.22 (0.28)	1.30 (1.89)

[a] Values are means of eight treatment plots (standard deviations in parentheses). Grams of soil on an oven-dryed-weight basis.
[b] Significant difference between flooded and unflooded or burned and incorporated treatments ($P < 0.05$).
[c] Significant difference between flooded and unflooded or burned and incorporated treatments ($P < 0.01$).

TABLE 5. Soil pH over time

Date	Soil pH[a]			
	No flooding	Flooding	Burning	Incorporation
Jan. 3	6.96 (0.29)	6.63[b] (0.28)	6.63 (0.32)	6.96[c] (0.24)
Feb. 2	6.77 (0.25)	6.61[c] (0.21)	6.57 (0.16)	6.81[d] (0.25)
Mar. 7	6.82 (0.17)	6.64[c] (0.26)	6.56 (0.30)	6.82 (0.18)
Mar. 30	6.82 (0.36)	6.63 (0.33)	6.53 (0.31)	6.92[b] (0.26)

[a] Values are means of eight treatment plots (standard deviations in parentheses).
[b] Significant difference between flooded and unflooded or burned and incorporated treatments ($P < 0.001$).
[c] Significant difference between flooded and unflooded or burned and incorporated treatments ($P < 0.05$).
[d] Significant difference between burned and incorporated treatments ($P < 0.01$).

following drainage of the flooded plots. This phenomenon is thus far unexplained and was not reflected in other measurements of the microbial community. Straw incorporation also resulted in consistently higher surface soil pH, which averaged 6.9 over all sample dates in incorporated plots, while burned plots averaged 6.6 (Table 5).

Soil water contents during the flooded period were approximately 10% higher in flooded plots than in nonflooded plots (Table 1). FDA-active bacterial counts were higher in flooded than in nonflooded plots; however, this difference was significant ($P < 0.05$) only on 3 January. A decrease in FDA-active hyphal lengths after 7 March flooding suggested depressed fungal activity; however, the high variability of these numbers did not permit further conclusions (Table 6). Significant increases were seen in respiration after flooding (Table 4) on all three sample dates during the flooded period. Flooding resulted in a decrease in pH from an average of 6.9 in unflooded plots to 6.6 in flooded plots during flooded sample dates. There was no difference in either microbial biomass carbon, microbial biomass nitrogen, or substrate-induced respiration among any of the treatments (data not shown).

Analysis of substrate utilization data from flooded sample dates. CCA of the combined data from sample dates within the flooded period is able to separate all treatment plots clearly on the basis of soil moisture, carbon inputs, and sample date as environmental variables (Fig. 1). Carbon inputs and soil moisture are highly significant ($P = 0.001$) in terms of explaining variation in substrate utilization data. The date of sampling is also highly significant but less important ($P = 0.003$). Variation due to the date of sampling is associated mainly with the third ordination axis. Its minor influence on the biplot of axes 1 and 2 (Fig. 1) is evidenced by the short arrow representing dates. All other soil parameters, including microbial biomass C and N, respiration rates, substrate-induced respiration, pH, FDA-active bacterial numbers, and FDA-active hyphal lengths, are not significant.

TABLE 6. FDA-active hyphal length over time

Date	FDA-active hyphal length (m/g of soil)[a]			
	No flooding	Flooding	Burning	Incorporation
Jan. 3	19.90 (4.98)	24.3 (5.87)	19.87 (4.61)	24.33 (6.14)
Feb. 2	5.25 (1.63)	4.73 (1.94)	4.44 (2.03)	5.54 (1.32)
Mar. 7	2.20 (1.31)	0.25[b] (0.32)	0.72 (0.68)	1.73 (1.74)
Mar. 30	13.32 (3.09)	14.01 (3.04)	13.38 (3.44)	13.95 (2.66)

[a] Values are means of eight treatment plots (standard deviations in parentheses). Grams of soil on an oven-dryed-weight basis.
[b] Significant difference between flooded and unflooded treatments ($P < 0.05$).

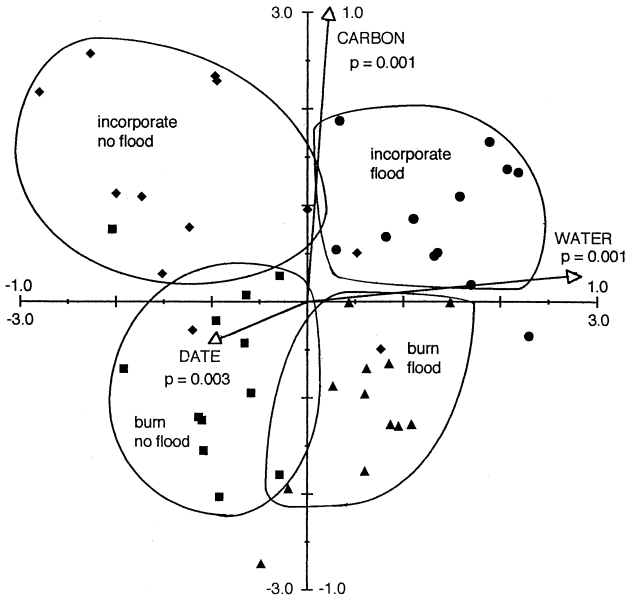


FIG. 1. CCA ordination biplot of treatment plot scores and significant environmental variables for the sample dates 3 January, 2 February, and 7 March from the Maxwell site. Treatment plots were as follows: ◆, straw incorporated, with no flooding; ●, straw incorporated, with winter flooding; ■, straw burned, with no flooding; ▲, straw burned, with winter flooding. Arrows indicate the directions and relative importance (arrow lengths) of the three environmental variables. CARBON, carbon inputs; WATER, soil water content; and DATE, sample date. The 1.0 scale refers to the environmental variables, and the 3.0 scale refers to treatment plot scores.

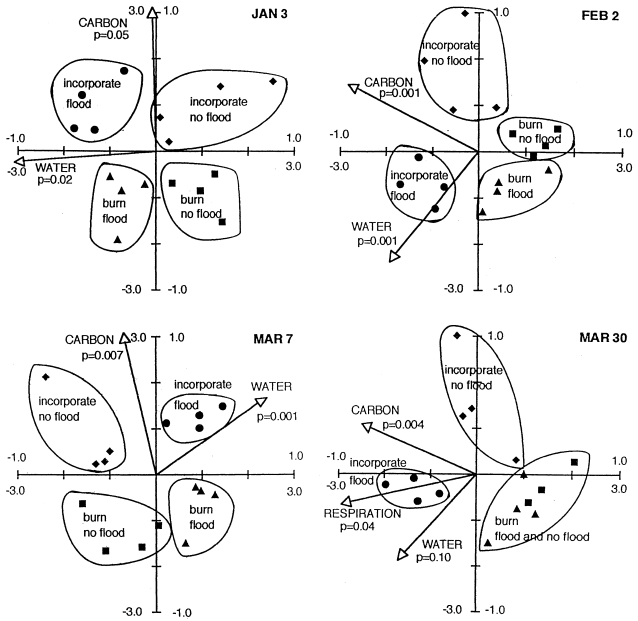


FIG. 3. CCA ordination biplot of treatment plot scores and significant environmental variables for sample dates 3 January, 2 February, 7 March, and 30 March from the Maxwell site. Treatment plots were as follows: ◆, straw incorporated, with no flooding; ●, straw incorporated, with winter flooding; ■, straw burned, with no flooding; ▲, straw burned, with winter flooding. Arrows indicate the directions and relative importance (arrow lengths) of the three environmental variables. CARBON, carbon inputs; WATER, soil water content; and RESPIRATION, soil basal respiration rates. The 1.0 scale refers to the environmental variables, and the 3.0 scale refers to treatment plot scores.

To identify the substrates which contribute to the separation of treatment plots on the ordination biplot (Fig. 1), the relationship of substrate utilization scores to environmental variables must be examined. A biplot of substrate scores (Fig. 2)

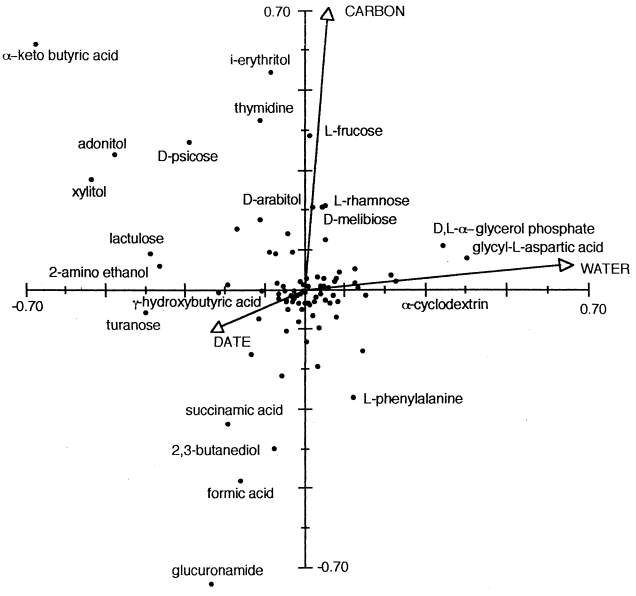


FIG. 2. CCA ordination biplot of Biolog substrates and environmental variables for sample dates 3 January, 2 February, and 7 March from the Maxwell site. Arrows indicate the directions and relative importance (arrow lengths) of the three environmental variables. CARBON, carbon inputs; WATER, soil water content; and DATE, sample date. Substrates with approximate correlations of ≥0.20 to either carbon inputs or soil water contents are labelled.

generated from the CCA depicted in Fig. 1 illustrates the distribution of substrate utilization data. The cloud of points in the center of the plot represents substrates which do not contribute to the variability in samples along these axes. From Fig. 2, it is possible to identify 22 substrates which are the most important in separating plots along the environmental axes. In this set of 22 substrates, eight carbohydrates, α-ketobutyric acid, and thymidine were utilized faster in rice straw-incorporated plots. Two amides, succinamic acid and glucuronamide, one amino acid, one alcohol, and one carboxylic acid were utilized more slowly in incorporated than in burned plots. Most substrates which separate plots by winter flooding were more slowly utilized by communities from flooded plots. These substrates include five carbohydrates, two carboxylic acids, an amide, and an amine. A polymer, an amino acid, and a phosphorylated chemical are all positively related to the flooding treatment.

Seasonal changes in substrate utilization patterns. There is excellent separation of treatments by CCA with water and carbon environmental variables on all sample dates (Fig. 3). A shift in the importance of the variables was seen over time as field conditions changed. On 3 January (Fig. 3), carbon and water are significant in explaining variations in the data (P = 0.02 and 0.05, respectively). On 2 February, both carbon and water variables are highly significant (P = 0.001), and the trend continues through 7 March. One month after flooding, on 30 March, carbon is still a highly significant variable (P = 0.004) and the soil respiration rate is also significant (P = 0.04). Water is only marginally significant (P = 0.10) at that time, because by this date there was no longer a flooded treatment and there was little difference among any treatments in soil water content. A biplot with these three variables (Fig. 3)

shows good separation between incorporated and burned samples; however, separation between flooded and nonflooded treatments disappeared in the burned plots. This is the only sample date for which a variable (i.e., respiration) other than carbon and soil water explains significant variation in the substrate utilization patterns. The importance of respiration may have become more evident as the importance of soil water content was diminished after drainage.

The particular substrates which are associated with the environmental gradients varied over the season (Fig. 4). On 3 January, high water contents in soil due to flooding were associated with less utilization of most substrates that contributed to the separation along the water axis. However, by 2 February, the metabolisms of certain amino acids, as well as of uridine, 2-3-butanediol, and D,L-α-glycerol phosphate, were enhanced in flooded plots. On 7 March, several carbohydrates were less utilized in flooded plots, but negative effects on carboxylic acid metabolism were no longer evident. High carbon inputs were associated with apparently faster utilization of certain carbohydrates and with slower utilization of carboxylic acids on all sample dates. Thymidine also showed a consistently positive response to carbon inputs. The responses of certain amides and amino acids were negatively associated with high carbon inputs, except on 30 March, when a number of additional substrates were related to separation of treatment plots along the carbon input axis. In addition to the set of substrates that change over time, there was a core group of important substrates reflected in the composite analysis which were consistent across dates (Fig. 2).

Comparison of substrate utilization data from the Maxwell and Pleasant Grove sites. The long-term experiment in Pleasant Grove, Calif., differed from the Maxwell experiment in that the soil type was an acid loam rather than a neutral clay, there were no flooded treatments, and rice straw incorporation had been repeated each fall from 1988 through 1993. CCA of substrate utilization data from this site indicated that straw incorporation is the only significant measured variable. A biplot (Fig. 5) shows separation along a significant axis ($P = 0.01$) which is linearly related to carbon inputs. All 10 carbohydrates which are positively related to carbon inputs at this site (Fig. 6) are also important in separating treatment plots along the carbon axis at the Maxwell site (Fig. 2). Of the 17 important substrates, only 2 (L-threonine and thymidine) show a negative relationship with straw additions. Because similar substrates are responsible for separating straw incorporated from straw burned plots at both experimental sites, the CCA of substrate utilization patterns from a combination of the two soils (Fig. 7) is able to separate plots subjected to different straw management along one highly significant carbon input axis ($P = 0.001$). The two soil types are also clearly separated from one another by differences in their Biolog substrate utilization patterns.

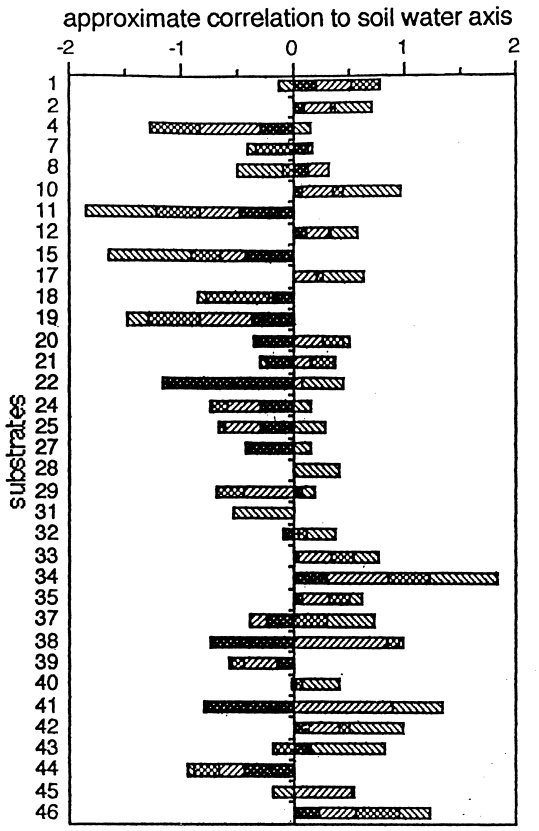
DISCUSSION

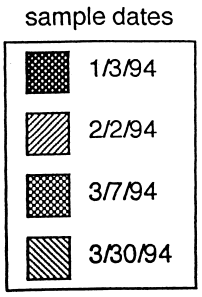
The metabolic diversity of the microbial community, as measured by Biolog analysis, was strongly impacted by field level carbon inputs and flooding. Treatment effects were significant irrespective of incubation time and were evident not only within independent sample dates but also when data from different sample dates were combined over a 3-month period. During the period of winter flooding, the sample date had less effect on substrate utilization patterns compared with the larger effects associated with the treatments. In contrast to the Biolog results, soil microbial biomass carbon and nitrogen were insensitive to carbon and water inputs. This is consistent with the results of Ritz et al. (11), who found that a single season's straw incorporation did not contribute to a sustained increase in microbial biomass. They suggested several years of straw incorporation are needed to change soil microbial biomass. A similar phenomenon was observed in agricultural fields in California, where consistent significantly higher microbial biomass carbon and nitrogen in organic rather than conventionally managed soils resulted only after the 3rd year of cover crop inputs (12).

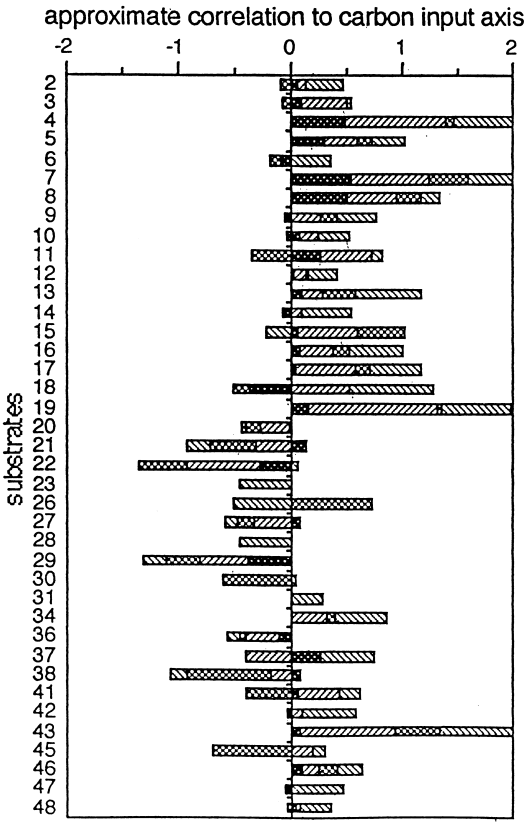
In the Biolog analysis, enhanced utilization of several carbohydrates was consistently associated with high carbon inputs at the Maxwell site. This result was repeatedly observed across sample dates and was confirmed on a very different soil type at the Pleasant Grove site. On the basis of substrate utilization patterns from late-March samples from both Maxwell and Pleasant Grove, rice straw inputs were generally associated with enhanced utilization of many of the substrates in Biolog plates (Fig. 4 and 6) and were rarely associated with less utilization of substrates. Since inoculum density was consistent among all samples, it is likely that the differences observed are due to changes in the microbial community composition. This result implies that microbial communities in these soils are, as is commonly presumed, limited by carbon, and that, thus, the metabolic diversity of the community was enhanced through high carbon inputs. This conclusion is consistent with field measurements of rice straw decomposition in which mesh bags were buried in all plots. Straw was found to degrade faster in straw-incorporated plots at both sites (data not shown), indicating increased activity of the microbial communities in incorporated plots.

Less utilization of several carbohydrates and carboxylic acids in the Biolog plates was associated with high soil moisture levels resulting from flooding. Although metabolism of some amino acids was enhanced in February and March, a general trend of reduced utilization of specific substrates under flooded conditions was seen (Fig. 2). A decrease in the metabolic potential of the community would be expected from flooding of soils, which leads to a reduction in the availability of suitable electron acceptors, resulting in lower rates of organic matter decomposition. However, in concert with the apparent decrease in metabolic diversity were increased respiration and numbers of active bacteria and breakdown of straw associated with flooded conditions. These increases may have resulted from other possible effects of flooding such as increased temperature buffering and increased physical contact between microorganisms and the straw under flooded conditions. In addition, Biolog plates measure aerobic respiratory activity-metabolic potential and growth, which can be expected to decline under oxygen-limited conditions. Obligate anaerobic bacteria, whose numbers and activity are likely to increase under flooded conditions, would not be detected by this assay.

Inoculum density has been demonstrated to be an important confounding variable in the interpretation of Biolog substrate utilization patterns (4, 6, 21). Canonical correspondence analysis demonstrated that the small population density differences (Table 2) observed in our study did not explain differences in substrate utilization patterns. Techniques to compensate for differences in initial density include division by average well color development for the whole plate (4) or use of a relative threshold, based on the highest absorbance for the plate, as a criterion for determining positive responses (6). Both techniques are potentially biased, because the amounts of carbon in the wells differ and, thus, when metabolized, result in different color endpoints. However, these correction methods may be useful if differences among treatments are quite sub-







SUBSTRATES

polymers

1 α-cyclodextrin
2 glycogen

carbohydrates

3 N-acetyl-D-galactosamine
4 adonitol
5 D-arabitol
6 cellobiose
7 i-erythritol
8 L-frucose
9 gentiobiose
10 α-D-lactose
11 lactulose
12 maltose
13 D-melibiose
14 β-methyl-D-glucoside
15 D-piscose
16 D-raffinose
17 L-rhamnose
18 turanoe
19 xylitol

esters

20 mono-methyl-succinate

carboxylic acids

21 acetic acid
22 formic acid
23 α-hydroxybutyric acid
24 γ-hydroxybutyric acid
25 itaconic acid
26 α-keto butyric acid
27 propionic acid
28 sebacic acid

amides

29 succinamic acid
30 glucuronamide
31 alaninamide

amino acids

32 D-alanine
33 L-alanyl-glycine
34 glycyl-L-aspartic acid
35 glycyl-L-glutamic acid
36 L-leucine
37 L-ornithine
38 L-phenylalenine
39 L-proline
40 D-serine
41 L-threonine

aromatics

42 uridine
43 thymidine

amines

44 2-amino ethanol

alcohols

45 2-3 butanediol

phosphorylated chemicals

46 D,L-α-glycerol phosphate
47 glucose-1-phosphate
48 glucose-6-phosphate

FIG. 4. Substrates which are important in separating treatment plots (approximate correlations of ≥ 0.25) and their positions on carbon input and soil water axes for each sample date. Substrate scores are based on separate CCA for each sample date.

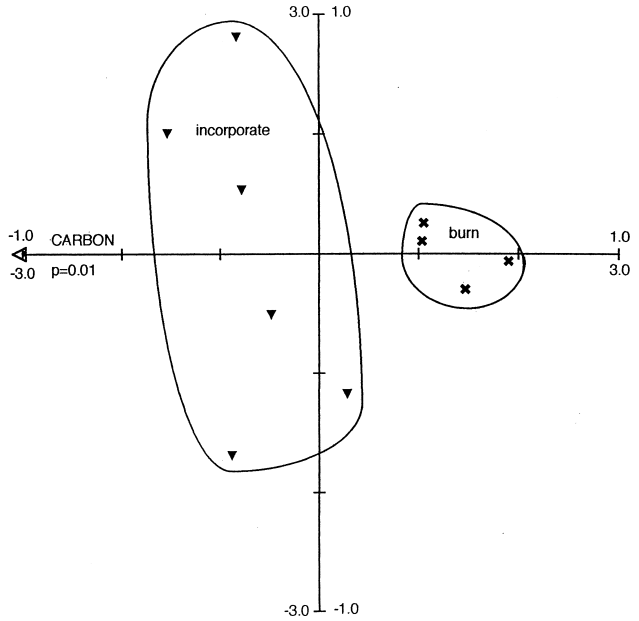


FIG. 5. CCA ordination biplot of treatment plot scores and the carbon input environmental variable for 22 March samples from the Pleasant Grove site. Treatment plots were as follows: ▼, straw incorporated; ✕, straw burned. The 1.0 scale refers to the environmental variables, and the 3.0 scale refers to treatment plot scores.

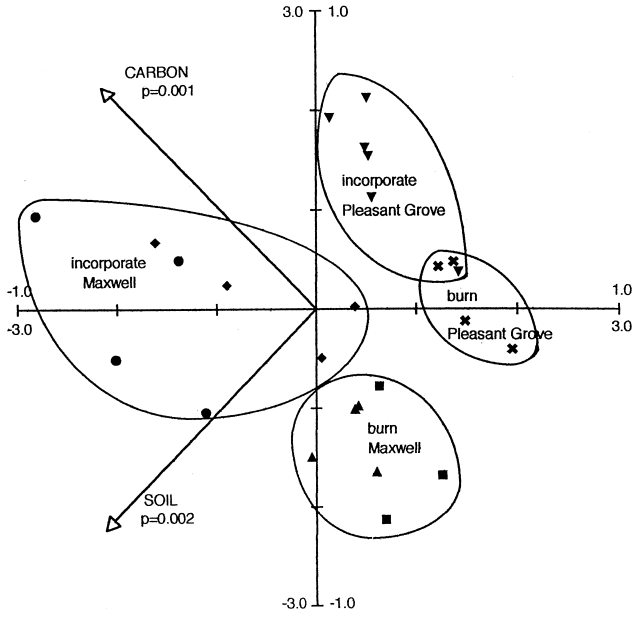


FIG. 7. CCA ordination biplot of treatment plot scores and significant environmental variables for the 30 March sample from the Maxwell site and for the 22 March sample from the Pleasant Grove site. Treatment plots from the Maxwell site are as follows: ◆, straw incorporated, with no flooding; ●, straw incorporated, with winter flooding; ■, straw burned, with no flooding; ▲, straw burned, with winter flooding. Plots from the Pleasant Grove site are as follows: ▼, straw incorporated; ✕, straw burned. Arrows indicate the directions and relative importance (arrow lengths) of the significant environmental variables. CARBON, carbon inputs; and SOIL, soil type. The 1.0 scale refers to the environmental variables, and the 3.0 scale refers to treatment plot scores.

stantial, which may occur in studies comparing distinctly different communities.

There are a number of statistical approaches for evaluating multivariate data such as Biolog substrate utilization patterns. Obviously, the particular objectives of a study will determine whether it is more appropriate to use a classification technique, such as cluster analysis, to place similar samples into discrete groups, or an ordination technique, such as CCA, to discriminate differences along a gradient. In our study, in which various treatments were imposed on a single microbial community, instead of comparisons of distinctly different communities being made, ordination analysis was the more useful approach. Application of a statistical approach, such as CCA, which was originally developed for evaluating environmental effects on plant community structure, was very fruitful in this study because we wanted to quantify the strength of association between microbial community properties and specific environmental variables. CCA was particularly powerful because it took advantage of the randomized complete block experimental design employed in this study. Other ordination and classification techniques are unable to do so.

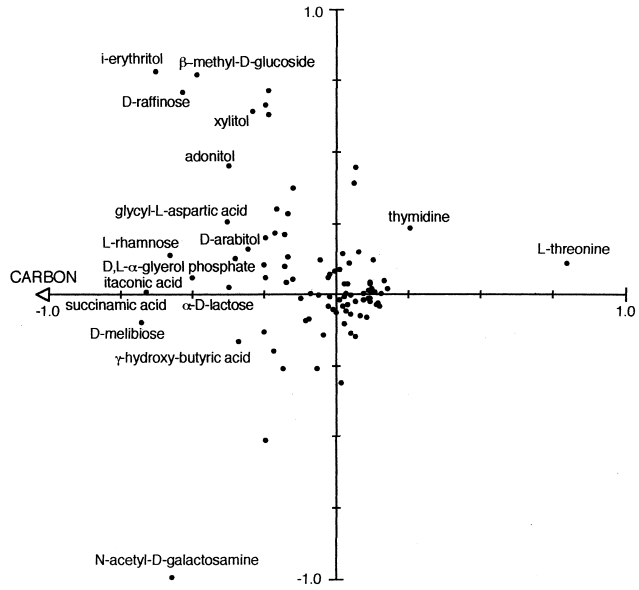


FIG. 6. CCA ordination biplot of Biolog substrates and environmental variables for 22 March sample dates from the Pleasant Grove site. Arrows indicate the directions and relative importance (arrow lengths) of the environmental variables. CARBON, carbon inputs. Substrates with approximate correlations of ≥0.25 to the carbon input axis are labelled.

Interpretation of Biolog analysis of microbial communities and of the relevance of Biolog patterns to ecological questions is still problematic. The significance of changes in utilization of specific substrates is difficult to interpret without a better understanding of how the interactions among the organisms constituting a community affect substrate utilization patterns. Haack et al. (6) listed a number of problems with the application of Biolog patterns, including the dependence of the patterns on inoculum density and the facts that different microbes using the same substrate may use them to different extents and that summation of the individuals does not predict the collective kinetic response of those individuals. They also demonstrated, as was suggested by Garland and Mills (4) and Zak et al. (23), that certain bacterial strains are unable to oxidize Biolog substrates and that their presence thus cannot be detected. The existence of organisms that are unable to metabolize substrates on Biolog plates, many of which are likely to be present in soils, will complicate any attempt to extrapolate

from community Biolog profiles to in situ community metabolic capacity (6). Nonetheless, the present study and others (4–6, 21) found Biolog patterns for specific communities to be highly reproducible.

It is tempting to try to make sense of the rich datum set which is the foundation of the Biolog patterns. However, we found that only 25 to 30% of the specific substrates responsible for separating treatment plots were stable by analysis of different time point readings, even though carbon input and soil water axes were significant throughout a 33- to 69-h incubation period. In addition, the patterns that we observed did not always correspond to what we expected on the basis of environmental treatments. Indeed, we found that utilization of the same carbohydrates was enhanced with straw additions in two very different soils. However, substrates that would have been expected to be enriched, such as fermentation products, did not show enhancement under flooding. Thus, patterns of specific substrates must be critically examined and used with caution, particularly because the substrates found to be responsible for separating treatment plots will change with incubation time. The patterns in substrate utilization suggested by the Biolog data should be considered hypotheses worth testing, e.g., in microcosm studies, rather than conclusions in themselves.

Nevertheless, we found that differences among microbial communities in their Biolog sole-carbon-source utilization patterns (i) could be explained by the environmental variables imposed upon the communities, (ii) exhibited seasonal fluctuations in direct correspondence to environmental fluctuations, and (iii) showed similar patterns at two independent locations. Using a rigorous experimental design with treatments imposed on a relatively uniform soil microbial community, we demonstrated that the differences among communities were not random but existed at the field scale.

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