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# Effects of Sieving, Storage, and Incubation Temperature on the Phospholipid Fatty Acid Profile of a Soil Microbial Community†

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**Disturbances typically associated with the study of soil microbial communities, i.e., sieving, storage, and subsequent incubation at elevated temperatures, were investigated with phospholipid fatty acid (PLFA) analyses. Treatment effects were quantified by statistical analyses of the mole percentage distribution of the individual fatty acids. Changes in the concentrations of individual fatty acids over a 7-week storage period at 4.5°C were generally not statistically significant. Sieving effects (mesh size, 4 or 2 mm) on $CO_2$ evolution and the PLFA profile were monitored over 3 weeks; the physical disturbance had only minor effects, although some damage to fungal hyphae by the first sieving (<4 mm) was suggested by a decrease in the signature fatty acid 18:2 ω6c. Temperature effects were investigated by incubating soil for up to 3 weeks at 4.5, 10, or 25°C. Principal component analyses demonstrated a significant shift in the PLFA composition at 25°C over the first 2 weeks, while changes at the other two temperatures were minor. Several of the changes observed at 25°C could be explained with reference to mechanisms of temperature adaptation or as a response to conditions of stress, including a decrease in the degree of unsaturation, an increased production of cyclopropyl fatty acids, and increased ratios of the branched-chain fatty acids *iso*-15:0 and *iso*-17:0 over *anteiso*-15:0 and *anteiso*-17:0, respectively. A decrease in the total amount of PLFA was also indicated.**

Many aspects of soil microbiology are studied at the community level. The characteristics of a microbial community will to some extent reflect the conditions of growth, i.e., nutrient availability and the physical environment prior to sampling, and these factors may therefore influence analyses of biomass size, community structure, and metabolic activities. Similarly, the disturbances introduced by sampling and subsequent handling (e.g., homogenization and adjustment of water content) as well as conditions of storage or incubation may affect the microbial community.

Phospholipid fatty acid (PLFA) analyses of microbial communities have only recently been introduced to the soil environment (3, 9). Phospholipids constitute an important part of all cell membranes, and thus the phospholipids of a given sample, as reflected in the composition of the associated long-chain fatty acids, are taken as a fingerprint of the microbial community. However, from pure culture studies it is well established that membrane lipids are much affected by environmental factors (31); therefore, species composition is not the only variable involved.

The environmental factor that most directly affects PLFA composition is temperature because of its impact on membrane function. The fluidity of the lipid bilayer will increase with an increase in temperature and may result in the formation of non-bilayer phases, which will affect membrane permeability (32). An increase in membrane fluidity will also affect many transport systems, the activities of which either increase or, in some cases, become inhibited (26). Adaptation of the PLFA composition in order to modify membrane fluidity is one of the mechanisms to compensate for such effects. However, if the organisms cannot adapt to a dramatic temperature change because of starvation or lack of nutrients, this may induce stress reactions or death.

The significance of environmental factors for the PLFA profile of a complex soil microbial community is not known. Disturbance effects, like those mentioned in the first paragraph, have not been studied in soil systems either; however, this information is of major importance for the interpretation of PLFA profiles, since handling and storage often precede the actual analysis.

The objective of the present study was to investigate effects of storage, sieving, and incubation temperature on PLFAs of a soil microbial community. Soil incubated at three different temperatures or with two levels of sieving was intensively sampled in two 3-week experiments. The significance of the observed changes was evaluated by multivariate statistical analysis.

## MATERIALS AND METHODS

**Soil sampling.** Soil was collected on 15 January 1993 from a field plot within the Long-Term Ecological Research field experiment located near the W. K. Kellogg Biological Station in southern Michigan. The treatment was a corn-soybean-wheat rotation with conventional till. The soil type of the experimental site was a Typic Hapludalf with 40 to 50% sand, 40 to 50% silt, and 12 to 20% clay. The plot sampled contained 0.9% C and 0.09% N and had a pH (KCl) of 5.1.

The soil was fallow and covered with ca. 20 cm of snow at the time of sampling, and the upper ca. 0 to 5 cm of soil was frozen. Therefore, soil for the experiments was collected from a depth of 5 to 15 cm and immediately stored at 4.5°C; on the first day, it was stored in open trays to reduce the water content prior to sieving. The final water content was 14.5% of the dry weight of

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the soil. After 3 days, the soil was passed through a 4-mm mesh screen and again stored at 4.5°C in plastic bags, which were vented regularly to avoid oxygen depletion. After 7 weeks, a portion of soil was sieved through <2 mm prior to the second experiment. With both levels of sieving, it was necessary to pass the soil forcibly through the screen.

**Glassware.** Centrifuge tubes and reagent bottles were acid washed in 10% HCl and rinsed three times in deionized water. Pyrex tubes and gas chromatography (GC) vials were burned in a muffle oven at 450°C for 4 h.

**Chemicals.** All reagents were high-performance liquid chromatography grade and supplied by Mallinckrodt (Paris, Ky.), except *n*-hexane (EM Science; Gibbstown, N.J.) and diethyl ether and toluene (J. T. Baker; Phillipsburg, N.J.). Lipid standards, phosphatidyl ethanolamine extracted from *Escherichia coli* and digalactosyl diglyceride from wheat, were obtained from Sigma (St. Louis, Mo.).

**Preparation of PLFAs.** Preparation of PLFAs consisted of three steps, i.e., an extraction of lipids, a separation of phospholipids by column chromatography, and a methylation of esterified fatty acids in the phospholipid fraction.

**(i) Lipid extraction.** Approximately 2 g (dry weight) of soil was weighed directly into 25-ml Corex centrifuge tubes equipped with Teflon-lined screw caps (Baxter, Romulus, Mich.). Then, phosphate buffer (50 mM; pH 7.4) was added to give a total aqueous volume of 2 ml, and then 7.5 ml of methanol-dichloromethane (DCM) (2:1 [vol/vol]) was added. Following extraction for at least 2 h, 2.5 ml of DCM and 10 ml of a supersaturated NaBr solution (0.8 g $ml^{-1}$) were added. The samples were left overnight for phase separation and then centrifuged at 7,500 × *g* for 10 min. The upper DCM phase containing the lipids could then be removed with a Pasteur pipette. Further details about this extraction procedure are given elsewhere (5, 28).

DCM was transferred to 10-ml Pyrex culture tubes with Teflon-lined screw caps (Baxter) and placed in a sand bath at 37°C, in which the samples were dried under a stream of nitrogen. The tubes were weighed before and after drying to calculate the fraction of the DCM phase analyzed.

**(ii) Isolation of phospholipids.** Polar lipids (mainly phospholipids) were isolated from the crude lipid extracts by preparative solid-phase extraction; columns with 100 mg of aminopropyl-bonded silica (Varian, Harbor City, Calif.) were applied by a procedure adapted from Kaluzny et al. (22). Up to 10 columns at a time were mounted on a vacuum manifold, which was connected to a pump via a sidearm flask for collection of solvents. The columns were conditioned by sequentially adding 1.5 ml of hexane (twice) and 1.5 ml of hexane-chloroform (1:1 [vol/vol]). Vacuum was applied to pull solvents slowly through the column material only when these had been added to all columns. Then, the dried lipid material was transferred to the solid-phase extraction columns in chloroform (three times, 100 μl). The chloroform was pulled through, and then lipids of low and intermediate polarities were eluted with 1.5 ml of chloroform-2-propanol (2:1 [vol/vol]) and then with 1.5 ml of 2% acetic acid in diethyl ether. A rack with clean Pyrex tubes was placed in the vacuum chamber before the phospholipid fraction was eluted with 1 ml of methanol (twice). It was later found that this column type, recommended by Varian and other companies for isolation of phospholipids, did not separate the relatively polar glycolipid digalactosyl diglyceride from the methanol fraction as effectively as silicic acid columns, even when 50 bed volumes of acetone (24) were included in the elution sequence. Glycosyl glycerides are primarily found in photosynthetic membranes but are also present in membranes of gram-positive bacteria (18, 27).

**(iii) Methylation of PLFAs.** The methanol collected was dried under nitrogen as described above, and then fatty acid methyl esters were prepared by a mild basic transesterification (8). One milliliter of methanol-toluene (1:1 [vol/vol]) and 1 ml of 0.2 M methanolic KOH were added to each Pyrex tube, which was capped, mixed, and placed in a water bath at 37°C for 15 min. Then, 2 ml of hexane, 0.3 ml of 1 M acetic acid, and 2 ml of demineralized water were added. After thorough mixing, samples were centrifuged and the organic phase was transferred to a clean Pyrex tube. After a second wash with 2 ml of hexane, the combined organic phase was dried under nitrogen as described above.

**GC analysis.** The fatty acid methyl esters were finally transferred to vials with inserts in 75 μl of hexane. The GC analysis was carried out by an automated procedure (the Microbial Identification System, Inc., Newark, Del.), which calibrates retention time using 24 different fatty acids ranging from 9 to 30 carbon atoms; peak identification requires a precision of approximately ± 1 s. More than 200 fatty acids could potentially be identified by combining information from existing libraries for pure culture analyses.

The instrument consisted of a Hewlett-Packard gas chromatograph (model 5890A) with a flame ionization detector, an automatic sampler, and an integrator. The injection volume was 2 μl, and a split ratio of 50 was employed. Normally, splitless injection is recommended (11), but this was not possible at the time since the instrument was also routinely used for pure culture analyses according to the Microbial Identification System procedure; therefore, the detection limit in this study was relatively high (see Results). The column was an Ultra 2 nonpolar fused silica capillary column (25 m by 0.2 mm by 0.33 mm) (Hewlett-Packard). Flow rates of $N_2$ (make-up), $H_2$ (detector-split vent-septum purge), and dry air were 30, 30/55/5, and 400 ml $min^{-1}$, respectively. The injection temperature was 250°C, and the detector temperature was 300°C. The oven temperature was initially 170°C and was programmed to increase 5°C per min until reaching 300°C, at which it remained for the rest of the 36-min run time. An equal detector response for all FAME's was assumed (11).

**Nomenclature of fatty acids.** Fatty acids are designated by the number of carbon atoms:number of double bonds and then by the position of the first double bond from the ω (methyl) end of the molecule. *cis* and *trans* isomers are indicated by *c* or *t*. Branched-chain fatty acids are indicated by prefixes *i* or *a* for *iso-* and *anteiso*-branchings, respectively. The fatty acid 10Me18:0 has a methyl group attached to the 10th carbon atom from the carboxyl end of the molecule. The prefix *cy* designates cyclopropyl fatty acids; the position of the ring structure is indicated after the name in Table 1. When present, the number of hydroxyl substitutions is also given.

**Experiments.** Two 3-week incubation experiments were carried out. The first experiment included soil sieved with a mesh size of 4 mm and incubated at 4.5 (the storage temperature), 10, or 25°C; PLFA profiles were determined after 0, 1, 4, 7, 14, and 21 days. Experiment 1 was initiated 10 days after soil sampling. In the second experiment, soil sieved at <4 mm was again incubated at 4.5, 10, or 25°C; a fourth treatment was included with soil sieved at <2 mm and incubated at 25°C. Carbon dioxide evolution was monitored in all treatments of experiment 2, with samplings after 0.5, 1, 2, 3, 4, 7, 10, 14, and 21 days, while PLFA profiles were determined after 0.5, 1, 3, 7, 14, and 21 days, but only for the two treatments incubated at 25°C. The major changes in PLFA composition observed at 25°C in experiment 1 were confirmed and will not be presented

TABLE 1. Mole percentage distribution of PLFAs after 10 days or 7 weeks of storage at 4.5°C[a]

| Fatty acid | Mol% ± SD ($n$ = 3) | |
|---|---|---|
| | 10 days | 7 wks |
| 14:0 | 0.26 (0.45) | —[b] |
| *i*15:1 | 0.65 (0.61) | — |
| *i*15:0 | 3.44 (0.97) | 5.14 (0.43) |
| *a*15:0 | 2.48 (0.71) | 3.12 (0.27) |
| *i*16:0 | 1.25 (0.19) | 1.50 (0.10) |
| 16:1 ω9*c*[c] | 2.09 (0.43) | 2.44 (0.27) |
| 16:1 ω7*c* | 9.62 (1.08) | 11.79 (1.23) |
| 16:1 ω5*c* | 3.70 (0.34) | 5.00 (0.43) |
| 16:0 | 10.84 (0.78) | 10.80 (0.64) |
| *i*17:1 | 7.85 (0.46) | 9.37 (0.43) |
| *i*17:0 | 1.16 (0.07) | 1.24 (0.08) |
| *a*17:0 | 1.64 (0.37) | 0.89 (0.77) |
| *cy*17:0 C9-10 | 3.09 (0.15) | 4.09 (0.57) |
| 16:1 2OH | 0.28 (0.49) | — |
| 18:3 ω4*c*[d] | 1.26 (0.50) | — |
| 18:2 ω6*c* | 7.13 (0.57) | 4.94 (0.95) |
| 18:1 ω9*c* | 8.19 (0.49) | 8.11 (0.14) |
| 18:1 ω7*c*[c] | 16.03 (5.50) | 15.28 (2.03) |
| 18:1 ω5*c* | 0.99 (0.87) | — |
| 18:0 | 5.07 (0.37) | 3.29 (0.36) |
| 10Me18:0 | 1.23 (0.23) | 0.42 (0.73) |
| *cy*19:0 C11-12 | 7.44 (0.84) | 8.09 (0.92) |
| 20:4 ω6*c* | 1.91 (0.09) | 2.32 (0.07) |
| 20:5 ω3*c*[f] | 0.89 (0.05) | 1.31 (0.06) |
| 20:0 | 0.72 (0.05) | 0.77 (0.71) |
| *cy*19:0 2OH | 0.81 (0.10) | — |

[a] Individual fatty acids were compared by Student's $t$ tests using a tablewise error rate of 0.05.
[b] —, Below the detection limit (see text).
[c] 16:1 ω9*c* and 16:1 ω11*c* could not be distinguished.
[d] Coelutes with *a*18:0; however, even-numbered *anteiso*-fatty acids are extremely rare (23).
[e] Coelutes with 18:1 ω9*t* and 18:1 ω12*t*.
[f] $P < 0.05/26$.

in detail. Experiment 2 was initiated 7 weeks after soil sampling.

**Soil preparation.** Soil portions (25 g [fresh weight]) were added to 70-ml glass beakers (for PLFA) or 100-ml glass flasks (for $CO_2$ measurements) and packed to a bulk density of 1.3 g $cm^{-3}$. All beakers were covered with Parafilm and incubated in the dark. The flasks for $CO_2$ measurements were covered with aluminum foil and closed with a septum.

**Sampling for PLFA.** At sampling, three replicates of each treatment group were removed and thoroughly mixed, and two (experiment 1) subsamples or a single (experiment 2) subsample of soil (ca. 2 g [dry weight]) were transferred from each replicate to centrifuge tubes. After 4 to 7 days, a few seedlings of the common weed *Chenopodium album* appeared in most replicates; the seedlings were carefully removed together with adhering soil before subsampling to avoid confounding effects from the rhizosphere microflora. Replicates from only one temperature treatment at a time were handled, and the extraction mixture was added as soon as subsampling had been completed. In experiment 1, there was no difference in the variation within and among replicates, so the subsamples of each treatment were subsequently treated as six replicate analyses.

**$CO_2$ measurements.** $CO_2$ production was measured repeatedly on three 25-g portions of soil per treatment. At each sampling, the headspace (ca. 88 ml) was equilibrated with the soil atmosphere by pumping with a 10-ml syringe; then, 100 to 300 μl of atmospheric air (depending on the concentration level in the different treatments) was injected, and the same volume of gas phase was removed for the $CO_2$ analysis. When the $CO_2$ concentration of the headspace exceeded 2%, the container was opened to the atmosphere for 30 min, and then the $CO_2$ concentration was measured as before to obtain a new starting value. $CO_2$ production rates, calculated for each interval as $\Delta CO_2/\Delta t$, were corrected for the dilution at sampling and for dissolved $CO_2$ assuming equilibrium between soil water and gas phase (25); temperature differences were taken into account (34).

$CO_2$ production was measured by infrared gas analysis spectrometry. The instrument was a Beckman model 865 infrared analyzer (Beckman, La Habra, Calif.); the flow rate of the carrier ($N_2$) was 50 ml $min^{-1}$.

**Statistical analyses.** The initial concentrations of PLFA in the two experiments, corresponding to 10 days and 7 weeks of storage, respectively, were compared by individual Student's $t$ tests and with a tablewise error rate of 0.05. Accordingly, an initial α level of $P < 0.05/k$ ($k$ = the number of tests) was used, and the tests were examined with the sequential Bonferroni test described by Rice (30). Temperature effects on the degree of unsaturation and summed areas per gram (dry weight) of soil, respectively, were tested for each of the 5 sampling days by Tukey's honestly significant difference multiple-comparisons test. Again, a tablewise error rate of 0.05 was used, corresponding to an initial α level of 0.01.

The analysis of data from the two experiments was restricted to 26 PLFAs that were consistently present in at least one treatment. Fatty acids with more than 20 carbon atoms, notably 22:0 and 24:0, could not always be quantified because of overlapping by other, unidentified peaks and were therefore not considered. A total of six samples were lost during the analytical procedure; a minimum of four replicates remained for all treatments and sampling dates.

The effects of sieving, incubation temperature, and incubation time were examined by principal component analyses (PCA) of the mole percentage distribution of PLFAs. Data that sum to a constant (in this case, 100%) are constrained, since correlations between individual variables are not free to vary between −1 and +1 (29). To overcome this problem, the percentages were transformed by the algorithm $x_i \rightarrow \log[x_i/g(x)]$, where $i = (1, 2, ..., n)$ and $g(x)$ is the geometric mean of the $n$ values summing to 100% (29). The covariance matrix of these so-called log ratios was used for the PCA. There is no easy way of handling zero entries (1), which in the present experiment occurred when a peak dropped below the detection limit. Therefore, it was necessary to delimit this analysis to 15 of the originally 26 PLFAs considered in this study, and 3 of 87 analyses were disregarded because of low total areas giving many zero entries. These 15 PLFAs, constituting between 80 and 89% of the total amount, were normalized to 100%.

All statistical analyses were carried out with the software package SYSTAT (version 5.2.1) for Macintosh (SYSTAT, Inc.; Evanston, Ill.).

## RESULTS

**Storage effects.** The PLFA distribution after storage at 4.5°C for 10 days or 7 weeks, respectively, is presented in Table 1. The detection limit of the GC analysis was relatively high (0.5 to 1%, depending on the amount of material), and this resulted in large coefficients of variation for some of the minor peaks, although most peaks had a coefficient of variation of 5 to 20%.

Six fatty acids (Table 1) dropped below the detection limit during storage, and several other differences that would each

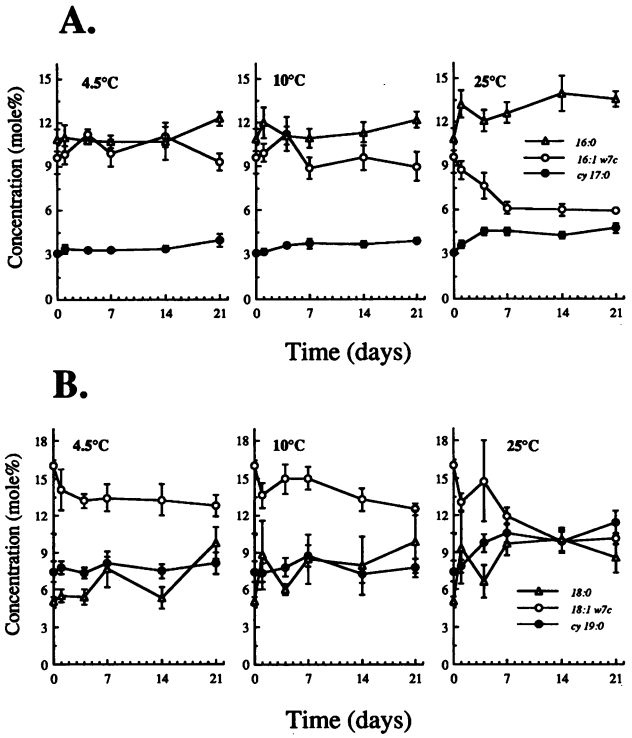


FIG. 1. Concentrations of three related fatty acids within the $C_{16}$ (A) and $C_{18}$ (B) clusters of PLFAs in soil incubated at 4.5, 10, or 25°C for up to 21 days ($n$ = 6).

have been significant at the $P$ = 0.05 level were observed. However, within a sequence of individual comparisons, several type 1 errors may occur by chance alone, unless the overall significance level is controlled (30). Therefore, the 20 tests were evaluated according to the sequential Bonferroni test (30). By this procedure, the only significant change in concentration was observed with 20:5 ω3*c*.

**Temperature effects.** In experiment 1, the effects of incubation temperature on PLFA were studied. The most dramatic shifts occurred within the clusters of $C_{16}$- and $C_{18}$-based fatty acids. Different double-bond locations were observed with both hexadecenoic (16:1) and octadecenoic (18:1) acids; however, major concentration changes occurred only with 16:1 ω7*c* and 18:1 ω7*c*. While these two fatty acids can be produced from 16:0 and 18:0, respectively, they are themselves precursors of cyclopropyl fatty acids *cy*17:0 and *cy*19:0, which are formed by a methylene bridging between the two carbons making up the double bond. In Fig. 1, the concentrations of the saturated, monounsaturated, and cyclopropyl fatty acids mentioned above are presented for the three different temperatures of incubation. The most dramatic changes occurred at 25°C, at which the concentrations of 16:1 ω7*c* and 18:1 ω7*c* dropped during the first part of the experiment, while there was a concomitant but smaller increase in *cy*17:0 and *cy*19:0. At all temperatures, 16:0 and 18:0 fluctuated over the 3-week period. A sudden increase of both 16:0 and 18:0 was observed after 1 day at 10 and 25°C, but not at 4.5°C; since saturated fatty acids cannot be formed by in situ modification of existing monounsaturated fatty acids (see Discussion), this suggested a phase of rapid lipid turnover at the two higher temperatures.

In many microorganisms, the number of double bonds in membrane lipids changes with temperature (18). *cis*-Unsaturated fatty acids have a lower melting point (or rather, phase transition temperature) than the corresponding saturated fatty acids; thus they counteract membrane crystallization at low temperatures, while a reduction in the degree of unsaturation at higher temperatures prevents excessive leakage. Survival is possible within a range of lipid compositions; optimizing the growth rate may be the most important factor behind this adaptation (18). The degree of unsaturation can be expressed (24) as

$$\Delta \text{mol}^{-1} = \Sigma(\% \text{ monoenes} + 2 \times \% \text{ dienes} + 3 \times \% \text{ trienes} \ldots)/100$$

TABLE 2. The degree of unsaturation in soil incubated at 4.5, 10, or 25°C for 21 days[a]

| Day | Degree of unsaturation | | |
|---|---|---|---|
| | 4.5°C | 10°C | 25°C |
| 0 | | 0.79 | |
| 1 | 0.76 | 0.75 | 0.70 |
| 4 | 0.73 | 0.76 | 0.71 |
| 7 | 0.74 ab | 0.79 a | 0.67 b |
| 14 | 0.72 a | 0.76 a | 0.58 b |
| 21 | 0.68 ab | 0.70 a | 0.57 b |

[a] Treatment groups were compared by Tukey's HSD multiple-comparisons test, with a tablewise error rate of 0.05; different letters within a row denote significance at this level.

The statistical analysis (Table 2) showed that the degree of unsaturation was significantly reduced at 25°C in the last part of the experiment, although the difference between 4.5 and 25°C after 21 days was not significant ($P$ = 0.016 [not significant]). Since there was a decrease in the average values at all three temperatures over the 3-week period, temporal effects were also tested within each treatment, but the decrease was significant only at 25°C.

Like *cis*-unsaturated fatty acids, branched-chain fatty acids have a lower phase transition temperature than the saturated straight-chain fatty acids with the same molecular weight. Seven different branched-chain fatty acids were observed in this study. No significant treatment effects or changes with time could be detected with $C_{14}$ and $C_{15}$ acids, which were both characterized by large coefficients of variation (20 to 40%). Minor relative increases were observed for *i*16:0 and *i*17:1 at 25°C, but only for *i*16:0 was the difference between treatments significant (data not shown). In contrast to *i*15:0 and *a*15:0, the concentration of *i*17:0 and *a*17:0 varied little among replicates (Fig. 2); *a*17:0 remained at a constant level at all three temperatures, while there was a significant increase of *i*17:0 in soil incubated at 25°C.

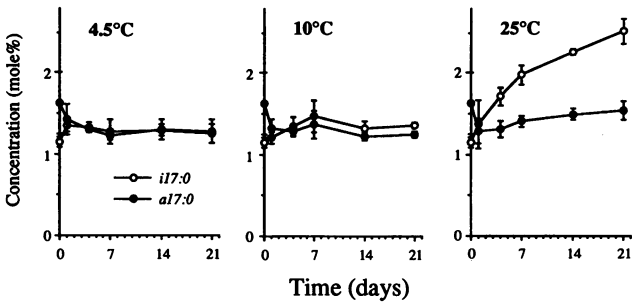


FIG. 2. Concentrations of *i*17:0 and *a*17:0 in soil samples incubated at 4.5, 10, or 25°C for up to 21 days ($n$ = 6).

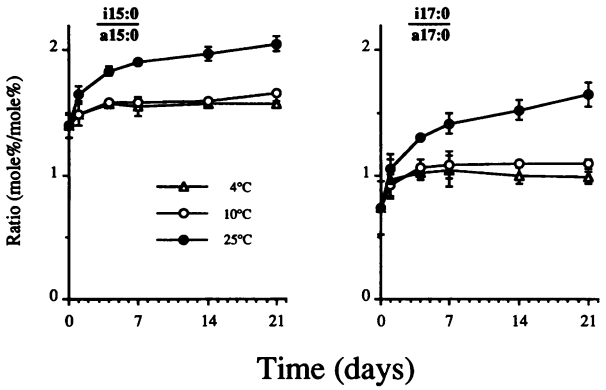


FIG. 3. Ratios of the branched-chain fatty acids *i*15:0 to *a*15:0 (left) and of *i*17:0 to *a*17:0 (right) at 4.5, 10, and 25°C.

Since pure culture studies have shown a relative increase of *iso*- over *anteiso*-fatty acids with an increase in temperature (23), the ratios of *i*17:0 over *a*17:0 and of *i*15:0 over *a*15:0 were calculated for the individual analyses (Fig. 3). The same very strong pattern emerged in both cases, despite the difference in variability; at 4.5 and 10°C, the ratio increased slightly and then remained constant, while at 25°C the ratio increased rapidly during the first 24 h but continued to increase throughout the 3-week period.

The relative abundance of all branched-chain fatty acids varied within a range of 16.3 to 21.7%; however, no consistent trends were observed (data not shown).

Most of the fatty acids mentioned above are found in a wide range of organisms and provide little information about community structure. However, there are fatty acids that are thought to be more or less exclusively associated with a well-defined subcomponent of the biomass. Examples are 10Me18:0 (*Actinomycetes*), and 18:3 ω4*c* and 18:2 ω6*c* (mainly fungi) (9, 36). Interpretation of PLFA profiles on the basis of such functional groups was discussed by Findlay and Dobbs (11) for sediments and by Federle (9) and Frostegård et al. (13) for soils.

In the present study, there were no clear trends or treatment effects with 10Me18:0. In contrast, in experiment 1 there was an interesting temporal pattern for 18:2 ω6*c* (Fig. 4), which dropped significantly during the initial phase at all three temperatures. In the second experiment, the concentration of 18:2 ω6*c* remained at the lower level. The concentration of 18:3 ω4*c* in experiment 1 was more variable; however, this fatty

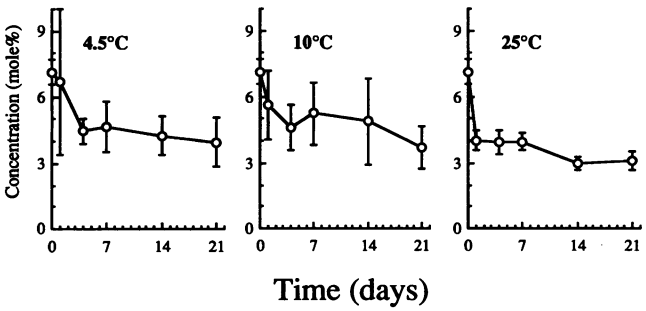


FIG. 4. Concentrations of 18:2 ω6*c*, mainly of fungal origin, in soil samples incubated at 4.5, 10, or 25°C for up to 21 days ($n$ = 6).

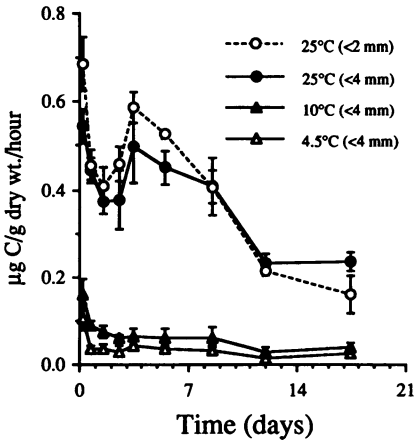


FIG. 5. $CO_2$ evolution rates in soil sieved with a 4-mm mesh sieve and incubated at 4.5, 10, or 25°C and in soil sieved <2 mm and incubated at 25°C ($n$ = 3). Correction was made for dissolved $CO_2$ and differences in temperature.

acid had dropped below the detection limit after 7 weeks of storage (Table 1).

**Sieving effects.** In experiment 2, the disturbance introduced by sieving was evaluated by comparing rates of $CO_2$ evolution from soil sieved at <2 or <4 mm and incubated at 25°C (Fig. 5); $CO_2$ evolution rates from soil sieved at <4 mm and incubated at 4.5 and 10°C are also shown. In all treatments, there was a maximum in activity after 12 h, even at the storage temperature (4.5°C), indicating that this high rate was in part due to release of $CO_2$ accumulated in the soil during previous storage. The rate from soil sieved at <2 mm was higher than from soil sieved at <4 mm during the first 12 h; however, this could also be due to a more rapid equilibration between dissolved $CO_2$ and $CO_2$ in the gas phase. Although slightly higher average $CO_2$ evolution rates were observed from <2-mm-sieved soil during the first week, the difference was mostly not significant, and the rate dropped below that from <4-mm-sieved soil during the last week; accumulated rates from the two treatments were not significantly different after 3 weeks. There were also apparently no qualitative differences in PLFA composition (see section below on PCA).

The respiratory activity dropped after the first 7 to 10 days, during which time the major shifts in PLFA composition also generally took place (see Fig. 1 to 4). If this initial phase of high $CO_2$ evolution rates was due to substrates released by sieving, a pronounced difference should have been observed between soil sieved at <4 mm 7 weeks prior to the experiment and soil sieved at <2 mm immediately prior to the experiment. However, $CO_2$ evolution rates from soil sieved at <2 and <4 mm were very similar, indicating that substrates were not released by sieving to any large extent.

**PCA.** In all PCA performed, the first two principal components always explained between 72 and 85% of the total variance. PLFA profiles of soil incubated at 4.5, 10, and 25°C were compared by a PCA for each sampling day, except that day 0 was included in the analysis of day 1. In Fig. 6, each number represents an individual PLFA profile, and the number indicates the incubation temperature; the clusters are delimited by stipulated lines. Initially (day 0 + 1), there was no separation of the three treatments; however, from day 4 onward, profiles of soil incubated at 25°C were clearly separated from those of soil incubated at 4.5 or 10°C. After 14 and

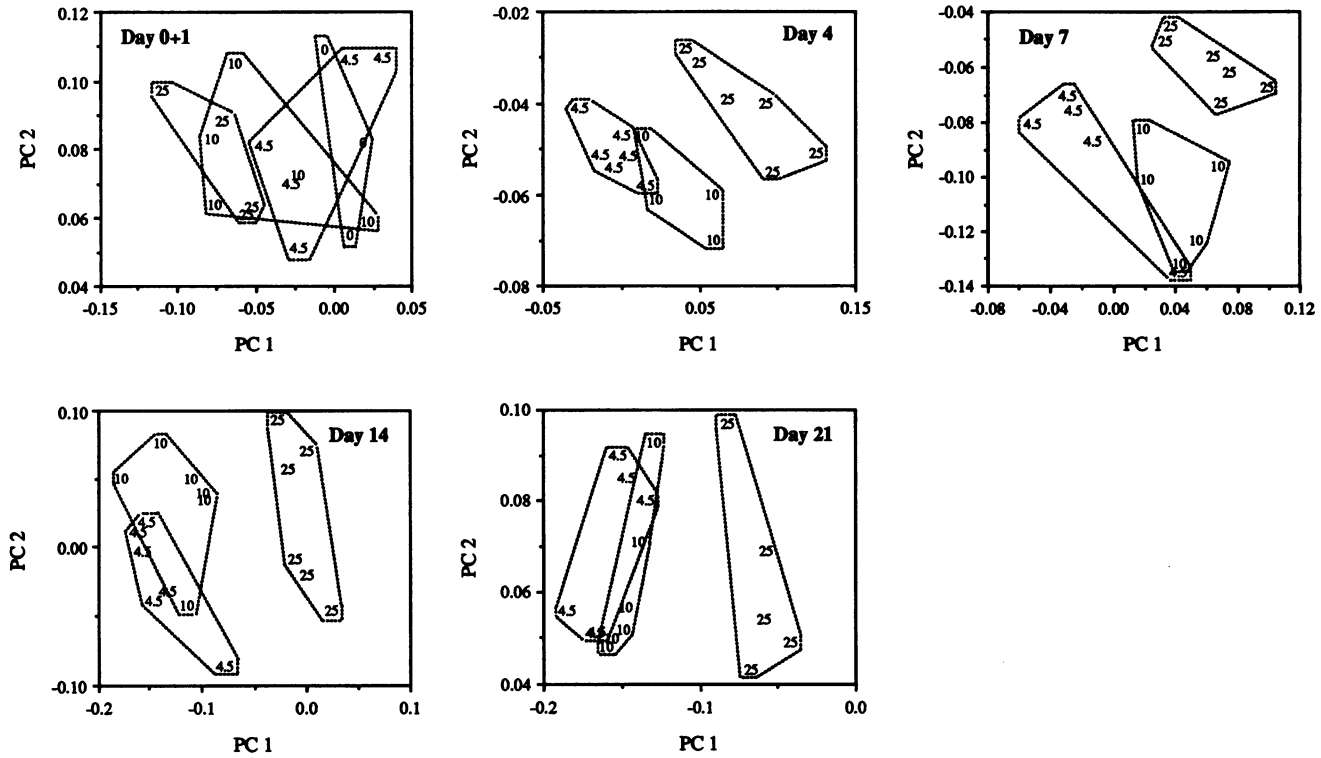


FIG. 6. Separate PCA of the mole percentage distribution of 15 PLFAs were made for each sampling day after a log ratio transformation (see text); numbers indicate the incubation temperatures. Percents of variance explained by PC 1 and PC 2: day 0 + 1, 50.9 and 24.0%; day 4, 64.7 and 17.0%; day 7, 50.2 and 33.3%; day 14, 48.6 and 37.1%; day 21, 51.6 and 20.4%.

21 days, the difference was mainly associated with the first principal component. Although PLFA profiles from 4.5 and 10°C, respectively, tended to group together, the clusters representing the two temperatures were never completely separated.

The pattern observed in Fig. 6 would suggest that significant changes in the PLFA profile of the microbial community mainly occurred at 25°C, while the other two treatments changed little. However, the analyses provided information not about temporal changes, but only about treatment effects. Therefore, a second set of PCA was performed, in which replicates from all sampling days of a given treatment were combined (Fig. 7). Some differences between the individual sampling days were suggested at 4.5 and 10°C; however, no clear trends were observed. In contrast, there was a strong pattern, with rapid initial changes along the first principal component (PC 1), in the clustering of profiles from soil incubated at 25°C. The cluster of day 21 falls completely within the cluster of day 14, indicating that the PLFA composition may have approached a new steady state.

At 4.5 and 10°C, only four fatty acids were represented by significant loadings on the first two principal components (*i*15:0, *a*15:0, 18:0, and 18:2 ω6*c*), while at 25°C several other fatty acids were also important (*i*17:0, 16:1 ω7*c*, 18:1 ω7*c*, and 20:4 ω6*c*). At 25°C, the following fatty acids generally had high positive loadings on PC 1: *i*15:0, *i*17:0, and 18:0. Instead, high negative loadings on PC 1 were found for 16:1 ω7*c*, 18:2 ω6*c*, and 18:1 ω7*c*. Thus, the PCA confirmed that the individual fatty acids emphasized above (Fig. 1 to 4) were in fact significant for the separation of PLFA profiles in response to temperature.

Figure 8 presents a PCA of sieving effects with the soil incubated at 25°C (experiment 2); the two levels of sieving were analyzed together but are presented separately to improve clarity. As in Fig. 7 (25°C), there was a rapid change away from the initial PLFA composition, and again a new steady state seemed to be reached after approximately 2 weeks. In this case, the shift mainly occurred along PC 2, where the highest positive loadings were found for *i*17:0 and 18:0 and high negative loadings were found for *a*15:0, 16:1 ω7*c*, 18:1 ω7*c*, and 18:2 ω6*c*.

**Quantitative changes.** The summed areas of the GC analysis were used as a relative estimate of the amount of PLFA for a comparison among the three incubation temperatures within each sampling day (Table 3). The variation was large; however, after 21 days the amount of PLFA had dropped significantly at 25°C relative to 4.5°C, while the drop was not significant relative to soil incubated at 10°C ($P = 0.035$ [not significant]).

## DISCUSSION

Ecological studies often require intensive sampling in order to capture spatial heterogeneity or temporal dynamics. It leaves the researcher with a practical problem of how to handle the samples until the analytical work can proceed. Federle and White (10) studied different methods of preserving estuarine sediments for lipid analyses. Direct extraction of the lipids was proposed as the ideal solution; however, they also found that a 5-day storage of sieved samples at 5°C, as well as long-term storage in Formalin, did not alter the PLFA composition significantly.

Although immediate extraction of the lipid material is no

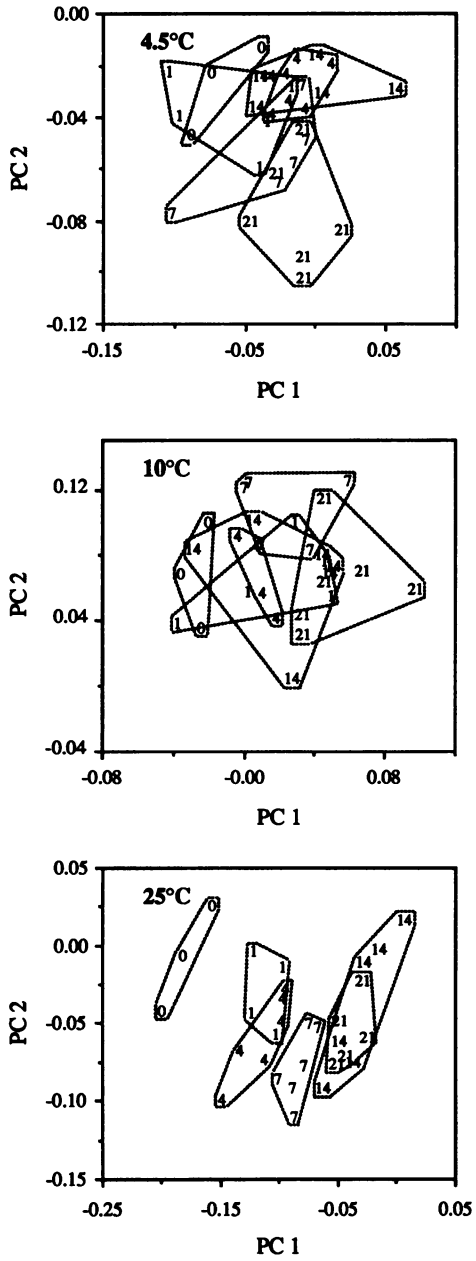


FIG. 7. Temporal changes in the mole percentage distribution of 15 PLFAs at 4.5, 10, or 25°C. The data were analyzed by PCA after log ratio transformation (see text); numbers indicate the sampling days. Percents of variance explained by PC 1 and PC 2: 4.5°C, 46.2 and 31.5%; 10°C, 40.7 and 37.1%; 25°C, 45.3 and 31.8%.

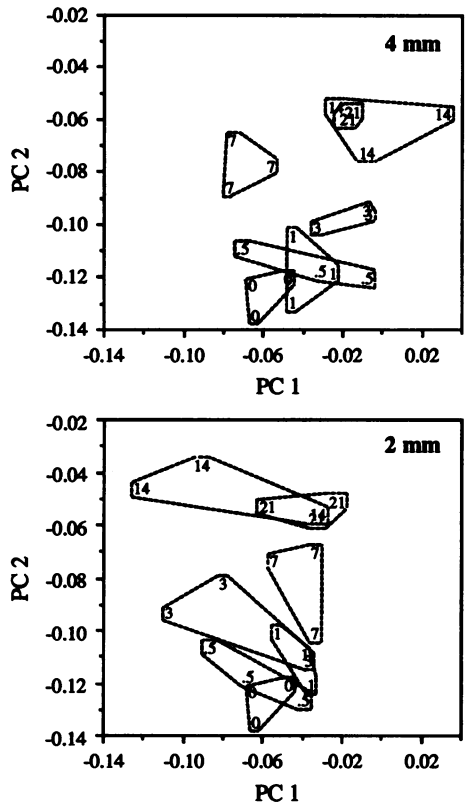


FIG. 8. Effects of sieving (2- or 4-mm mesh) soil subsequently incubated at 25°C on the mole percentage distribution of 15 PLFAs. All data were analyzed by a single PCA after log ratio transformation (see text); numbers indicate the sampling days. Although the two treatments were combined for the analysis, they are presented separately. Percents of variance explained by PC 1 and PC 2: 42.0 and 38.6%.

doubt preferable, storage cannot always be avoided. In this study, any changes in the concentrations of individual fatty acids during the almost 6-week storage period between experiments 1 and 2 (Table 1) were generally not significant, although a number of peaks dropped below the detection limit and therefore could not be tested. Unfortunately, the power (i.e., the probability of detecting significant differences) of a test involving many individual comparisons is low. Findlay et al. (12), who faced a similar analysis with 29 different fatty acids, chose an $\alpha$ level of 0.02 for the individual tests but had to accept a 46% chance of having one or more type 1 errors. Using this approach, we would have found significant changes also for 16:1 $\omega$5*c*, *i*17:1, 18:0, and 20:4 $\omega$6*c*. Fungal hyphae may have been damaged by sieving (see below), so it is probably advisable to store the soil without sieving. The storage temperature (4.5°C) was not very different from the in situ temperature in this study, but if sampling had taken place at a time where the average soil temperature was, for example, ca. 20°C, more dramatic changes in the PLFA composition might occur upon storage at ca. 4°C.

Homogenization of the soil may release a pool of substrate

TABLE 3. Summed areas ($10^3$) of PLFA per gram (dry weight) in soil incubated at 4.5, 10, or 25°C for 21 days[a]

| Day | Summed areas ($10^3$) | | |
|---|---|---|---|
| | 4.5°C | 10°C | 25°C |
| 0 | | 134 | |
| 1 | 106 | 96 | 105 |
| 4 | 109 | 120 | 106 |
| 7 | 97 | 84 | 89 |
| 14 | 99 | 96 | 74 |
| 21 | 96a | 86 ab | 56 b |

[a] Treatments were compared for each sampling day with Tukey's HSD multiple-comparisons test. The tablewise error rate was 0.05; different letters within a row denote significance at this level.

by breaking up aggregates and/or by damaging cells. The $CO_2$ evolution rates (Fig. 5) suggested that sieving the soil at <2 mm did not release much extra substrate compared with sieving at <4 mm, and the comparison by PCA of soil sieved at <4 and <2 mm, respectively, did not reveal any clear differences. This agrees with the findings of Craswell and Waring (6), who studied sieving effects on mineralization in 10 different soil types. It thus appears that the changes observed in PLFA composition were only to a small extent associated with physical disturbance. A possible exception is the concentration of 18:2 ω6*c*, mainly of fungal origin, which in experiment 1 dropped significantly at all temperatures (Fig. 4), perhaps because filamentous fungi were more susceptible to damage by sieving than single-celled organisms. Since the drop also occurred at the storage temperature, the concentration of this fatty acid may have been even higher than the initial value of ca. 7% at the time of sampling.

Federle (9) found that the concentrations of 18:2 ω6*c* in 47 different species of fungi were normally distributed, and he suggested that the average value, 43%, could be used to estimate the contribution of fungi to the total biomass. With this approach, he found that 13 to 33% of PLFAs in four surface soils of different textures were of fungal origin, while Frostegård et al. (13) estimated that 25 to 36% of the PLFAs were fungal in two forest soils. In the present study, fungal PLFAs constituted 16.5% of all PLFAs at the start of the first experiment, decreasing to ca. 7% after 3 weeks. Although the concentration of 18:2 ω6*c* may have decreased before the first experiment was started, these percentages are much lower than estimates based on respiratory activity using specific inhibitors (2); however, the relationship between membrane lipids and the metabolically active cytoplasmic volume is not straightforward because of differences in surface-volume ratio between filamentous organisms and single cells, as well as the difference in size class distribution (33) and the presence of cell organelles in eukaryotes, but also of lamellar structures in, e.g., nitrifying bacteria.

The limited effect of sieving suggests that temperature was the major factor behind the changes observed. Among microorganisms, membrane lipids are regulated in response to temperature changes by one of two main strategies: by changing the degree of unsaturation or, less commonly, by changing the concentration of branched-chain fatty acids (23). The incubation temperatures were all higher than the in situ temperature, which was close to the freezing point. The expected response would be a decrease in the concentration of *cis*-unsaturated and branched-chain fatty acids and a corresponding increase in the concentration of straight-chain saturated fatty acids, which have higher phase transition temperatures. This, however, is complicated by the fact that very few organisms are capable of removing a double bond by hydrogenation of intact membrane lipids (17); therefore, the conversion of unsaturated to saturated fatty acids can take place only in connection with membrane lipid turnover or growth. This is probably also true for the regulation of branched-chain fatty acids (18). Other adaptations, such as regulation of the average chain length or the proportions of different lipid classes, are also associated with lipid synthesis. Since biosynthetic reactions require that the organisms have access to an energy source, a large part of the soil community may not be able to adapt to a dramatic change in temperature, or may only be able to do so at the expense of other cell constituents. On the other hand, failure to adapt will increase energy requirements for maintenance of the proper intracellular environment because of impaired membrane function. This energy demand in an energy-limited soil environment may lead to stress reactions or death for part of the biomass.

The observations made in this study are consistent with the interpretation described above of how a soil microbial community will respond to a dramatic temperature increase. The degree of unsaturation decreased at 25°C within 1 to 2 weeks, while no decrease occurred at 4.5 or 10°C (Table 2). Within the group of branched-chain fatty acids, a large increase was observed at 25°C for *i*17:0, while minor increases were seen for *a*17:0, *i*16:0, and *i*17:1 at this temperature. The ratios of *i*15:0 and *i*17:0 over *a*15:0 and *a*17:0 remained nearly constant at 4.5 and 10°C but increased at 25°C (Fig. 3). This is in accordance with pure culture studies, mainly of *Bacillus* species, in which the ratio between *iso*- and *anteiso*-fatty acids increased with increasing temperature (23). This reflects the difference in melting points between *iso*- and *anteiso*-fatty acids, which is in the order of 25°C (23).

During the first week, the cyclopropyl fatty acids *cy*17:0 and *cy*19:0 increased at 25°C, while the two precursors, 16:1 ω7*c* and 18:1 ω7*c*, decreased. This pattern, i.e., an increase in *cy*17:0 and *cy*19:0 and concomitant decrease in 16:1 ω7*c* and 18:1 ω7*c*, was also observed by Guckert et al. (15) in sediment slurries incubated anaerobically. Cyclopropyl fatty acids, which are mainly found in gram-negative bacteria (18), are formed by modification of existing membrane lipids, often as the organisms enter the stationary phase. Factors such as oxygen depletion, decreasing pH, a high concentration of $Mg^{2+}$, high temperature, and poor nutrient quality have been shown to stimulate their production in pure culture studies (14, 16, 18), and thus it appears that this class of PLFAs is formed under stressful conditions. In the present study, the production of *cy*17:0 and *cy*19:0 increased with temperature. Although a relative increase in the number of gram-negative bacteria is possible, the accompanying decrease in the corresponding monounsaturates makes it more likely that this was mainly a response to the temperature increase.

The total amount of PLFA, as reflected in the summed area per gram (dry weight) of soil, apparently decreased at 25°C relative to 4.5 or 10°C during the 3-week incubation (Table 3), although a significant difference was observed only after 21 days. Petersen et al. (28) found that lipid P in soil from six plots in two field experiments decreased by 25 to 57% over 3 weeks. An initial decline of approximately 20% in microbial biomass C, determined by the chloroform fumigation direct extraction method, was also observed by Joergensen et al. (21 [Fig. 4a in this reference]) after 10 days of incubation at 25°C (following storage at 10°C), although these authors emphasized the long-term development over 240 days. Other studies of similar or smaller temperature effects on biomass C and N were quoted. The decline may be caused by cell death or, as earlier suggested, may reflect degradation of storage material or cell constituents, including membrane lipids (35), in response to higher energy requirements at the elevated temperature.

Several analytical techniques require incubation at 20 to 25°C prior to or as a part of the actual measurement. Notably, these include most chloroform fumigation-based methods for quantification of the microbial biomass, which generally employ between 2 and 7 days of preincubation at 25°C to "stabilize the biomass" (e.g., see references 5a, 7, 19), although direct extraction after storage at 4°C has also been used (4). With the fumigation incubation technique (20), it is assumed that $CO_2$ produced by the control during a 10-day incubation at 25°C comes exclusively from substrates being released from dead organic matter in the soil. While this may be largely true after a 7-day preincubation, the present study has provided evidence of temperature adaptation and possibly a decrease in

the total biomass within a period of 1 to 2 weeks in soil incubated at 25°C, which would change the characteristics of the microbial biomass both qualitatively and quantitatively. This possible effect of preincubating the soil was recognized by Jenkinson and Powlson in the original description of the method (20); however, generally such changes are not considered.

There is still little information about the significance or time scale of variability caused by environmental factors on PLFA profiles in natural systems. White (37) suggested that the conditions of growth allowing survival in a highly competitive consortium of microorganisms would severely restrict this kind of variability. However, in surface soils there may be significant temperature variations, to which large parts of the microbial community respond in a similar way. The range of temperatures experienced over the year in temperate regions may not be very different from the range applied in this study. In the soil studied here, there was a significant separation of PLFA profiles within this temperature range, as reflected in the PCA; whether similar shifts can be observed in situ, as a seasonal variation, awaits further investigation.

Temperature adaptation within a complex microbial community has, to our knowledge, not previously been studied with PLFA analyses. Although a number of changes could be explained as such with reference to pure culture studies, the possible influence of nutrient availability on the shifts occurring must be acknowledged. Firstly, the temporal pattern is probably influenced, since starved cells would not be able to adapt as quickly as well-fed cells. Secondly, different PLFA changes may occur within a given species, depending on the quality of the carbon source (14). Thirdly, the inability of some organisms to adapt may lead to cell death and thereby induce successional changes fuelled by substrates released from the organisms that died. This interaction between temperature adaptation and nutritional status adds a new level of complexity to the characterization of microbial communities in their natural environment, and it may significantly affect the outcome of methods that involve several days of incubation at an elevated temperature.

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