12 Interpretation of Fatty Acid Profiles of Soil Microorganisms

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12.1 Obtaining Fatty Acid Profiles from Soil Samples

This work focuses on the calculations performed on the peak areas obtained by gas chromatography (GC). All the steps of soil sampling, lipid extraction and fractionation, derivatization, and capillary GC have been repeatedly reviewed, and will only be briefly mentioned (a bibliography of work done in this laboratory is available at http://cba.bio.utk.edu/director_peerfull.html, and an extensive bibliography of methods is provided by Dr. William Christie's group, Mylnefield Research Services Ltd. at http://www.lipidlibrary.co.uk/lit_surv.html).

Sampling is the most important step in sample analysis, and is often delegated to the most junior member of the lab or to site specialists not associated with the lipid laboratory, such as a subsurface sediment drilling crew. Besides sampling location, the sample's consistency, integrity, and appearance should be recorded. In order to obtain deep subsurface samples, the use of drilling equipment and drilling mud is usually required, and methods have been developed to prevent and detect drilling mud contamination of samples (Griffin et al. 1997; Phelps et al. 1989).

Capillary GC with flame ionization detection (FID) is a powerful analytical method – simpler in operation, of greater linear range, and more sensitive, reliable, and reproducible than most analytical instrumentation available. The users' manuals for the chromatograph and data system are the primary references for their operation. If you won't read the manual, you shouldn't touch the equipment. There are also many excellent reviews of capillary chromatography of polar lipid fatty acids (PLFA) available (for example, Grob and Barry 1995).

Capillary GC-MS is a necessary adjunct to GC-FID for the identification of fatty acid peaks (Christie 2003). Various chemical methods are also available to help with specific identification problems such as silver ion chromatography to separate saturates, monounsaturates, and polyunsaturates

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Soil Biology, Volume 5
Manual for Soil Analysis
R. Margesin, F. Schinner (Eds.)

(Momchilova and Nikolova-Damyanova 2000), and special derivatization methods to determine the position and geometry of monounsaturation, such as MS of dimethyldisulfide adducts (Nichols et al. 1986). MS of picolinyl esters provides more informative fragmentations than GC-MS of the methyl ester (Christie et al. 1991; Harvey 1992).

This work presupposes some knowledge of Microsoft Excel (Microsoft Corp., Redmond, WA), which is used to manipulate chromatographic results in many laboratories. The on-line help system is the basic reference for Excel, such as it is. A novice user will benefit from one of the many introductory books available at a bookstore. Also assumed is some background in the statistical procedures commonly applied to PLFA data, including analysis of variance (ANOVA) and factor analysis.

12.2 Transforming Fatty Acid Peak Areas to Total Microbial Biomass

Gas chromatography provides a peak area proportional to the amount of the compound in the sample responsible for the peak. A known concentration of an internal standard, usually 19:0 or 21:0, is added to the sample before analysis to allow calculation of absolute amounts (see Sect. 12.5 for the naming of fatty acids). The equation used to calculate the total amount of fatty acids in a sample is,

$$FA = \frac{(sum\ A_{FA}/A_{IS}) \times IS \times X}{Y} \tag{12.1}$$

FA total picomoles of fatty acids per gram dry mass of sample (pmol/g dry mass)

sum A_{FA} sum of the areas of all identified fatty acid peaks excluding the internal standard

A_{IS} area of the internal standard peak

IS concentration of internal standard used (50 pmole/μL)

X volume of internal standard used to dilute the fatty acid methyl esters (μL)

Y mass of sample extracted (g soil dry mass). In some instances, rather than grams dry mass as the divisor, it will be volume of water (L), surface area in meters squared, or some other extensive variable.

Many analysts calculate the pmol/g dry mass for each fatty acid, then add them together to get the total pmole/g dry mass. This is not good practice, since the pmol/g dry mass for each fatty acid is not then of use in further analysis, and the more complicated calculation makes more work and opportunities for error.

The total moles of membrane fatty acids is proportional to the total microbial biomass. The constant of proportionality used in our laboratory is 2.5×10^4 cells/pmol PLFA (Balkwill et al. 1988; White et al. 1996 and references therein). This conversion factor was derived from measurements on laboratory cultures, so the number of cells will be underestimated for environments populated by smaller bacterial cells, such as oligotrophic environments.

Researchers who count cells, with automated cell counting instruments or by microscopy, are often uncomfortable with measurements of viable biomass expressed as moles of PLFA or grams dry mass of cells. In order to estimate cell counts from moles of PLFA requires knowledge of the distribution of cell sizes in the sample and the amount of PLFA per cell for different sizes, information which is not usually available. It makes more sense to transform cell counts to moles PLFA or from the latter to grams dry weight of cells, since the cell counting can provide the data on cell size distribution.

For most sample sets, the biomass will not be normally distributed, that is, a histogram of the biomass data will be skewed with a long tail toward the higher biomasses. This can be tested for by using the standard f-test for normality. Also, in most biomass data sets, the variance of biomass increases with the absolute value of the biomass. This violates the assumptions of parametric statistics, including ANOVA and factor analysis, and lowers the power of any statistical test employed. These problems can be solved by a $\log(X + A)$ transformation, where X is the mole percent of the fatty acid, and A is a small constant. The small constant is added so that zero values give a real solution when the log transform is applied. The most common value used for A is one, which gives a value of zero for the transform when X is zero, since $\log(0 + 1) = 0$.

There are two approaches to proving the value of applying a log transform to biomass data, the theoretical and the practical. The theoretical explanation involves the scaling of the forces affecting microbial biomass (Magurran 1988) and the fractal structure of microbial environments (Mandelbrot 1982), and is beyond the scope of this work. The practical reason for the log transform is that it works; applying a log transformation to the data is perfectly legitimate, and results in more significant differences on statistical tests.

12.3 Calculation and Interpretation of Community Structure

After the biomass, the next most important information to extract from a PLFA profile is the community structure. But where the biomass is a single value for each sample with a straightforward interpretation, the community structure data is multivariate with many options in its interpretation. A "standard" method for presenting community structure data, how to create a custom method for community structure, and factor analysis will be presented.

12.3.1 Standard Community Structure Method

In the standard method for community structure analysis of PLFA profiles, chemically related fatty acids are grouped as in Table 12.1. A PLFA profile may contain, for example, from 18 to 92 fatty acids. The standard community structure approach summarizes that in six variables, which are just the sum of the mole percents of each of the fatty acid groups. The use of a standard community structure analysis method allows comparison between/among experiments.

Table 12.1. Groups of chemically related fatty acids used in the standard community structure analysis

Group name	Rule	Examples	Microbiota represented
Saturates	Saturated straight-chain fatty acids	12:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0	All organisms
Monounsaturates	Fatty acids with a single unsaturation plus cyclopropyls	14:1ω5c, 16:1ω7c, 16:1ω7t, 18:1ω7c	Proteobacteria
Mid-chain branched	Any mid-chain branched fatty acid	10Me16:0, 10Me18:0	Actinomycetes, sulfate-reducers
Terminally branched	*Iso-* and *anti-iso-* branched saturated fatty acids	i14:0, i15:0, a15:0, i16:0, i17:0, a17:0	Gram positive bacteria
Polyunsaturates	Any fatty acid with more than one unsaturation	18:2ω6c, 18:3ω3c	Eukaryotes
Branched unsaturates	Any branched monounsaturate	i17:1ω7c	Anaerobes

The standard community structure breakdown was originally developed on marine sediments, and has been successfully applied to microbial communities from many environments, including, for example, marine macrofaunal burrows (Marinelli et al. 2002), a subsurface zero-valent iron reactive barrier for bioremediation (Gu et al. 2002), marine gas hydrates (Zhang et al. 2002), soils contaminated with jet fuel (Stephen et al. 1999), and to a comparison of subsurface environments (Kieft et al. 1997).

12.3.2 Custom Community Structure Methods

When examination of the chromatograms or the mole percent table shows differences with treatment, but no significant differences are found in the standard community structure groups, some other way of grouping the fatty acids may be more useful. For example, if samples differ in the proportions of Cyanobacteria and Eukaryotic algae, it may be useful to separate the polyunsaturates with 18 or fewer carbons characteristic of Cyanobacteria (Øezanka et al. 2003) from those typical of Eukaryotic algae with 20 or more carbons (Erwin 1973).

There are several methods for developing alternative community structure groups. The manual method uses the pattern recognition power of the human eye. The PLFA chromatograms are printed on the same scale and spread out on a large table. Similar-looking chromatograms are grouped together and different-looking ones are placed in separate groups. While very low-tech, this works remarkably well. This same approach can be applied to a mole percent table by printing it out, cutting out a strip for each sample, and sorting the samples by similarity. Once the samples have been sorted into similar groups, the fatty acids responsible are summed to form new community structure groups.

Given access to statistical software, a triangular table of Pearson's r correlation coefficients is usually available as an output option. Visual examination of this table will locate fatty acids with high correlations, which are then grouped together to form new community structure groups.

12.3.3 Factor Analysis

Factor analysis includes several related methods, including principal-components analysis. The virtue of this method is that it automatically constructs fatty acid groups reflecting the differences in community structure, rather than applying a preconception of fatty acid groups. The data determines the fatty acid groups, rather than the analyst. Factor loadings greater

than 0.7 indicate fatty acids with "significant" effects on the results. The factor scores are new variables that are linear combinations of the original values. These new variables can be submitted to statistical tests such as ANOVA like any other variable. Examples of the application of factor analysis to PLFA profiles include storage perturbation of soil microbial communities (Haldeman et al. 1995; Brockman et al. 1997), soils at different temperatures (Zogg et al. 1997), and soils from different ecosystems (Myers et al. 2001).

The results of factor analysis are usually improved by applying the log(X+1) transformation to the mole percent data before factor analysis. A rough method to determine whether the mole percent data is normally distributed is to calculate the maximum, average, and the minimum not equal to zero for each fatty acid. The formulas for these in Excel are "= max(b2.b45)", "= average(b2.b45)", and "= min(if(b2.b45 = 0, 100, b2.b45))", where b2.b45 is the range containing the data. The formula for min 0 is what Excel terms an array formula; you have to hold down the Shift and Control keys while you press Enter to enter the formula. If the difference between the maximum and average is greater than the difference between the average and the minimum 0 for most of the fatty acids, then the data is not normally distributed and the log(X+1) transformation will probably improve results.

There are theoretical reasons to advocate the arcsin[square root(X)] transformation over the log(X+1) transformation, but very little difference is found in practice, and the log(X + 1) is simpler to apply and explain. Similarly, there are theoretical reasons to prefer factor analysis *sensu stricto* over principal components analysis, and vice versa, which can, and have been, argued for days to no conclusion. In practice, the two methods give very similar results.

12.4 Calculation and Interpretation of Metabolic Stress Biomarkers

The membrane of the bacterial cell handles all of its interactions with its environment, and bacteria have many strategies to deal with stressful environmental conditions, including modifying the fatty acids used in the membrane. This is illustrated in Eq. (12.2), where S stands for the substrate fatty acid and P for the product fatty acid induced by metabolic stress, namely, a *trans* monounsaturate or cyclopropyl fatty acid.

$$\begin{array}{c} \text{S} \rightarrow \text{P} \\ \textit{cis} \text{ monounsaturate} \rightarrow \textit{trans} \text{ monounsaturate} \\ \textit{cis} \text{ monounsaturate} \rightarrow \text{cyclopropyl} \end{array} \qquad (12.2)$$

cyclopropyl fatty acids by prefix "Cy." For example: 18:1ω7c is 18 carbons long with one double bond occurring at the 7th carbon atom from the ω end, and the unsaturation is in the cis conformation. Also, 16:0, i16:0, a16:0, and br16:0 are all 16-carbon fatty acids, while 10Me16:0 and Cy17:0 both contain a total of 17 carbons, not counting the carbon of the methyl ester moiety.

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