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Measuring soil microbial community diversity using polar lipid fatty acid and denaturing gradient gel electrophoresis data ☆

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Abstract

The possibility of calculating useful microbial community diversity indices from environmental polar lipid fatty acid and 16S rDNA PCR-DGGE data was investigated. First, the behavior of the species richness, Shannon's, and Simpson's diversity indices were determined on polar lipid fatty acid profiles of 115 pure cultures, communities constructed from those profiles with different numbers of species, and constructed communities with different distributions of species. Differences in the species richness of these artificial communities was detected by all three diversity indices, but they were insensitive to the evenness of the distribution of species. Second, data from a field experiment with substrate addition to soil was used to compare the methods developed for lipid- and DNA-based diversity indices. Very good agreement was found between indices calculated from environmental polar lipid fatty acid profiles and denaturing gradient gel electrophoresis profiles from matched samples (Pearson's correlation coefficient $r = 0.95$–0.96). A method for data pre-treatment for diversity calculations is described. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Diversity; Shannon's index; Simpson's index; 16s rDNA; Denaturing gradient gel electrophoresis; Polar lipid fatty acids

1. Introduction

Diversity is an important concept in ecology, often applied in environmental monitoring (Hosmani, 1987) and conservation management (Bourgeron, 1989). High diversity may correlate with ecosystem resistance to stresses such as pollution (Nevo et al., 1986). Extreme conditions (temperature, pH, salt concentration) can reduce the diversity of affected communities (Strom, 1985), and a more diverse

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physical environment often produces a higher community diversity (McArthur et al., 1988). Each of these statements is highly controversial, as is the measurement of diversity itself (Magurran, 1988; Peet, 1974; Pielou, 1975; Robinson and Sandgren, 1984).

Diversity is an intuitively obvious concept which is difficult to define and quantify. There are two parts to diversity: the number of species and the evenness of the distribution. A sample with more species is more diverse; while an evenly distributed community is more diverse than an unevenly distributed community with the same number of species.

Ecology has a lively literature on how diversity is measured on species-abundance data, but for practical reasons this type of data on bacterial communities has been unavailable or unreliable. Bacterial species are not, in general, distinguishable by microscopic examination (Woese, 1987). Culturing methods for enumerating the composition of microbial communities are also not effective since the vast majority of individuals present are not currently culturable under laboratory conditions (Torsvik et al., 1990a,b). Antibody-based detection methods are highly specific and reliable, but an antibody reagent only responds to a predetermined range of cultured strains or species, while there are estimated to be thousands of microbial species in a few grams of soil (Torsvik et al., 1990a,b; Øvreås and Torsvik, 1998). A very promising method for estimating microbial diversity is based upon the reassociation kinetics of DNA isolated from the entire community (Torsvik et al., 1990a,b), but the method is too expensive and time consuming for routine use.

Polar lipid fatty acids (PLFA) have been validated for the measurement of microbial biomass, community structure, and metabolic status of environmental samples (Ringelberg et al., 1989). Their utility as a measure of viable biomass is based upon their presence in the membranes of all living cells, with the exception of the Archaea (Langworthy, 1985), and their rapid turnover upon cell death (White et al., 1979). There are two conceptual approaches to the use of PLFA profiles for determining community structure. The entire PLFA profile can be used as a 'fingerprint' of the microbial community, using statistical methods such as clustering or factor analysis to detect differences with treatments. Alternatively, signature lipid biomarkers for specific phylogenetically or metabolically defined groups can be reported as proportions of the total PLFA (Findlay et al., 1990).

There are a number of reasons for believing that some function of the PLFA profile could be used as an indication of microbial community diversity. First, the PLFA profile of an environmental sample is indicative of the entire viable microbial community (Ringelberg et al., 1989). Second, bacterial species are routinely distinguished by their PLFA profiles (Welch, 1991), with the difference in their profiles increasing with phylogenetic distance (Guckert et al., 1985; Kohring et al., 1994). Finally, each species in the microbial community contributes to the PLFA profile in proportion to its biomass.

The requirements of a function of the PLFA profile to be used as a measure of diversity are: that it should bear a recognizable relation to species-based measures of community diversity, it should be able to distinguish between treatments in an experiment, and it should be easily interpretable.

A second biomarker suitable for community-level diversity analysis is the 16S rRNA gene. Homologues of this gene are present in all known organisms, and again, phylogenetic distances are reflected in the level of difference in 16S rRNA gene sequence. Fragments of 16S rRNA genes can be recovered from environmental samples by extraction of total DNA followed by amplification using the polymerase chain reaction targeting sequences which are conserved within Bacteria, but differ in the kingdoms Eukarya and Archaea (Tsai and Olson, 1992). Separation of such mixed amplification products on the basis of melting point within a gel matrix generates a banding pattern (Muyzer et al., 1993). Measurement of the number of bands, their positions, and their relative intensities provides data that is amenable to standard diversity analyses (Eichner et al., 1999; Kowalchuck et al., 2000).

In this paper, three commonly used diversity indices were applied to pure culture bacterial fatty acid profiles generated in silico, and the profiles of artificial communities constructed from them. These same diversity indices were then applied to PLFA and DGGE data from an in situ substrate amendment experiment, in which shifts in the soil microbial community induced by the injection of milk-whey

were monitored. An earlier application of PLFA profiles to the calculation of a diversity index is available (Korner and Laczkó, 1992).

2. Materials and methods

2.1. Diversity measures: S, H, and D

Of the many diversity measures that have been suggested in the ecological literature, three were selected which are representative and widely recognized: species richness, Shannon's H', and Simpson's D. Species richness, S, is the number of species counted in the sample, which monotonically increases with the true number of species in the community (Magurran, 1988). Herein, S(PLFA) is the number of the PLFA detected and S(DGGE) the number of 16S rRNA bands detected.

Shannon's H', originally developed in information theory (Shannon and Weaver, 1949), and later applied to ecological diversity (Margalef, 1957), is calculated from

$$H' = -\Sigma[p_i \times \ln(p_i)]$$

where p_i is the proportion of the community represented by species i, and the summation is over all species. H' represents the uncertainty in predicting the species of an individual chosen at random (Ludwig and Reynolds, 1988, p. 96). H(PLFA) and H(DGGE) was calculated similarly using the mole fraction of each fatty acid or band.

Simpson's λ was the first diversity measure proposed (Simpson, 1949). It is the probability that two individuals chosen at random will belong to the same species (Ludwig and Reynolds, 1988, p. 96). Subtracting it from 1 forces it to range from 0 to 1, and to increase with diversity.

$$D = 1 - \lambda = 1 - \Sigma\ p_i^2$$

S, H', and D are successively less sensitive to the biomass of the sample and more sensitive to the evenness (Magurran, 1988). All three increase with increasing diversity. While the species richness, S, increases with the number of species in the sample, H and D also respond to the evenness of the distribution, D more so than H.

These diversity indices were applied to: PLFA data for pure cultures, communities constructed from the pure culture data with perfect evenness and different numbers of species, constructed communities with geometric distributions having different evenness, and data from environmental samples.

2.2. Normalizing to common biomass

In ecological studies using species-abundance data, a method called rarefaction (Magurran, 1988) is used to normalize samples to a common sample size, that is, the same number of individuals counted. The following procedure was used in order to normalize either PLFA or DGGE data from a set of samples to a common analytical sensitivity so that their diversity indices could be compared.

For each sample, the values of the PLFA or DGGE peaks expressed as a percentage of the total were sorted into descending order. The minimum percent reported in each sample is noted, and the largest of these is chosen for the cutoff point. The largest minimum percent reported in any sample represents the analysis with the least sensitivity, the sensitivity that all the samples need to be corrected to for their diversity indices to be compared. (If all peaks less than the cutoff value were simply deleted, it is possible that a few values that should have been retained will be deleted.) If the two lowest values for a sample were both lower than the cutoff point, the lower value was deleted, and the sample re-normalized such that the sum of the remaining values equals 100%. This was repeated until the lowest two values were above and below the cutoff point. If the lower of these values was closer to the cutoff point than the second lowest, then the lowest value was retained. Otherwise, the lowest value was deleted and the sample again re-normalized.

2.3. Bacterial fatty acid database

A database of fatty acid profiles was collected from the literature (see Appendix I for bibliographic citations). Profiles were included in the database if: (1) they were bacterial polar lipid fatty acids or Gram-positive total lipid fatty acids; (2) if the capillary gas chromatographic peaks had been subject to mass spectral verification; (3) if the least

abundant reported fatty acid was at most 1 mol%; and (4) there were no more than two bacteria from the same species included. Polar lipid fatty acids were specified, although the fractionation of the polar lipid from the total lipid removes information from the sample. Most environmental samples contain significant amounts of 'fossil' fatty acids from dead organisms that mask the signal from the viable microbial community, necessitating the additional purification step to obtain the polar lipids. Gram-positive bacteria are a large and important group which do not significantly differ in their polar lipid fatty acid and total lipid fatty acid profiles (O'Leary and Wilkinson, 1988), so total lipid profiles were included from that group. Polar lipid fatty acid profiles were chosen from organisms selected to represent a broad range of species. One hundred and fifteen bacterial fatty acid profiles were included, from nine of the 16 division-level rankings of cultured bacteria, with an approximately equal distribution of Gram-positive and Gram-negative species (Maidak et al., 1999).

2.4. Microbial communities constructed from literature data

Two different types of community were constructed: even distributions with varying numbers of species, and geometric distributions of 115 species. For the even distributions, five or 10 species were chosen at random, their PLFA profiles were averaged, and the minimum truncated to a common value, as described above. This was repeated 5 times for both species counts. An additional even distribution was calculated using all 115 species in the database.

For the geometric series, the proportion of the biomass, p_i, in each species, i, was calculated from

$$p_i = [1 - (1 - k)^S]^{-1} \cdot k \cdot (1 - k)^{i-1}$$

where k is the fraction of available resources utilized by each succeeding species and S is the total number of species (Magurran, 1988). Each of the 115 pure cultures were randomly assigned to a position in the community, and the community PLFA profile calculated. Before calculation of diversity indices, the PLFA and species abundance data were truncated to a sensitivity of 0.1%. This procedure was repeated 4 times to construct four different communities, each with 10 different values for the evenness component of diversity.

2.5. Whey barrier experiment data: PLFA

Samples were obtained from a site located near the Michigan Integrated Remediation Technology Laboratory (MIRTL) at the former Wurtsmith Air Force Base (WAFB) in Oscoda, MI. Redox conditions were adjusted through the in situ injection of an aqueous suspension of milk solids (whey), inducing a sub-surface iron-reducing or methanogenic zone ('whey barrier'). This zone was allowed to develop for 30 days prior to sampling. Samples from within the whey barrier as well as background controls from up-gradient of the barrier were obtained using a Geoprobe piston corer (Geoprobe Systems, Salina, KS). Each core was sectioned into vadose (2.13 m), bridging water table (2.43 m), and saturated zones (2.74 m). Samples on dry ice were sent overnight to the laboratory for storage at −80°C.

All solvents used for lipid analysis were of GC grade and were obtained from Fisher Scientific (Pittsburgh, PA). Samples for lipid analysis were approximately 70 g (average, 68 g, S.D.=4 g). Lipids were extracted using the modified Bligh and Dyer extraction (White et al., 1979). The organic layer was fractionated into glyco-, neutral- and polar-lipids and the latter then transesterified to their methyl esters (Guckert et al., 1985). The methyl esters were separated, quantified, and identified by gas chromatography–mass spectrometry (Guckert et al., 1985), using a Hewlett-Packard HP5890 series II gas chromatograph interfaced with a HP5972 series mass selective detector (Hewlett Packard, Wilmington, DE). Fatty acids were designated as described by Ringelberg et al. (1989).

2.6. Whey barrier experiment data: PCR-DGGE

Direct nucleic acid extraction was performed using a bead-beating system adapted from Stephen et al. (1999) with minor modifications. Soil (0.5 g), sodium phosphate buffer (425 μl; 0.12 M, pH 8.0), chaotropic reagent (175 μl; CRSR, Bio-101, Vista, CA, USA), and 0.17-mm glass beads (0.5 g) were agitated in a 2-ml screw-capped microcentrifuge tube using a 'FastPrep' bead-beating system (Bio 101) set

at maximum speed for 45 s. The sample mixture was centrifuged at 13 000×*g* for 5 min and the supernatant collected. Chloroform (300 μl) was added to the soil pellet, mixed thoroughly, and centrifuged at 13 000×*g* for 5 min. The aqueous supernatant was collected and combined with the first supernatant fraction. DNA was precipitated from the aqueous phase with an equal volume of 100% isopropanol in an ice bath for 30 min. DNA was pelleted by centrifugation at 13 000×*g* at 4°C for 15 min, washed with 80% ethanol (1 ml) twice, air-dried, and re-dissolved in TE buffer (200 μl; pH 8.0). The DNA extract was purified by extraction twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v), followed by a glass-milk DNA purification protocol using a Gene Clean™ Spin-kit (Bio-101) as described by the manufacturer.

PCR amplification of 16S rDNA gene fragments was performed as described (Muyzer et al., 1993) with modifications. Thermocycling consisted of 35 cycles of 92°C for 45 s, 55°C for 30 s and 68°C for 45 s, using 1.25 units of Expand polymerase (Boehringer Mannheim, Indianapolis, IN, USA), 10 pmol each of the forward primer 341f-GC and reverse primer 534r in a total volume of 25 μl performed on a 'Robocycler™' PCR block (Stratagene, La Jolla, CA, USA). The final extension period was performed for 7 min at 68°C. DGGE employed a D-Code 16/16 cm gel system (BioRad, Hercules, CA) maintained at a constant temperature of 60°C in 0.5× TAE buffer (20 mM Tris–acetate, 0.5 mM EDTA, pH 8.0). Gradients were formed between 15 and 65% denaturant (100% denaturant was defined as 7 M urea, 40%, v/v, formamide) and the gels run at 35 V for 16 h. Gels were stained in purified water (Milli-Ro™, Millipore, Bedford, MA, USA) containing ethidium bromide at 0.5 mg/l, and de-stained twice in 0.5× TAE. Images were captured by use of an Alpha Imager™ system (Alpha Innotech, San Leandro, CA, USA). Quantification of ethidium bromide fluorescence of DGGE bands and scoring of band positions were carried out using the '1D Multi' function of the accompanying image software.

3. Results and discussion

3.1. Diversity indices applied to pure culture data

S(PLFA) calculated from the literature database of pure cultures was highly variable, averaging 15.6 fatty acids with a standard deviation of 7.8, and a maximum and minimum of 42 and 3, respectively (Table 1). *H*(PLFA) and *D*(PLFA) were each successively less variable. When the 115 pure culture PLFA profiles were divided into three groups according to

Table 1
Behavior of diversity indices on pure culture bacterial fatty acid profiles from the literature[a]

	n	*S*(PLFA)				*H*(PLFA)				*D*(PLFA)			
		Avg	%SD	Max	Min	Avg	%SD	Max	Min	Avg	%SD	Max	Min
Pure cultures:	115	15.6	50	42	3	1.73	27	2.94	0.42	0.73	18	0.93	0.16
Minimum %:													
High, 0.6–1.1	27	9.3	34	15	3	1.52	31	2.33	0.42	0.67	23	0.88	0.23
Med., 0.12–0.51	38	12.3	31	22	6	1.69	25	2.29	0.42	0.72	19	0.87	0.16
Low, 0.0006–1	50	21.5	36	42	7	1.86	25	2.94	0.70	0.76	14	0.93	0.36
Truncated data:	115	10.2	34	23	4	1.62	28	2.75	0.36	0.71	19	0.92	0.14
Minimum %:													
High, 0.6–1.1	27	9.6	33	15	4	1.50	32	2.33	0.42	0.67	24	0.88	0.23
Med., 0.12–0.51	38	9.6	27	16	5	1.60	26	2.19	0.36	0.71	20	0.87	0.14
Low, 0.0006–1	50	11.0	36	23	4	1.71	26	2.75	0.57	0.74	15	0.92	0.33
Even dist., 5 sp.	5	17.4	30	24	13	2.42	13	2.83	2.08	0.88	4	0.92	0.83
Even dist., 10 sp.	5	21.4	7	23	19	2.73	3	2.82	2.63	0.91	2	0.93	0.89
Even dist., 115 sp.	1	25				2.91				0.92			

[a] The diversity indices *S*(PLFA), *H*(PLFA), and *D*(PLFA) were calculated before and after truncation of the profiles to a common sensitivity, as described in Section 2. Constructed communities with an even distribution and either 5, 10, or 115 species are also presented. *n*, number of cases; Avg., average; %SD, percent relative standard deviation; Max, maximum value; Min, minimum value.

the sensitivity of analysis, as indicated by the minimum value reported, the values of *S*(PLFA) were seen to vary strongly with sensitivity of analysis. More sensitive PLFA analyses detected more PLFA. The same trend was seen in *H*(PLFA) and *D*(PLFA), though less dramatically. However, by truncating the PLFA profiles to a common sensitivity of 1 mol%, the differences between the averages of the diversity measures on the high, medium, and low sensitivity PLFA profiles were greatly decreased (Table 1).

3.2. Constructed communities: even distribution

Constructed communities with even distribution and only five or 10 species had much less variation in the diversity indices (Table 1), when compared with the truncated pure culture data. The percent relative standard deviation for *S*(PLFA) went from 34% for truncated pure cultures to 30% for an even distribution of five species, and to 7% for 10 species; *H*(PLFA) from 28–13 to 3%, and *D*(PLFA) from 19–4 to 2%. If the database truncated to a minimum of 1% is considered a collection of communities of one species each, then we have four points showing how the diversity measures respond to increasing number of species: 1, 5, 10, and 115 species. The numerical value of all three diversity measures increased with increasing number of species in an even distribution, and the variation within a number of species went down.

3.3. Constructed communities: geometric distribution

Fig. 1 shows the behavior of *S*(PLFA) on communities constructed according to the geometric series. On the *x*-axis, the number of species above 0.1% of the community is plotted, as *S*(PLFA) is on the *y*-axis. The four different randomly chosen mixtures of species are represented by different lines. The even distribution is identical for all four mixtures of bacterial species, as shown by the point in the upper right of the graph where the four lines meet. The value of *S*(PLFA) monotonically increased with increasing evenness of the distribution. Different mixtures of bacteria give slightly different values of *S*(PLFA), but each community responded to increasing evenness with increasing *S*(PLFA). At unrealistically high degrees of evenness, *S*(PLFA) approaches a constant. Most real microbial communities will be in the range of rapidly increasing slope on the left side of the graph.

In Figs. 2 and 3, *H*(PLFA) and *D*(PLFA) also

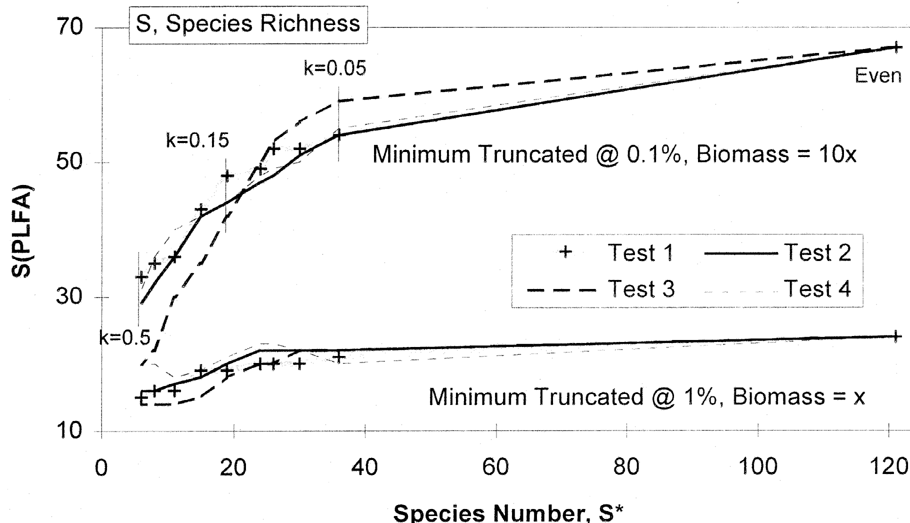

Fig. 1. *S*(PLFA) as a function of the true species number, *S**, for constructed communities with different degrees of evenness. Tests 1–4 represent different randomly chosen combinations of bacterial species.

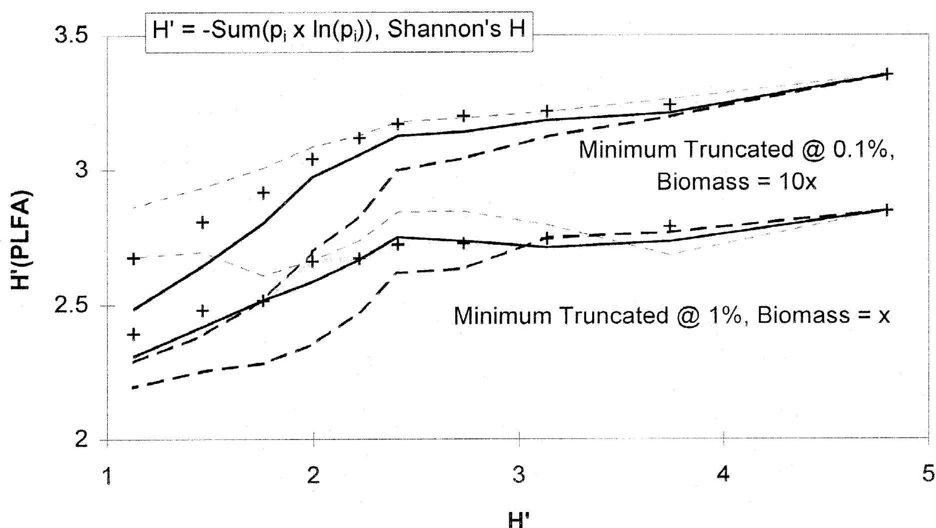

Fig. 2. H'(PLFA) as a function of Shannon's diversity index, H', for constructed communities with different degrees of evenness.

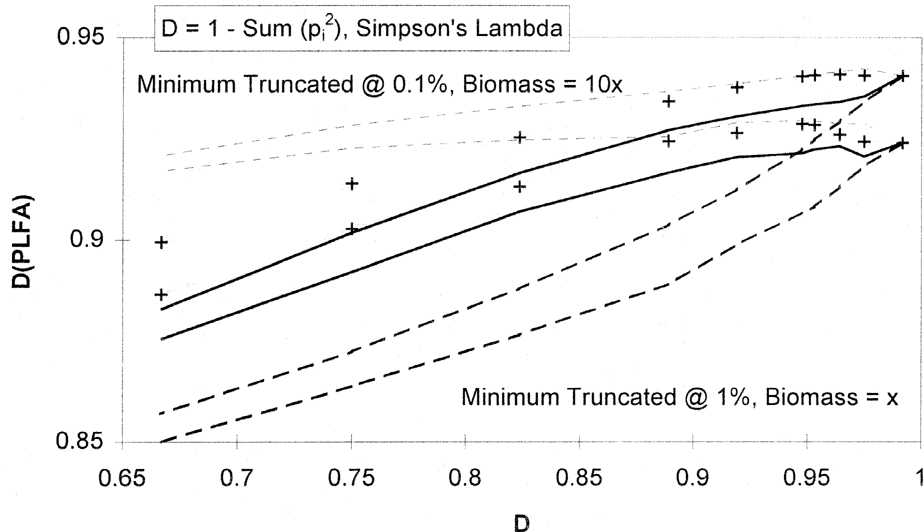

Fig. 3. D(PLFA) as a function of Simpson's diversity index, D, for constructed communities with different degrees of evenness.

increased monotonically with increasing evenness. Also, the differences between different combinations of bacterial species at a particular level of evenness was greatest for D(PLFA), less for H(PLFA), and least for S(PLFA).

In a species-abundance count, species richness should not have changed with different evenness of the distribution. To model analytical results on real samples however, the PLFA profiles of the constructed communities had to be truncated to a common sensitivity. It would not be a realistic analytical result to report PLFA detected in the parts per million range. PLFA from the less abundant species in the distribution were preferentially eliminated in the more uneven distributions, lowering S(PLFA). The truncation had the effect of lowering

the number of species detected in the uneven distribution.

In species-abundance counts, a species represented by a single individual in the community has a reasonable chance of being counted. In fact, the most common species count found is usually one individual per species. On the other hand, a microbial species represented by a single individual in a sample has no chance of being detected in a PLFA or DGGE profile. Since the majority of species have no effect on these indices (the large number of rare species representing a minority of the biomass) information provided by these indices only considers that part of the distribution above the analytical sensitivity cutoff point. So while species-abundance counts tend to give more information on the least abundance species, chemical measures such as PLFA and DGGE profiles give more information on the most abundant species. There is a cut-off point in species abundance, determined by the analytical sensitivity, below which there is effectively no information available. Changes in the evenness of the distribution of species push individual species above or below this cutoff point, increasing or decreasing the species richness. Information on the evenness of the distribution of the most abundant part of the community is confounded with the species abundance information in all three indices. The result is that these diversity indices, *S*(PLFA), *H*(PLFA), and *D*(PLFA), are all different measures of the diversity of the most abundant part of the microbial community: that part of the community above the analytical sensitivity.

3.4. PLFA and DGGE data from a substrate amended site

The species richness values calculated from the PLFA and DGGE data from sub-surface sediments are compared in Fig. 4. *S*(DGGE) values are on the *x*-axis and *S*(PLFA) on the *y*-axis. Before whey treatment, the four sampled soil horizons decreased in species richness in the order vadose>water table>saturated zone 1>saturated zone 2. After whey treatment, the species richness for the three depth horizons sampled all increased to very similar values. The data are offset from the *x*-axis, due to each microbe contributing several fatty acids. The

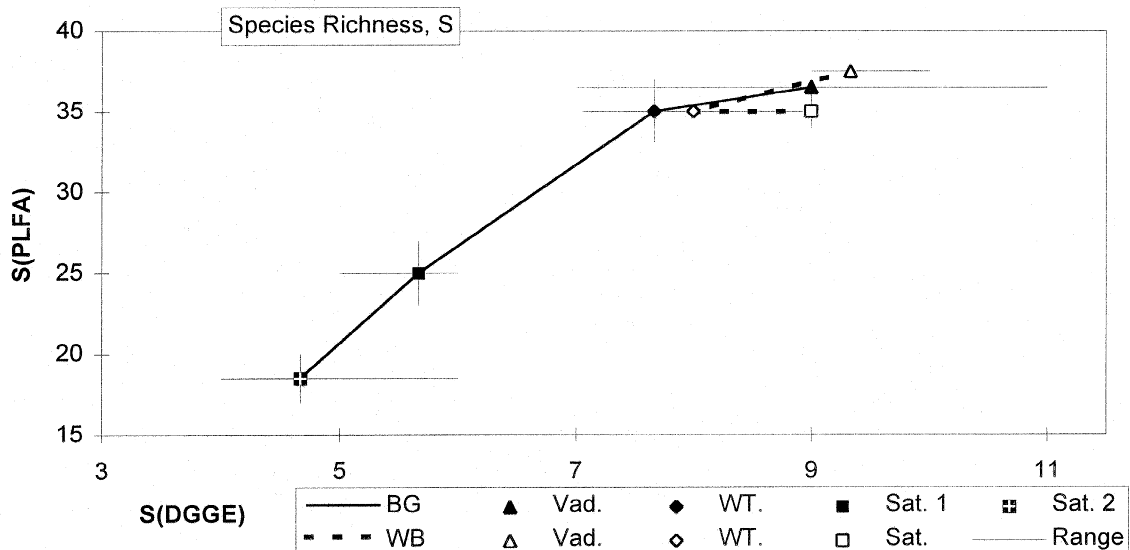

Fig. 4. *S*(DGGE) compared with *S*(PLFA) for samples from a bioremediation site. Background (BG) samples are connected by solid lines, whey-treated samples (WH) by dashed lines. Vadose (Vad.), water table (WT), and saturated zone (Sat.) samples were collected before and after whey treatment. Thin horizontal and vertical lines represent the range of values determined at each site. There is no vertical error bar for the whey-treated water table sample, since only one was analyzed for PLFA.

curvature of the data are due to decreasing response of *S*(PLFA) with greater number of species in the sample. Error bars in Fig. 4 represent the range of values. The much greater width of the *S*(DGGE) error bars may be due to the much smaller sample size analyzed for DGGE than for PLFA (0.5 vs. 68 g), and would be an indication of the local small-scale spatial variation in the microbial community. Additionally, the smaller absolute values of *S*(DGGE) as opposed to *S*(PLFA) contributed to the error. Importantly, however, random drift in amplification events during the early stages of PCR may induce differences in product from identical complex templates, (Wagner et al., 1994; Polz and Cavanaugh, 1998; Chandler et al., 1997), inducing artifactual variations between replicates. Generally, the species richness calculated from the DGGE data agreed very well with those calculated from the PLFA data, as shown by all the points falling along a line with a Pearson's correlation $r = 0.96$.

H(PLFA) also agreed very well with *H*(DGGE), as well as *D*(PLFA) with *D*(DGGE), for the subsurface sediment samples (Figs. 5 and 6). Pearson's r for H' was 0.95, and for D was 0.96. The control samples from before whey injection lay upon a straight line for both indices. The vadose samples after whey injection were on the same line, while the water table and saturated zone samples were above and below that line, respectively.

3.5. The different natures of species-abundance count and molecular data

The nature of the data collected by an ecologist counting species and individuals per species is different from that determined by a lipidologist measuring biomass and proportions of fatty acids, which is again different from a molecular biologist measuring the proportions of target gene sequences. Species-abundance data like that collected in ecological studies of eukaryotes consists of a list of species and counts of individuals in each species. In real environments, there are many more rare species than common ones. The minimum count is one, and all values are integers. Rarely is an entire population counted (usually only a small fraction of the individuals are collected), and the experiment has to be carefully designed so the data are representative of the community (Magurran, 1988). Ecologists are very aware of the difference between the population and the sample counted, as well as the effect of sample size on the diversity measures. A procedure

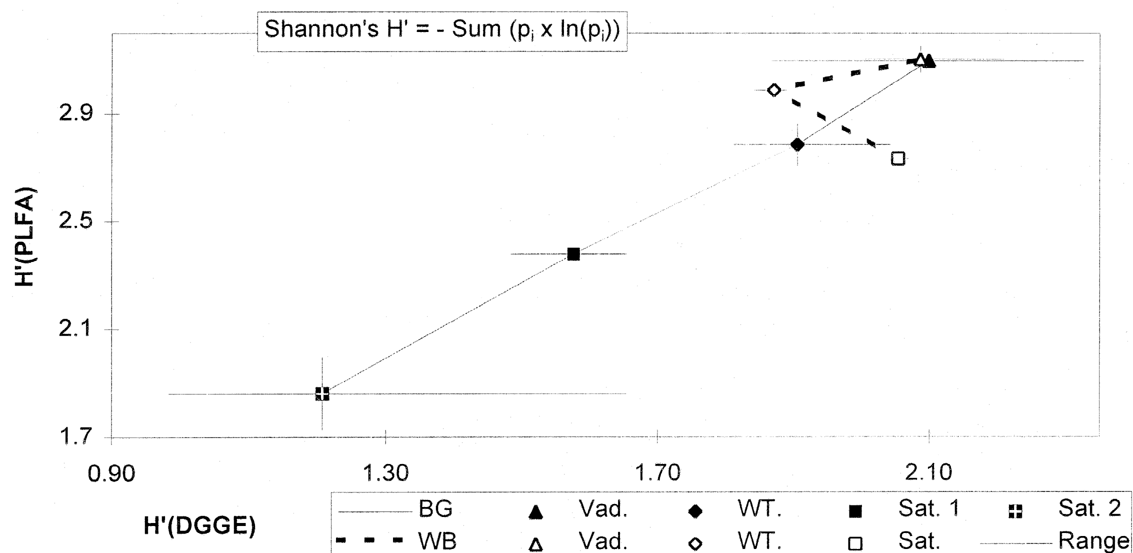

Fig. 5. H'(DGGE) compared with H'(PLFA) for samples from a bioremediation site. Symbols as in Fig. 4.

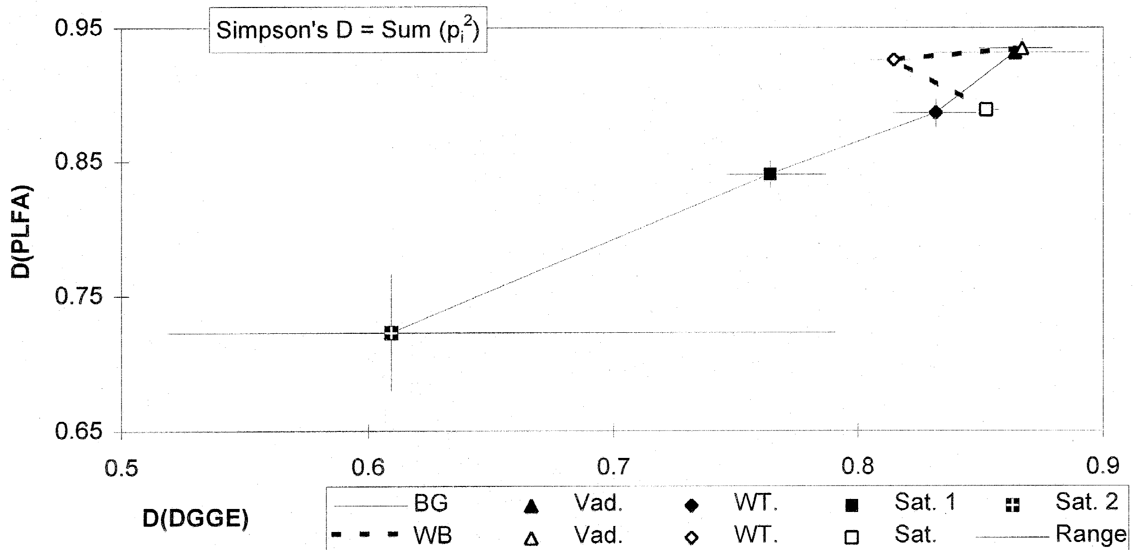

Fig. 6. *D*(DGGE) compared with *D*(PLFA) for samples from a bioremediation site. Symbols as in Fig. 4.

called rarefaction is used to correct species enumeration data to a common sample size. A measure of relative biomass is always available by multiplying counts of each species by an estimated average weight. The sensitivity of analysis is determined by the number of individuals counted.

Environmental polar lipid fatty acid (PLFA) profiles consist of a list of fatty acids and the amount of each normalized to some extensive variable, typically nanomoles per gram dry weight in soil studies. This is usually expressed as mole percent of each fatty acid and the total PLFA per gram. Fatty acid patterns contain a great deal of information about microbial communities, and total PLFA per gram has been shown to be a very good estimate of viable soil biomass (Ringelberg et al., 1989; Guckert et al., 1985). The major limitations of PLFA data for the measurement of diversity is that each species contributes a group of PLFA to the profile, and many species have similar PLFA profiles. Sensitivity of analysis is determined by amount of sample analyzed, dilution factors, and instrumental parameters of the capillary gas chromatographic analysis.

Denaturing gradient gel electrophoresis (DGGE) data consists of the relative abundances of bands separated in a polyacrylamide matrix on the basis of their melting points. As PCR-DGGE is a qualitative analysis, only the abundance of each band relative to the total detectable community are normally measured. Potential sources of error include incomplete DNA recovery, variable rDNA gene number per cell, (Farrelly et al., 1995), differences in primer efficiency on different species (Polz and Cavanaugh, 1998), unequal amplification efficiencies of different sequences (Felske et al., 1998) and, as this is a single-dimension chromatographic procedure, co-migration of different gene fragments. PCR-DGGE based measures of changing community structure are likely to be most appropriate when cell growth rates are high, and community changes rapid, thus minimizing interference from free DNA (Lorenz and Wackernagel, 1987). Very specific information on microbial community components can be recovered by sequencing individual bands (e.g., Macnaughton et al., 1999). Sensitivity of analysis is chiefly limited by the resolving power of the DGGE gel and the imaging system employed.

In a species-abundance count, the vast majority of species are represented by only one or a few individuals, and many individuals must be counted in order to get reasonable estimates of the proportions of the major species. In an environmental PLFA profile, the many minor species contribute small proportions to the PLFA detected, and the PLFA profile as a whole is reflective of the species in the sample in proportion to their abundance. The total

signal in a DGGE banding pattern represents the total biomass amenable to the primers used (Chang et al., 2000), in the sample, but only the major species give quantifiable bands (Muyzer et al., 1993; Stephen et al., 1999).

Since the nature of the data provided by these three methods (species-abundance counts, PLFA, DGGE) are so different, and the determination of diversity on species-abundance counts remains so controversial after 50 years of work, the methods developed for species-abundance data cannot be directly applied to chemical measures such as PLFA or DGGE data. The nature of the data must be respected, and the data treatment methods modified accordingly.

4. Conclusions

Upon whey treatment, *S*, *H*, and *D* calculated from PLFA or DGGE data increased for the sub-surface and water table samples. Only a minority of the bacterial population would be able to exploit the resources in the whey. Those bacteria multiplied, making the distribution of species more uneven and decreasing the diversity. This pushed more species above the detection limit, increasing the measured species richness. The total number of species in these subsurface samples is not expected to change, but number of species above the detection limit increased.

Differences in biomass between samples greatly affects the number of PLFA detected, and can also have an effect on the number of DGGE peaks detected. Before the calculation of diversity indices, PLFA or DGGE data should be adjusted to a common sensitivity across all samples, analogously to the rarefaction method applied to species count data. A method to adjust data to a common sensitivity is presented in this paper which could be automated with a spreadsheet macro or programming language.

S(PLFA) may not show differences with treatment on some PLFA data sets, due to high biomass of the sample. Either *H*(PLFA) or *D*(PLFA) can be used, or a smaller sample can be analyzed. *D*(PLFA) is much more affected by differences in the species making up the community. In general, all three indices should be calculated and compared to obtain the most reliable information. Similarly, the use of DGGE data in diversity measurements is limited in the complexity of the systems that can be analyzed. A given target sequence must represent approximately 1–2% of the target community to be detected, meaning that in theory, samples generating a maximum of 50–100 distinct bands are amenable to analysis (Muyzer et al., 1993). Communities carrying a larger number of evenly distributed dominant organisms are expected to generate an unresolved 'smear' of DNA fragments. In practice, we find accurate quantification of band intensities problematic in soil when the technique reveals over approximately 25 bands due to interference from the background 'smear' of less abundant sequences.

That the sub-surface and water table samples gave increased measures of diversity after whey treatment, while the species count stayed the same and the evenness component of the diversity decreased, argues for much caution in the application of these measures. Simply calculating *S*, *H*, or *D* for a data set may very well give misleading results. Differences in biomass of the samples and sensitivity of analysis must be controlled for, and consideration given to what is actually being measured.

The nature of the data collected by ecological species-abundance counts is different from that obtained from chemical measures such as PLFA or DGGE. There is a need for new diversity indices and/or distribution models to deal with molecular data.

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Appendix I. References for the fatty acid database

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