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FEMS Microbiology Ecology 33 (2000) 129–137

FEMS MICROBIOLOGY Ecology

www.fems-microbiology.org

Comparison of methods to investigate microbial populations in soils under different agricultural management

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Received 22 February 2000; received in revised form 22 May 2000; accepted 23 May 2000

Abstract

The microbial community in sludge-treated and nearby untreated soils was investigated using direct microscopic counting, plate culture, carbon substrate utilisation (Biolog™), and fatty acid methyl esters. Long-term sewage sludge applications had resulted in higher concentrations of organic carbon and had altered other factors in the soil, including the concentration of metals. Bioluminescence was inhibited in assays of the sludge-treated soil, although microbial counts were similar in all soils. A detailed analysis of carbon substrate utilisation patterns and fatty acid methyl esters showed qualitative differences in the microbial populations. This work shows that a variety of approaches are required to assess microbial communities in soil where, despite large differences in land management, the populations are similar in size and overall composition.

Keywords: Soil microbial population; Biolog™; Fatty acid methyl ester; Bioluminescence; Sewage sludge

1. Introduction

Soils are reported to contain 10^5–10^8 bacteria, 10^6–10^7 actinomycetes and 10^5–10^6 fungal colony-forming units g^{-1} [1]. These high numbers together with the limitations of conventional plating techniques pose a great problem in measuring microbial populations in soils. Bacteria that are able to grow on agar plates typically account for only around 1% of the bacteria in the soil [2]. Also, the presence of an organism in the environment does not necessarily mean that it is contributing significantly to soil processes. Methods using direct approaches, such as cloning and sequencing DNA extracted from soil are very labour-intensive and inform of the presence of an organism, not its function and activity. Fatty acid methyl esters (FAMEs) and phospholipid fatty acids (PLFAs) extracted directly from soil indicate the presence of groups of organisms: FAMEs represent all lipids in the soil; PFLAs are a subset that arise from the living portion of the biomass [3,4]. The Biolog™ system has been used widely in assessing the metabolic diversity of soil microbe populations, with no consensus on the best method for interpreting or analysing such data [5]. All of these methods are subject to their own biases, nevertheless, combinations of the different approaches can provide valuable information. Differences in microbial population size, respiration, culturable bacterial numbers, FAMEs and PFLAs, and substrate utilisation by Biolog™ have all been investigated in relation to soil organic matter and the concentration of heavy metals [6,7]. However, the studies were mostly carried out in controlled experiments and did not compare long-term effects in agricultural systems.

The factors affecting the size and composition of microbial populations in soils are poorly understood. The size, abundance and difficulty in differentiating between growing and reproducing and dormant cells, make it impossible

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0168-6496/00/$20.00
PII: S0168-6496(00)00053-2

to relate observations to many of the ecological theories expounded in more established work on animal and plant ecology [8]. Furthermore, the genetic flexibility which allows microbial populations to rapidly exploit new niches makes it difficult to identify species by using phenotypic identification systems that provide useful but not comprehensive databases (API, bioMériuex sa, France; Biolog™, USA). These techniques rely on the isolation and culturing of bacteria, so are not appropriate for the majority of non-culturable soil micro-organisms. Estimation of microbial diversity in soil based on DNA approaches or other cellular identifying markers, such as PFLAs, FAMEs, lipopolysaccharides and ergosterol have not so far answered the underlying question of function in soil [8].

In studies on grasslands, it has been shown that the more diverse the community of plants, the more stable the size of the biomass overall, despite the fact that individuals within this community, and the biomass they produce, fluctuate independently of the overall diversity [9]. This is, in turn, reflected in the soil microbial biomass [10]. Thus, the complex interactions of individuals with each other and their environment makes monitoring the impact of different management regimes difficult. A number of techniques have been suggested as suitable candidates, including Biolog™ [11] and the exploitation of assays involving bioluminescence [12], as routine monitoring techniques especially for farmers wishing to benefit from improvements to soil fertility by the addition of sewage sludge. However, sewage sludge contains not only carbon and phosphates, but also other compounds including metals, which can be detrimental to plant productivity and/or microbial functions if allowed to accumulate in soil [13].

In this study, a farm in central England, where sewage sludge has been disposed of for the last 100 years, has been investigated. The site chosen had high total metal concentrations resulting from the addition of sewage sludge, but the effect of management practices, in particular liming the soil, decreased metal availability. Although sewage sludge contains heavy metals, availability is limited by the pH, high levels of organic matter, and phosphates that are also present. Methods to compare microbial population size and structure at sites under different management regimes: grass; or arable with the application of sewage sludge, were assessed. The chemical properties of the soils were investigated, together with the microorganism population size, diversity and activity. Also the impact of continued sewage application, and its associated addition of contaminants, such as metals, was investigated using a luminescence bioassay in soil pore water with a *lux* marked *Pseudomonas fluorescens* [14].

2. Materials and methods

2.1. Soil collection

Soils subject to regular application of sewage sludge, were collected on 5 May 1997 from a site on an alluvial sandy soil overlying the Trent river gravels and Keuper Marl in Nottinghamshire [15]. The site is an operational sewage farm where sludge has been frequently injected into the soil from 1975 to the present, although it had been applied to the site using more traditional methods for the last 100 years. Three sites were chosen, within the same field, to which sludge had been applied and which had been under arable crop rotation (sites T1, T2 and T3). Two nearby grassland sites that had no sludge applied were also sampled (sites U1 and U2). The sludge-treated areas had ground limestone applied at 5 t ha^{-1} approximately every 5 years to increase the soil pH and decrease the availability of heavy metals. Five kilograms of soil from each site were passed through a 2-mm plastic sieve and stored at 4°C for 2 weeks before use. All microbiological analyses were done on the same day except a preliminary plate count to normalise the number of bacteria used to inoculate the Biolog™ plates.

2.2. Chemical analysis

All assays were done in triplicate. Soil solution pH was measured using an Orion 720A pH meter (Orion, Cambridge, UK). The dissolved organic carbon was measured using a TOC200 analyser (UV Developments, Cambridge, UK), the % N and C assessed using a LECO FP2000 combustion analyser (LECO, Stockport, UK), according to the manufacturers' protocols. The total metal in the soils was measured according to the method of McGrath and Cunliffe [16], and the ammonium nitrate extractable

Table 1
Soil characteristics for the samples

Treatment	Soil solution pH	DOC (mg) l^{-1}	Total (mg kg^{-1})		Extractable (1 M NH_4NO_3 mg kg^{-1})		% Carbon	% Nitrogen
			Zn	Cu	Zn	Cu		
T1	6.8	26.0	432	92	0.26	0.59	3.01	0.27
T2	6.4	25.8	397	79	0.70	0.45	3.66	0.38
T3	6.7	50.3	1230	473	1.40	3.87	8.35	0.77
U1	5.7	45.0	153	32	1.10	0.17	3.30	0.31
U2	7.6	44.1	606	112	0.36	0.98	4.07	0.31

DOC, dissolved organic carbon.

metals according to DIN 19730 [17]. Metal concentrations were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES; Accuris, Fisons Instruments, East Grinsted, UK).

2.3. Microbial estimations

The total number of soil bacteria was determined by the direct microscopic acridine orange method of Fry and Zia [18]. Soil micro-organisms were also cultured at 28°C on three types of agar media: 1/10th strength tryptone soy broth agar (TSBA, Oxoid, UK) for heterotrophic bacteria; nutrient agar (NA, Oxoid, UK) pre-treated at 80°C for 2 h before incubation for heat resistant spore-forming bacteria; and potato dextrose agar (PDA, Difco, UK) for fungi. Soil, 10 g, suspended in 90 ml Ringers solution (Oxoid, UK) was shaken with 10 g of sterile glass beads (1.5 mm diameter) for 2 h before plating on the agar media. Bacterial counts were made 24 h after plating, fungal counts after 5 days. Each soil sample was analysed in triplicate and the dilution series plated onto five replicate plates of each medium. TSBA plates containing approximately 100 bacterial colonies were selected and up to 96 (or as many as possible) individual colonies transferred into 100 μl of full strength TSBA in microtitre plates. After incubation overnight, 100 μl 50% glycerol (BDH, UK) was added to the cultures, which were then stored at −70°C. These were plated on pseudomonad selective agar containing the CFC antibiotic supplement at a final concentration in the agar of 10 μg ml^{-1} centrimide, 10 μg ml^{-1} fucidin and 50 μg ml^{-1} cephaloridine (PSA, Oxoid, UK) to estimate the number of fluorescent pseudomonads in the total culturable bacterial heterotrophic population.

2.4. Substrate utilisation assays

A substrate utilisation assay was done to assess the catabolic diversity of microbial populations in the soil samples. Biolog™ EcoPlates (Don Whitley Scientific, UK) with 31 carbon sources were used according to the manufacturers instructions. Samples were normalised for the number of bacteria using information from a preliminary count on TSBA before inoculation avoiding the need to correct results by deducting the average well colour development (AWCD) [19], a disputed but common practice [20]. Soil suspension (150 μl) containing 10^4 CFU, was added to the wells, incubated at 28°C and measured at 590 nm using a Multiskan RC reader (Labsystems, Basingstoke, UK) approximately every 12 h for 140 h, until most wells had reached maximum colour development.

2.5. FAME analysis

The FAMEs were analysed to assess the diversity of organisms in the soil samples (predominantly fungi and bacteria). They were extracted directly from the soil samples according to the method of Buyer and Drinkwater [21] based on the MIDI procedure for bacterial isolates (Microbial ID, Newark, DE, USA). Soil samples, 5 g, were lyophilised overnight and saponified, methylated and extracted according to the MIDI protocol. Samples were centrifuged at $700 \times g$ and the top layer of the extract removed and the peaks identified and standardised to the MIDI standard library. Only fatty acids comprising at least 1% of the total in at least one replicate were included in the analysis.

2.6. Luminescence bioassay

An acute toxicity study of heavy metals in the soils was performed using a luminescence-based bioassay utilising a *lux* marked construct of *Pseudomonas fluorescens* 10586rs pUCD607 [22]. The construct has the full *lux* cassette integrated as a multi copy plasmid. The strain was resuscitated from freeze-dried vials following the protocol of Paton et al. [12,23] and 50 μl of the bacterial suspension was added to 450 μl of the soil solution: luminescence of the

Table 2
Microbial numbers, substrate utilisation indices estimated from Biolog™, bioluminescence inhibition and relative biomass estimated from fatty acids extracted from the five sites

Site	Microbiological counts (g^{-1} dry weight soil)					Substrate utilisation indices		Toxicity bioassay	Biomass
	Total bacterial count ($\times 10^6$)	Viable aerobic heterotrophic bacteria $\times 10^3$	% Pseudomonads (as % of viable aerobic heterotrophic bacteria)	Viable spore-forming bacteria ($\times 10^3$)	Viable fungi ($\times 10^3$)	Shannon's Index	Gini coefficient	% Inhibition in bioluminescence compared to control	Relative amount of fatty acid methyl esters extracted (g^{-1} dry weight soil)
T1	45.4 (1.9)	9.97 (2.9)	31.8	8.35 (1.09)	6.99 (0.7)	3.20 (0.02)	0.44 (0.01)	17.6 (1.4)	1.56
T2	58.5 (1.6)	18.7 (0.6)	36.8	29.1 (3.9)	9.91 (0.2)	3.33 (0.01)	0.38 (0.01)	50.9 (0.6)	1.56
T3	109 (15.9)	22.5 (10.1)	9.1	22.9 (3.3)	8.41 (1.3)	3.22 (0.04)	0.40 (0.02)	45.5 (5.9)	1.30
U1	53.8 (4.3)	7.45 (1.3)	9.1	9.73 (2.1)	8.44 (0.7)	3.30 (0.01)	0.38 (0.01)	8.0 (1.2)	1
U2	68.0 (7.7)	n.d.	n.d.	18.2 (17.1)	7.15 (0.8)	3.28 (0.03)	0.38 (0.02)	10.0 (1.1)	1.22

Standard error of the mean is given in parentheses. n.d., not determined.

Table 3
Linear regression analysis of bacterial community indicators and measures against soil chemical properties (explanatory variable)

Response variable	pH	DOC×0.1	Total		Extractable	
			Zn[a]	Cu[a]	Zn[a]	Cu[a]
Viable aerobic heterotrophic bacteria simple regression	$P>0.20$	$P>0.20$	0.44 (0.19) [+36%] $P=0.044$	0.32 (0.15) [+25%] $P=0.061$	$P>0.20$	0.25 (0.15) [+19%] $P=0.122$
% Pseudomonads in viable heterotrophic community simple regression	$P>0.20$	−0.235 (0.037) [−42%] $P<0.001$	$P>0.20$	$P>0.20$	−0.76 (0.23) [−41%] $P=0.008$	$P>0.20$
Viable spore-forming bacteria simple regression	$P>0.20$	$P>0.20$	$P>0.20$	$P>0.20$	0.63 (0.37) [+55%] $P=0.117$	$P>0.20$
Viable fungi simple regression	−0.062 (0.029) [−13%] $P=0.054$	$P>0.20$	$P>0.20$	$P>0.20$	0.140 (0.070) [+10%] $P=0.069$	$P>0.20$
Total bacterial count simple regression	$P>0.20$	0.082 (0.026) [+21%] $P=0.008$	0.332 (0.087) [+26%] $P=0.002$	0.284 (0.060) [+22%] $P<0.001$	0.32 (0.10) [+25%] $P=0.008$	0.242 (0.058) [+18%] $P=0.001$
Multiple regression	–	0.038 (0.016) [+9%] $P=0.043$	0.267 (0.054) [+20%] $P<0.001$	–	0.200 (0.061) [+15%] $P=0.008$	–
Reduction in bio-luminescence simple regression	$P>0.20$	$P>0.20$	0.67 (0.26) [+59%] $P=0.023$	0.54 (0.20) [+46%] $P=0.015$	0.50 (0.29) [+41%] $P=0.116$	0.33 (0.19) [+26%] $P=0.111$
Multiple regression	–	−0.266 (0.033) [−85%] $P<0.001$	0.75 (0.11) [+68%] $P<0.001$	–	0.96 (0.12) [+95%] $P<0.001$	–

Regression coefficient with standard error in parentheses (if $P<0.20$). Values in square brackets illustrate % change in the response variable with either a one-unit increase in pH and DOC ×0.1 or a doubling of Zn or Cu. P is the probability that the coefficient is different from zero under the null hypothesis of no association between responses.
[a]Variable was $\log_{10}$-transformed before linear regression.

cells was measured in a Bio-Orbit 1253 luminometer after 10 min exposure. The bioassay was carried out in triplicate in soil pore water extracted using the method of Knight et al. [24] and the luminescence expressed as a percentage reduction of the luminescence measured from a standard KCl solution. A previous study showed that the bioluminescence response of the biosensors was stable across the soil solution pH range 4.5–7 [14].

2.7. Experimental design and statistical analysis

Three separate sub-samples of the soil collected from each site were used to assess bacterial numbers and were analysed for fatty acids. These were treated as replicates when analysing the Biolog™ results in each soil.

All statistical analyses were carried out using Genstat 5 Release 3.2 [25]. The relationship between bacterial numbers and physical/chemical data was assessed by linear regression, after $\log_{10}$-transformation of metal concentration and the bacterial counts to stabilise their variance approximately. The association of each potential explanatory variable with each of five response variables was assessed by simple linear regression, and the joint effects by multiple linear regression when there was sufficient evidence. The explanatory variables were, by the nature of the experiment, strongly correlated: in particular, it is not possible to distinguish between effects associated with total Zn, total Cu and extractable Cu in this analysis.

There was concern that the systematic arrangement of the carbon source on the Biolog™ plates could bias the results. In particular, the sources along the edges of the plate may be more susceptible to evaporation when the plates are stacked. This was checked by a multiple logistic regression of all the Biolog™ measurements, including effects of site, time and carbon source, and an edge effect (sources along the edge compared to other sources). Logistic regression was used rather than linear regression to take account of the binomial-like reduction in variation of individual Biolog™ measurements near the extremes (no colour development and full development, respectively). This gave an estimate of less than 2% increase in the edge measurements on average, which was not statistically significant ($P>0.20$).

The Biolog™ measurements were adjusted by subtracting the average of the measurements from the blank wells, containing no carbon source (and set to 0 if less than the blank). The adjusted data for each sample at the 11 successive time-points were summarised by estimating the area under the substrate utilisation curves, using the method of Gill and Miller [26]. From these areas, we calculated

Shannon's diversity index and the Gini coefficient [27] across the 31 carbon substrates. We report (1-Gini) rather than Gini so that the coefficient varies in the same way as the Shannon's index.

The carbon substrates were clustered using the average linkage method, from a similarity matrix formed from the areas for each soil sample using the city-block metric.

3. Results

3.1. Abiotic and biotic soil properties

The results of soil analysis, with considerable differences in a number of soil characteristics, are shown in Table 1. Sites T1 and T2 were similar in all the characteristics examined; T3 was of similar pH but higher in the other parameters. However, the bioassay (Table 2) indicated similar luminescence inhibition in T2 and T3 with less inhibition in T1 and much less in U1 and U2. The grassland site U1 had lower pH than the sludge treated soils; U2 had the highest pH and, surprisingly, the second-highest total Zn and Cu indicating that sludge application had occurred at some time in the past. Culturable bacterial and fungal numbers were similar in all sites and were about three orders of magnitude lower than the total microscopic counts (Table 2). Qualitative differences in growth of the microbial populations due to differential utilisation of carbon sources in Biolog™ [28] were not apparent from the Shannon's diversity index or the 1-Gini coefficient (Table 2).

Because there were no clear overall relationships between soil abiotic and biotic parameters, the data were investigated further using statistical methods.

3.2. Regression analysis

Simple linear regression indicated an association be-

Table 4
Biolog™ carbon sources characterised by classes and the curve characteristics for each of the classes

Substrate utilisation group	Carbon source	Characteristics of the graph
Group 1	σ-hydroxybutyric acid D-mannitol L-asparagine L-arginine D-galacturonic acid L-serine	Slow growth by all extracts even slower growth for T3 and U1, generally not reaching OD 2.5 before 40 h. T1, T2 and U2 have very similar utilisation patterns
Group 2	D-glucosaminic acid 4-hydroxy benzoic acid Itaconic acid D-xylose *N*-acetyl-D-glucosamine	Long time to reach maximum colour development, between 60 and 80 h
Group 3	L-threonine 2-hydroxy benzoic acid D,L-α-glycerol phosphate α-keto butyric acid	Very slow or no use of substrates by U2 and T1: either no or slight use by T2. U1 and T3 generally fairly rapid utilisation except D,L-α-glycerol phosphate at OD 2–2.5
Group 4	Phenylethyl-amine	All extracts grew very slowly and generally achieve OD 1.8–2.2
Group 5	Glucose-1-phosphate	No growth T1 very little growth U2, OD reaching maximum at 40–70 h none getting to 2.5
Group 6	Putrescine	Minimum density T3, maximum OD below 1.8 reached by T1 and T2, long lag very slow growth
Group 7	D-malic acid	Poor use of T3 and U1 maximum OD of 2 very varied carbon use response
Group 8	I-erythritol L-phenylalanine Glycyl-L-glutamic acid	Long lag (40 h) in all soil with exception T3 and U1 in L-phenylalanine and I-erythritol
Group 9	β-methyl-D-glucoside	Maximum OD 2 long lag except T2 and U2
Group 10	α-D-Lactose D-galactonic acid lactone Pyruvic acid methyl ester α-cyclodextrin Tween 40 Tween 80 Glycogen D-cellobiose	Takes up to 110 h (instead of 60 h) to reach a maximum

tween total Zn and the counts of heterotrophic bacteria. Although the size of some effects was large, more data would be needed to establish these effects with the observed level of variability. A doubling of the total Zn was associated with a 36% increase in the counts (Table 3). The same association was evident with total Cu and extractable Cu, which were highly correlated with total Zn.

Dissolved organic carbon (DOC) was strongly associated with the percentage of pseudomonads from the heterotrophic community, an increase of 10 units of DOC was associated with a 42% reduction in the %pseudomonads (Table 3). Similarly, a doubling of the extractable Zn was associated with a 41% reduction in %pseudomonads. However, effect of DOC and extractable Zn on the heterotrophic community was not significant ($P > 0.20$), although the correlation between these two variables did not allow their joint effect to be reliably determined.

Only extractable Zn was significantly associated with the number of heat resistant spore-forming bacteria. The effects of pH and extractable Zn on fungal counts were almost significant statistically, but the size of the effects was small (Table 3). All the explanatory variables except pH were significantly positively associated with the total bacterial counts. A multiple regression model, excluding total and extractable Cu because of the high correlation mentioned above, showed each of the remaining variables apparently contributing to the increases in counts, with total Zn showing the strongest association (Table 3).

Both total Zn and total Cu were significantly associated with inhibition of the bioluminescence assay. Multiple regression indicated that the major effects were exerted by total and extractable Zn, and opposed DOC, although due to the correlation between these explanatory variables, and the small number of observations, this result should be treated with caution.

The amount of fatty acids extracted (Table 2), as well as the average well colour development from the Biolog™ plates (data not shown), showed no association with the explanatory variables.

3.3. Carbon substrate utilisation patterns

Although there was no difference in the Shannon's diversity index or the 1-Gini coefficient which represent the number of different Biolog™ carbon sources utilised (Table 2), the possibility that these differed between soils was investigated. The city-block matric was used to cluster the carbon sources into utilisation groups (dendrogram not shown). Table 4 shows how the groups are characterised from the growth rate curves and indicates that the substrates were used very differently by the soil extracts, e.g. glucose-1-phosphate supports no growth of T1 and U2, whereas putrescine did not allow growth of T3 and showed a long lag and slow growth of T1 and T2. The general responses of the microbial communities of the soil samples can be summarised: U2 and T2 have similar carbon utilisation patterns; T3 and U1 show some similarity; otherwise the soil extracts show different patterns. However, there are no obvious explanations for these relationships, in terms of the soil characteristics shown in Table 1.

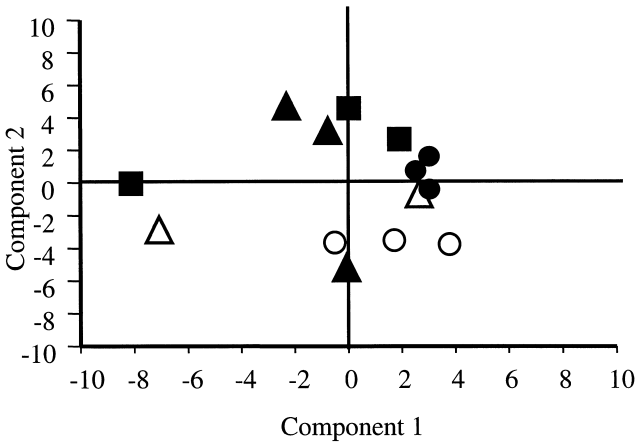

Fig. 1. Principal components analysis of the fatty acids derived directly from soil whose peak area was greater than 1%. The *x*-axis is the first principal component (accounting for 64% of the variance), the *y*-axis is the second principal component (accounting for 11% of the variance). The different soils are represented by different symbols: ●, T1; ■, T2; ▲, T3; ○, U1; △, U2.

3.4. Fatty acid analysis

The MIDI system identified peaks representing 32 individual fatty acids in T1, 39 in T2, 31 in T3, 21 in U1 and 48 in U2, with 62 occurring in at least one replicate of all samples. All the fatty acids present in U1 and U2 were also present in the sludge-treated sites, but 13 were unique to the sludge-treated sites. Many of the peaks were very small and some could not be identified by name, but 38 fatty acids that comprised at least 1% of the total fatty acids extracted in at least one replicate from any treatment were plotted using principal component analysis (PCA). This showed grouping of the replicates from sample T1 only (Table 5, Fig. 1). The fatty acids known to be characteristic of Gram-negative and Gram-positive bacteria and eukaryotes including fungi were also plotted but showed no obvious differences (data not shown). The Gram-negative fatty acids from U1 and T1 formed clusters but otherwise there was no differentiation between groups. Similarly the Gram-positive and eukaryotic fatty acids from T1 formed a group, but other samples were more diverse and there is no clear differentiation between the soils (data not shown).

4. Discussion

A number of measures were used to assess differences in the microbial communities in this soil type. The sizes of

Table 5
Fatty acids extracted from soils

Fatty acid name[a]	Treatment				
	T1	T2	T3	U1	U2
10:0	0.00	1.64 (0.03)	2.26 (0.22)	0.00	0.00
10:0 3OH	3.21 (0.05)	4.87 (0.12)	2.97 (0.29)	0.00	2.02 (0.12)
11:0 ISO	1.42 (0.01)	2.85 (0.10)	0.88 (0.17)	0.00	1.53 (0.54)
11:0 ISO 3	1.61 (0.16)	6.49 (0.71)	1.27 (0.24)	3.31 (0.32)	10.23 (3.14)
11:0 3OH	1.79 (0.17)	0.80 (0.15	0.00	0.00	0.83 (0.29)
12:0	4.83 (0.04)	6.23 (0.13)	7.82 (0.64)	3.25 (0.23)	5.23 (0.43)
13:0	1.49 (0.14)	0.00	0.00	0.00	0.85 (0.30)
13:0 ISO	2.17 (0.06)	1.17 (0.11)	0.85 (0.08)	0.00	0.78 (0.27)
12:0 ISO	0.00	1.21 (0.23)	0.44 (0.08)	1.01 (0.01)	2.28 (0.81)
12:0 ISO 3	1.53 (0.17)	0.00	0.00	0.00	0.51 (0.18)
14:0 ISO	2.91 (0.11)	1.92 (0.19)	0.88 (0.09)	1.54 (0.03)	1.51 (0.53)
14:0	4.59 (0.04)	4.25 (0.19)	4.22 (0.04)	3.99 (0.21)	3.30 (0.28)
15:0 ISO	5.20 (0.19)	4.43 (0.25)	2.48 (0.09)	4.82 (0.14)	3.54 (0.04)
15:0 ANTEISO	6.24 (0.16)	4.95 (0.60)	3.81 (0.14)	5.85 (0.12)	2.80 (0.99)
15.549 unknown	1.69 (0.01)	2.04 (0.09)	10.79 (1.96)	5.53 (0.37)	0.52 (0.18)
SUM in Feature 3	1.85 (0.01)	2.67 (0.06)	1.57 (0.15)	0.64 (0.12)	0.90 (0.32)
15:0 ISO 3	0.00	1.60 (0.03)	0.98 (0.10)	0.00	0.00
15:0	1.49 (0.07)	0.59 (0.11)	1.63 (0.16)	1.75 (0.07)	0.61 (0.21)
16:0 ISO	1.46 (0.04)	0.98 (0.10)	0.00	2.43 (0.034)	0.78 (0.28)
16:1 CIS 9	4.51 (0.06)	5.02 (0.12)	3.48 (0.08)	6.66 (0.24)	2.86 (1.01)
16:1 CIS	2.53 (0.05)	1.30 (0.13)	3.13 (0.49)	4.81 (0.18)	3.16 (0.23)
16:0	10.49 (0.21)	7.90 (0.18)	10.84 (0.33)	13.71(0.17)	9.83 (0.08)
17:1 ISO G	2.01 (0.03)	0.00	0.00	2.36 (0.04)	0.94 (0.33)
17:1 ANTEISO	0.00	1.20 (0.07)	0.00	1.95 (0.38)	0.00
17:1 ISO H	0.00	2.16 (0.42)	0.89 (0.17)	0.86 (0.17)	4.20 (1.48)
16:2 OH	0.00	0.89 (0.17)	0.00	1.05 (0.11)	0.00
16:0 2OH	0.00	0.38 (0.07)	0.96 (0.01)	0.00	0.00
16:0 3OH	1.15 (0.01)	1.80 (0.10)	1.05 (0.11)	0.81 (0.08)	0.54 (0.19)
18:3 CIS 6,12,14	1.28 (0.03)	0.00	9.56 (1.84)	2.22 (0.43)	0.63 (0.22)
SUM in Feature 6	3.63 (0.03)	4.67 (0.12)	1.20 (0.12)	4.61 (0.07)	7.81 (1.09)
18:1 CIS 9	5.52 (0.09)	3.02 (0.31)	5.60 (0.17)	7.14 (0.12)	2.80 (0.99)
SUM in Feature 7	2.72 (0.07)	1.50 (0.15)	1.67 (0.05)	3.44 (0.05)	1.27 (0.45)
18:0	2.17 (0.07)	2.67 (0.21)	2.48 (0.08)	3.58 (0.09)	4.33 (0.82)
17:0 ISO 3OH	1.40 (0.02)	1.52 (0.15)	0.95 (0.09)	0.00	0.60 (0.21)
17:0 3OH	0.00	1.54 (0.30)	0.64 (0.12)	1.10 (0.11)	2.95 (1.04)
SUM in Feature 9	5.05 (0.03)	6.52 (0.13)	2.24 (0.22)	9.32 (0.59)	10.67 (0.45)
18:0 2OH	1.79 (0.08)	1.13 (0.11)	0.80 (0.08)	1.41 (0.14)	1.30 (0.46)
19:1 TRANS 7	2.45 (0.24)	0.00	0.00	0.00	1.31 (0.46)

Values given are % total fatty acids extracted, mean of three replicates (two replicates for U2), standard error of the mean in parentheses.
[a]Names of fatty acids are as given by the MIDI software, no name could be assigned to the unknown fatty acid at position 15.549 although it appeared consistently. 'Sum in Feature' are unresolved peaks of known fatty acid composition, recognised by the MIDI software.

the total microbial populations were similar, but there were indications of differences in community structure. These structural differences were shown by the substrate utilisation profiles obtained with Biolog™, by the numbers of groups of culturable microbes measured by plate counts and selective plating; and by the direct extraction of fatty acids from the soils. Samples taken from sites with high sludge additions might be expected to be altered not only in the composition of the microbial communities, but also in population size and total biomass [29]. In the sludge-treated soils T1, T2 and T3, the heavy metal concentrations increased with DOC and indicated a higher rate of sewage application to T3, which had by far the highest organic matter, and consequently metal, content. Total microscopic counts and the number of culturable heterotrophic bacteria were significantly higher in T3, but the proportion of fluorescent pseudomonads was lower, indicating a difference in community structure (Table 2). This may or may not be due to the long-term treatments, as we cannot exclude the possibility that the populations were different before the sludge additions commenced. The indicator organism for the acute toxicity bioassay was also a pseudomonad, which showed a reduction in bioluminescence in all the sludge-treated soil compared with the grassland soils (Table 2).

Bååth and Arnebrant [7] found no effect of application of lime on the direct count of bacteria in soils, but did observe an increase in the ratio of bacteria that grew on agar plates to the total bacterial count with increasing pH. Thus, the comparison of the sludged and grassland soils in

our study could be influenced by liming and also by increased organic carbon, metals and phosphates in the sludge as well as other factors. Smit et al. [30] showed no differences from direct counting, but did find lower diversity of isolates in soils contaminated with 750 kg ha^{-1} added Cu compared to uncontaminated grassland soil.

The total extracted FAMEs show a similar trend to the bacterial plate counts (Table 2), with higher values in the sludge-treated plots. The method used for FAMEs also extracts fatty acids from non-microbial sources and dead cells, but in this case, the FAMEs appear to reflect the living bacterial biomass. In soils that contained approximately twice as much carbon and a maximum concentration of Zn and Cu of 1230 and 473 mg kg^{-1}, respectively (Table 1) we have shown little effect on the microbial community as measured by FAMEs. The differences may be attributed to the effect of liming. Frostegård et al. [31] showed that liming and the resultant increase in pH, changed PLFA composition in the soil. Much of this change was attributed to a greater availability of carbon [32]. Studies where changes measured by PFLAs were attributed to metals in the soil were shown in soils with much higher concentrations of metals [4,11,33].

The Shannon's and Gini indices of diversity derived from the analysis of Biolog™ indicated no significant difference between the soils (Table 2). However, closer examination of the patterns of carbon substrate utilisation showed that microbial activities in the soil extracts were not identical and so the similarity between the indices is due to retention of the overall community structure, while changes may have occurred in the frequencies of individual species. It was important to consider not just the responses of the soil extracts to individual substrates at any one time, but also to compare growth curves. This highlights the importance of examining information carefully before assuming that an index is providing a true picture of the information.

There has long been an interest in the relationship between community diversity and the stability of communities responding to perturbations, such as toxic challenges. This interest has been sharpened recently by the work of Tilman [9] on plant communities confirming that biodiversity stabilises community and ecosystem processes, but not population processes (i.e. individual populations are less stable). In our study, the response of bacterial communities to sewage sludge in soils was considered, investigating the taxonomic (FAMEs) and catabolic (Biolog™) diversity alongside the total and viable bacterial numbers. Our results indicate that diversity was relatively stable, while individual species and functions underwent limited fluctuations, such as the decline in fluorescent pseudomonads and different carbon substrate utilisation patterns. The addition of sewage sludge with its associated land-management practices may have influenced the numbers of micro-organisms, and their catabolic and taxonomic profiles, but it is difficult to identify the most relevant comparisons to make when undertaking this type of field monitoring. There were no unlimed sludged sites available, and the grassland sites chosen had completely different management. A controlled long-term experiment would need to be set up to look at changes due specifically to the treatments. Nevertheless, it is interesting that the two grassland sites did not show great differences when compared with the sludged sites. However, the considerable spatial variability at the site, in terms of soil C and metals, means that the samples cannot be viewed strictly as with and without sludge. Variability in some soil properties (Table 1) was as great within the treated arable and untreated grassland areas as it was between them.

This work highlights the need for integrated approaches when looking at microbial population structures in any soils, whether they are under different management or perturbed in some way. A number of different techniques, both biological and chemical, are needed when monitoring soils for impacts of land management practices on microbial communities. Our comparison of similar soil types with different managements showed no great impact on the microbial populations by all the techniques tested. However, it also indicated that there were differences in specific microbial parameters and populations and that it is necessary to use a number of different techniques including acute assays and measures of community diversity and function. The results do indicate that fluorescent pseudomonads are particularly sensitive indicators of soil perturbations that affect microbial communities.

Acknowledgements

This work was supported in part by the United Kingdom Ministry of Agriculture Fisheries and Food. IACR-Rothamsted receives grant-aided support from the UK Biotechnology and Biological Sciences Research Council. We would like to thank Severn Trent Water for access to the site, and for the assistance provided.

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