Sheep-urine-induced changes in soil microbial community structure

Naoise Nunan, Brajesh K. Singh, Eileen Reid, Brian Ord, Artemis Papert, Julie Squires, Jim I. Prosser, Ron E. Wheatley, Jim W. Mcnicol, Peter Millard

▶ **To cite this version:**

Naoise Nunan, Brajesh K. Singh, Eileen Reid, Brian Ord, Artemis Papert, et al.. Sheep-urine-induced changes in soil microbial community structure. FEMS Microbiology Ecology, 2006, 56, pp.310-320. 10.1111/j.1574-6941.2006.00072.x . bioemco-00136989

HAL Id: bioemco-00136989

https://hal-bioemco.ccsd.cnrs.fr/bioemco-00136989v1

Submitted on 16 Mar 2007

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Sheep-urine-induced changes in soil microbial community structure

Naoise Nunan[1a], Brajesh Singh[2], Eileen Reid[2], Brian Ord[2], Artemis Papert[3], Julie Squires[4], Jim I. Prosser[3], Ron E. Wheatley[4], Jim McNicol[1], Peter Millard[2]

1. Biomathematics and Statistics Scotland, Scottish Crop Research Institute, Dundee DD2 5DA, UK.

2. Environmental Sciences, Macaulay Institute, Craigiebuckler, Aberdeen, AB15 8QN, UK.

3. School of Medical Sciences, University of Aberdeen, Institute of Medical Sciences, Foresterhill, Aberdeen, AB25 2ZD, UK.

4. Plant-Soil Interface, Scottish Crop Research Institute, Dundee DD2 5DA, UK.

a. present address: Laboratoire de Biogéochimie des Milieux Continentaux, CNRS, Bâtiment EGER, aile B, Institut National Agronomique Paris-Grignon, 78850 Thiverval-Grignon , France.

Author for correspondence:

Naoise Nunan

Laboratoire de Biogéochimie et Ecologie des Milieux Continentaux, CNRS, Bâtiment EGER, aile B, Institut National Agronomique Paris-Grignon, 78850 Thiverval-Grignon, France.

Email: nunan@grignon.inra.fr Tel: 00-33-1.30.81.59.88

Short title: Heterogeneity in upland grassland microbial community structure

Date submitted: 04-04-2005

This manuscript contains 32 pages, including 2 Tables and 4 Figures.

Abstract

Soil microbial communities play an important role in nutrient cycling and nutrient availability, especially in unimproved soils. In grazed pastures, sheep urine causes local changes in nutrient concentration which may be a source of heterogeneity in microbial community structure. In the present study, we investigated the effects of synthetic urine on soil microbial community structure, using physiological (community level physiological profiling; CLPP), biochemical (phospholipid fatty acid analysis, PLFA) and molecular (denaturing gradient gel electrophoresis; DGGE) fingerprinting methods. PLFA data suggested that synthetic urine treatment had no significant effect on total microbial (total PLFA), total bacterial or fungal biomass. However, significant changes in microbial community structure were observed with both PLFA and DGGE data. PLFA data suggested that synthetic urine induced a shift towards communities with higher concentrations of branched fatty acids. DGGE banding patterns derived from control and treated soils differed, due to a higher proportion of DNA sequences migrating only to the upper regions of the gel in synthetic urine-treated samples. The shifts in community structure measured by PLFA and DGGE were significantly correlated with one another, suggesting that both datasets reflected the same changes in microbial communities. The changes caused by synthetic urine addition accounted for only 10-15% of the total variability in community structure, suggesting that, overall microbial community structure was reasonably stable and that changes were confined to a small proportion of the communities. Synthetic urine treatment caused a significant increase in the average well colour development of Biolog® microplates, suggesting an increase in bacterial population density that was corroborated by the 100-fold increase in bacterial culturable cell concentrations. Rhizosphere-C source utilisation was preferentially stimulated. Significant correlations between DGGE, PLFA and CLPP principal components were observed but, with the exception of relationship between the first DGGE principal

component and the third PLFA principal component, the scatter diagrams were highly dispersed. These data provide evidence that urine deposition contributes to heterogeneity in microbial community structure in upland grassland soils.

Introduction

Acidic grasslands are common in the uplands of NW Europe. In these soils the availability to plants of nutrients such as N and P can depend heavily on microbial activity, as they tend not to receive inputs of nutrients in the form of fertilizers. As a result, microbial communities of temperate upland grasslands have been widely studied across a range of scales in order to identify the factors that regulate their community structure. Factors that have been shown to cause shifts in community structure range from management practices such as fertiliser inputs and grassland improvement to geographic location [1,2,3]. Influences are thought to be exerted through rhizodeposition, litter and root diversity and soil physiochemical properties [4,1]. Seasonal changes in microbial community structure have also been observed [5] and Ritz et al. [6] found that community structure was subject to a high degree of local spatial variability. Clegg et al. [7] also found that differences in genetic composition of microbial communities from the same site could be as great as those among communities from sites separated by several hundred kilometres. This raises the question of the ecological importance of the relationships between microbial community structure and the factors that have been found to play a regulatory role, such as inputs of carbon and nitrogen: how strong are these relationships in light of the temporal and spatial variability that is present?

Ritz et al. [6] reported a high spatial variability in biomass, phospholipid fatty acid and community level physiological profiles in a previous study of the soils of Sourhope upland

grasslands, but little change in the background genetic structure of the microbial community. They hypothesised that the spatial variations could be caused by some significant localised input or redistribution of nutrients, as would occur with sheep urine. Urine excretion represents an important flux of nutrients in extensively grazed grasslands and causes local changes in soil solution chemistry such as increases in nutrient concentration (N and P) and soil pH [8]. Williams et al. [9] found that carbon utilisation patterns of soil microbial communities in upland pastures were altered by treatment with urine, generally leading to an increase in substrate utilisation 2 to 5 weeks after urine addition. However, they did not quantify the impact of urine deposition in relation to the overall variability of microbial community structure at the sites studied. To investigate whether such disturbances do in fact impact on soil microbial community structure, we treated an upland pasture with synthetic urine and then measured short-term effects on such communities, using physiological, biochemical and molecular fingerprinting. The impact of urine deposition on soil microbial community structure in an extensive upland soil is described and quantified. .

Materials and Methods

Soils and sampling

Samples were collected from Fasset Hill, Sourhope (55^{o} 28' 30" N; 2^{o} 14' W), an upland grazed grassland at Sourhope Research Station in the Scottish Borders [10]. The underlying soil is a brown ranker (Haplumbrept; FAO/UNESCO, 1994) derived from old red sandstone, with a pH of 3.3 - 6.4 and organic C content of 11.4%. The site is a permanent *Festuca ovina - Agrostis capillaris - Galium saxatile* unimproved grassland, National Vegetation Classification - U4a [2], at 370 m above sea level. The grassland has been freely grazed by sheep during the summer months for at least 30 y but received no N, P or K

fertiliser. Average annual rainfall for the site is 975 mm y^{-1} and the minimum and maximum temperatures are -10°C and 27°C, respectively.

Synthetic sheep urine

Synthetic sheep urine (SU) was used for the experiment as it was easier to obtain than natural sheep urine (NU) and provided a well-defined medium for the experiment. The composition of NU is variable, depending on the physiology of the individual animals, their diet and water intake [11] and can also change with storage. The composition of the SU used was as described by Haynes and Williams [12] and comprised: urea 21.4 g l^{-1}, potassium bicarbonate ($KHCO_3$), 23.1 g l^{-1}, potassium chloride (KCl) 3.8 g l^{-1}, potassium sulphate (K_2SO_4) 1.9 g $^{-1}$and glycine 10.7 g l^{-1}. To prevent hydrolysis of urea during storage the solutions were prepared and mixed immediately before use.

Field experiment

Twelve 1 m^2 plots were selected, each bounded on all sides by a 1 m guard strip. Due to the exceptionally dry summer, prior to application of the treatments, the site (all 12 plots) was irrigated daily with 1000 l of water, applied through a mist hose, for one week. Five litres of water (control) or SU were then applied evenly to each of six replicate plots of 1 m^2, using a watering can fitted with a medium rose. This provided the equivalent of 499.2 kg urea derived N ha^{-1}, excluding the nitrogen content from glycine. Subsequently, 5 l water was added to every plot to wash the treatment into the soil. Two adjacent soil cores from each plot were sampled prior to SU or water application, one day and one, two and four weeks after application. The cores were typically 8 cm long; with a 3 cm thatch layer and a 1-2 cm organic horizon, the remainder being mineral soil. One core was analysed for pH (1: 4 soil:vol ratio in 0.01 M $CaCl_2$), moisture content, mineral N and dissolved organic carbon

(DOC) content to relate to the population measurements made on the other soil core. The upper 1 cm of the organic horizon was used for all analyses.

Phospholipid fatty acid profiles

Lipids from samples were extracted from 1 g of soil samples using the method of Frostegård et al. [13]. The extracted fatty acid methyl esters were identified and quantified from the retention time of chromatograms and mass spectral comparison on a Hewlett Packard 5890 II gas chromatograph equipped with a 5972A mass selective detector (MSD II), using standard qualitative bacterial acid methyl ester mix (Supelco; Supelco UK Poole, Dorset, UK) that ranged from C11 to C20. Standard fatty acid nomenclature was used as described before [14]. An internal standard (C19:0) was used to estimate the total amount of PLFA. Microbial biomass was estimated from total amounts of PLFA [13]. PLFA analyses were not performed on soils collected at the final harvest (day 29) because the samples did not contain enough soil. Total bacterial PLFA and a fungal biomarker were chosen as in Grayston et al. [2].

Community level physiological profiles

CLPP were constructed using Biolog® GN microplate (Biolog Inc., Hayward, CA, USA) together with exudate profile microplates, prepared using Biolog® MT plates containing an additional 46 ecologically relevant carbon sources identified mainly as plant root exudates [15]. Soil dilution was adjusted to a similar inoculum density of approximately 10^4 colony forming units (cfu) ml^{-1} (based on the growth on 1/10 strength Oxoid tryptone-soy agar) and 150 µl inoculated into each well. Microplates were incubated for five days at 15^oC and colour development (carbon utilisation) was measured daily as absorbance at 590 nm (A_{590}), using a microplate reader (Emax, Molecular Devices, Oxford, UK). Two forms of CLPP data were

analysed: untransformed raw difference data, in which the colour response of the control well was subtracted from the colour of each of the C source wells, and transformed data, where colour values of individual wells were divided by the average well colour development (AWCD) of each Biolog® GN and MT plate, to account for differences in average colour development (see Results section). Raw data were analysed to determine whether SU treatment had an effect on AWCD profiles. Colour development in individual wells was measured as the area under the colour development profile [16]. This measure was chosen over single point absorbance readings at a given AWCD [17] and curve fitting [16] because it is more independent of incubation time than the former [18] and does not suffer from problems of models of the latter not fitting the data adequately [16]. The area under the colour development profile is sensitive to the inoculation density and care was taken to ensure the inoculum density was similar for all samples.

Nucleic acid extraction and PCR amplification of DNA

Nucleic acids were extracted from 0.5 g of soil as described in Griffiths et al. (2000) [19]. Eubacterial primers were used to amplify 160 bases of the V3 hypervariable region of the bacterial 16S rRNA gene from extracted DNA. A nested PCR approach was used, with primers 27f [20] and Pfr [21] as forward and reverse primers, respectively, for first round amplification, followed by a second PCR-round using primers 357f, with GC-clamp and 530 r [22]. Amplification reactions for both PCR rounds were carried out in a 50 µl PCR mixture (final volume) containing: 1xNH_4 Reaction Buffer (Bioline, London, UK), 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 4 µM of each forward and reverse primer, 0.25 µg T4 gene 32 protein (Roche Diagnostics, Lewes, UK), 0.5 µl formamide, 1 U Biotaq DNA polymerase (Bioline), 1.2 U Bio-X-ACT polymerase (Bioline) and 1 µl DNA template. Template was either genomic DNA or 1:50 diluted PCR product from the first round. T4 gene 32 protein

was used to neutralise the effects of the PCR inhibitors that were present in the DNA extracted from the root fragments [23]. PCR amplification and yields were checked under UV light on ethidium bromide stained agarose gels (1.2%, w/v). The PCR program used was as follows: 5 min at 95 ºC (1 cycle); 30 s at 94ºC, 30 s at 55ºC, 180 s at 72ºC (10 cycles); 30 s at 92ºC, 30 s at 55ºC, 180 s at 72ºC (25 cycles); 10 min at 72ºC (1 cycle). Extension time was reduced to 45 s for the second PCR round.

DGGE analysis of amplified DNA

Amplified 16S rRNA gene fragments were analysed by denaturing gradient gel electrophoresis (DGGE) as described by Muyzer et al. [22]. Gel characteristics were: 8% (w/v) bis-acrylamide, denaturing gradient of 42% to 72% (100% denaturing being defined as 40% v/v formamide and 7 M urea). Electrophoresis was performed for 12 h at 100V and 60ºC on a DCode System (Bio-Rad laboratories, Hercules, USA). After electrophoresis, gels were stained with silver nitrate and scanned at a 1200 dpi resolution with an Epson GT-9600 scanner. The staining procedure was as follows (S. Mahmood, pers. comm. adapted from [3]): gels were fixed by shaking for 2 h in a fixing solution (10% ethanol (v/v), 0.5% glacial acetic acid (v/v), 89.5% water). Gels were then incubated with shaking in silver nitrate solution (0.2% w/v, in fixing solution) for 20 min. Following rinsing of the silver nitrate solution, gels were shaken in a developing solution (3% NaOH (w/v) and 1.3% formaldehyde, prepared in water) until DNA bands appeared (30 - 60 min). Gels were then preserved in ethanol-glycerol preservative solution (25% ethanol, 10% glycerol, 65% H_2O) and stored in sealed plastic bags at room temperature.

DGGE banding patterns of the digitised gel images were analysed with Phoretix 1D advanced software (version 4.01, NonLinear Dynamics Ltd., Newcastle-upon-Tyne, UK). Bands were identified visually and the relative intensity of each band, within individual

profiles of different samples, was quantified by the software. Bands in each gel were normalised for variations in DNA loading by detecting the lane with the lowest amount of DNA (i.e. lowest total band intensity) and then the faintest band within that lane. The percentage intensity of the faintest band in the lane with the lowest loading was taken as the limit of detection. Bands with a lower percentage of total band volume in all other lanes were excluded from the analysis. The intensity of each band was then calculated by determining the proportion of the total band intensity in a particular lane, and the resulting normalized data was used in multivariate analysis. A simple binary matrix describing the presence or absence of bands at each position was also analysed.

Plate counts

Culturable cell concentration was determined by serial dilution of cell suspensions used to inoculate the CLPP plates to 10^{-1} in ¼ strength Ringer's solution and spread plating onto tryptone soy agar plates (0.1 strength, Oxoid) containing cycloheximide (50 mg l^{-1}). Plates were incubated at 25°C and counted regularly until no new growth was observed. No new growth was observed during the second week of incubation and therefore the counts at the end of the first week were used for analysis. Cell concentrations were expressed as the number of colony forming units (cfu) g^{-1}.

Statistical analysis

Underlying patterns in PLFA, DGGE band intensity and CLPP data were investigated using principal component analysis (PCA) to determine whether microbial community structure and carbon utilisation patterns varied among the different levels of time, SU treatment and their interaction. Although widely used, the appropriateness of PCA for ecological data has been questioned and it has been suggested that correspondence analysis

may be more suitable (e.g. [24]). We analysed the data using both PCA and correspondence analysis and these resulted in substantially the same conclusions (i.e. similar treatment effects accounting for a similar proportion of total variance or inertia, respectively, and similar fatty acids and DGGE bands responsible for discrimination). For this reason, only PCA results are presented.

Principal component analysis forms linear combinations of the variables with the aim of maximising the inter-sample variance. In general a small number of combinations, or components, accounts for most of the sample variation and the statistical analysis can proceed using the values of the samples for these components. These are called the sample scores. Often most of the interesting biological information is contained in the first few principal components. In our case these new sample scores were used as the variables to answer the basic biological questions, such as determining treatment effects or finding relationships with other variables (e.g. pH). The similarity of the biological information contained in the different analyses of microbial communities was also assessed using the sample scores.

The fatty acids, carbon sources and DGGE bands most responsible for the discrimination were identified by reference to the principal component loadings. In total, 49 fatty acids were detected. However, one of these (17:1ω8t) was absent from all but eight of the samples and was removed before PCA. An initial analysis showed that there were large differences in microbial biomass (total PLFA) among samples. All but four fatty acids (12:0, 13:0, 20:4(6, 9, 12, 15) and 20:4(2, 6, 10, 14)) were significantly correlated ($P < 0.05$) with total PLFA, suggesting that these four fatty acids were not of microbial origin. They were also removed before PCA.

Patterns in DGGE data were also investigated using the binary matrix of presence or absence of bands at each location. The similarity between pairs of samples was calculated using the simple matching coefficient of similarity, which is based on the proportion of

positions in which there is a match between samples (i.e. presence-presence or absence-absence of bands). Principal co-ordinate analysis (PCO) was used to identify underlying patterns in the similarity data by deriving PCO scores for the samples.

Two-way analysis of variance (treatment x time) was carried out on the first three PLFA and CLPP principal components to identify statistically significant differences between the main experimental factors. In order to account for the large differences in microbial biomass (total PLFA) among samples, analysis of variance was carried out on individual fatty acids with total PLFA as a covariate. To avoid problems associated with gel to gel variation only 36 samples were analysed by DGGE on a single gel, resulting in an unbalanced dataset. Therefore, DGGE principal components and principal coordinate scores were analysed by Residual Maximum Likelihood (REML) to identify significant differences between experimental factors.

All statistical analyses were performed with Genstat 7 (VSN International Ltd, UK).

Results

Soil pH and NH_4^+-N concentrations were significantly greater (P<0.001) in those plots amended with artificial urine than in plots receiving only water (Table 1), demonstrating that the urea application had been successful. NO_3^--N levels were moderate in all plots, probably due to dry/wet deposition rather than nitrification. Dissolved organic carbon (DOC) showed a significant (P<0.001) increase in SU-treated plots between days 8 and 15.

PLFA profiles

No significant effects were found in the two-way analysis of variance (treatment x time) for total PLFA (Fig 1), for total bacterial PLFA or for the fungal biomarker 18:2(9, 12). A SU treatment effect was found for five fatty acids, nine fatty acids changed significantly with time and three showed significant treatment x time interaction (Table 2). The abundance

of the branched fatty acids i14:0, a15:0 and i17:0, indicative of Gram positive bacteria [25], was significantly greater in the SU-treated plots than in the control plots. The fatty acids a15:0 and i17:0 also showed significant treatment x time interaction: control levels remained constant throughout the sampling period, whilst levels in SU-treated soils increased significantly. The abundance of monounsaturated fatty acids was largely unaffected by SU treatment, with the exception of trans16:1ω11, which increased significantly, and 17:1ω7, which decreased significantly (Table 2). Fatty acids showing significant changes in abundance with time and generally increased after incubation for 8 days. Exceptions to this were i17:0 and a15:0, which increased after incubation for 1 day, and 16:0 and 20:1, which decreased after incubation for 8 days. The ratios of monounsaturated to saturated fatty acids were 0.61 for control samples and 0.55 for SU-treated samples and were not significantly different.

The first 3 principal components (PC) accounted for 76% of the total variance among samples (58, 12 and 6%, respectively). There were significant effects of SU treatment ($P < 0.014$) and time of sampling ($P < 0.007$) on mean PC scores of the third PC (Fig 2a). The third PC also showed significant positive correlation with pH ($r^2 = 0.36$, $P < 0.001$), with moisture content ($r^2 = 0.34$, $P < 0.001$) and with ammonium concentration ($r^2 = 0.27$, $P < 0.001$). Analysis of the loadings of individual fatty acids indicated that most of the differences in the third PC were due to higher concentrations of branched (a15:0, i17:0, 10Me16:0, i14:0 and 12Me16:0), of cyclopropyl (cy17:0), of monounsaturated (19:1ω8, 14:1ω9c, 14:1ω9t) and of polyunsaturated (18:3(6, 8, 13)) fatty acids, and lower concentrations of the fungal biomarker (18:2(9, 12)), of cyclopropyl (cy19:0) and of monounsaturated (17:1ω7, 18:1ω9 and 18:1ω10or11) fatty acids in SU-treated samples. Most of the PLFA loadings for the first PC were positive and similar in magnitude, suggesting that

the first PC was related to the total amount of PLFA. A highly significant linear relationship (r^2 = 0.99; P < 0.001) was found between total amount of PLFA and the first PC (Fig 1).

DGGE analysis

For DGGE profiles derived from SU-treated and control soils, the first three principal components accounted for 30% of the total variability in the dataset (12, 10 and 8%, respectively). Significant treatment effects were found in the first PC (P = 0.016; Fig 2b). The loadings of DNA sequences on the first PC indicated that the separation between treated and control soils was due to a higher proportion of sequences in the latter migrating further in the gel. In general, DNA fragments with a high G+C content will migrate further because they are harder to denature. However, other factors can also influence the migration of sequences and sequence information is required for more detailed analysis of reasons for separation of control and synthetic urine treated soils. No effect of time of sampling was observed. Principal components scores were not correlated with pH, moisture content, or ammonium or nitrate concentrations.

CLPP – Raw data

The first PC of the raw CLPP data, accounting for 34% of the total variability, was significantly correlated with the AWCD of all 141 C-sources (P < 0.001, r^2 = 0.99; data not shown). Inspection of the loadings of individual C-sources indicated that root exudate C-sources, mainly in the Biolog MT plates, contributed less to the first PC than the majority of C-sources in the Biolog® GN plates, suggesting that the relationship between the AWCD and C-sources in the two plates differed. This was confirmed by performing PCA on each plate separately. Whilst the relationship between the first PC for the Biolog® GN plate and the AWCD of all 141 C-sources was virtually unchanged, that between the first PC for the Biolog

MT data changed significantly. Furthermore, the mean AWCD of Biolog® GN for all samples was significantly higher (P <0.001) than that of Biolog® MT and the first PC for each dataset was significantly correlated with the respective AWCD (P <0.001, r^2 = 0.99 for Biolog GN plates and P <0.001, r^2 = 0.87 for Biolog MT plates). These data suggest that there were two populations of variables and that it would be inappropriate to analyse these together without prior normalisation to account for the differences in colour development in the different plates. Therefore, individual well colour development values were transformed by dividing by the AWCD of the plate to which they belonged, before further multivariate analysis.

Average well colour development for SU-treated samples was significantly higher than for control samples in each of the datasets (Biolog® GN and Biolog® MT plates analysed individually or together). A greater amount of variance in the AWCD of Biolog® MT plates was attributed to treatment differences (F = 10.46, P =0.009) than in the AWCD of Biolog® GN plates or of both plates combined (F = 9.43, P = 0.012 and F = 10.05, P <0.01, respectively). Furthermore, there was a significant treatment x day interaction in the AWCD of the Biolog® MT plates (F = 4.43, P = 0.005). The AWCD of SU-treated samples increased until day 15 and started to decline on day 29, whilst the AWCD of control samples was stable over time. No significant interaction was observed in the other measurements of AWCD.

CLPP – transformed data

The first 3 PCs of AWCD-transformed CLPP data accounted for 21% of the total variability (8, 7 and 6%, respectively). A significant treatment effect (P < 0.001) was found in the second PC (Fig 2c). Sample scores on the first and second PCs were weakly correlated with moisture content and with ammonium concentration and pH, respectively. However, scatter diagrams showed that the data were highly dispersed. The loadings of individual C-

sources on the first PC indicated that differences between treated and control samples were mainly due to an increase in the use of amino acids (proline, glutamine, alanine, and serine) and long chain aliphatic acids (oleic and linolenic acid), and a decrease in the use of phenolic acids in SU-treated samples. Utilisation of some carboxylic acids (cis-aconitic acid, glycyl-L-glutamic acid, D,L -lactic acid, malic acid and citric acid) was stimulated in SU-treated samples, whilst utilisation of others (succinic acid, bromosuccinic acid and oxalic acid) was depressed. Differences observed in the second PC were primarily due to an increase in utilisation of amino acids (glycine, lysine and aspartic acid) and nucleic acid bases (cytosine and guanidine) in SU-treated samples. The main factor discriminating treated from control samples in the third PC was increased utilisation of sugars and a decreased utilisation of amino acids in SU-treated samples.

Plate counts

Bacterial culturable cell concentrations increased significantly ($P < 0.001$) in the SU-treated soils from 2 x 10^7 cfu g^{-1} soil, in samples taken on day 0, to a maximum of 1.1 x10^9 cfu g^{-1} soil in samples taken after incubation for 15 days (Fig 3). Sampling day also had a significant effect on bacterial plate counts ($P = 0.001$) and there was a significant treatment x day interaction ($P = 0.014$). There were significant increases in plate counts in SU-treated soils, while plate counts in control samples did not change significantly during the incubation (Fig 3).

Comparison of methods

There were significant correlations between the principal components of the DGGE, PLFA and CLPP data. The first PC of the DGGE data was positively correlated with the third PC of the PLFA data ($r^2 = 0.47$, $P < 0.001$; Fig 4) and negatively correlated with the first PC

of the CLPP data (r^2 = 0.25, P = 0.002). Sample scores on the first PC of CLPP data were also negatively correlated with scores on the second PC of the PLFA data (r^2 = 0.32, P < 0.001). Significant correlations between other DGGE-PCs, CLPP-PCs and PLFA-PCs were found, but scatter diagrams showed that the data were also highly dispersed.

Discussion

PLFA and DGGE profiles

Differences among samples in total PLFA accounted for 57% of the total variability (Fig 1). To ensure that treatment effects were not masked by this large variability, total PLFA was included as a covariate in the analysis of variance of individual fatty acids and important microbial groups. Total PLFA, which represents microbial biomass [13,26], and the fungal biomarker 18:2(9, 12) were not affected by SU addition, which is consistent with results obtained at this and another upland grassland site [9]. The lack of treatment effect on total bacterial PLFA contrasts with the increase in bacterial colony forming units found here and reported previously [9]. This probably reflects the fact that different portions of bacterial populations were measured by PLFA extraction and plate counts. Plate counts only measure the culturable bacterial types whereas bacterial PLFA gives a measure of total communities, including the majority of the community that will not be detected on plates. The significant effect of SU addition on culturable bacterial types was not reflected in total PLFA measurements, presumably because only a small proportion of soil bacteria are culturable.

Branched fatty acids are commonly, though not exclusively, found in Gram-positive bacteria while monounsaturated fatty acids are often associated with Gram-negative bacteria [27]. The content of a number of individual branched fatty acids was significantly higher in SU-treated than in control soils, whilst the effect of SU treatment on monounsaturated fatty acids was more variable. These data may indicate that SU treatment caused an increase in the

relative abundance of Gram-positive bacteria. A previous study at this site found that the level of many branched fatty acids were affected by the nutrient status of samples, greater nutrient availability being associated with high levels of branched fatty acids [6].

Changes in pH and mineral N content have previously been reported to influence PLFA profiles. Increases in pH, similar in magnitude to those observed here, have caused shifts in bacterial community fatty acids indicative of more Gram-negative and fewer Gram-positive bacteria [14]. Fertilisation with N/P/K resulted in greater proportions of fatty acids suggesting increases in Gram-negative bacteria [28]. These results are not consistent with the shifts in PLFA profiles observed here. However, Clegg et al. [29] found that branched fatty acids were most responsible for the discrimination of undrained, N-fertilised soil and drained soil.

Principal components analysis was used to identify fatty acids that varied in combination and to detect changes in underlying patterns in the PLFA profiles caused by SU application that may not have been apparent from analysis of individual fatty acids or groups of fatty acids representative of large taxonomic groups. This might arise if the effects of an environmental factor on a subset of the microbial community were masked by more important effects of other environmental factors. PCA also permits assessment of the importance of shifts in community structure in relation to the total variability in the data. The first PC reflected differences in total PLFA among samples. Subsequent PCs reflect shifts in microbial community structure. The second PC was not affected by SU application and may have been associated with factors such as vegetation composition or the physical habitat of the microorganisms. Significant differences in the abundance of individual fatty acids and in fatty acid patterns associated with different vegetation classes have previously been found at this site [6]. Scores in the third PC reflected increased abundance of branched fatty acids and a variable response of monounsaturated fatty acids in SU-treated samples, corroborating

analysis of individual fatty acids. The fatty acids 18:1ω9c, 18:2(9, 12) and 18:3(5, 10, 12) are common in fungi [30]. Their variable response to SU treatment suggests that different fungal groups may have responded differently to the treatment. Similarly, the two cyclopropyl fatty acids made inverse contributions to the difference between control and SU-treated samples. Gram negative bacteria contain cyclopropyl fatty acids but rarely together or in similar amounts [30,14]. Different groups of Gram negative bacteria responding differently to SU application could therefore result in the observed changes in cyclopropyl fatty acids. However, the third PC only accounted for 6% of the total variability in PLFA profiles, equivalent to approximately 14% of total variability in community structure.

Shifts in microbial community structure due to SU were also reflected in the DGGE band intensity patterns, but not in the presence or absence of bands at each position in the gel. Therefore, SU treatment did not result in the appearance of new DDGE bands but rather a change in the relative abundance of bands, with an increase in intensity in the upper regions of the gel. It is unlikely that the observed changes in relative band intensity were an experimental artefact because the positions of treated and control samples on the gel were chosen at random. The first PC of the DGGE data and the third PC of the PLFA data were significantly correlated (Fig 4), both showing significant treatment effects and accounting for a similar amount of total community structure variability. This suggests that these PCs reflected the same changes in microbial community structure. Thus, treatment with synthetic urine favoured microorganisms with high concentrations of branched fatty acids and DNA sequences that did not migrate as far as sequences from control soils. It is possible that these data are indicative of a shift in microbial community structure towards Gram positive bacteria with a low G+C content, although confirmation would require sequence analysis of DGGE bands. However, the changes only accounted for 10-15% of total community structure variability, suggesting that microbial community structure was reasonably stable despite the

significant changes in soil solution chemistry. The relative importance of the effect of SU addition in each of the analyses was different and this is reflected in the fact that the significant effect of SU addition is seen in the first PC of DGGE data (suggesting that it was the single most important factor influencing band intensity variability) but seen in the third PC of the PLFA data (suggesting that other factors are more important, environmental factors, intrinsic biological variation or experimental error for example).

Community level physiological profiles

The first PC of the raw sole carbon source utilisation data shows that plate AWCD or overall carbon use was significantly greater in the SU-treated samples that in the control samples. Plate AWCD is related to the inoculation density of total bacterial cells and of total active cells [17], suggesting that SU treatment stimulated bacterial growth or increased the proportion of active bacterial cells. This is consistent with the increase in bacterial colony forming units observed here and in a previous experiment at this site [9]. In the present experiment, colony forming units in treated samples had increased between 10- and 100-fold 15 days after SU application (Fig 3).

Comparison of AWCD values suggested that SU treatment preferentially stimulated bacteria with an ability to use rhizosphere C-sources and in particular the neutral amino acids glycine and valine. Differential substrate availability is believed to cause shifts in microbial community structure and in substrate utilisation profiles [31,32,33]. For example, carbohydrate use was enhanced but amino acid and polymer use impaired in soils amended with glucose, whilst soils amended with hydroxyproline and gelatin showed enhanced use of amino acids, polymers and carbohydrates, suggesting that microbial communities adapted to the type of available C-source [32]. The SU applied in this experiment contained glycine, reflecting the composition of natural sheep urine [8], which would explain the enhanced use

of glycine in SU-treated samples. Furthermore, SU is known to cause a release of organic forms of C, N and P into the soil solution [8], potentially leading to further selection. None of the PCs of the AWCD-transformed data accounted for a large part of the total variability, suggesting no dominant influence on C-source utilisation patterns but rather a number of different factors exerting different pressures on the microbial communities. In grasslands, plant density is very high, with a variety of species co-existing in close proximity which, in combination with differences in soil physiochemical properties, can result in a wide variety of local environments in which different microbial communities can develop. A high degree of local variability in microbiological properties has previously been identified at this site [6].

The correlation between the first CLPP-PC and the second PLFA-PC indicates that the microorganisms or groups of microorganisms responsible for the PLFA pattern may have been more adapted to the use of amino acids and long chain aliphatics than phenolic acids. However, caution must be exercised as the correlation was weak. The fact that these PCs were not affected by SU treatment may reflect a number of factors, e.g. local plant composition, potentially influencing microbial community activity but unaffected by SU application. There was significant divergence between CLPP and PLFA profiling. CLPP indicated a significant effect of SU treatment on the density of active bacteria although neither total PLFA nor total bacterial PLFA were affected by SU treatment. CLPP patterns were also less sensitive to moisture content that PLFA profiles. Bossio and Scow [34] also concluded that PLFA profiles responded more readily to soil water content than CLPP patterns and Grayston et al. [2] reported differences between CLPP and PLFA profiles. CLPP profiles are biased towards microorganisms that grow rapidly under laboratory conditions in an aqueous, nutrient-rich environment and may not represent dominant populations in the inoculum [35], whereas PLFA profiles are likely to reflect dominant microbial types. DGGE profiles appeared to be more closely related to PLFA profiles than to CLPP profiles, probably for similar reasons.

Synthetic urine treatment caused significant changes in the microbial community structure detected by PLFA profiles, DGGE banding patterns and C-source utilisation. The data indicated a shift towards bacteria with higher concentrations of branched fatty acids and a higher proportion of DNA sequences migrating to the upper regions of the gel. The shift observed only accounted for 10-15% of the total variability in community structure, suggesting that, while the effect was significant, it was limited in magnitude. SU treatment also preferentially stimulated bacteria adapted to utilise rhizosphere C-sources. The consequences of the changes in microbial community structure for subsequent soil function and plant growth were not determined but many of the changes in community structure observed here are consistent with the results of Ritz et al. [6], and thus support the hypothesis that urine addition is a significant cause of heterogeneity in upland grassland soils. However, the data also show that the effect of urine was not large and that there were no overarching controlling factors.

Acknowledgements

This work was funded by the Scottish Executive Environment and Rural Affairs Department under their Flexible Funding Scheme and is part of the MICRONET Co-ordinated Programme of Research. The authors would like to thank two anonymous referees for helpful discussions.

References

[1] Grayston, S. J., Griffith, G. S., Mawdsley, J. L., Campbell, C. D., and Bardgett, R. D. (2001) Accounting for variability in soil microbial communities of temperate upland grassland ecosystems. Soil Biol. Biochem. 33, 533-551.

[2] Grayston, S. J., Campbell, C. D., Bardgett, R. D., Mawdsley, J. L., Clegg, C. D., Ritz, K., Griffiths, B. S., Rodwell, J. S., Edwards, S. J., Davies, W. J., Elston, D. J., and Millard, P. (2004) Assessing shifts in microbial community structure across a range of grasslands of differing management intensity using CLPP, PLFA and community DNA techniques. Appl Soil Ecol 25, 63-84.

[3] McCaig, A. E., Glover, L. A., and Prosser, J. I. (2001) Numerical analysis of grassland bacterial community structure under different land management regimens by using 16S ribosomal DNA sequence data and denaturing gradient gel electrophoresis banding patterns. Appl. Environ. Microbiol. 67, 4554-4559.

[4] Brodie, E., Edwards, S., and Clipson, N. (2002) Bacterial community dynamics across a floristic gradient in a temperate upland grassland ecosystem. Microb. Ecol. 44, 260-270.

[5] Griffiths, R. I., Whiteley, A. S., O'Donnell, A. G., and Bailey, M. J. (2003) Influence of depth and sampling time on bacterial community structure in an upland grassland soil. FEMS Microbiol. Ecol. 43, 35-43.

[6] Ritz, K., McNicol, W., Nunan, N., Grayston, S., Millard, P., Atkinson, D., Gollotte, A., Habeshaw, D., Boag, B., Clegg, C. D., Griffiths, B. S., Wheatley, R. E., Glover, L. A., McCaig, A. E., and Prosser, J. I. (2004) Spatial structure in soil chemical and microbiological properties in an upland grassland. FEMS Microbiol. Ecol. 49, 191-205.

[7] Clegg, C. D., Ritz, K., and Griffiths, B. S. (2000) %G+C profiling and cross hybridisation of microbial DNA reveals great variation in below-ground community structure in UK upland grasslands. Appl Soil Ecol 14, 125-134.

[8] Shand, C. A., Williams, B. L., Smith, S., and Young, M. E. (2000) Temporal changes in C, P and N concentrations in soil solution following application of synthetic sheep urine to a soil under grass. Plant Soil 222, 1-13.

[9] Williams, B. L., Grayston, S. J., and Reid, E. J. (2000) Influence of synthetic sheep urine on the microbial biomass, activity and community structure in two pastures in the Scottish uplands. Plant Soil 225, 175-185.

[10] Ridgeway, K. P., Duck, M. J., and Young, P. W. (2003) Identification of roots from grass swards using PCR-RFLP and FFLP of the plastid trnL (UAA) intron. BMC Ecology 3, 8-

[11] Shand, C. A., Williams, B. L., Dawson, L. A., Smith, S., and Young, M. E. (2002) Sheep urine affects soil solution nutrient composition and roots: differences between field and sward box soils and the effects of synthetic and natural sheep urine. Soil Biol. Biochem. **34**, 163-171.

[12] Haynes, R. J. and Williams, P. H. (1993) Nutrient Cycling and Soil Fertility in The Grazed Pasture Ecosystem. Adv Agron 49, 119-199.

[13] Frostegård, Å., Tunlid, A., and Bååth, E. (1991) Microbial Biomass Measured As Total Lipid Phosphate in Soils of Different Organic Content. J. Microbiol. Meth. 14, 151-163.

[14] Frostegård, Å., Bååth, E., and Tunlid, A. (1993) Shifts in the Structure of Soil Microbial Communities in Limed Forests As Revealed by Phospholipid Fatty-Acid Analysis. Soil Biol. Biochem. 25, 723-730.

[15] Campbell, C. D., Grayston, S. J., and Hirst, D. J. (1997) Use of rhizosphere carbon sources in sole carbon source tests to discriminate soil microbial communities. J. Microbiol. Meth. 30, 33-41.

[16] Hackett, C. A. and Griffiths, B. S. (1997) Statistical analysis of the time-course of Biolog substrate utilization. J. Microbiol. Meth. 30, 63-69.

[17] Garland, J. L. (1996) Analytical approaches to the characterization of samples of microbial communities using patterns of potential C source utilization. Soil Biol. Biochem. 28, 213-221.

[18] Guckert, J. B., Carr, G. J., Johnson, T. D., Hamm, B. G., Davidson, D. H., and Kumagai, Y. (1996) Community analysis by Biolog: Curve integration for statistical analysis of activated sludge microbial habitats. J. Microbiol. Meth. 27, 183-197.

[19] Griffiths, R. I., Whiteley, A. S., O'Donnell, A. G., and Bailey, M. J. (2000) Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. Appl. Environ. Microbiol. 66, 5488-5491.

[20] Lane, DJ (1991) 16S/23S rRNA Sequencing. In: Nucleic acid techniques in bacterial systematics. (Stackebrandt, E and Goodfellow, M, Eds), pp.115-175. John Wiley & Sons,NJ.

[21] Edwards, U, Rogall, T, Blocker, H, Emde, M, and Bottger, EC (1989) Isolation and direct complete nucleotide determination of entire genes. characterization of a gene coding for 16S ribosomal RNA. Nucl. Acids Res. 17, 7843-7853.

[22] Muyzer, G., Dewaal, E. C., and Uitterlinden, A. G. (1993) Profiling of Complex Microbial-Populations by Denaturing Gradient Gel-Electrophoresis Analysis of Polymerase Chain Reaction-Amplified Genes-Coding for 16S Ribosomal-RNA. Appl. Environ. Microbiol. 59, 695-700.

[23] Tebbe, C. C. and Vahjen, W. (1993) Interference of Humic Acids and DNA Extracted Directly from Soil in Detection and Transformation of Recombinant-DNA from Bacteria and A Yeast. Appl. Environ. Microbiol. 59, 2657-2665.

[24] Fromin, N., Hamelin, J., Tarnawski, S., Roesti, D., Jourdain-Miserez, K., Forestier, N., Teyssier-Cuvelle, S., Gillet, F., Aragno, M., and Rossi, P. (2002) Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. Environmental Microbiology 4, 634-643.

[25] Zogg, G. P., Zak, D. R., Ringelberg, D. B., MacDonald, N. W., Pregitzer, K. S., and White, D. C. (1997) Compositional and functional shifts in microbial communities due to soil warming. Soil Sci. Soc. Am. J. 61, 475-481.

[26] Zelles, L. (1999) Identification of single cultured micro-organisms based on their whole-community fatty acid profiles, using an extended extraction procedure. Chemosphere 39, 665-682.

[27] Bai, Q., Gattinger, A., and Zelles, L. (2000) Characterization of microbial consortia in paddy rice soil by phospholipid analysis. Microb. Ecol. 39, 273-281.

[28] Steer, J. and Harris, J. A. (2000) Shifts in the microbial community in rhizosphere and non- rhizosphere soils during the growth of Agrostis stolonifera. Soil Biol. Biochem. 32, 869-878.

[29] Clegg, C. D., Lovell, R. D. L., and Hobbs, P. J. (2003) The impact of grassland management regime on the community structure of selected bacterial groups in soils. FEMS Microbiol. Ecol. 43, 263-270.

[30] Zelles, L. (1997) Phospholipid fatty acid profiles in selected members of soil microbial communities. Chemosphere 35, 275-294.

[31] Grayston, S. J., Wang, S. Q., Campbell, C. D., and Edwards, A. C. (1998) Selective influence of plant species on microbial diversity in the rhizosphere. Soil Biol. Biochem. 30, 369-378.

[32] Schutter, M. and Dick, R. (2001) Shifts in substrate utilization potential and structure of soil microbial communities in response to carbon substrates. Soil Biol. Biochem. 33, 1481-1491.

[33] Baudoin, E., Benizri, E., and Guckert, A. (2001) Metabolic fingerprint of microbial communities from distinct maize rhizosphere compartments. Eur. J. Soil Biol. 37, 85-93.

[34] Bossio, D. A. and Scow, K. M. (1998) Impacts of carbon and flooding on soil microbial communities: Phospholipid fatty acid profiles and substrate utilization patterns. Microb. Ecol. 35, 265-278.

[35] Smalla, K., Wachtendorf, U., Heuer, H., Liu, W. T., and Forney, L. (1998) Analysis of BIOLOG GN substrate utilization patterns by microbial communities. Appl. Envron. Microbiol. 64, 1220-1225.

Table 1. Influence of synthetic sheep urine application on selected properties of grassland soil.

	pH		Moisture (% dry weight)		DOC (µg g^{-1})		NH_4-N (µg g^{-1})		NO_3-N (µg g^{-1})	
Day	control	urine	control	urine	control	urine	control	urine	control	urine
0	3.3	3.4	66	67	nd[b]	51	8	88	6.5	7.1
1	3.4	5.3	57	121	693	115	14	1390	4.9	7.9
8	3.7	6.3	110	117	972	189	24	1527	3.6	12.0
15	3.7	6.4	86	149	912	2285	20	1612	5.5	28.4
29	3.6	5.1	161	176	929	1023	17	966	4.2	39.0
s.e.d.[a]	0.5		24		322		370		9	

[a]s.e.d.: standard error of the difference

[b]n.d.: not determined

Table 2. Significance level of urine treatment effects (residual degrees of freedom = 10), effects of time of sampling and treatment x time interaction (residual degrees of freedom = 29) found for individual PLFAs determined by analysis of covariance with total PLFA as a covariate, where significant effects were apparent.

Variable	Treatment	Time	Treatment x Time
	P	*P*	*P*
i14:0	0.040	n.s.	n.s.
cis14:1ω9	n.s.*	<0.001	n.s.
i15:0	n.s.	0.016	n.s.
a15:0	0.022	0.013	0.045
i16:1	n.s.	0.023	n.s.
trans16:1ω11	0.028	n.s.	n.s.
cis16:1ω7	n.s.	0.005	n.s.
16:0	n.s.	0.009	0.022
i17:0	0.007	n.s.	0.013
17:1ω7	0.007	n.s.	n.s.
12Me17:0	n.s.	0.033	n.s.
18:3(6,8,13)	n.s.	0.002	n.s.
20:1	n.s.	0.020	n.s.

*n.s.: not significant

Fig 1. Relationship between the first principal component and total amount of microbial-specific PLFA. The number of days after water or urine application for control (normal type) and urine-treated (bold) samples, respectively, are indicated. No separation between treatment and control samples is observed.

Fig 2. Mean principal component scores for control (black bars) and urine-treated (grey bars) samples on each sampling day in the third principal component of PLFA data (a), the first principal component of DGGE data (b) and the second principal component of CLPP data (c). Bars are standard error of the mean.

Fig 3. Effects of synthetic urine on total bacterial plate counts (cfu g^{-1}). Control samples are black bars and urine-treated samples are grey bars. Error bars are standard error of the means.

Fig 4. Relationship between sample scores on the first principal component of DGGE banding patterns and on the third principal component of PLFA profiles.

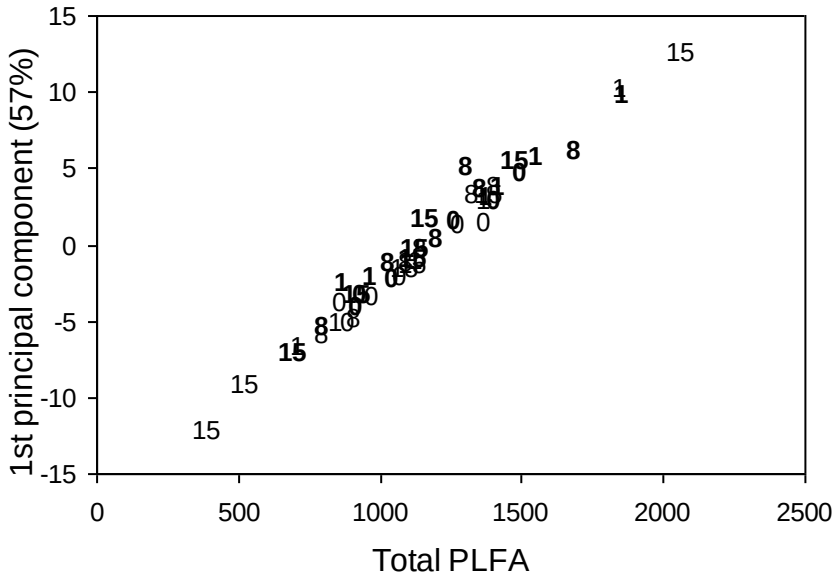
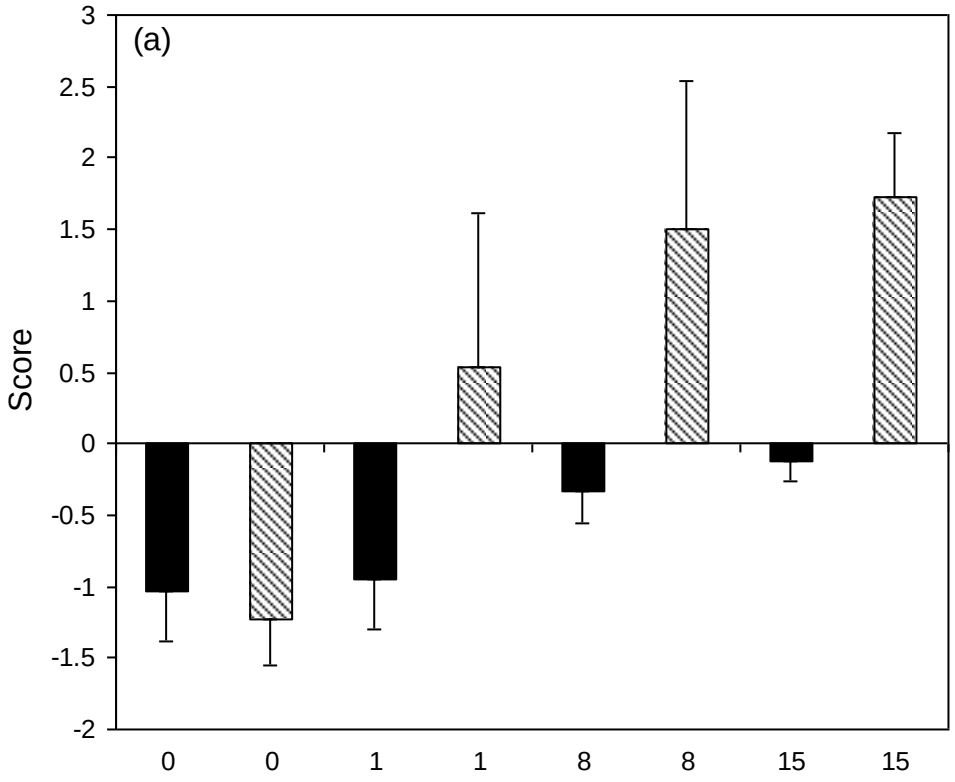
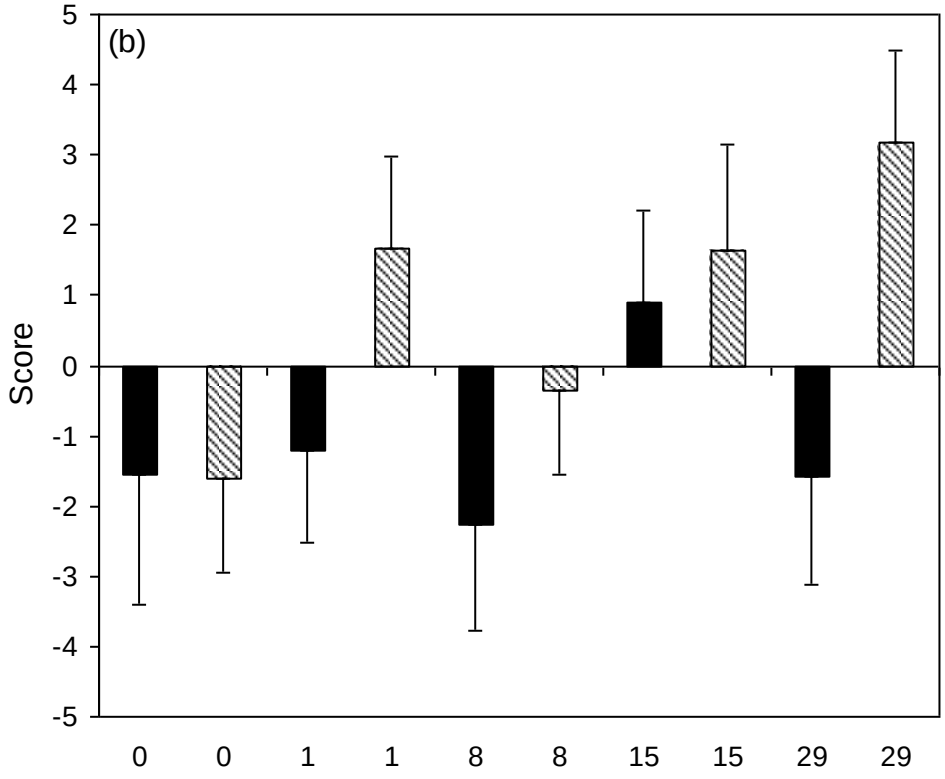


Fig 1. Relationship between the first principal component and total amount of microbial-specific PLFA. The number of days after water or urine application for control (normal type) and urine-treated (bold) samples, respectively, are indicated. No separation between treatment and control samples is observed.


(a)
Score
3
2.5
2
1.5
1
0.5
0
-0.5
-1
-1.5
-2
0
0
1
1
8
8
15
15


(b)
Score
5
4
3
2
1
0
-1
-2
-3
-4
-5
0
0
1
1
8
8
15
15
29
29

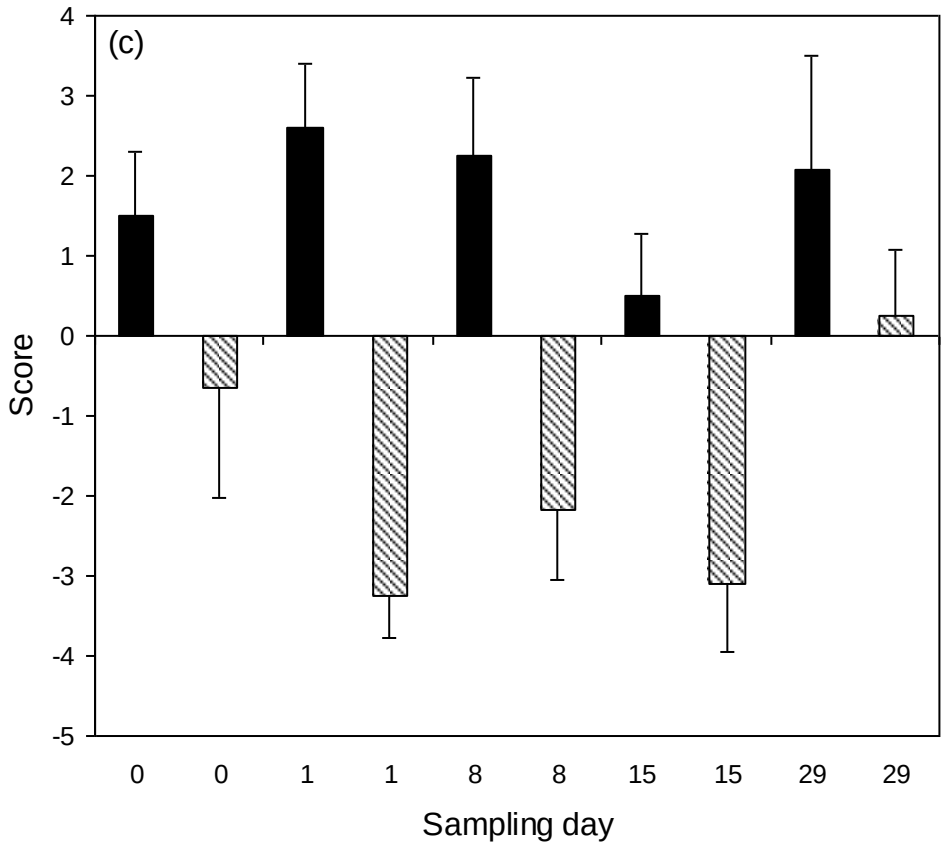


Fig 2. Mean principal component scores for control (black bars) and urine-treated (grey bars) samples on each sampling day in the third principal component of PLFA data (a), the first principal component of DGGE data (b) and the second principal component of CLPP data (c). Bars are standard error of the mean.

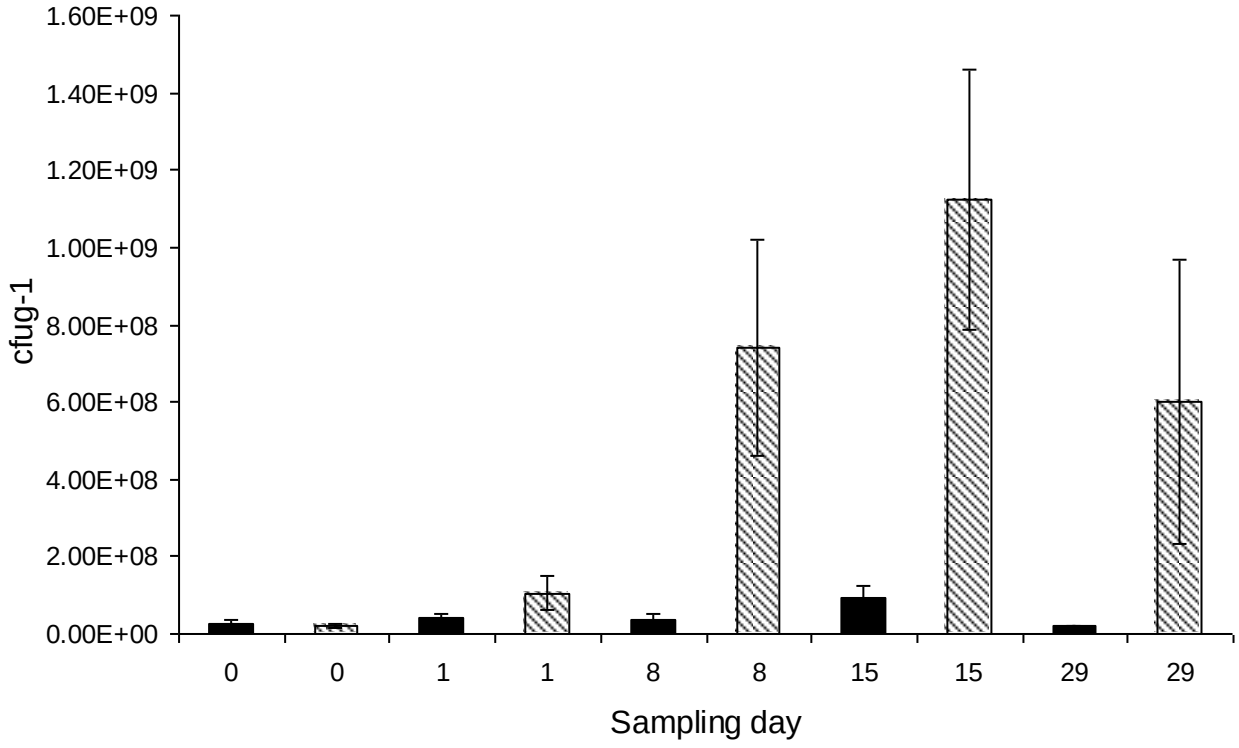


Fig 3. Effects of synthetic urine on total bacterial plate counts (cfu g^{-1}). Control samples are black bars and urine-treated samples are grey bars. Error bars are standard error of the means.

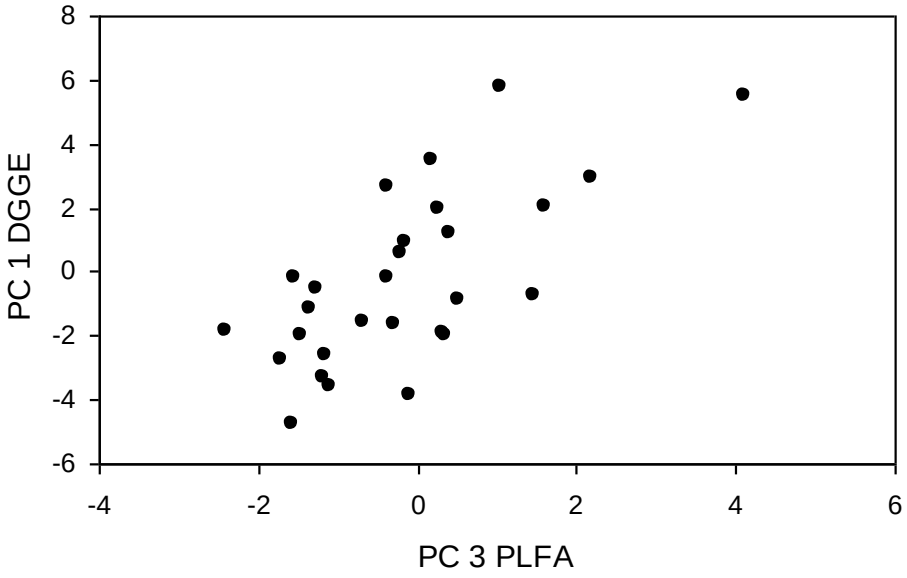


Fig 4. Relationship between sample scores on the first principal component of DGGE banding patterns and on the third principal component of PLFA profiles.