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Use of earthworm casts to validate FT-IR spectroscopy as a 'sentinel' technology for high-throughput monitoring of global changes in microbial ecology

John Scullion[1]*, Geoff N Elliott[1], Wei E Huang[1], Roy Goodacre[1], Hilary Worgan[1], Robert Darby[1], Michael J. Bailey[2], Dylan Gwynn-Jones[1], Gareth W. Griffith[1], Michael K. Winson[1], Peter A. Williams[3], Christopher Clegg[4] and John Draper[1]

[1] Institute of Biological Sciences, University of Wales Aberystwyth, Ceredigion SY23 3DE, UK
[2] Centre for Ecology and Hydrology, Mansfield Road, Oxford OX1 3SR, UK
[3] School of Biological Sciences, University of Wales Bangor, Gwynedd LL57 2UW, UK
[4] Institute of Grassland and Environmental Research, North Wyke Research Station, Oakhampton, Devon EX20 2SB, UK

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Summary

This study aimed to evaluate metabolic fingerprinting by Fourier transform infrared (FT-IR) spectroscopy as a technique for investigating microbial communities and their activities in soil. FT-IR spectra from earthworm casts, and other 'biosamples', were compared using multivariate cluster analyses. The work formed part of a wider study to quantify the risk of horizontal gene flow and to assess ecological impacts associated with the release of GM crops or recombinant micro-organisms.
A range of samples, including pure cultures of similar soil bacteria, plant materials and earthworm casts of various ages and feeding regimes were analysed. A subset of the cast FT-IR data was compared with DGGE analysis of extracted DNA/RNA. Cluster analysis of FT-IR spectra was capable of differentiating between different bacterial, litter and cast samples. There was congruence between FT-IR and DGGE clustering for food type but not for cast age. Further detailed work on the microbial populations will be needed to investigate relationships between microbial and spectroscopy data.

Key words: FT-IR, metabolic fingerprinting, micro-organisms, earthworms

Introduction

Spatial and temporal heterogeneity of microbial communities in environmental samples presents problems when monitoring changes in these communities. The particular issue underlying our work was the transfer of novel genetic combinations from crops/crop residues to soil organisms.

Microbial population dynamics have been investigated using several methodologies. PCR-based approaches such as denaturing gradient gel electrophoresis (DGGE) (Muyzer et al. 1993) can be used to produce a genetic profile for each environmental sample, indicative of the presence or absence of differing

*E-mail corresponding author: jos@aber.ac.uk

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species of ribosomal DNA/RNA. More generalised 'chemical fingerprinting', such as soil fatty acid analysis (Tunlid & White 1992), is also widely applied. However, the resolution of these methods may be restricted to a limited subset of the soil biota, and extraction and analysis procedures can be time consuming, labour intensive and have a low throughput.

In contrast, spectroscopic analysis of complex biological samples is comparatively non-selective. Fourier transform infra-red spectroscopy (FT-IR) produces 'fingerprints' made up of the vibrational features of microbial cell components (Naumann et al. 1991). FT-IR allows the chemically-based discrimination of intact microbial cells, without their destruction, and produces complex biochemical fingerprints which are not only reproducible and distinct for different bacteria and fungi (Goodacre et al. 1998; Timmins et al. 1998), but can be an automated and very rapid method of analysis (Winson et al. 1997).

FT-IR based approaches give an overall "organic chemical fingerprint" of soil and leaf litter. The ability to analyse metabolites, in addition to more abundant macromolecules, makes it possible to detect subtle shifts in microbial populations interacting with plant litter.

Work reported here formed part of a wider investigation into potential risks of horizontal gene transfer, from plants to soil microorganisms (Nielsen et al. 1998). There is a need to establish sampling and analytical protocols for monitoring such processes where they are most likely to occur under field conditions. There is evidence for enhanced HGT in the gut and faeces of Lumbricidae (Clegg et al. 1995). The diversity of transconjugant bacteria may also be greater because of macroscopic blending of organic material with micro-organism/soil mixtures and increased contact between donor and recipient bacteria (Daane et al. 1996). Sampling of earthworm cast material therefore, would appear to be consistent with a precautionary approach. The specific aim of the present work was to evaluate metabolic fingerprinting by FT-IR spectroscopy, coupled with multivariate cluster analyses, for detecting variations in the chemistry of casts from different earthworm species feeding on various substrates.

Materials and Methods

Samples from a series of experiments were analysed by FT-IR to evaluate this procedure as a means of distinguishing between suspensions of common soil bacteria, earthworm food types, casts of *L. terrestris* and *L. rubellus*, and casts of differing ages. A subset of these data was also subject to DGGE analysis and compared with FT-IR data.

Pot trials

Earthworms were incubated in 2.2L pots (8 replicates) containing a mix of sand, moss peat and kaolin (OECD 1984). An excess of food (processed oat grain (as a 'control'), fresh or aged tobacco (*Nicotiana tobaccum*), or fresh or aged *Arabidopsis thaliana*) was added to the surface of these pots. Plant litter was 'aged' by storing fresh leaves at 28 °C for 7 days. Samples of different food types were taken for FT-IR analysis prior to their being added to pots.

After 2 weeks, pot surfaces were cleared of casts, fresh food was added and all casts collected over the next three days. Some casts were analysed immediately whilst the remainder were aged for up to 8 days and sampled at intervals (1, 2, 3, 4 and 8 days). Cast incubations were carried out in the dark at 15 °C in a humid atmosphere.

FT-IR analysis

Bacterial suspensions for FT-IR analysis were grown, washed and resuspended in sterile physiological saline (0.9 % NaCl) prior to analysis. Litter and cast samples were mixed with 30 ml liquid nitrogen and ground in a mortar before being collected with sterile 1.5 ml micro tubes and suspended in 0.6 ml saline. Final concentrations were 10^9 cells/ml, 75 mg/ml and 125 mg/ml saline for bacterial, litter and cast suspensions respectively.

FT-IR analysis was performed (Goodacre et al. 1998, 2000) with 10 µl aliquots of sample suspensions added onto each well of an aluminium plate. Before analysis, samples were dried at 50 °C for 30 min. Each sample was run in triplicate. FT-IR data were collected with Opus software (version 2.1). The scan range of spectra was 4,000 to 600 cm^{-1} and resolution was 4 cm^{-1}; each spectrum was represented by 882 points.

DGGE analysis

Nucleic acids were extracted from casts (Griffiths et al. 2000), but using less material (0.1g wet weight) than normal, with extraction confirmed and quantified by gel electrophoresis. PCR amplification was performed (Whiteley & Bailey 2000) using primers 530R and GC338F previously designed to target the V3 region of 16SrDNA. DGGE analysis was performed by loading ca. 1 µg of the resultant PCR products onto a 10 % (wt/vol) acrylamide gel containing a 30 %–60 % gradient of denaturant (100 % denaturant – 7M urea and 40 % (vol/vol) formamide) parallel to the direction of electrophoresis, using the INGENY phorU system (Ingeny International, Goes, Netherlands). Gels were stained following DGGE using Sybr Gold (Molecular probes, Eugene, Oreg.) and the banding profiles analysed using Phoretix one-dimensional analysis software (Newcastle-upon-Tyne, UK).

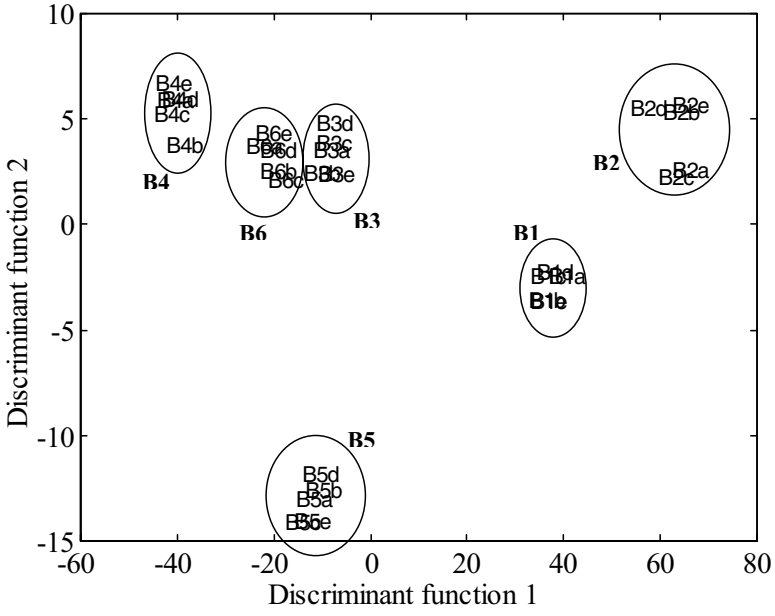

Fig. 1. Cluster analysis (PC-DFA) of FT-IR data derived from bacterial suspensions.
(Key: B1 – *Serratia rubidaea* Pink NM6P; B2 – *Serratia rubidaea* NM8P; B3 – *Chromobacterium violaceum* CVWT; B4 – *Aeromonas hydrophila* NM11W; B5 – *Pseudomonas* sp. NCIB9816; B6 – *Acinetobacter* sp. ADP1)

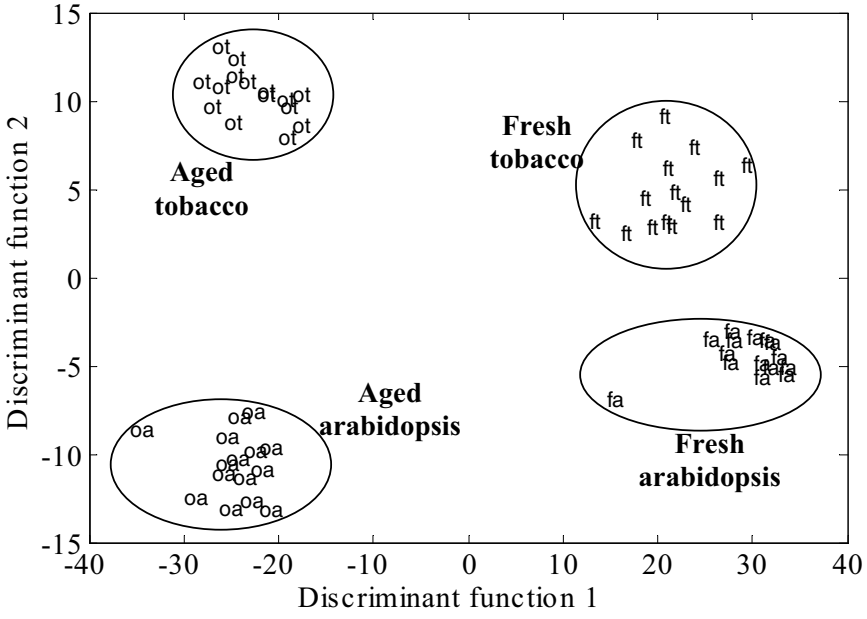

Fig. 2. Cluster analysis (PC-DFA) of FT-IR data derived from different leaf litter material. (Key: fa – fresh arabidopsis; oa – aged arabidopsis; ft – fresh tobacco; ot – aged tobacco)

Cluster analysis

ASCII data were exported from the Opus software and imported into Matlab version 5 (The MathWorks, Inc., 24 Prime Par Way, Natick, MA, USA). To minimize baseline shifts (Timmins et al. 1998), spectra were first normalised with the smallest absorbance set to 0 and the highest to +1 for each spectrum. Smoothed first derivatives of these normalised spectra were then calculated using the Savitzky-Golay algorithm (5-point smoothing).

Cluster analysis (PC-DFA) was used to analyse the FT-IR data (Goodacre et al. 1998; Timmins et al. 1998). Principal components analysis (PCA; Jolliffe 1986) reduced the dimensionality of the FT-IR data from 882 to 20 PCs (> 99 % of the total explained variance). Next discriminant function analysis (DFA; Manly 1994) discriminated between groups based on these retained PCs and the *a priori* knowledge of which spectra were replicates, a process that does not bias the analysis.

Results

FT-IR separated six pure bacterial strains found naturally in soils following PCA and DFA (Fig. 1). Successful differentiation of leaf materials destined for use in future experiments was also found, with good PC-DFA separation between *Arabidopsis* and Tobacco, and between aged and fresh material of both types (Fig. 2). The first discriminant function (DF1), which is extracted to explain the most variance, appeared to separate on plant type, whilst DF2 discriminated on food age.

Analysis of *L. rubellus* and *L. terrestris* casts, following their feeding on aged and fresh plant materials, indicated a less defined clustering than found for pure strains and for leaf material analysis (Fig. 3). However, the DFA did show some limited separation between the different food types, with clear separation between the

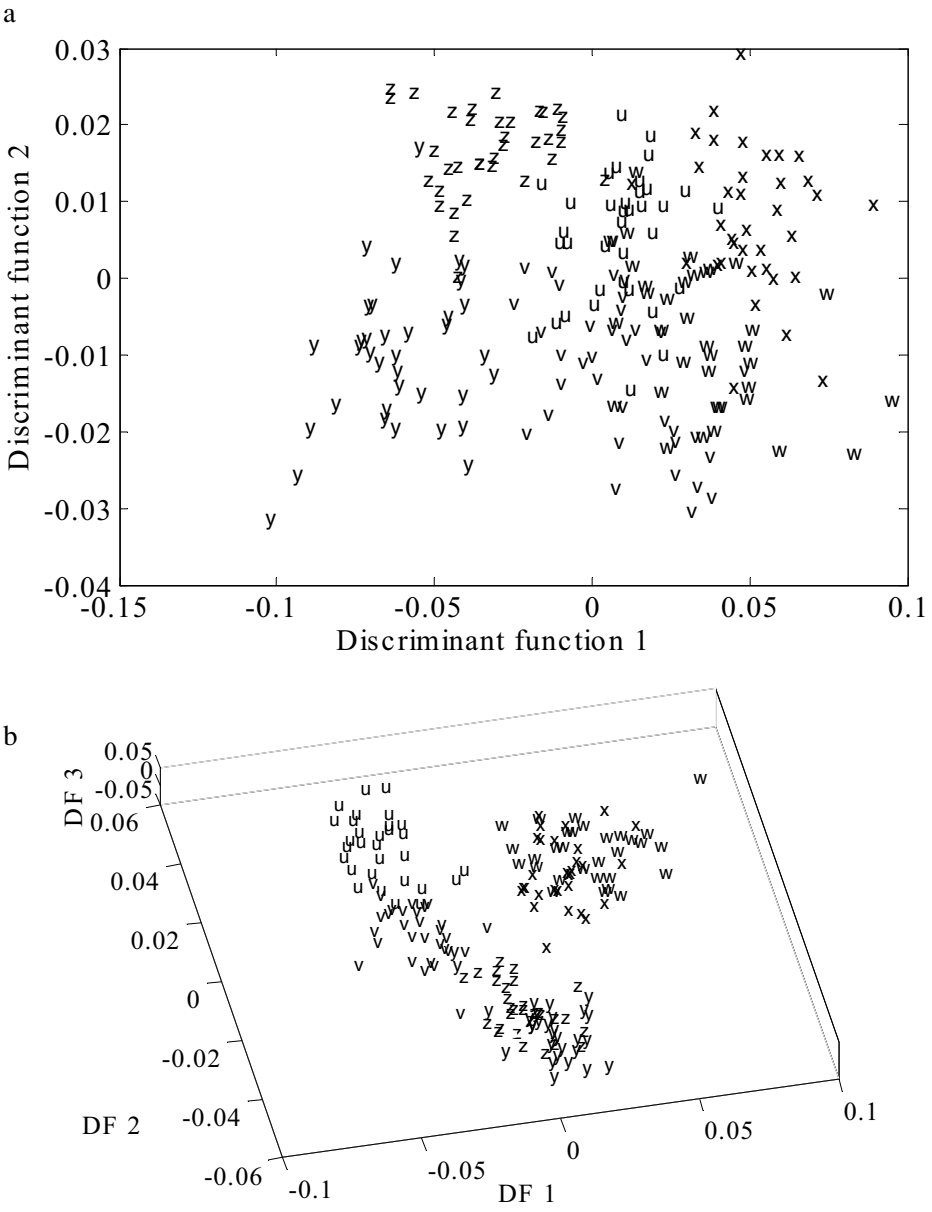

Fig. 3. Cluster analysis (PC-DFA) of FT-IR data derived from casts of *L. terrestris* (a) and *L. rubellus* (b) fed on different food material. (Key: u – aged tobacco leaves; v – fresh tobacco leaves; w – aged arabidopsis leaves; x – fresh arabidopsis leaves; y – processed oat grain; z – control (no additional food)

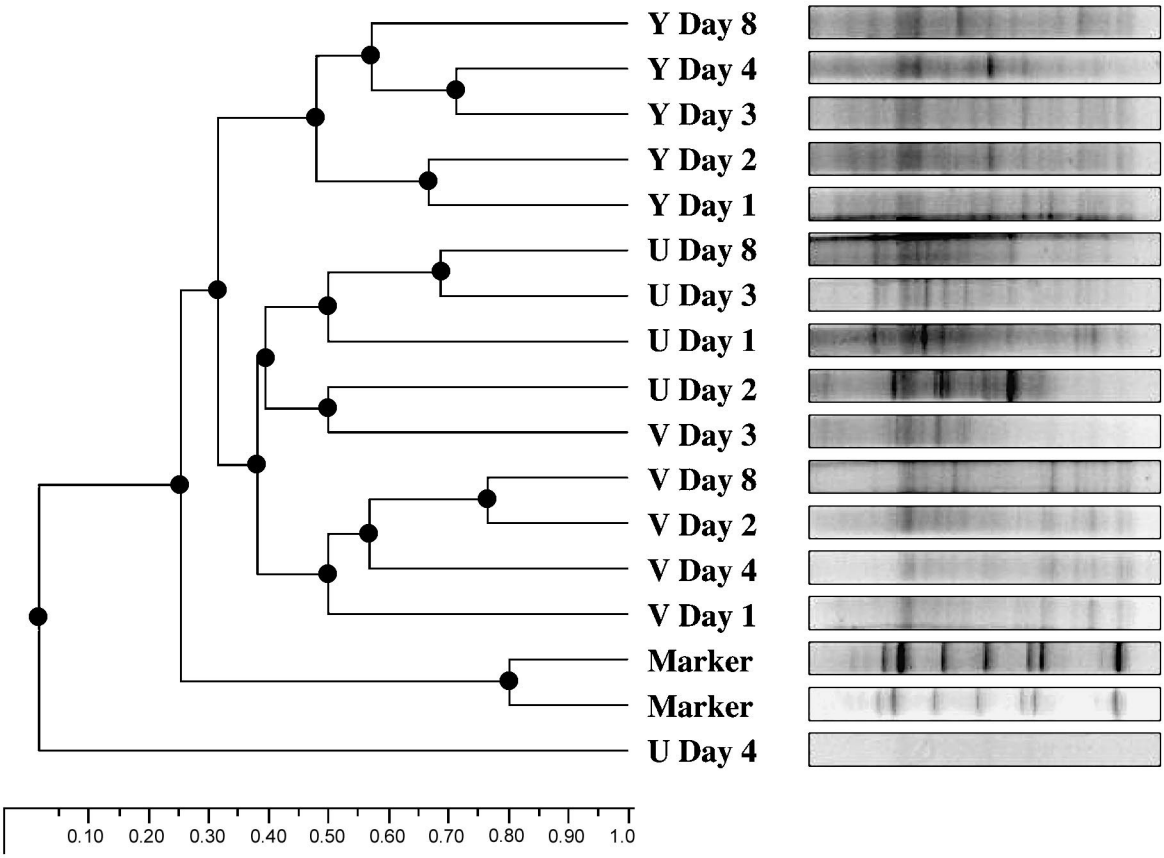

Fig. 4. Dendrogram derived from DGGE profile data from ageing casts of *L. terrestris* fed on different food material (unweighted pairwise grouping method with mathematical averages used to formulate dendrogram). (Key: V – fresh tobacco leaves; U – aged tobacco leaves; Y: processed oat grain; Day 'n': cast material aged for 'n' days)

casts from plant species and those from oat grain/control. Differentiation between aged and fresh *Arabidopsis* was least defined. These results, coupled with an apparent lack of *Arabidopsis* palatability for earthworms, led to the removal of both *Arabidopsis* data sets from further analysis.

DGGE profiling of 16S rDNA from *L. terrestris* cast material was performed for both tobacco and oat grain (Fig. 4) over our cast ageing period. Profile analysis revealed clustering of oat grain profiles separately from tobacco profiles. Four out of five tobacco cast profiles clustered together for both aged and fresh tobacco data sets, although there was no apparent relationship with respect to cast age.

FT-IR analysis was also performed using *L. terrestris* cast material for both tobacco data sets and for the oat grain data set (Fig. 5). Similar to the DGGE results, samples of the same type clustered by food type rather than by days aged (Fig. 5a). However, when only specific regions of FT-IR spectra, rather than the entire spectrum, were used for construction of PCA and therefore DFA plots, results were found to differ markedly (Fig. 5b). When only putative fatty acid peak (2800 to 3050 cm^{-1}) and putative amide peak (1500 to 1750 cm^{-1}) wave number regions (Naumann et al. 1996) were made available for analysis, the number of days aged became the more prominent feature, with casts from differing food groups clustering together with cast age.

Discussion

Although other studies (Naumann et al. 1991; Goodacre et al. 1998) have used FT-IR based approaches to differentiate bacteria, findings reported here indicate that our technique separated similar isolates of native soil bacteria. FT-IR analysis coupled with multivariate cluster analyses is a rapid technique also capable of distinguishing between complex biological samples, including leaf and casts (Figs. 2, 3, 5) at different stages

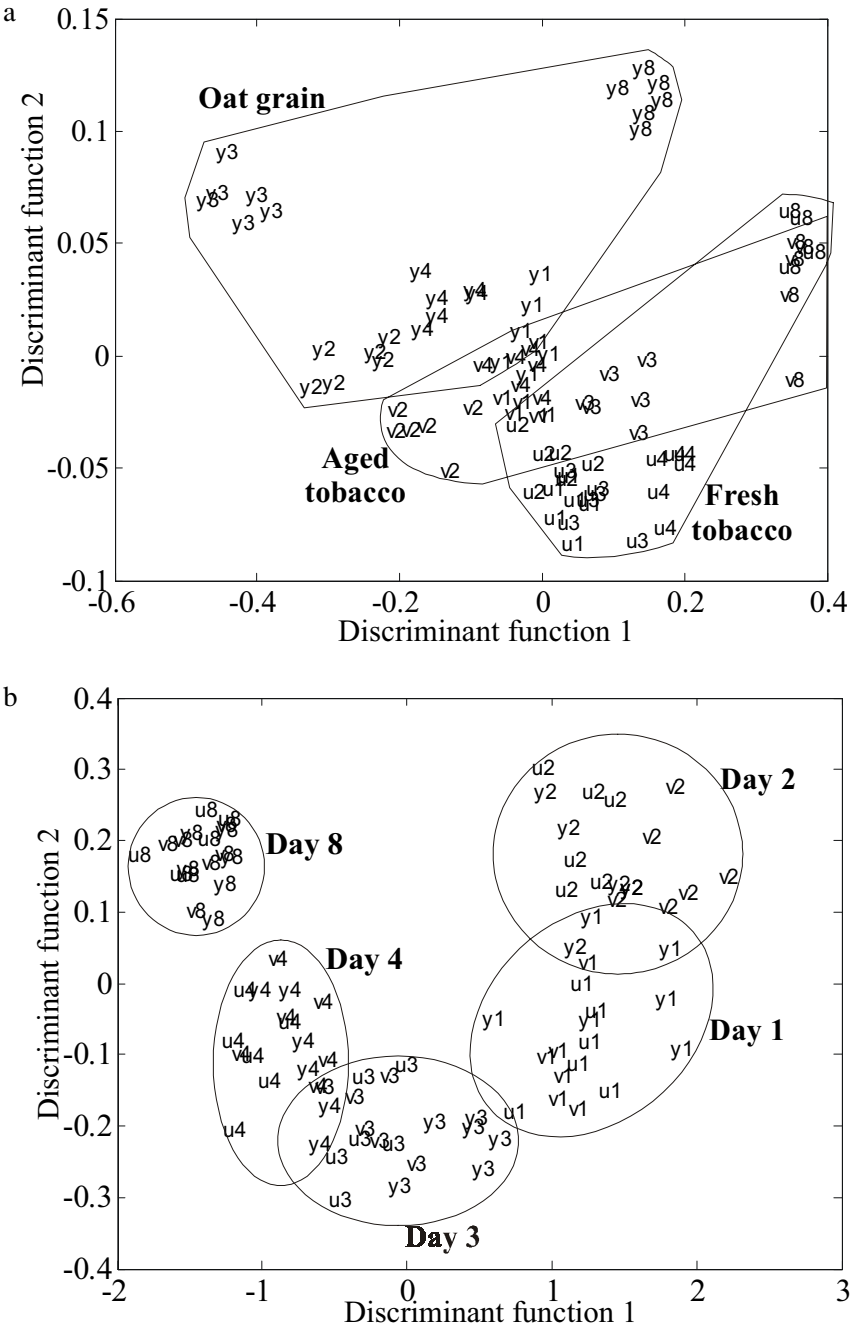

Fig. 5. Cluster analysis (DFA) of FT-IR data derived from ageing casts of *L. terrestris* fed on different food material. (a) Analysis using full FT-IR spectra data.
(b) Analysis using selected FT-IR spectra data (ranges 1500 to 1750 cm^{-1} and 2800 to 3050 cm^{-1}). (Key: u – aged tobacco leaves; v – fresh tobacco leaves; y – processed oat grain. Numbers following letters indicate number of days cast has been aged)

of microbial colonisation and decomposition. The possibility of restricting the wavelength ranges analysed to those particularly relevant to biological samples may allow different aspects of the same systems to be assessed (Fig. 5).

Comparison of FT-IR and DGGE patterns reveals some similarities and some differences. Both DGGE and full spectral analysis grouped cast samples by food type. The weaker correspondence between techniques when casts of differing ages were considered is perhaps not surprising. Oberreuter et al. (2002) concluded that diversification of 16S rDNA sequences and the micro-evolutionary change of the overall cellular characteristics measured by FT-IR were not necessarily coupled.

Investigations into relationships between FT-IR and DGGE profiling are ongoing. Initial findings indicate the potential of FT-IR spectroscopy, coupled with cluster analyses, for monitoring responses in biological systems to perturbations. One important application of

these techniques may be to detect impacts of potential gene transfers. The possibility of horizontal gene transfer (HGT) is a concern relating to GM organisms. Soil heterogeneity, low densities of recipient cells and lack of microbial substrates mitigate against efficient plasmid transfer (Derore et al. 1994). Without high throughput tools to quantify the low risk of horizontal gene flow events, we are unable to assess the ecological impacts associated with the release of GM crops or recombinant micro-organisms.

The above approach to monitoring microbial communities may facilitate other investigations into soil responses to perturbations, such as differences in litter quality resulting from variable exposure to UV-B radiation. It might also be used to study relationships between vegetation and soil microbial diversity. In all such studies, earthworm casts provide an appropriate sample for analysis since they provide one of the main initial sites for transfer of plant-derived materials into the soil carbon cycle.

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