Functional Diversity of Soil Decomposer Organisms in Grasslands Subject to Different Long-Term Management Regimes

A thesis submitted for the degree of

Doctor of Philosophy

at the University of Wales Cardiff

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2004

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Table of Contents

Summary i

Abbreviations ii

Species Names and Authorities iii

Publications, Posters and Presentations v

Acknowledgements vi

Chapter 1. Introduction 1

1.1 Grassland Management and Restoration 1

1.2 Effect of Plant Diversity on Function 4

1.2.1 Effect of Diversity on Ecosystem Stability 7

1.3 Management Effects 10

1.3.1 Structure, Biomass, Respiration - Influence of Management Regime .10

1.3.2 Soil Microbial Community Structure, Biomass, Respiration - Influence of Season/Sampling Time 13

1.3.3 Implication of Qualitative Change in Litter, Roots and Rhizodeposition, on the Soil Microbial Community 14

1.3.4 The Direct Effect of Fertiliser 16

1.4 Effects of Grazing on the Soil Microbial Community 18

1.5 Mutualistic Interactions 21

1.6 Spatial Scales in Soil Ecology: Physical Properties, Heterogeneity and Sample Size *26*

1.7 Techniques for the Investigation of Microbial Community Structure and Function *28*

1.7.1 Community Structure **29**

1.7.2 Community Function **32**

1.8 Aims *34*

Chapter 2. The Field Site **37**

2.1 Situation and Management History *37*

2.2 Plant Diversity *40*

2.3 Fungal Fruit Body Diversity *42*

2.3.1 Introduction **42**

2.3.2 Method **44**

2.3.3 Results **46**

2.3.4 Discussion **47**

2.4 Soil Chemistry *51*

2.4.1 Soil Moisture **51**

2.4.2 Soil pH **52**

2.4.3 Soil Nutrients **54**

2.5 Microbial Activity, Measured as Dehydrogenase Activity *56*

2.5.1 Introduction **56**

2.5.2 Method..56

2.5.3 Results and Discussion 58

2.6 Meteorological Data *60*

Chapter 3. Community Structure: Phospholipid Fatty Acid Analysis.62

3.1 Introduction *62*

3.1.1 Viable Microbial Biomass...62

3.1.2 Indicators of Physiological Status 65

3.1.3 Community Structure 65

3.2 Methods.. *68*

3.2.1 Soil Samples 68

3.2.2 PLFA Procedure..70

3.2.3 Fatty Acid Nomenclature...72

3.2.4 Statistical Analysis..72

3.3 Results.. *74*

3.4 Discussion *77*

3.4.1 Sample Recovery 77

3.4.2 Seasonal Variation...78

3.4.3 Microbial Community Structure 80

3.5 Conclusions... *81*

Chapter 4. Litterbag Decomposition Experiments 82

4.1 Introduction *82*

4.2 Methods *87*

4.2.1 Substrate Preparation **87**

4.2.2 Experimental Design: Seasonal Experiments **88**

4.2.3 Analyses of Substrates **90**

4.2.4 Experimental Design: Extended Decomposition Experiment **91**

4.2.5 Microclimate and Soil Chemistry **93**

4.2.6 Statistical Analysis **93**

4.3 Results *94*

4.3.1 C:N Ratio of Field Leaf Litters: Seasonal Experiment **94**

4.3.2 Field Litter Decomposition: Seasonal Experiment **95**

4.3.3 Cellulose Decomposition: Seasonal Experiments **97**

4.3.4 Field Litter Decomposition: Extended Decomposition Experiment **99**

4.3.5 Cellulose Decomposition: Extended Decomposition Experiment **100**

4.3.6 Oak Leaf Decomposition: Extended Decomposition Experiment **101**

4.3.7 Wood Veneer Decomposition: Extended Decomposition Experiment **103**

4.3.8 Nutrient Status of Field Litters and Oak Leaf Litterbags During the Decomposition Process **103**

4.4 Discussion *109*

4.4.1 C:N Ratios of Field Litters Prior to Decomposition **109**

4.4.2 Seasonal Decomposition **111**

4.4.3 Substrate Quality **113**

4.4.4 Litter C, N and P Content During Decomposition **116**

4.5 Conclusions *118*

Chapter 5. Assessment of Community Functional Diversity through Sole-Carbon-Source-Utilisation Profiling **120**

5.1 General Introduction *120*

5.2 Determination of Appropriate Inoculum Dilution *122*

5.2.1 Introduction **122**

5.2.2 Methods **123**

5.2.3 Results and Discussion **124**

5.3 Selection of Antibiotics for Fungal Plates *127*

5.3.1 Introduction **127**

5.3.2 Methods **127**

5.3.3 Results and Discussion **131**

5.4 Functional Diversity of the Traditional and Improved Field Soil Microbial Communities *132*

5.4.1 Introduction **132**

5.4.2 Methods **132**

5.4.3 Results **139**

5.4.4 Discussion **161**

Chapter 6. Synthesis....175

6.1 Site Conditions....175

6.2 Community Structure....178

6.3 Community Function....181

6.4 Structure and Functional Status of the Microbial Communities in Relation to Plant Diversity and Management History....185

6.5 Future Work....188

6.6 Conclusions....189

6.6.1 Techniques for the Investigation of Soil Microbial Community Function....189

6.6.2 Conclusions for the Management of Plant and Microbial Diversity in the Pentwyn and Bush Meadows....190

Appendix I. Field Site Data Corresponding to Chapter 2. .... **215**

I.I Phosphorus Concentration of Soil or Plant Litter Samples *215*

I.II Dehydrogenase Activity *216*

I.III Meteorological Data *216*

Appendix II. Phospholipid Fatty Acid Analysis **219**

II.I Extraction and Analysis *219*

II.I.I Soil Samples **219**

II.I.II First-Phase Extraction **219**

II.I.III Modified Bligh/Dyer **220**

II.I.IV Silicic Acid Column Chromatography **221**

II.I.V Preparation of Fatty Methyl Esters from Esterified Lipids **222**

II.I.VI Separation, Quantification and Identification of Organic Compounds **222**

Appendix III. Litterbags **227**

III.I Litterbag Decomposition Statistical Results Tables *227*

Appendix IV. Potential Function: Biolog Analysis. **231**

IV.I Biolog Soil Moisture Compensation Calculation *231*

IV.II Soil Extract Agar Medium *231*

IV.III Determination of Soil Background OD *232*

IV.IV Biolog Data Processing *233*

IV.V *Soil Microclimate*..234

IV.VI *Bacterial Biolog Plate Colour development*..................................235

IV.VI.I **Endpoint Well Colour of Bacterial and Fungal Biolog Plates.....236**

IV.VI.II **Categorised r-Parameter Data**..**244**

IV.VII *Principal Component Analysis (PCA) of Biolog r-Parameter Data Sets*..252

Appendix V. **Publication**..**256**

Table of Figures (in Chapters)

Figure 2.1 Pentwyn farm site map,....................39

Figure 2.2 Examples of the field site vegetation,. 42

Figure 2.3 Fruit body positions in Traditional Field 48

Figure 2.4 Fruit body positions in the Improved Field....................49

Figure 2.5 Soil moisture 53

Figure 2.6 Soil pH 53

Figure 2.7 Soil nutrients. 55

Figure 2.8 Dehydrogenase activity with soil depth....................59

Figure 2.9 Local temperature and rainfall data 61

Figure 3.1 Layout of fixed sampling areas (A-D) for soil cores. 69

Figure 3.2 Microbial group PLFA concentrations. 75

Figure 3.3 Concentration of individual PLFAs....................76

Figure 4.1 Layout of litterbags within 1 m^2 quadrats....................89

Figure 4.2 C:N ratios of field litter (prior to decomposition)....................94

Figure 4.3 Decomposition of field litters in TF and IF in four seasonal experiments. ...96

Figure 4.4 Cellulose decomposition in TF and IF over four seasonal experiments........96

Figure 4.5 Extended decomposition of all substrates....................102

Figure 4.6 Nutrient concentrations in TF and IF litters during decomposition............104

Figure 4.7 C:N ratios, prior to decomposition, extended decomposition experiment...107

Figure 4.8 Change to C:N ratio and total organic and ammonium N of oak leaves. 108

Figure 5.1 Colour development, Biolog inoculum dilution tests 126

Figure 5.2 Fungal growth following addition of antibiotics combinations to control bacterial levels in the soil inoculum for SF-N2 plates. 130

Figure 5.3 Chemistry of a Biolog plate well containing a tetrazolium dye..................144

Figure 5.4 Biolog GN2 bacterial plates, PCA (correlation matrix)..............................151

Figure 5.5 PCA (correlation matrix), bacterial plates combined seasonal data. 155

Figure 5.6 Biolog SF-N2 fungal plates, PCA (correlation matrix) 157

Figure 5.7 Comparison of mean plate lag period 159

Figure 5.8 PCA (correlation matrix) of the lag period in all bacterial plates 160

Figure 6.1 Summary of soil conditions 177

Figure 6.2 Microbial activity 179

Figure 6.3 Local temperature and rainfall for the period Feb 2002 to Jan 2003..........179

Table of Figures (Appendices)

Figure I.I Phosphorus calibration curve........215

Figure I.II Trimethyl formazan calibration curve, dehydrogenase assay........216

Figure II.I Chromatogram of FAMEs........226

Figure IV.I End response utilisation of Biolog GN2 plates........232

Figure IV.II Solver data entry spread sheet........233

Figure IV III Solver constraints........233

Figure IV.IV Solver constraints........234

Figure IV.V Example of total plate colour development........235

Figure IV.VI April 2002 bacterial plates Endpoint OD........236

Figure IV.VII July 2002 bacterial plates Endpoint OD)........237

Figure IV.VIII November 2002 bacterial plates Endpoint OD........238

Figure IV.IX January 2003 bacterial plates Endpoint OD........239

Figure IV.X April 2002 Fungal plates Endpoint OD........240

Figure IV.XI July 2002 Fungal plates Endpoint OD........241

Figure IV.XII November 2002 Fungal plates Endpoint OD........242

Figure IV.XIII January 2003 Fungal plates Endpoint OD........243

Figure IV.XIV April 2002 r-parameter categorised bacterial plates........244

Figure IV.XV July 2002 r-parameter categorised bacterial plates........245

Figure IV.XVI November 2002 r-parameter categorised bacterial plates........246

Figure IV.XVII January 2003 r-parameter categorised bacterial plates........247

Figure IV.XVIII April 2002 r-parameter categorised fungal plates........248

Figure IV.XIX July 2002 r-parameter categorised fungal plates........249

Figure IV.XX November 2002 r-parameter categorised fungal plates........250

Figure IV.XXI January 2003 r-parameter categorised fungal plates........251

Figure IV.XXII Biolog GN2 April 2002 bacterial plates, PCA (covariance matrix)....252

Figure IV.XXIII Biolog SF-N2 April 2002 fungal plates, PCA (covariance matrix)...253

Figure IV.XXIV Biolog GN2 Jan 2003 bacterial plates, PCA with correlation matrix. Score plots for PC1 & PC2 (a). Component loadings plots for PC1 & PC2 (b)...253

Figure IV.XXV Fungal plates PCA (correlation matrix) combined seasonal data.......254

Figure IV.XXVI PCA with correlation matrix of bacterial plate *r*-parameters, from data adjusted for both background only, and well A1 and background.......................255

Table of Tables (in Chapters)

Table 1.1 Functions of the major groups of soil organisms 5

Table 2.1 Summary of TF and IF. 38

Table 2.2 Plant species present in TF and IF. 41

Table 2.3 Fruit body records for TF and IF 45

Table 3.1 Fatty acids chosen to represent the major soil microbial groups 73

Table 4.1 Breakdown of the main macromolecular components of plant tissues 85

Table 4.2 Comparison of field litter decay curves in seasonal experiments. 97

Table 4.3 Comparisons of cellulose decomposition rates 99

Table 4.4 Average decay rates of substrates in each experiment. 114

Table 5.1 Dilutions of antibiotics tested for bacterial control in SF-N2 plates 128

Table 5.2 Antibiotic combinations tested 129

Table 5.3 Mean soil moisture content and fresh weights of soil. 134

Table 5.4 Mean soil temperatures of TF and IF 139

Table 5.5 Substrate list for Biolog GN2 and SF-N2 plates 142

Table 5.6 Biolog plate uncatabolised C-sources 145

Table 5.7 Percentage of variation in data explained by principal component analysis using both the correlation and covariance coefficients for Biolog plate data. 149

Table 5.8 Carbon source significance 164

Table of Tables (Appendices)

Table I.I Mean temperature and rainfall data for the local Monmouth area compared with 30 yr averages....217

Table I.II Monthly average temperature and rainfall data 2000-2003 for the England & Wales region, compared with 30 yr averages....218

Table II.I Preparation of buffers and solvents required for PLFA analysis. 224

Table II.II Literature and corresponding fatty acids used to assess soil microbial community structure....225

Table III.I Seasonal litterbag experiments, fitted curves for field litter decomposition data. 227

Table III.II Regression models fitted to cellulose seasonal decomposition data and comparison of regressions....228

Table III.III Extended decomposition experiment results of regressions from field litter, cellulose, oak leaves and wood veneer decomposition....229

Table III.IV Regressions and comparisons of C:N and absolute total N in oak leaves decomposing in TF and IF....230

Table IV.I Soil temperature compared between TF and IF....234

Table IV.II Soil moisture compared between TF and IF 234

Summary

Agricultural intensification of hay meadows has resulted in a severe reduction in grassland plant diversity and associated wildlife. Failures in the restoration of grassland plant diversity, comparable to that of the historically traditionally managed hay meadows, has been attributed to a concomitant reduction in the diversity of the soil microbial community and thus, ecosystem function.

Two neighbouring hay meadows were studied, one of which had been traditionally managed for over 80 years, the other had previously been intensively managed and agriculturally improved, but had been returned to a traditional management system in 1996. Thus the aim was to compare the microbial community structure and functional diversity of the two meadows, which lay at opposite ends of a plant diversity gradient, and determine whether the agricultural improvement and reduced plant diversity had resulted in a microbial community that was less able to metabolise a range of recalcitrant substrates and sole-carbon-sources, producing a lower rate of turnover and mineralisation than the community under traditional management.

Abiotic soil conditions were compared and related to community structure and function. Intensive management had reduced soil organic matter and water holding capacity, resulting in significant differences in microbial activity between meadows. Both structure and function were affected by season. In general, the traditional meadow soil contained a higher proportion of fungal fatty acids and differences in the species of fruiting fungi were recorded (although fruiting species richness was similar). However, rates of decomposition of recalcitrant substrates were equivalent between fields. The same sets of sole-carbon-sources were utilised by both communities, however, the traditional field bacterial community catabolised carbohydrates more rapidly than the improved field community, on some sample dates, indicating either a larger size or greater activity of the traditional meadow's r-selected bacterial community. Overall, however the two microbial communities appeared to be functionally equivalent.

Abbreviations

AM	Arbuscular mycorrhiza
Amp	Ampicillin
ATP	Adenosine 5′-triphosphate
AWCD	Average well colour development
BAP	Biodiversity action plan
C	Carbon
CCW	Countryside Council for Wales
cfu	Colony forming unit
DF	Degrees of freedom
DGGE	Denaturing gradient gel electrophoresis
FA	Fatty acid (phospholipid fatty acid)
FAME	Fatty acid methyl ester
Gent	Gentamycin
GN2	Bacterial Biolog Microplate™
GWT	Gwent Wildlife Trust
IF	Improved field
IGER	Institute of Grassland Environmental Research
Kan	Kanamycin
MTT	Dimethylthiazolyl-diphenyl-tetrazolium bromide
N	Nitrogen
NADH	Nicotinic adenine dinucleotide + H
NVC	National vegetation classification
OD	Optical density
P	Phosphorus
PCA	Principal component analysis
PCR	Polymerase chain reaction
PLFA	Phospholipid fatty acid
RDL	Red data list
Rif	Rifampicin
SCSU	Sole-carbon-source-utilisation
SF-N2	Fungal Biolog Microplate™
SIR	Substrate induced respiration
SSSI	Site of special scientific interest
Strep sulph	Streptomycin sulphate
TCC	2,3,5-triphenyltetrazolium formazan
TF	Traditional field
TPF	Triphenyl formazan
T-RFLP	Terminal restriction fragment length polymorphism

Species Names and Authorities

Plants	
Achillea millefolium	L.
Agrostis capillaris	L.
Ajuga reptans	L.
Anthoxanthum odoratum	L.
Bellis perennis	L.
Briza media	L.
Bromus hordeaceus	L.
Carex flacca	Schreber
Centaurea nigra	L.
Cerastium fontanum	Baumg.
Chrysanthemum leucanthemum	L.
Cirsium arvense	(L.) Scop.
Conopodium majus	(Gouan) Loret
Crepis capillaris	(L.) Wallr.
Crepis vesicaria	L.
Cynosorus cristatus	L.
Dactylis glomerata	L.
Dactylorhiza fuchsii	(Druce) Soo
Dactylorhiza maculata	(L.) Soo
Festuca ovina	L.
Festuca rubra	L.
Helianthemum nummularium	(L.) Miller
Heracleum sphondylium	L.
Holcus lanatus	L.
Holcus mollis	L.
Hyacinthoides nonscripta	(L.) Chouard ex Rothm.
Hypericum tetrapterum	Fries
Hypochoeris radicata	L.
Lathyrus pratensis	L.
Leontodon autumnalis	L.
Leontodon hispidus	L.
Lolium perenne	L.
Lotus corniculatus	L.
Luzula campestris	(L.) DC.
Orchis mascula	(L.) L.
Orchis morio	L.
Pimpinella saxifraga	L.
Plantago lanceolata	L.
Poa annua	L.
Poa pratensis	L.
Polygala vulgaris	L.
Potentilla sterilis	(L.) Garcke
Primula veris[1]	L.
Prunella vulgaris	L.
Pteridium aquilinum	(L.) Kuhn
Ranunculus acris	L.
Ranunculus bulbosus	L.
Ranunculus repens	L.
Rhinanthus minor	L.
Rumex acetosa	L.
Senecio jacobaea	L.
Taraxacum officinale	Wigg.
Tragopogon pratensis	L.
Trifolium pratense	L.

Trifolium repens	L.
Veronica chamaedrys	L.
Vicia cracca	L.
Vicia sativa	L.
Fungi	
Armillaria mellea (chunky form)	Vahl. Ex Fr.
Boletus porosporus	Imler
Clavaria argillacea	Fr.
Clavaria fumosa	Fr.
Clavulinopsis cinerioides	Atk.
Clavulinopsis leuto-alba	Rea
Clitocybe rivulosa	(Pers.: Fr.) Kummer
Conocybe pseudopilosella	(Kühn.) Kühn & Romagn
Coprinus lagopus	(Fr.) Fr.
Entoloma porphyrophaeum	(Fr.) Karst.
Galerina mycenopsis	(Fr.: ex Fr.) Kühn
Hygrocybe calyptriformis	(Berk. & Broome) Fayod
Hygrocybe ceracea	(Fr.: Fr.) P.Kumm.
Hygrocybe citrinovirens	(J.E. Lange) Jul. Schäff.
Hygrocybe coccinea	(Schaeff.: Fr.) P.Kumm.
Hygrocybe conica var. chloroides (yellow capped form)	(Schaeff.: Fr.) P. Kumm.
Hygrocybe fornicata (pale form)	(Fr.) Singer
Hygrocybe glutinipes var.glutinipes	(J.E. Lange) R. Haller Aar.
Hygrocybe insipida	(J.E. Lange) M.M. Moser
Hygrocybe pratensis	(Pers.: Fr.) Murril
Hygrocybe psittacina	(Schaeff.: Fr.) P.Kumm.
Hygrocybe unguinosa	(Fr.: Fr.) P. Karst
Hygrocybe virginea	(Wulfen: Fr.) P.D.Orton & Watling
Hygrocybe vitellina	(Fr.) P.Karst.
Laccaria laccata	(Scop. ex Fr.) Cke.
Lepiota konradii	Huijsman ex. Orton
Lepiota procera	(Scop. ex Fr.) S.F. Gray
Lepista saeva	(Fr.) Orton
Leptonia lazulina	(Fr.) Quél.
Mycena leptocephala	(Pers. ex Fr.) Gillet
Mycena fibula	(Bull. ex Fr.) Kühner
Mycena flavo-alba	(Fr.) Quél.
Nolanea hirtipes	(Schum.: ex Fr.)
Nolanea staurospora	Bres.
Omphalina postii	(Fr.) Sing.
Panaeolus rickenii	Hora
Pleurotus acerosus	(Fr.) Konrad & Maubl.
Psilocybe semilanceata	(Fr. ex. Secr.)
Scleroderma areolatum	Her.
Stropharia pseudocyanea	(Desm. Fr.) Morgan
Trichoglossum hirsutum	(Pers. ex Fr.) Boud.

Publications, Posters and Presentations

Publications	**Preston-Mafham J**, Boddy L, Randerson PF, Analysis of microbial community functional diversity using sole-carbon-source utilisation profiles - a critique. FEMS MICROBIOL ECOL 42 (1): 1-14 OCT 2002
Posters	Silwood ecology forum 2001: *Functional Diversity of Soil Decomposer Organisms in Grasslands Subject to Different Long-Term Management Regimes.* BES spring meeting 2003: *A Comparison of Fungal and Bacterial Functional Diversity in a Traditional and Improved Hay Meadow.*
Presentations:	BEPG mini symposium, Cardiff 2002: *A Comparison of Fungal and Bacterial Functional Diversity in a Traditional and Improved Hay Meadow.* All Wales microbiology meeting, Gregynog 2004: *Functional Diversity of Soil Decomposer Organisms in Grasslands Under Different Management Regimes.*

Acknowledgements

Firstly I would like to thank Gwent Wildlife Trust and Cardiff University, for providing the funding for this project, and my supervisors, Lynne Boddy, Hilary Rogers and Peter Randerson for their encouragement and assistance over the past few years.

A special thanks must go to Laurienne Tibbles, who survived being soaked to the skin and nearly being trapped in the car in a quite substantial puddle, after putting out litterbags into the fields. Also I am very grateful to the others who had to withstand various trips to the field to gather litterbags and spot mushrooms whatever the weather: Paul Wald, Julie Hunt, George Tordoff, Lynne (and, Hannah......a caterpillar counts for two you know), and the rest of the lab, Melanie Harris, Catherine Eyre, Deborah Johnston, Julie Harris and Damian Donnelly who have all put up with my constant moaning about it being too hot or too cold (mostly too cold).

Ian Vaughan and Jes Hynes have been of particular help with computer programming, to make the data analysis possible, particularly Jes who was always there to write a macro when my hand couldn't take any more mouse clicks. Finally thank you to everyone else who has helped along the way.

Chapter 1. Introduction

1.1 Grassland Management and Restoration

Over the last 50 years much of the diversity of British grasslands has been lost (Porter, 1994) due to modern intensive agricultural methods, such as: replacement of grasslands with agricultural crops or fodder grasses (e.g. *Lolium perenne*), the intensification of management to increase the output of existing hay meadows or grasslands by the use of fertilisers, herbicides, increased drainage and grazing intensification, and the implementation of early season hay cuts or multiple silage cuts (Hopkins *et al.*, 1999). However, cessation of management or abandonment of hay meadows has also resulted in significantly reduced diversity (Hellstrom *et al.*, 2003). This severe reduction in plant diversity is also reflected in a reduction of habitat for associated populations of interacting species that are dependent on the grassland ecosystem, from microbes and invertebrates to birds and mammals.

The timing of grazing and hay cuts are critical to the maintenance of plant diversity. In terms of seed production, the traditional 21st July hay cut prevents many of the late flowering perennial grasses from setting seed, yet is late enough to have allowed many of the forb species to set seed (Smith *et al.*, 2000). Many grassland plants regenerate from a short-lived seed bank, consequently the timing of gap creation in the sward is also critical (Smith *et al.*, 2000). Aftermath cattle grazing in the autumn thins the top growth and creates patches of bare ground through trampling, which enables the transient seed bank to germinate the following spring (Smith *et al.*, 2000). Spring sheep grazing then maintains the reduced density of the sward allowing the establishment of seedlings by keeping vigorous spring growth in check.

Attempts to restore diverse (i.e. plant species rich) grasslands have not been completely successful. Natural diversity only increases slowly after ceasing intensive agricultural treatments, and has been predicted to take up to 100 years (Smith *et al.*, 2000). Even then the original diversity may never be recovered due to constraints on restoration, which include a reduced seed bank and propagule dispersal (Bakker & Berendes, 1999; Smith *et al.*, 2000; van der Putten *et al.*, 2000), above- and below-ground herbivore activity (van der Putten *et al.*, 2000), together with abiotic conditions, e.g. changes in soil fertility and particularly pH (acidification) and hydrology (Bakker & Berendes, 1999). Following the abandonment of sown fodder grasslands, the original swards (typically *Lolium perenne* and *Trifolium repens*) may be replaced by vigorous self sown grasses such as *Agrostis capillaris, Dactylis glomerata, Festuca rubra, Holcus mollis* and *Poa pratensis* (Marriott *et al.*, 2003) which may inhibit the regeneration of further plant species. The reinstatement of annual cutting and well timed grazing (Hellstrom *et al.*, 2003) or severe annual cutting (Marriott *et al.*, 2003), can increase the diversity but not necessarily to levels equivalent to adjacent traditionally managed diverse grasslands.

The optimal regime for producing and maintaining plant diversity appears to be the traditional approach of: no fertilisation, autumn and spring grazing with a late July hay cut. This regime significantly increased plant diversity over a period of 8 years on an upland meadow field trial, although, diversity remained lower than that found on original traditionally managed meadows (Smith *et al.*, 2000). In fact Smith *et al.* (2000) estimated that it would take 20 years for reinstatement of traditional management to generate a sward consisting of a reasonable number of long term traditionally managed

meadow species if the rate of re-establishment were linear. However, re-establishment was not a linear process, there being an initial 4 year period during which few seeded species were integrated into the sward.

Reseeding is a commonly suggested option (Hopkins *et al.*, 1999; Marriott *et al.*, 2003; Smith *et al.*, 2003; Smith *et al.*, 2000; Walker *et al.*, 2004) and together with disturbance (see below) of the established swards works for a number of species but not all. Of 44 species seeded onto the plots by Smith *et al.* (2000) only 18 species established themselves, even though 21 of the unsuccessful species were found on traditionally managed meadows in the same region. Although residual fertility may have slightly compromised the habitat conditions for these species, the traditional management systems would have been expected to create a suitable environment for them (Smith *et al.*, 2000). Also, Hopkins *et al.* (1999) found that while some plant species can be successfully reintroduced by at least one trial method (sowing of site-specific seed mix directly into the mown undisturbed sward; strip seeding; over-sowing following disturbance by light harrowing, light rotivation or deep rotivation [50% bare ground]; turf removal to 3cm depth; transplant of plug plants into existing sward), a number of species e.g. *Helianthemum nummularium, Pimpinella saxifraga* and *Rhinanthus minor* could not be established by any of the methods used, including plug plants, onto most or all of the 6 sites across England and Wales. This was even though all sites had been managed in a low intensity manner (mown and without intensive fertilisation or grass reseeding) for the previous 10 years.

The failure of some species to re-establish on abandoned improved pasture or arable land, in regions where they otherwise exist naturally, even with the removal of

competition pressures through severe sward disturbance, suggests that some underlying mechanism is affecting the success of plant reestablishment. This may be enduring alterations to the soil chemistry (Gastine *et al.*, 2003) or associated alteration of the microbial community resulting in the persistence of unfavourable soil conditions (e.g. acidification due to oxidation of ammonia by nitrifying bacteria). Alternatively, direct interactions between reintroduced plant species and the soil microbial community (e.g. the presence of pathogens or absence of mutualistic symbionts), may be inhibiting certain plant species (van der Putten *et al.*, 2000).

1.2 Effect of Plant Diversity on Function

Concomitant changes to ecosystem functions may result from the loss of hay meadow plant diversity, through intensification of agricultural management or cessation of traditional management regimes. Since, it has been suggested that plant litter plays a critical part in determining ecosystem properties and function due to its effect on the soil community, greater plant species diversity would be expected to enhance the functioning of the ecosystem (Wardle *et al.*, 1997).

Ecosystem functions such as primary productivity, nutrient cycling (quality, ability and rate of turnover) substratum formation and structural condition (in the case of soils), and transfers between trophic positions (Chapin *et al.*, 1997; Grime, 1997; Jones & Bradford, 2001) are performed by functional groups which may consist of a variety of organisms (Table 1.1) (Brussaard *et al.*, 1997). In particular, soil microbial communities (bacteria and fungi) are responsible for maintaining mineralisation, thus, the rate at which nutrients are released back into the soil and made available for plants is dependent on a combination of which soil organisms are present and the climatic

conditions. Thus damage to the soil community may result in a shift towards a less productive system as a result of a lower rate of organic matter turnover (Dighton & Jones, 1994).

Table 1.1 Functions of the major groups of soil organisms (after Brussaard *et al.*, 1997)

Organism groupings	Related function
Free-living bacteria	Immobilisation and mineralisation of elements; breakdown of anthropogenic chemical inputs; biofilm formation; humic production; soil aggregation; food source for grazers; mutualistic intestinal associations; plant/soil-animal pathogens; plant growth promoters; assist mycorrhizal associations
Rhizobial bacteria	Additional nitrogen source to associated plants; food supply for root-feeding nematodes and other grazers
Non-mycorrhizal fungi	Immobilisation and mineralisation of essential elements; accumulation of toxic materials; breakdown of anthropogenic chemical inputs; mutualistic and commensal associations; nutrient source for grazers and some other fungi; nutrient translocation; humic production; detrital conditioning/breakdown; soil aggregation and pore formation; promotion and alteration of niche development; arthropod and nematode parasitism; production of environmental biochemicals (antibiotics, enzymes and imunosuppressants)
Mycorrhizal fungi	Competitive advantage to associated plants through sequestering and transport of nutrients and water to roots; plant to plant translocation of essential elements and carbohydrate; regulation of water and ion movement through plants and regulation of photosynthetic rate; regulation of below-ground C allocation; reduced seedling mortality; protection of roots against pathogens and herbivores; promotion and alteration of niche development; creation of root mycosphere for bacteria; high-quality resource for mesofaunal and microfaunal grazers; production of environmental biochemicals (antibiotics, enzymes and imunosuppressants)
Protozoa	Graze bacteria and fungi; enhance microbial growth and C and N availability to higher trophic levels; resource for nematodes and mesofauna; bacterial pathogen host; parasitic to higher-level organisms
Nematodes	Graze bacteria fungi and roots; plant parasites; predators/parasites of micro, meso and macrofauna; enhance microbial growth and C and N availability to higher trophic levels; facilitate dispersal of bacteria and fungi; resource for meso and macrofauna
Mites	Graze bacteria fungi and roots; consume detritus; micro ecosystem engineers; predators of nematodes and insects; facilitate dispersal of bacteria and fungi; disperse and vector helminthe parasites; host for protozoan parasites; parasitic on insects and other arthropods; resource for macrofauna
Insects – General	Grazing and dispersal of microorganisms; predators of soil fauna; consumption of plant and animal matter
Insects – Root herbivores	Modification of plant performance below and above-ground by root herbivory; impact on above-ground herbivory through plant physiological response to root herbivory
Insects – collembola	Graze micro-fauna and –flora especially in rhizosphere; consumption of detritus; micropredators of nematodes, tardigrades and rotifers; dispersal of microorganisms, helminthes and cestode parasites; parasite hosts; prey for macrofauna; microsystem engineers
Insects – ants	Bioturbators; enhancement of microbial growth; keystone species for inquilinous fauna and plants associated with anthills
Enchytraeids	Bioturbators; enhancement of microbial growth; fragmentation of plant litter; dispersal of microorganisms
Earthworms	Bioturbators; enhancement of microbial growth; dispersal of microorganisms and algae; hosts of protozoan and other parasites

However, like plant communities (Rosenfeld, 2002), a level of functional redundancy has been suggested to occur at a microbial level. For example, in a comparison of unimproved and improved grassland soil communities all fungi demonstrated protease activity, 2/3 were capable of cellulytic activity and 1/2 showed activity of lignin degrading enzymes (Donnison *et al.*, 2000b). Again changes in community structure were recorded without any significant alteration to functional diversity (enzyme activity) by Lovell *et al.* (1995). Carbon cycling is affected to a lesser extent by microbial community composition so may experience more functional redundancy than nitrogen and phosphorus cycling (Chapin *et al.*, 1997), and Griffiths *et al.* (2000) also observed that common processes such as respiration, decomposition and denitrification were less affected by depletion of a microbial community than nitrification and methane oxidation, which are undertaken by a smaller number of species.

It is not only plants, fungi and bacteria that are involved in the decomposer feedback, the grazing of microorganisms by protozoans and mesofauna such as nematodes and collembola leads to increased rates of nutrient turnover and greater nitrogen and phosphorus availability to plants (Chapin *et al.*, 1997; Spehn *et al.*, 2000) due to the death and recycling of the microbes. Hence, not only does a reduction in plant biomass have potentially direct effects on microbial losses, but also a knock on effect on mesofauna and macrofauna due to the reduction of their food supply. The potential effect of plant diversity reductions on earthworm biomass was recorded by Spehn *et al.* (2000) in which it decreased with both plant species diversity and richness of plant functional groups probably due to an above-ground reduction in plant biomass or the

relative abundance of ecological groups. Earthworm species richness did not differ between plant diversity treatments. Stephan *et al.* (2000) also observed a strong positive correlation between plant diversity and earthworm abundance.

Many experiments investigating the affect of plant diversity on the soil community are run as all else being equal, but this is unrealistic and over simplified, since an additional influence, e.g. fertiliser input, is driving plant loss and may also be directly impacting the soil community (Donnison *et al.*, 2000a).

1.2.1 Effect of Diversity on Ecosystem Stability

Ecosystem stability represents two aspects: (1) **resistance**, the ability to withstand a disturbance or stress and (2) **resilience**, the time taken to recover to pre-disturbance levels. Stresses may also be sub divided into those which are persistent, e.g. heavy metal contamination or other persistent chemical inputs, and regular repeat disturbances like agricultural tillage, and those which are transient, e.g. heat stress (from the passage of fire) soil movement (whether anthropogenic or natural) or antibiotics (from livestock) and other less persistent pesticides. A stress will always be selective for adapted species and while a transient disturbance may ultimately allow ecosystem function to return to its former state, a persistent stress results in a permanently altered state (Müller *et al.*, 2002).

The main ecosystem stability from diversity debate has classically proposed that ecosystem stability is positively correlated with diversity. In fact from the studies focussing on plant community functioning, the evidence seems to suggest that effects of higher diversity, such as increased productivity and weed suppression, are due to the

"chance effect", where the chance that more influential (more productive and disturbance resistant) species occur in the community is increased by a higher diversity (Chapin *et al.*, 1997; Lepš *et al.*, 2001). However, Chapin *et al.* (1997) also suggest that more complimentarity (interspecific differences in resource partitioning) might result from the mixing of species from a larger number of functional groups. In microbial communities, however, it has been suggested that changes to the community structure of organisms within functional groups that process the more complex carbon sources (e.g. lignin, phenolics and tannins), may damage ecosystem processes to a greater extent than changes to the composition of functional groups responsible for degrading simple carbon sources (e.g. carbohydrates, amino acids) due to there being fewer species with the capacity to degrade complex substrates (Waldrop & Firestone, 2004). Microbial functional groups that degrade labile substrates (xylose, starch and vanillin) have been shown not to differ between plant community type (oak woodland compared with grassland), whereas groups responsible for the degradation of a more complex substrate (pine litter) did (Waldrop & Firestone, 2004).

Although it appears that high plant diversity does not necessarily automatically equal stability, some level of stability with diversity does appear to occur in soil microbial communities (Griffiths *et al.*, 2000). This may be because microbial communities are large, experience functional redundancy and many are fast growing so respond rapidly to environmental disturbance expanding rapidly into empty niches (Müller *et al.*, 2002). For example, the resistance and resilience of a soil microbial community was affected by the resultant reduced diversity following chloroform fumigation (Griffiths *et al.*, 2000). The more diverse soil communities showed greater resistance (decomposition rates) to heavy metal (persistent stress) than less diverse soil communities, however,

neither showed any resilience, no recovery being observed after 2 months. While under a transient heat stress, greater resilience was clearly shown by the more diverse community demonstrating that loss of soil community diversity may impair recovery from transient stress or reduce resistance to severe stress (Griffiths *et al.*, 2000). It is possible that reduction in diversity from an initial stress can be compensated for by a level of functional redundancy, however, continued reduction in diversity results in permanent functional losses at least for microbial systems (Griffiths *et al.*, 2000). In a follow up field based study, pairs of fields chosen because of their different levels of soil biodiversity, were again exposed to persistent copper stress and transient heat stress (Griffiths *et al.*, 2001). Resistance to stress was greater in more diverse soils, however, resilience was greater from the least diverse soils (long-term monoculture experimental plot and polluted industrial soil) indicating the "versatility of populations from stressed habitats". Thus highlighting the fact that stresses select for adapted, or in this case adaptable species.

Physiological responses also occur under stress situations for example, microbial communities under environmental stress incorporate a reduced amount of carbon into their biomass and increase respiration as indicated by the increased respiration observed from 24h fumigated soil (Griffiths *et al.*, 2000). This increase, in what is referred to as the soil metabolic quotient (qCO_2), has been shown to occur in microbial communities from immature (rather than mature) systems where it is thought to indicate physiological stress (Grayston *et al.*, 2001). qCO_2 was found to be higher in improved grasslands possibly indicating physiological stress, however it is possible that it reflects the larger proportion of bacteria which have a lower biomass but higher respiration rate than that of fungi (Grayston *et al.*, 2001).

1.3 Management Effects

Up to 90-95% of the total heterotrophic metabolism of soils is undertaken by bacteria and fungi (Donnison *et al.*, 2000a). It has been suggested (Bardgett *et al.*, 1996; Bardgett & McAlister, 1999) that in low input traditionally managed grasslands decomposition is driven to a greater extent by fungi. If management intensification, primarily the addition of mineral fertiliser, or drainage leads to changes in the microbial community composition, it may impact upon the function of the system in terms of decomposition and nutrient cycling which can in turn influence the productivity of the ecosystem.

1.3.1 Structure, Biomass, Respiration - Influence of Management Regime

Grassland management intensification (regular inorganic fertiliser additions and reduced plant diversity) has commonly been found to result in the alteration of the relative proportions of the microbial community by reducing the biomass of fungi relative to bacteria in the soil community (Bardgett *et al.*, 1999a; Donnison *et al.*, 2000b; Grayston *et al.*, 2004; Grayston *et al.*, 2001). These studies have all been made on well established grasslands or long term experimental field trials comparing a gradient of improved, semi-improved and unimproved sites.

While field studies agree that management intensification results in a shift from fungal to bacterial dominated soil microbial communities, management type seems to exert little influence over microbial biomass (total phospholipid fatty acid [PLFA]) and activity (basal respiration of CO_2) which tend to correlate more closely with soil moisture than field management type (Donnison *et al.*, 2000b; Grayston *et al.*, 2001).

Bardgett *et al.* (1999a) did find that microbial activity was significantly affected by management type, being lowest in the improved grassland although equivalent across the range of the three less intensively managed fields. It was also, however, significantly affected by sample date suggesting a seasonal influence of moisture and temperature. The moisture content of the soil also varied significantly with management intensity, being highest in the unimproved grassland and lowest in the most intensive grassland. Hence the correlation of microbial activity with management intensity may be an artefact of soil moisture since their statistical analysis only tested for differences between the main experimental factors, management and sampling date. An effect of management intensity on microbial biomass was detected by Lovell *et al.* (1995), where grasslands with no past history of mineral fertilisation had consistently higher microbial biomass than fertilised swards. In general, microbial biomass is reported to be greater in unimproved fields (e.g. Bardgett *et al.*, 1999a; Grayston *et al.*, 2004; Grayston *et al.*, 2001) although this is likely to be because unimproved grassland soil contains more organic matter and received a greater level of shading by broad level plants than is experienced in grass dominated fields, thus providing a more moist environment. However, the cause of lower microbial biomass due to long term high fertiliser additions may be through reduced inputs of root carbon, due to reduced sward root mass which is often not matched by the increased return of above-ground material to the soil (Lovell *et al.*, 1995). No pH driven changes were observed (Donnison *et al.*, 2000b) even though pH is thought to be one of the primary limiting factors in grassland restoration from agricultural land (Bakker & Berendes, 1999).

Differences in fungal species composition, between management levels ranging from improved to semi-improved and unimproved, have been recorded together with little

difference in enzymatic activity indicating a level of functional redundancy within the culturable portion of the fungal community (Donnison *et al.*, 2000b). Similarly, within a range of intensive fertilisation treatments (mineral and organic), bacterial community structure was altered by treatment differences (but not eukaryotic community structure or fungal biomass) and again no associated changes in enzyme activity occurred with the change in community structure (Donnison *et al.*, 2000b; Marschner *et al.*, 2003). Donnison *et al.* (2000b) also found no difference in the diversity of culturable fungi, however, Grayston *et al.* (2001) recorded that the diversity of culturable fungi was higher in unimproved grasslands, while culturable bacteria diversity was higher in improved grasslands. These bacterial data are supported by Lovell *et al.* (1995) who were only able to culture 4 times fewer bacterial strains from unfertilised soils compared with fertilised soils. Culturable bacteria tend to favour high nutrient conditions therefore this may indicate a shift towards more r-selected community. In particular, there were greater numbers of *Pseudomonas* spp. in the improved grasslands; this group responds quickly to any increase in nutrient availability (Grayston *et al.*, 2001). Lovell *et al.* (1995) found that fungal cfu (colony forming units) were not affected by the fertilisation level however, this may purely indicate the limits of culturing fungi.

Alteration of fertiliser applications can produce rapid effects on the soil microbial community. Over the short term (1 year), withholding fertiliser from previously fertilised pasture or the addition of mineral fertiliser to a previously unfertilised field had no significant effect on microbial biomass C and N, however, measurements of ATP (activity proportional to microbial biomass) and respiration were significantly affected by these treatments (Lovell *et al.*, 1995). ATP increased with addition or

decreased with removal of fertilisation, whereas respiration increased with fertiliser application but did not decrease with the withdrawal of fertilisation.

1.3.2 Soil Microbial Community Structure, Biomass, Respiration - Influence of Season/Sampling Time

Sampling date has a significant impact on most soil parameters, including community structure potentially due to a combination of environmental factors such as temperature and moisture. For example, wetting and drying of leaf litter increased and decreased microbial biomass and respiration respectively (Schimel *et al.*, 1999), and although biomass and respiration were not found to be significantly correlated with sampling date, they were correlated with soil moisture which was (Grayston *et al.*, 2001). However, if temperature and moisture are experimentally controlled using constant environment growth chambers, microbial biomass can be strongly positively correlated with plant productivity (root and shoot biomass) (Bardgett *et al.*, 1999b). Peaks in the activity of the microbial community in spring correspond with increasing soil temperature and mobilisation of winter accumulated organic matter, correspondingly, root growth and turnover also increase in spring and so may increase the availability of root exudate carbon sources (Grayston *et al.*, 2001; Lovell *et al.*, 1995). On the other hand, grazing organisms such as protozoa and nematodes peak in autumn, possibly accelerating the reduction of microbial activity together with decreasing temperature (Grayston *et al.*, 2001). Thus investigations of microbial community structure, function, biomass and activity need to take into account temporal variations such as those described above. The experimental control of environmental variables, e.g. temperature and moisture, in microcosm systems, may allow other influences that were previously masked, e.g. plant productivity, to be revealed.

1.3.3 Implication of Qualitative Change in Litter, Roots and Rhizodeposition, on the Soil Microbial Community

Observed changes to the relative proportion of fungal:bacterial biomass, community structure and activity may be related indirectly to fertiliser additions through qualitative change in litter quality resulting from altered plant community composition and plant productivity (Donnison *et al.*, 2000a; Grayston *et al.*, 2001).

Soil nutrient status can affect the litter quality of different plant species in different ways. Concentrations of lignin and %N in beech leaf litter from trees growing on soils with a high nutrient status were greater than those growing on low nutrient soils, however the opposite was found to be true for oak litter (Sariyildiz & Anderson, 2003). Differences in decomposition rates of oak and beech litters from the different soil types were explained for the most part by initial lignin concentrations. In grassland systems, high concentrations of N fertiliser can lead to a reduction of root mass (Bardgett *et al.*, 1999b) and consequently rhizodeposition and root turnover (both aspects of root associated carbon supply) (Lovell *et al.*, 1995), the rate of root exudation being higher in smaller barley roots associated with N fertilisation (Bardgett *et al.*, 1999b). Changes to rhizodeposition may therefore alter competition between, and relative abundance of, microbial groups (Bardgett *et al.*, 1999b).

Litter quality (chemical substrates provided through plant litter) can determine the structure of the microbial community, grassland microbes being suited to the degradation of a range of labile substrates, while the microbial community of an oak woodland primarily degraded a more recalcitrant pine litter substrate (Waldrop &

Firestone, 2004). This indicates the importance of litter quality in determining microbial community structure. More labile substrates tend to have a lower C:N ratio (i.e. more available nitrogen). Meadow plants for example have a higher concentration of nitrogen than conifers and this is reflected in the activity of the denitrifying portion of the microbial community, which can be an order of magnitude greater in meadow compared with coniferous forest soils (Rich *et al.*, 2003)). Shifts in the relative abundances of denitrifying genotypes, determined by terminal restriction fragment polymorphism profiles (T-RFLP), were also recorded. Thus changes to the C:N ratio of plant material, through the effects of management, may result in changes to the structure of different functional groups of microbes. Effects may be seen both within functional groups and in the structure of the whole microbial community within an ecosystem.

Significant changes to microbial community structure may also result from the production of breakdown products such as aromatic dissolved organic compounds, released by the microbial degradation of lignin, which are difficult to degrade and potentially toxic (Marschner *et al.*, 2003). In a study of long term soil treatment by five fertilisation methods: mineral fertiliser only, mineral fertiliser + plant residues, manure, sewage sludge or straw (with additional nitrogen), Marschner *et al.* (2003) found that bacterial community structure appeared to be affected by the higher concentration of aromatic dissolved organic compounds that occurred in the straw and manure (contains straw) treatments. These communities differed structurally from that found in the sewage sludge treatment, although, the authors also considered that bacteria, which would have been added with the sewage sludge, may also have contributed to the difference. However, if this were the case then indigenous bacteria in the manure might

also be expected to distinguish this community from the other two but no significant difference was observed between the straw and manure community structure.

A number of studies suggest that changes in litter type (plant species composition) in particular, together with changes in plant productivity and species composition influence the microbial community to a greater extent than the direct effects of fertiliser (Bardgett *et al.*, 1999b; Bardgett & Shine, 1999; Wardle *et al.*, 1997). However, the effects of litter type can be unpredictable. For example, only one species of six commonly isolated litter fungi produced a significantly altered growth rate in response to litter from an unimproved traditional grassland (Donnison *et al.*, 2000a). Thus, Donnison *et al.* (2000a) suggested that fertiliser was more responsible for changing structure of the fungal community than was litter type of improved fields but the extent of the response differs depending on the fungal species.

In general, improved grasslands seem to supply larger resources of easily accessible C-sources suited to bacterial growth, while unimproved or semi-improved grasslands produce more complex, recalcitrant humus that is predominantly decomposed by fungi (Grayston *et al.*, 2001).

1.3.4 The Direct Effect of Fertiliser

The application of inorganic fertilisers to intensively managed or improved grasslands may directly affect the microbial community. On agricultural land, mineral fertilisers (including a treatment of mineral fertiliser with farmyard manure) compared with organic (manure and slurry) treatments result in reduced: arbuscular mycorrhizal (AM) diversity, AM infection potential, microbial diversity and biomass, faunal diversity

(earthworms and carabids), floral diversity (weeds), soil respiration rates, enzyme activities, amount and turnover of microbially bound P, potential for organic P mineralisation and soil aggregate stability (Oehl *et al.*, 2004).

In grasslands inorganic fertiliser may have a significant effect on fungal activity. Culture and microcosm based studies of growth and activity of six commonly isolated fungi of hay meadows under different litter and fertiliser treatments showed a species specific response to fertiliser additions; some increasing growth rate while others were negatively affected (Donnison *et al.*, 2000a). It was suggested that these varied responses might be due to specific extracellular enzymes being promoted, e.g. cellulases, or inhibited, e.g. carboxymethylcellulases and peroxidases, by the presence of nitrate or ammonium ions from inorganic fertilisers, while they may also provide a source of limiting nutrients, e.g. P to *Mucor* spp.. Acidification may also be a factor although no significant evidence of pH reduction was found.

Carbon to nitrogen ratio is thought to affect decomposition whereby excessive levels of carbon would result in reduced microbial activity and hence slow the decomposition rate. It was therefore expected that the addition of nitrogen to high carbon low nitrogen systems would compensate and increase decomposition rate. However, it would appear that the opposite reaction is a relatively common phenomenon (Fog, 1988). The source of the nitrogen seems not to be crucial, urea, ammonium salts, and nitrate salts all having given negative results (Fog, 1988). Complex organic N sources give more positive results than inorganic, but this may be due to combined effects from vitamin or carbon sources. Positive effects tend to occur in the earlier stages of a trial, but produce an overall negative effect over the longer term. Natural substrates produce an

unpredictable effect with no apparent correlation with C:N ratios. For example, easily degradable deciduous leaves (low C:N ratio) have shown positive effects of added N, while the decomposition rate of some high C:N substrates has become even slower after addition of N. The degradation of different C substrates appears to be variable, cellulose tends to be accelerated by N additions while lignin is slowed. Fog (1988) suggests three possible explanations for this: (1) that basidiomycetes that decompose lignocellulose, and ascomycetes that decompose cellulose, at low N levels alternate in a community decomposing each substrate in turn resulting in greater overall decomposition; (2) under increased N levels, bacteria in particular have a greater growth rate leading to negative interactions with the basidiomycetes and reducing the overall decomposition of lignin, alternatively; (3) certain lignin degrading enzymes may only be produced by basidiomycetes under the exhaustion of available N (ammonia metabolite repression). Evidence also suggests that N additions cause depolymersation products to react with amino compounds forming products that are unavailable for further biological decomposition, so reducing the formation of insoluble humus leading to changes in the soil structure. Water-soluble substances are formed from the surplus of N and lignin, which are presumably leached from the soil. The negative effects of N addition can last for several years, possibly because the dissipation of the amino products and depletion of carbohydrate supplies that induce lignin decomposition is only very slow (Fog, 1988).

1.4 Effects of Grazing on the Soil Microbial Community

Herbivory by large vertebrates is considered one of the major influences on the soil microbial community of grasslands world wide. The most likely cause of this is the rapid processing of the majority of the vegetation in the animal gut, rather than slow soil

decompositional steps, after which it is returned to the system as urine and faeces providing labile substrates for rapid microbial processing (Bardgett *et al.*, 1997). Foliar herbivory may also affect litter quality and quantity (Bardgett *et al.*, 1997) which in turn affects microbial abundance and activity (Donnison *et al.*, 2000a). Significant affects of grazing intensity on soil C:N ratio are likely to be in part related to the quality of litter inputs, soil C:N being lowest in heavily grazed grasslands and highest in long term ungrazed grasslands (indicating better input quality under grazed conditions) (Bardgett *et al.*, 2001), although root exudates and nitrifiers will also contribute to the ratio.

Defoliation may also result in a reduction in soil temperature and moisture (Bardgett *et al.*, 1997), due to reduced organic matter input. Rhizodeposition rates can alter, since it is suggested that herbivory stimulates C-exudate from roots and encourages the growth or accumulation of soil fauna and microbes resulting in increased grazing on microbes by nematodes etc. (Bardgett *et al.*, 1998). Short term shifts in carbon allocation from shoot to root may also occur (Bardgett *et al.*, 1998) as a means of 'safe storage' until the threat has passed.

Grazing may produce negative or positive effects on soil organisms depending on whether herbivory results in increased root exudates, reduced litter quality or *vice versa*. Litter quality may be altered through the stimulation of plant defence compounds (secondary metabolites) (Kögel-Knabner, 2002). Also selective feeding may reduce dicotyledonous plants in grassland (dicotyledonous litter generally contains more N, so is considered better quality). However, the avoidance of certain unpalatable species, e.g. *Senecio jacobaea*, may produce localised areas of good litter quality in a sea of poorer quality grasses (Bardgett *et al.*, 1998).

Sheep grazing has been shown to significantly increase total microbial biomass compared with no grazing (Bardgett *et al.*, 2001; Bardgett *et al.*, 1997); the abundance of fungal PLFA was significantly reduced following the cessation of grazing, although this was more pronounced depending upon soil type (Bardgett *et al.*, 1997). The level of grazing intensity also determines the total microbial biomass, being highest under light grazing but reducing under moderate or heavy grazing intensity (Bardgett *et al.*, 2001). This corresponds with fungal activity, which over a range of grazing intensities was greater in the lower intensity pastures. Conversely, greater bacterial activity was associated with more highly grazed pasture, indicating that frequent defoliation results in the release of rapidly metabolised labile substrates into soil that favours bacterial growth (Bardgett *et al.*, 1998). This is also substantiated by microcosm experiments in which *Pseudomonas* spp. and other culturable bacteria increased under defoliation (Mawdsley & Bardgett, 1997) indicating that factors, other than manure inputs from grazing animals, increase the microbial levels.

Nematodes are important grazers of microorganisms that facilitate the dispersal of resources through soil and enhance microbial growth (Miller, 1995), therefore changes to the nematode community could have a knock on effect on other functional groups within the whole soil community and higher trophic levels. A reduction in nematode numbers following the removal of grazing from a heavily sheep-grazed site has been suggested to relate to a reduction of microbial biomass (Bardgett *et al.*, 1997), however, no correlation was observed between nematode abundance and microbial biomass or respiration. In a subsequent study the variation in abundance of nematodes along a gradient of grazing intensity was found to vary between sites, however, variable

responses from different trophic groups of nematodes may be responsible for the lack of consistency (Bardgett *et al.*, 2001) as is seen with different intensities of fertiliser application (Sarathchandra *et al.*, 2001). Thus Bardgett *et al.* (2001) suggest that further analysis of nematode trophic groups at different sampling times would be required to clarify the influence of grazing on nematode abundance.

Lower intensity of grazing consistently shows peak levels of microbial biomass (Bardgett *et al.*, 2001; Bardgett *et al.*, 1997; Bardgett *et al.*, 1998), which is in general agreement with plant based studies which find optimum levels of plant diversity (Smith *et al.*, 2000) and ecosystem productivity (primarily primary productivity) at moderate levels of grazing (Bardgett *et al.*, 2001). The cessation of grazing, resulting in reduced microbial activity and changes to microbial community structure, could potentially have a severe effect on decomposition and nutrient cycling within grassland systems (Bardgett *et al.*, 1997).

1.5 Mutualistic Interactions

The primary mutualistic interactions occurring in grassland ecosystems are those of the nitrogen fixing bacteria, e.g. *Rhizobium* with legumes, and the association of mycorrhizal fungi with plant roots from around 80-85% of terrestrial plant species (van der Heijden *et al.*, 1998). The growth of legumes can be inhibited by a lack of *Rhizobium* available in the soil to infect their roots. While arbuscular mycorrhizal (AM) fungi are particularly important for promoting the uptake of phosphorus (both inorganic and organic (Read & Perez-Moreno, 2003)) by plants (Klironomos *et al.*, 2000; van der Heijden *et al.*, 1998), water relations and pathogen protection (Daniell *et al.*, 1999;

Smith *et al.*, 1999) and in return are provided with carbon fixed by the plant (Smith & Read, 1997).

Mycorrhizal hyphae act as extensions to the root system (Harley, 1989; Klironomos *et al.*, 2000) and in terms of material investment require 100 times less material than plant roots to produce an equivalent surface area for absorbance (Harley, 1989). This makes them particularly useful for collecting less mobile ions such as phosphate and ammonium which, due to their slow diffusion rates, become rapidly depleted around roots and hyphae requiring continuous extension into uncolonised soil (Harley, 1989). AM fungi also increase soil aggregation and the water stability of soil aggregates through the production of the insoluble glycoprotein, glomalin (Rillig & Steinberg, 2002).

There are vastly fewer species of AM fungi than the number of plants that form associations with them. It has thus been suggested that AM fungi have low host specificity (Bever *et al.*, 1996; Harley, 1989; Johnson *et al.*, 2003). However, the extent of the benefit gained by plant and mycorrhiza may depend on the fungus-host combination (Bever *et al.*, 1996; Smith & Read, 1997). Due to the lack of host specificity, a number of AM fungi may form associations with a single plant at the same time, plants of the same and different species may also be linked by a common mycelium (Smith & Read, 1997). This may incur important consequences for intra- and inter-specific interactions, whereby one plant may contribute more carbon to the mycelium, while another plant gains more from the mineral uptake and contributes less, therefore profiting more from the association (Harley, 1989; Smith & Read, 1997). Root

competition, for water and nutrients, may also be limited to plants that do not share a common fungal association (Harley, 1989).

The community structure, abundance and diversity of AM fungi has been shown to influence plant competition and community diversity (van der Heijden *et al.*, 1998; Smith *et al.*, 1999; Johnson *et al.*, 2003). van der Heijden *et al.* (1998) proposed that "the species composition and diversity of AM fungal communities have the potential to determine plant biodiversity in natural ecosystems". For example, the suppression of mycorrhizas can result in competitive release, where a decrease in abundance of dominant plant species with high mycorrhizal associations (high percentage of root length colonised) occurs, thereby allowing the abundance and diversity of sub-dominant plant species to alter and potentially increase in productivity (Smith *et al.*, 1999). The growth of individual plant species from calcareous grassland, in microcosm experiments, have been found to be highly dependent on mycorrhizal fungi being present (van der Heijden *et al.*, 1998). However, the extent of the dependency was species specific. Again it appears that the biomass of the sub-dominant species were affected to a greater extent by the composition of the AM fungal community used, altering individual biomass more greatly than total plant community biomass. Hence AM associations can significantly influence both the plant productivity and diversity of an ecosystem. Limiting resources may be utilised more fully by plants with mycorrhizal associations because the mycorrhizas extend the foraging area available to the roots, this may enable the maximum level of plant productivity (biomass) to be reached at a lower level of plant diversity than if mycorrhizas were not present (Klironomos *et al.*, 2000). However, the level of productivity is also dependent on the amount of carbon extracted from the plant by the mycorrhiza (Klironomos *et al.*, 2000).

Mycorrhizal community structure affects the productivity of plants and, in turn, the community structure of plants in experimental microcosms has been shown to affect AM community structure. In a comparison of bare soil, *Festuca ovina* (high AM association) monoculture, *Carex flacca* (low AM association) monoculture and a 12 plant species mix (mixed range of associations), subsequent AM colonisation of biological assay plants (*Plantago lanceolata*) showed different reproducible assemblages of AM fungi when planted in each microcosm treatment (Johnson *et al.*, 2003). AM diversity was greatest in bare soil because of the availability of a wide range of spore propagules and lowest in *F. ovina* microcosms due to rapid colonisation of roots by the dominance of AM fungal species with high host specificity to *F. ovina* (Johnson *et al.*, 2003).

The application of mineral or organic fertilisers can also directly affect the AM community of agricultural fields. Particular AM species may be completely absent from fields that undergo mineral fertilisation (even in combination with organic inputs of slurry or manure) while the abundance of other species is altered depending on the type of fertilisation (Oehl *et al.*, 2004). Organic and non-fertilised treatments recorded greater species diversity and more spores per gram soil than treatments containing mineral fertiliser, and the proportion of root length colonised by AM fungi in bioassay plants was greater in organic and non-fertilised soils (Oehl *et al.*, 2004). In high nutrient soils with high concentrations of soluble very mobile nitrate, mycorrhizal extension to the absorbance area of mycorrhizal plants does not enable these plants to absorb nitrogen any faster than non-mycorrhizal plants, therefore in terms of N absorption,

there is no advantage to being mycorrhizal under highly fertilised conditions (Harley, 1989).

The interaction between AM fungi and plant hosts also affects other soil microbes. Four times as much carbon can be allocated to mycorrhizal compared with non-mycorrhizal roots (Harley, 1989). Thus mycorrhizas may, by increasing the carbon allocation to roots and ultimately the soil system upon decomposition, enhancing the activity of microbial generalists (Read & Perez-Moreno, 2003). AM fungi also alter the release of root exudates so affecting the flow of labile carbon to other microbes. They can also impact upon conidial germination and growth of bacteria through unidentified exudates, which incur pathogenic control (Hodge, 2000). In nodulated plants that additionally have AM associations the AM facilitation of phosphorus uptake by the plant may benefit N_2 fixation by the nodule bacteria, increasing N_2 fixation so promoting plant growth which in turn benefits the AM fungus (Hodge, 2000). Other microbes such as *Trichoderma* species and pseudomonads may have neutral, positive or negative affects on AM development and establishment of AM-plant associations (Hodge, 2000).

The productivity and community structure (both above- and below-ground) of grassland ecosystems may be significantly affected by AM-plant associations. In systems where AM diversity has been reduced, few plant species with high association for the remaining AM species may dominate. Enhancement of the productivity of the dominant plant species may render it almost impossible for less vigorous species to establish without intervention to disturb the dominant AM-plant association. Resultant effects on plant or AM diversity due to management regimes, such as mineral fertilisation, may impact upon ecosystem function. Since the diversity of AM fungi is reduced in

agricultural fields (Oehl *et al.*, 2004; van der Heijden *et al.*, 1998) this may contribute to the reason why restoration of diverse plant communities on reclaimed agricultural land is so difficult.

1.6 Spatial Scales in Soil Ecology: Physical Properties, Heterogeneity and Sample Size

The physical properties of a soil system dictate the rate and range of ecological processes for example, negatively charged clay particles in soils tend to adsorp positively charged nutrient cations on to their surface. Ion exchange readily releases the nutrients back into the soil solution (Killham, 1994) making them available for uptake by plants and microbes, thus, adsorption reduces nutrient leaching rates from clay soils. Clay particles also act as sites for the adsorption of soil enzymes. Enzymes bound in this way are stabilised against denaturing, however, while adsorped their activity is reduced (Killham, 1994). The cation exchange capacity of different clay minerals depends on their structurally available surface area (Courtney & Trudgill, 1984). Therefore, different types of clay mineral render different affects on the nutrient flow in soils (Killham, 1994). The structure of humus is also particularly important in soil fertility since its cation exchange capacity is about twice that of pure clay (Courtney & Trudgill, 1984). Gas permeation of the soil structure is affected by water regime, for example, in a state of water saturation CO_2 diffuses through soil pores at approximately 1cm per day. If the pores are empty CO_2 will diffuse at about 1cm in less than an hour (Young *et al.*, 2003).

Soil is a massively complicated ecosystem. 1g of soil encompasses a surface area of approximately 20m^2 covered by something in the region of 10 000 protozoa, 10^7

bacteria and 5km of fungal mycelium (Young *et al.*, 2003). However, this covers less than 10^{-4}% of the surface area, approximately equivalent to the proportion of the earth's surface populated by humans (Young *et al.*, 2003). Given that such a small quantity of soil can contain such a large microbial community operating within the many niches available within the complex micro-architecture, the concept of soil heterogeneity becomes an almost overwhelming hurdle, which the soil ecologist must consider within an experimental design.

Scale and sample-size are an important issue. In a complex heterogeneous environment, the larger the sample-size the more heterogeneity that is encompassed within the sample and therefore the poorer the resolution of the experimental results. The scale at which the environment is observed should depend upon the questions being asked (Jones & Bradford, 2001). For example, at the microbial scale the distribution of resources within the microenvironments of the soil structure affect/reflect the distribution of soil organisms (Nicol *et al.*, 2003). Very small sample sizes may find domination by a species due to a single fragment of organic matter. If the scale is expanded and a larger sample taken, there are likely to be more fragments of organic matter within it with one or more species dominating them. Between the fragments may be species processing the bi-products of these species e.g. nitrifiers (Grundmann & Debouzie, 2000). So at the smallest scale the samples might each produce totally different results, on a larger scale the perception of heterogeneity of the environment is reduced by including more niches per sample. Hence, 0.01 and 0.1g soil sample sizes showed greater variation in bacterial community between replicates than 1 and 10g samples (Ellingsøe & Johnsen, 2002). While for archaeal communities, 10g samples were similar while 0.1g and 1g samples

differed. The resolution of 10g samples however was reduced, as they could not be differentiated to grassland type (Nicol *et al.*, 2003).

Soil communities are not randomly distributed as previous studies may have assumed, rather, bacterial patchiness occurs at scales below 1mm (Nunan *et al.*, 2003). Structural variation differs between soil types and gradients of bacterial numbers are observed around organic resource patches and also soil pores (although in the latter case bacterial numbers increased and the decreased again with distance from the pore due to the location of the most favourable conditions) (Nunan *et al.*, 2003). Soil consists of a mosaic of regions of high and low cell densities, the effect of substrate supply and accumulation of metabolites or signalling molecules, upon the cells within these regions may ultimately impact community function (Nunan *et al.*, 2003).

The mobility of soil organisms should also be considered, while some bacteria may lead a generally sedentary lifestyle, experiencing apparent periods of starvation while waiting for resources to appear (Lavelle, 2002), other microorganisms such as protozoa are more mobile depending upon environmental conditions, and larger organisms such as macro-fungi actively seek out resources. Resources may be dispersed through the soil by rhizodeposition and subsequent leaching, the activities of invertebrates or root and mycelial growth.

1.7 Techniques for the Investigation of Microbial Community Structure and Function

Classical ecology has is the past concentrated on the communities of macrohabitats. However, the importance of microhabitat underlying this, such as the fungal and

microbial communities of soil which are key to nutrient cycling, are increasingly being recognised. The analyses of biological communities, by ecological studies, may include observations of community structure (e.g., species diversity, relative proportions of major groups by PLFA abundance, Genotype structure by T-RFLP etc.) and/or function (e.g., the presence or rates of specific processes) (Bossio & Scow, 1995) such as primary productivity, nitrogen mineralisation, nitrate leaching and litter decomposition (Chapin *et al.*, 1997; Grime, 1997).

Studying the function of soil communities is not simple due to the rapid growth rates of some groups within the community. Like higher organisms different species are affected by different selection pressures, and r- and K- selective theories (Grime, 1979; MacArthur & Wilson, 1967) may be applied. For example, in a stable undisturbed soil community, K-selected species may be established which are strong enough to hold their own among the competition. Once that community is disturbed however, r-selected species may be able to take advantage of the gap that is created by the disturbance and multiply rapidly until they are out competed by the slower growing K-selected species again. This kind of population dynamics creates difficulties with trying to make any type of functional assessment, because by removing a soil sample the conditions/community are disturbed/altered, therefore, only potential community function can be assessed by this type of method. Techniques to investigate the structure and function of these communities are still being developed.

1.7.1 Community Structure

Investigation of environmental microbial communities is fraught with difficulty since only around 1% of soil bacteria can be successfully cultured on agar (Lawlor *et al.*,

2000) and only around 5% of fungi have been described (Hawksworth, 1997) many also remaining unculturable, particularly non-saprotrophic species such as the arbuscular mycorrhizas. Culturing techniques and time consuming cell counts have traditionally been used, but traditional fungal plating methods (e.g. dilution plates or soil plates (Warcup, 1950) favour r-selected fungi (Frankland *et al.*, 1990). Basidiomycetes are particularly difficult to isolate from soil, even though they are commonly found fruiting in grasslands and woodland soils of temperate regions (Clark, 1980). Even when organisms are cultured, identification is difficult due to the lack of reproductive structures, since many species do not fruit, or only form abnormal fruiting structures, in culture (Frankland *et al.*, 1990; Thorn *et al.*, 1996).

Molecular techniques involving the extraction of DNA from soil have the advantage of being independent of culturing and, depending on the sensitivity of the method, may be used to detect species, genera, families or higher taxonomic levels (Nannipieri *et al.*, 2003). The extraction of DNA from soil samples and its subsequent amplification by polymerase chain reaction (PCR) may be inhibited by common soil compounds such as humic acids and polyphenols, in many cases the initial extraction and cleanup of the DNA has had to be optimised since with potentially low concentrations of sample DNA, dilution to reduce inhibitor levels may be unfeasible (Bridge & Spooner, 2001). Identification to high resolution, e.g. genus or species level requires the amplification of extracted DNA by genus or species specific primers, followed by cloning and sequencing. This is a relatively long-winded approach if looking at a diverse sample such as that from the soil community and is relatively expensive. Amplification of DNA by PCR does not necessarily reflect the relative abundance of the species of the community since the most abundant DNA tends to be amplified over less abundant

species (Bridge & Spooner, 2001). Quantitative PCR techniques are available but are time consuming and expensive. Lower resolution techniques such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) analysis are available for comparisons of community composition. In DGGE the banding pattern, following electrophoresis, of denatured DNA fragments obtained from PCR can be compared to give an idea of the number of species in a sample (Nannipieri *et al.*, 2003). T-RFLP on the other hand, uses enzyme-digested flourescently labelled PCR products that are size separated and detected by DNA sequencing (Nikolcheva *et al.*, 2003), again the fragment fingerprint can be compared to indicate differences between samples. Both techniques are relatively simple, but experience similar problems in that PCR biases are still a factor and more than one species may produce the same patterns of fragmentation (Müller *et al.*, 2002; Nikolcheva *et al.*, 2003). DNA extraction also does not produce an accurate picture of the viable community, since the DNA pool contains both viable and non-viable sources (depending on the conditions DNA can persist after death for a relatively long period of time). However, molecular biology is a rapidly evolving area of science and techniques are constantly being improved. As sequence databases improve, molecular profiling of communities potentially enables the determination of community structure to the highest resolution.

The phospholipid-fatty-acid (PLFA) profile of a sample can be used to provide a quantitative measure of viable microbial biomass since (unlike DNA) PLFA deterioration is rapid following cell death due to hydrolysis of the phosphate groups by cellular enzymes (White, 1995). Identification of community components from PLFAs depends upon the specificity of those lipids to individual species or species groups.

However, the specificity of these compounds is debatable (Zelles, 1997) and it is more reliable to use them to distinguish larger groupings, such as to determine relative proportions of fungal and bacterial biomass in a community. This is discussed in further detail in Chapter 3.

1.7.2 Community Function

Community function, i.e. the actual catabolic activity expressed, is a very difficult area to study since it would require the community to remain completely undisturbed by the researcher, any disturbance resulting in some alteration to the community structure. Therefore community functional diversity, i.e. potential function, the capacity of the community to adapt its metabolism and structure to cope with variable substrates and conditions is the area of function that most experiments assess. Various techniques are available for the assessment of potential function and in general they involve exposing the community to a range of carbon resources. One of the classic techniques is the exposure of mesh bags containing leaf litter or other complex carbon substrates to the soil community by burying them or pinning them to the surface of the soil. This has the advantage that the substrates are being exposed to a relatively undisturbed community existing in its natural conditions. Also, by using various mesh sizes different groups of decomposers can be excluded (Gray, 1990). However, moisture and temperature are strong dictators of decomposition rates, which will vary with sampling time within and between years. Different mesh sizes can affect moisture conditions within the bags (Gray, 1990). Also buried substrates are not presented to the soil community in the state in which they would normally reach it since some preliminary invertebrate and microbial action would occur prior to the incorporation of the organic matter into the

soil horizon. In a comparative sense though, decomposition rates can provide information about the relative functional abilities and activity rates of communities.

The majority of functional assessments require the removal of soil samples. This unfortunately creates a large disturbance of the community, exposing populations that might normally be isolated from each other and resulting in competitive interactions and subsequent changes to community structure. Therefore the application of these techniques is best done as rapidly after removal as possible. Various techniques can be applied. Traditional substrate induced respiration (SIR) (Anderson & Domsch, 1978) estimates microbial biomass by recording CO_2 evolution from the soil (above basal levels) following the application of glucose. Enough glucose is added to saturate the enzyme systems so that the initial rate of CO_2 production, prior to the formation of new biomass, is proportional to microbial biomass (Gray, 1990). However, biomass may be underestimated due to the different response of anaerobes and microbes that may not utilise glucose (Gray, 1990) and it may favour r-selected species able to respond rapidly to the nutrient flush. A relatively recent modification of this technique was made by Degens and Harris (1997), in order to measure microbial potential function, in which a range of soluble carbon sources were added to vials containing soil samples and the subsequent respiration recorded for each substrate.

A method for the assessment of potential function, which is similar in principle to SIR is sole-carbon-source-utilisation using Biolog Microplates^{TM}. The details of the Biolog method are discussed later in Chapters 5, however, in brief Biolog plates use redox dye technology (rather than CO_2 evolution) to measure the catabolic response of the inoculum to the 95 different individual carbon sources contained in the wells of the

microtitre plate. The pattern of resource use and degree of colouration of each well can be used to compare the range of C-sources that each community can potentially utilise. Again this technique suffers from the same problems of community disturbance and change, but easily provides an assessment of the potential for use of a wide range of carbon sources.

Microbial activity can also be assessed by measuring the activities of various extracellular enzymes that are produced by the microbial community. These can be divided into groups of enzymes, which are responsible for various metabolic processes e.g., carbon, phosphorus or nitrogen metabolism or intracellular metabolism (Schinner *et al.*, 1996). However, the soil structure, particularly the adsorption of enzymes to clay particles may render them inaccessible to the assay (Stemmer, 2004), and enzymes may remain active for extended period if bound to clay or organic matter particles so may not be directly proportional to current levels of microbial activity (Schinner *et al.*, 1996).

1.8 Aims

Between 1991 and 1996, Gwent Wildlife Trust acquired a set of hay meadows at Pentwyn Farn, Pennalt, near Monmouth, South Wales (site description, Chapter 2). The site consists of the Pentwyn meadows, a designated SSSI of high plant diversity, and the Bush meadows a range of less diverse hay meadows that in the past have been more intensively managed. The Wildlife Trust ultimately aim to bring the Bush meadows up to the same standard of conservation value as the older more diverse Pentwyn SSSI. The current method of achieving this goal is to simply manage the fields in the same traditional low intensity manner under the expectation that this will increase floral and

subsequently faunal diversity. Given the difficulties of imposing restoration upon previously intensively managed grasslands, is it possible to recover the diversity in the Bush fields without greater intervention?

The Bush meadows field (IF) used for this study is the most plant species poor of the improved fields, however, it has not been intensively managed for at least 10 years. The previous management systems have had a lasting impact on the plant community diversity. Thus, the aim of the current project is to determine whether the soil microbial community structure and function also differs, between the traditionally managed Pentwyn meadow (TF) and the agriculturally improved Bush meadow (IF) (Chapter 2, Figure 2.1). If soil function and fungal:bacterial ratios in IF are recovering towards that in TF, then the recovery of plant species diversity may be possible through natural re-seeding or interventional re-seeding and plant introductions. However, if the microbial communities are different the plant community composition and microbial function may be locked into a cycle, which prevents the successful establishment of new species, and more drastic treatments may need to be considered to enable species recovery.

This study concentrated on microbial community function since a partner project attempted to identify the structure of the fungal communities using molecular techniques (Hunt, 2003). In summary this research project compares microbial community structure and function in TF and IF through: (1) visually recording differences in fungal fruit bodies (Chapter 2), (2) assessing fungal:bacterial ratios by phospholipid fatty acid analysis (Chapter 3) and (3) determining levels of microbial activity and potential function from decomposition rates (Chapter 4) and sole-carbon-

source-utilisation (Chapter 5) and finally the information from all of the individual techniques is discussed in Chapter 6.

Chapter 2. The Field Site

2.1 Situation and Management History

Pentwyn Farm and its surrounding lands lie 4km south east of Monmouth at an altitude of 120-200m. The Pentwyn Meadows (Figure 2.1) were purchased by the Gwent Wildlife Trust in 1991 and were designated an SSSI in May 1993. The Bush meadows (Figure 2.1) were acquired by the trust in 1996 with the intent to enhance their conservation value to bring them in as an integral part of the Pentwyn farm SSSI. Details of the fields used for the study are summarised in Table 2.1.

Pentwyn meadows (Figure 2.1) are considered *Centaureo - Cynosuretum* neutral grasslands with a *Lathyrus pratensis* sub community (MG5a unimproved lowland grassland (National Vegetation Classification)). Bush Meadow Field 6 has been classified as MG6 semi-improved lowland grassland (IGER, 1999). The two fields chosen for this project lie at opposite ends of the management and plant community composition spectrum. Pentwyn Meadow Field 3 (Figure 2.1) (Grid ref. SO 523 093) has been managed using a traditional low intensity regime, and consequently displays a range of grasses and forbs including some less common species such as the green winged orchid (Table 2.2). This meadow will be referred to as the traditional field (TF). Although there are no official records of the management system, the lands were previously owned by the same family since the 1920s. Gwent Wildlife Trust records that Field 3 (TF) has remained unploughed since this time. The meadows as a whole were grazed and/or cut for hay, fertilisers were last applied in 1983 and it is thought that no pesticides have ever been used. Bush meadow field 5 (Figure 2.1) (Grid ref. SO 523 092) subsequently here referred to as (IF), has been more intensively managed and after

1985 was used for grazing sheep with no annual hay cut. Although there are no records, it is likely that prior to 1985 artificial fertilisers and pesticides were applied. As a result of the more intensive management system, the plant community diversity is much more limited, being dominated by red fescue (*Festuca rubra*) and Yorkshire fog (*Holcus lanatus*) (Table 2.2).

Table 2.1 Summarised details of the particular fields, at Pentwyn and Bush Meadows, used in this study.

	Pentwyn Meadow (TF)	**Bush Meadow (IF)**
Field No. (from Figure 2.1)	3	5
Grid ref.	SO 523 093	SO 523 092
Vegetation classification	MG5a	MG6
Pre-1996 management	Traditional low intensity regime, no fertiliser or pesticide and not ploughed since 1920s	Last fertilised before 1985, after 1985 sheep grazed so no hay cut
Current management	Traditional low intensity regime	Traditional low intensity regime
Approx. No. Plant species	53, mixture of forbs and 11 grasses	9, dominated by grasses
Area (km^2)	1.3	0.9

Following their purchase by the Trust Pentwyn and Bush Meadows have been managed in line with traditional methods (Smith *et al.*, 2003). Sheep are allowed to graze in spring through until May, the hay crop is then allowed to develop, and is cut at the beginning of July (or as soon after, weather permitting) followed by aftermath grazing by cattle. No mineral fertilisers or pesticides are used. This continued until 2001 when Foot and Mouth Disease prevented the movement of animals so that the sheep left to graze in the spring could not be removed from IF until around August. Hence IF experienced very heavy grazing and no hay crop or cattle grazing that year. TF received

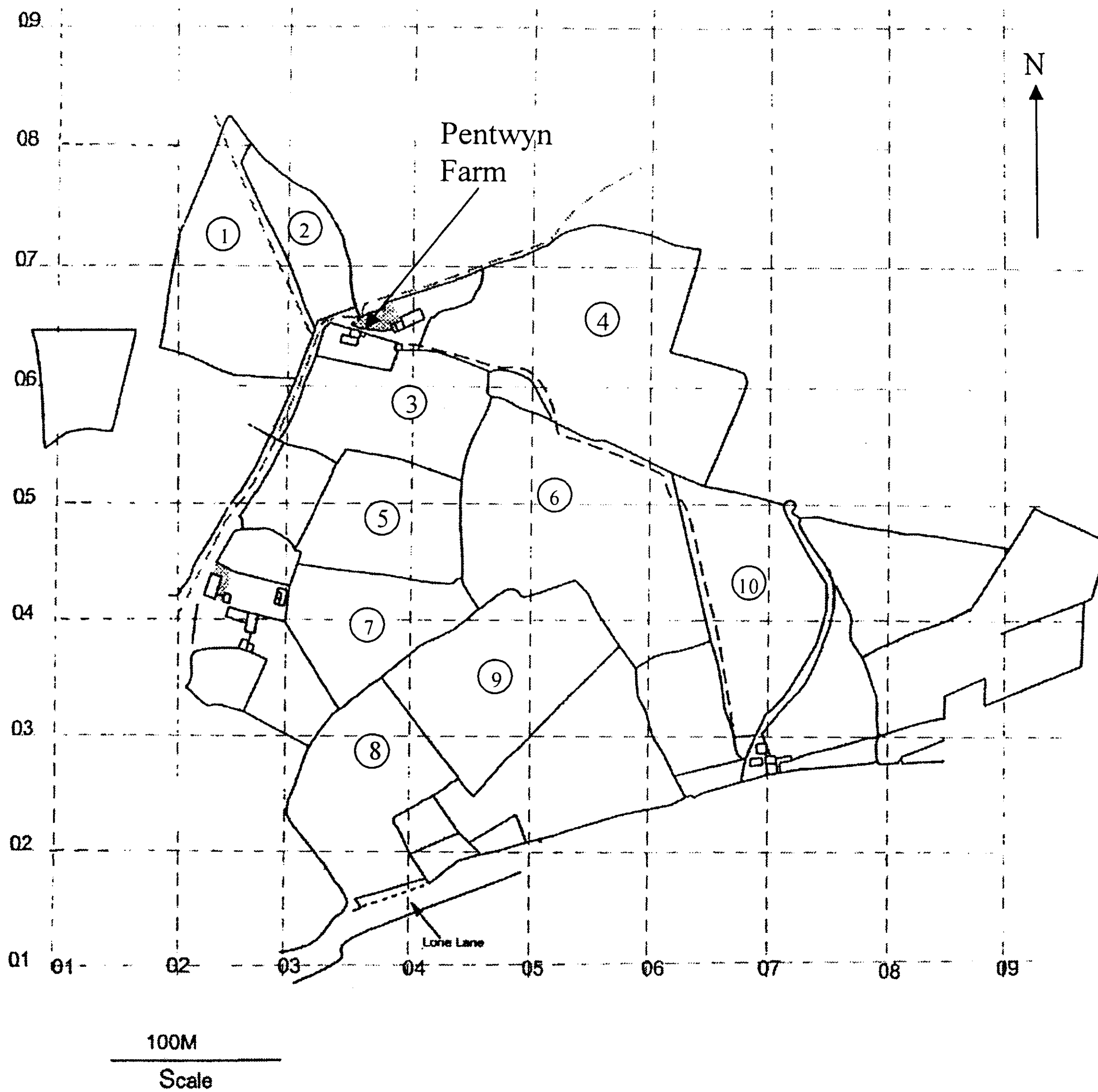

Figure 2.1 Pentwyn farm site map, fields 1-4 are Pentwyn meadow SSSI, Fields 5-10 are Bush meadows (Field 10 not owned by GWT but management agreement obtained). Field 3=TF, Field 5=IF. 4 and 6 are fields being used by IGER (Institute of Grassland and Environmental Research) for Project BD1415 (IGER, 1999).

no grazing at all during 2001. In 2002 no spring sheep grazing occurred, the hay crops in both fields were cut late in August due to bad weather, although both fields were briefly cattle grazed late in the autumn. In spring 2003 again no sheep grazing was instated. The TF was however chain harrowed to remove some of the moss layer. Hay on both fields was cut late July early August although no aftermath grazing occurred during the autumn.

2.2 Plant Diversity

Plant diversity distinctly differs between the two fields. TF contains a high plant diversity (Figure 2.2a), the 1991 surveys by the CCW and GWT recording 53 species in total, with only 11 of these being grasses. This shows that it is a mixture of many species of forbs and a variety of grasses providing a wide range of habitat and food plants for invertebrates and small mammals. Some of the species recorded in TF are typical of older grasslands, such as *Conopodium majus*. The orchid *Dactylorhiza maculata* tends not to be found in cultivated areas, while *Orchis morio* has declined due to ploughing of old grasslands and is now considered an uncommon species.

The diversity of IF is quite poor (Figure 2.2b), being dominated by about 9 species (Table 2.2) although no formal survey of the plant community has been carried out. Of the Bush meadows, IF is the most agriculturally improved and appears to have been ploughed and sown with animal fodder grasses at some point in the past. It contains a less diverse plant community than the other Bush meadows (IGER, 1999) which are considered an MG6 plant community (NVC).

Table 2.2 Plant species present in TF and IF, data combined from Countryside Council for Wales survey carried out on 5th & 26th June 1991 and GWT survey fom 18th May & 11th June 1991. Abundances within the field recorded as: R=Rare, O=Occasional, F=Frequent, A=Abundant. Since no NVC survey has been carried out on IF, the major component species of the plant community are marked with an asterisk. [1]Species not recorded in 1991 survey but recorded in following years.

Abundances TF (CCW)	TF (GWT)	IF	Species	Common name
F	F		*Achillea millefolium*	Yarrow
	O		*Ajuga reptans*	Bugle
R	O		*Bellis perennis*	Daisy
F	A		*Centaurea nigra*	Black knapweed
R	O	*	*Cerastium fontanum*	Common mouse ear
O	A		*Chrysanthemum leucanthemum*	Oxeye daisy
	R	*	*Cirsium arvense*	Creeping thistle
R			*Conopodium majus*	Pignut
O	O		*Crepis capillaris*	Smooth hawk's-beard
	O		*Crepis vesicaria*	Beaked hawk's-beard
	O		*Dactylorhiza fuchsii*	Common spotted orchid
	O		*Dactylorhiza maculata*	Heath spotted orchid
R			*Euphrasia sp.*	Eyebright
O	O		*Heracleum sphondylium*	Hogweed
R	R		*Hyacinthoides nonscripta*	Bluebell
	O		*Hypericum tetrapterum*	Square stalked St Johns-wort
F	A	*	*Hypochoeris radicata*	Common catsear
F	F		*Lathyrus pratensis*	Meadow vetchling
O	F		*Leontodon autumnalis*	Autumn hawkbit
A	A		*Leontodon hispidus*	Rough hawkbit
F.A	F		*Lotus corniculatus*	Bird's foot trefoil
F	O		*Luzula campestris*	Field wood rush
R			*Orchis mascula*	Early purple orchid
	*		*Orchis morio*[1]	Green winged orchid[1]
F	F	*	*Plantago lanceolata*	Ribwort plantane
O	O		*Polygala vulgaris*	Common milkwort
O			*Potentilla sterilis*	Barren strawberry
O	*		*Primula veris*[1]	Cowslip[1]
P	O		*Prunella vulgaris*	Selfheal
	O		*Pteridium aquilinum*	Bracken
O	F	*	*Ranunculus acris*	Meadow buttercup
F	F		*Ranunculus bulbosus*	Bulbous buttercup
	R		*Ranunculus repens*	Creeping buttercup
F	A		*Rhinanthus minor*	Yellow rattle
O	O		*Rumex acetosa*	Common sorrel
O	F		*Taraxacum officinale*	Dandelion
R			*Tragopogon pratensis*	Goat's beard
F	A	*	*Trifolium pratense*	Red clover
	O	*	*Trifolium repens*	White clover
R	O		*Veronica chamaedrys*	Germander speedwell
F	R		*Vicia cracca*	Tufted vetch
R	O		*Vicia sativa*	Common vetch
A	F		*Agrostis capillaris*	Common bent
A	A		*Anthoxanthum odoratum*	Sweet vernal grass
R			*Briza media*	Quaking grass
R			*Bromus hordeaceus*	Soft brome
F	F		*Cynosorus cristatus*	Crested dog's tail
F	F		*Dactylis glomerata*	Cock's foot
A	A	*	*Festuca rubra*	Red fescue
R	F	*	*Holcus lanatus*	Yorkshire fog
R			*Lolium perenne*	Rye grass
	O		*Poa annua*	Annual meadow grass
R			*Poa pratensis*	Meadow grass

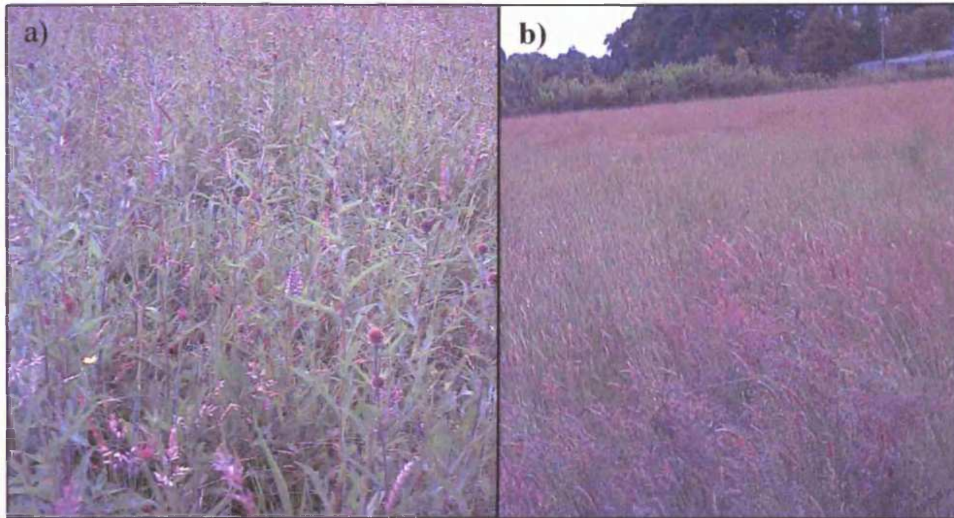

Figure 2.2 Examples of the field site vegetation in spring, a) diverse traditionally managed meadow TF and b) grass dominated improved grassland IF.

2.3 Fungal Fruit Body Diversity

2.3.1 Introduction

The plant communities in the TF and IF meadows are visually very different and this can also be seen in the range of fungal fruit bodies that occur. In general, TF sports a range of larger fruit bodies from a number of what are considered to be rare species indicative of undisturbed older grasslands such as *Hygrocybe* spp., Entolomaceae, members of the Clavariaceae and Geoglossaceae (Rotheroe, 1999). IF also sports a variety of these species, however they tend to be those with smaller fruit bodies that are recorded more frequently in Britain and are mixed in with a range of common species such as *Mycena* spp. and *Psilocybe* spp..

The classification of a site's conservation value has traditionally focussed on the plant community. However, increasingly the importance of the fungal fruiting diversity is being recognised and, in 1996, the British Mycological Society (BMS) initiated surveys of fungi that characterise unimproved meadows and grasslands (Rotheroe, 1999). This work has produced a classification (also called the CHEG profile) based upon the numbers of the species of:

Clavaroid fungi (fairy clubs)	**C**
Hygrocybe (waxcaps)	**H**
Entolomataceae grassland spp. (pink-gills)	**E**
Geoglossaceae (earth-tongues)	**G**

Thus, a site recording 3 species of fairy clubs, 6 waxcaps, 1 *Leptonia* and 1 earth-tongue, would be described as C3, H6, E1, G1. This simple quantification method has been combined by Rotheroe (1999) with the Rald (Rald, 1985) classification, which uses the total number of *Hygrocybe* spp. recorded from a site to define it as of:

National Importance (I)	17-32 (11-20 during a single visit)
Regional Importance (II)	9-16 (6-10 during a single visit)
Local Importance (III)	4-8 (3-5 during a single visit)
No Importance (IV)	1-3 (1-2 during a single visit)

By combining the two systems, sites are initially ranked in order of importance according to their Rald (1985) data. The CHEG classification and presence of any Red Data List species (Ing, 1993) then allows refinement of the conservation priority when a large number of sites are being compared. It should be stated that this type of assessment is based upon fruiting data alone and this can vary significantly year to year depending upon the weather conditions (Straatsma *et al.*, 2001) as was observed on the

site during this study. Therefore a full evaluation can only be made following several years assessment.

2.3.2 Method

Fungal fruit bodies were surveyed over 4 years by systematically walking the field up and down at about 2m intervals (visual scanning area considered to be about 1m side to side). The start point was rotated to each corner since the samplers concentration tends to be more focussed during the initial passes. The presence of fruit bodies was recorded and where necessary were taken for identification. The approximate numbers of larger or more important species and positions within the fields were mapped (Figure 2.3 & Figure 2.4).

The initial survey on 26th October 2000 was just a single visit at the start of the project to assess the site. However, in the following years, repeated visits were made during the main fruiting period (Sept-Dec), 10th & 18th Oct 2001, 9th, 18th, 31st Oct, 5th, 15th & 28th Nov 2002, 9th, 24th Sept, 15th, 30th Oct, 13th Nov, 2nd & 9th Dec 2003. Any fruiting of important species that was observed during the rest of the year was also noted.

Table 2.3 Fruit body records for TF and IF from autumn 2000 – winter 2003. Species identification numbers assigned for use in Figure 2.3 and Figure 2.4. Both annual species richness and cumulative totals included. RDL = Red data list species, BAP = Biodiversity action plan species.

					2000	2001		2002								2003					
	ID No.	Species	Status as reported in Keys/Guide	No. records in BMSFRD	26-Oct	10-Oct	18-Oct	30-Sep	09-Oct	18-Oct	23-Oct	31-Oct	05-Nov	15-Nov	28-Nov	29-May	12-Jul	12-Aug	13-Nov	02-Dec	09-Dec
Traditional Field at SO523093.5	1	*Armillaria mellea* (chunky form)	Very common	246						✓											
	2	*Clavaria argillacea*	Occasional	259								✓									
	3	*Clavaria fumosa*	Rare	317	✓																
	4	*Clavulinopsis cinerioides*	Rare	137 RDL Vulnerable						✓											
	5	*Coprinus lagopus*	Occasional	944	✓																
	6	*Entoloma porphyrophaeum*	Uncommon	549	✓	✓	✓						✓	✓							
	7	*Galerina* spp. (poss. *mycenopsis*)	Rare	[339]								✓		✓					✓	✓	✓
	8	*Hygrocybe calyptriformis*	Rare	770 BAP	✓		✓														
	9	*Hygrocybe citrinovirens*	Rare	155	✓	✓	✓			✓					✓						
	10	*Hygrocybe coccinea*	Occasional	2029	✓		✓	✓					✓	✓	✓						
	11	*Hygrocybe conica var. chloroides* (yellow capped form)	Frequent	3104	✓	✓	✓							✓							
	12	Hygrocybe pratensis	Frequent	2544		✓								✓	✓						
	13	*Hygrocybe unguinosa*	Rare	961		✓															
	14	*Hygrocybe virginea*	Common	3282		✓	✓							✓	✓						
	15	*Hygrocybe vitellina*	Rare	182	✓									✓							
	16	*Laccaria laccata*	Common	5446			✓														
	17	*Lepiota konradii*	Rare	166		✓			✓		✓		✓						✓	✓	
	18	*Lepiota procera*	Frequent	1168			✓														
	19	*Leptonia lazulina*	Occasional	251		✓	✓														
	20	*Marasmius* sp.								✓									✓		
	21	*Mycena* (beige)			✓	✓				✓										✓	
	22	*Mycena* (grey striate) (poss. *leptocephala*)		[1407]	✓	✓	✓			✓		✓		✓					✓	✓	
	23	*Mycena flavo-alba*		730								✓								✓	✓
	24	*Nolanea hirtipes*	Occasional	156			✓														
	25	*Paxina* sp.	uncommon/ occasional									✓									
	26	*Panaeolus rickenii*	Frequent	982	✓	✓					✓	✓		✓					✓		
	27	*Pleurotus acerosus*	Uncommon	209								✓									
	28	*Psilocybe semilanceata*	Frequent	1288		✓															
	29	*Scleroderma areolatum*	Occasional	610		✓	✓			✓	✓										
	30	*Tricholoma* sp.			✓																
	31	*Conocybe* sp. 4-5cm cap					✓														
	32	Unknown (ochre cap striate, gills free ochre elipsoid 7umx4um)										✓									
	33	Unknown (cream/white 6cm cap diam, concave dimple in cap, mat silkey distinct rim. Fine gills, decurrent cream)					✓														
	34	Unknown (Large 10cm diam brown cap with unbo, viscid texture, lots of salmon pink gills)					✓														
	Cumulative species total	34	Annual species totals		12	21		20								6					
Improved Field at SO523092.5	35	*Boletus porosporus*	Rare	325		✓															
	36	*Clavulinopsis leuto-alba*	Common	459		✓				✓		✓									
	37	*Clitocybe rivulosa*		512	✓																
	38	*Conocybe pseudopilosella*	Uncommon	56									✓								
	6	*Entoloma porphyrophaeum* (small cluster)	Uncommon	549	✓		✓						✓	✓							
	7	*Galerina* sp.								✓		✓									
	39	*Hygrocybe ceracea*	Occasional	1127		✓	✓														
	9	*Hygrocybe citrinovirens*	Rare	155	✓																
	10	*Hygrocybe coccinea*	Occasional	2029					✓												
	40	*Hygrocybe fornicata* (pale form)	Rare	346			✓					✓									
	41	*Hygrocybe insipida* or *H. glutinipes var.glutinipes* (yellow-orange-red)	Frequent /rare	937 [351]		✓			✓					✓	✓		✓	✓			
	12	Hygrocybe pratensis	Frequent	2544		✓					✓	✓									
	42	*Hygrocybe psittacina*	Occasional	2537 BAP		✓				✓		✓		✓	✓	✓					
	14	*Hygrocybe virginea*	Common	3282																✓	
	16	*Laccaria laccata*	Common	5446	✓		✓						✓	✓							
	17	*Lepiota konradii*	Rare			✓															
	43	*Lepista saeva*	Frequent	428									✓	✓							
	21	*Mycena* (beige)			✓	✓	✓			✓										✓	
	22	*Mycena* (grey striate) (poss. *leptocephala*)		[1407]	✓	✓	✓			✓		✓	✓	✓	✓				✓	✓	✓

44	*Mycena fibula*	Common	2129						✓			✓								
23	*Mycena flavo-alba*	Frequent	730		✓	✓					✓	✓	✓						✓	✓
45	*Nolanea staurospora*	Common	1971		✓	✓						✓	✓							
46	*Omphalina postii*	Rare	33		✓															
26	*Panaeolus rickenii*	Frequent	982	✓							✓	✓	✓	✓						
28	*Psilocybe semilanceata*	Frequent	1288	✓							✓		✓							
29	*Scleroderma areolatum*	Occasional	610			✓				✓										
47	*Stropharia pseudocyanea*	Scarce/ rare	248									✓	✓							
48	*Trichoglossum hirsutum*	Occasional	316		✓															
Cumulative species total	28	Annual species totals		8	17		20								6					
Recorder				Juliet Hynes & Julie Hunt			Juliet Hynes													
Determiner				Juliet Hynes & Julie Hunt			Juliet Hynes, Except H. coccinea, citrinovirens and fornicata which were confirmed through Dr Gareth Griffith, University of Wales, Aberystwyth													

2.3.3 Results

A range of common and conservationally more important species indicative of old undisturbed grasslands were found in both fields including *Hygrocybe* spp., Entolomaceae and clavarioid species (Table 2.3). Using the combined Rald (1985)-Rotheroe (1999) classification (Section 2.3.1) the conservation value of the two fields in terms of the cumulative fungal fruit body data is:

TF: C3, H8, E3, G0. Site of regional importance

IF: C1, H7, E2, G1. Site of regional importance

TF can be classified of greater conservation value than IF because it has a higher CHEG rating and also records an occurrence of a RDL species *Clavulinopsis cinerioides*. Cumulative species richness in TF is greater being 34 species compared with 27 in IF.

The approximate positions of the fruit bodies within the fields were mapped from 2001-2004. Unfortunately although the species observed in the preliminary site visit were recorded their positions were not mapped. However, in autumn 2000 the biodiversity action plan (BAP) species *Hygrocybe calyptriformis* was in approximately the same position in TF as it was the following year (Figure 2.3). Also in TF in 2000, clusters of good-sized fruit bodies of both *H. conica* and *H. citrinovirens* were in the region in front of the gate where the field begins to widen. Although both fields contain a

selection of fruiting *Hygrocybe* spp. which occur in both fields (Table 2.3), some species, *H. calyptriformis, H. conica, H. unguinosa* and *H. vitellina* were recorded only in TF, while *H. ceracea, H. fornicata, H. insipida/glutinipes* and *H. psittacina* (BAP species) fruited only in IF.

2.3.4 Discussion

Due to fungal fruit body surveys being observations of reproductive structures, it is difficult to justify the use of normal ecological indices of abundance and evenness. An individual fungal isolate may cover a very large area, for example *Armillaria ostoyae* is thought to be the largest organism in the world, a single clone covering 965 hectares (Ferguson *et al.*, 2003). Therefore it cannot be assumed that on a site, two clusters of fruit bodies of the same species are in fact from individual mycelial networks, or are from the same mycelium which covers a large area. In essence, counting the number of fruit bodies is equivalent to counting the number of individual flowers on a site. This would be greatly biased towards annuals producing many small flowers, which would outweigh the number of flowers from perennials that invest more in fewer larger structures. Hence, here the only ecological index used is that of species richness. However, even this is biased towards those species that produce visible fruit bodies over the course of the sampling period and it is clear from the 4 years over which these fields were surveyed, that not all species fruit every year. This shows the importance of the molecular assessment of species richness which is increasingly being employed to avoid such fruiting biases (see Chapter 1, Section 1.7.1).

Fruiting appears to be highly weather dependent (Straatsma *et al.*, 2001), a combination of warmth and dampness being required to induce fruit body production. The 4 year

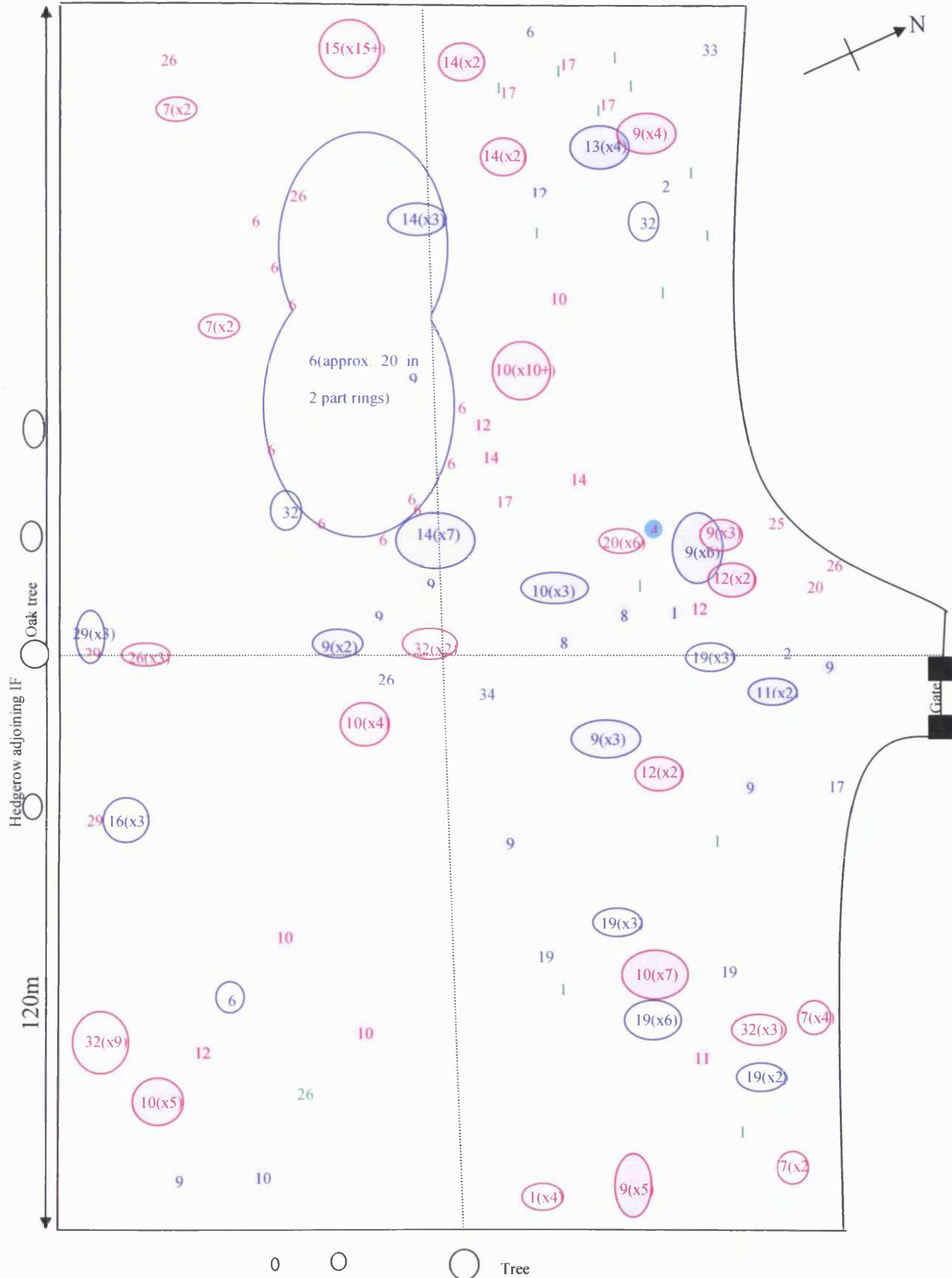

Figure 2.3 Fruit body positions in Traditional Field (not including common *Mycena* spp.), coloured by year: 2001, 2002 and 2003. Numbers match species in Table 2.3, circles indicate groups, numbers in brackets indicate numbers of fruit bodies. Grey shading denotes *Hygrocybe* spp., blue shading indicates Red Data List species.

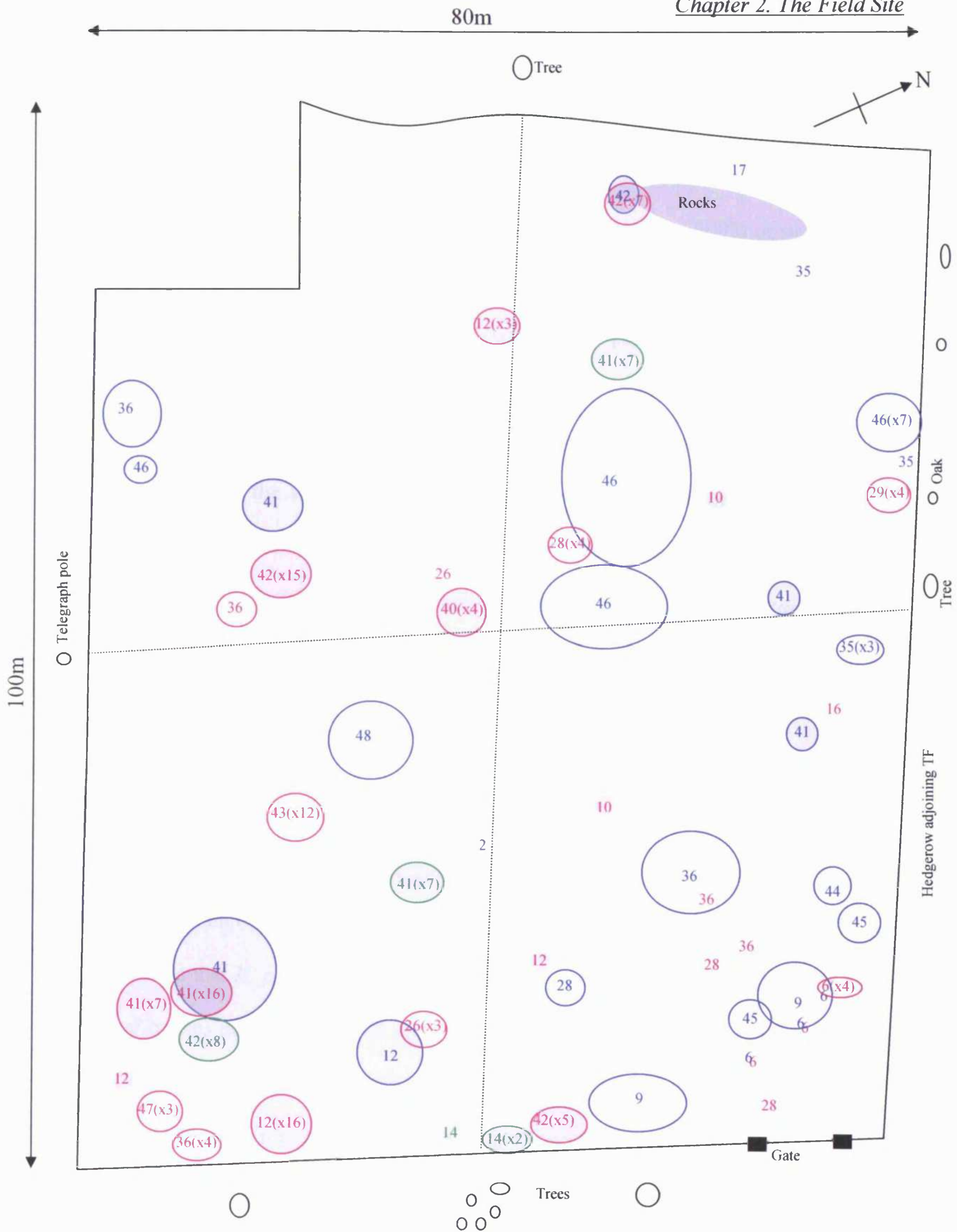

Figure 2.4 Fruit body positions in the Improved Field (not including common *Mycena* spp.), coloured by year: 2001, 2002 and 2003. Numbers match species in Table 2.3, circles indicate groups, numbers in brackets indicate numbers of fruit bodies. Grey shading denotes *Hygrocybe* spp..

period over which these surveys were taken experienced extremes of wet and dry, February 2002 being particularly wet and September 2002 and June - late October 2003 being particularly dry (Section 1.1). Dry autumns result in reduced fruit body size (due to late development of the primordia) and delayed fruiting (e.g. 2002) or no fruiting if the dry period continues for long enough (e.g. 2003). However, autumnal style weather (a hot period in April 2003, followed by cooler damp period, comparable to summer passing into autumn) resulted in May fruiting of *H. psittacina* and *H. insipida/glutinipes* in IF, although no autumnal fruiting was observed due to the prolonged dry summer. Repeat fruiting in the same position over consecutive years did occur while fruiting conditions were favourable, particularly with *Entoloma porphyrophaeum* in both the ring formation in TF (Figure 2.3) and the small patch in IF. Also *H. psittacina* fruited repeatedly by the rocky patch in IF (Figure 2.4), but, other patches of *H. psittacina* did not fruit consecutively.

Both fields sport a good selection of *Hygrocybe* spp. and others indicative of undisturbed grasslands. The species found in IF, such as *H. psitticina* and *H. virginea*, are quite common on grasslands where fertilisation has ceased quite recently and it has been reported that *H. psitticina* and *H. conica* reappear after about 10 years, while more sensitive species may take over 30 years (Griffith *et al.*, 2002). Within a relatively short period, only 10 years or so, since tillage and fertilisation were ceased IF can be assigned the status of a site of regional importance. This shows how rapidly an important fungal community can develop. In fact the fungal community appears to be able to change much more rapidly than the plant community which remains species poor and would certainly not be considered of any conservational significance.

Even though IF recorded almost as many important species of fungi as TF, the spread of these fruit bodies in IF was patchy and the larger fruit bodied species tended to be restricted to very small numbers. TF, on the other hand, sported a greater number of species with large sporocarps, which were widespread across a large proportion of the field (Figure 2.3). The longer period of time over which TF has been left undisturbed (at least 80 years), particularly the lack of tillage, has allowed these presumably slow growing species to establish and become widespread. *Entoloma porphyrophaeum,* for example, when grown at 15°C on Potato Dextrose Agar or 2% Malt Extract Agar produces a mean growth rate of 34.35 and 43.66mm year^{-1}, respectively. If this is representative of the growth rate in soil, the 4m diameter rings in TF could have taken around 45 years to reach that size. Obviously mycelium with such slow growth rates would not survive in tillage disturbed grasslands.

2.4 Soil Chemistry

2.4.1 Soil Moisture

Soil moisture (% oven dried weight) was measured as part of the sole-carbon-source-utilisation (SCSU) experiment (Chapter 5) on 3rd April, 9th July and 5th November 2002 and 21st January 2003. Soil cores from the 0-10cm soil horizon were removed, 12 per field (3 per field quadrant, see Figure 3.1), using screw-capped 15ml plastic centrifuge tubes. The samples were capped immediately to prevent moisture loss. Individual soil samples were transferred to oven dry glass flasks and dried over night at 105°C. The results are shown in Figure 2.5, the TF soil was consistently more moist than the IF soil.

2.4.2 Soil pH

Soil pH was measured as part of the SCSU experiments (Chapter 5), briefly the method used was soil:water 1:2 v/v (Allen, 1989) left overnight to equilibrate. Measurements were made during April 02, July 02, Nov 02 and Jan 03 on sub samples from the bulked soil samples taken for phospholipid and SCSU (Chapter 2, Section 3.2.1). From the four bulked samples per field, three sub samples were measured (to control for experimental variability) and the mean of the three replicate sub samples used in statistical analysis (n = 4 per field). At all time points the pH in TF was significantly higher than in IF (Figure 2.6), overall average of TF pH=6.07 and IF pH=5.85. Within TF, the pH varied significantly between the seasons (one-way-ANOVA, $F_{3,12}$=155.52, P≤0.001), except July and Jan. Within IF there was no significant difference between the seasonal data. These are less acidic values than were obtained by IGER (1999) who found Fields 4 (Pentwyn) and 6 (Bush) to be pH 4.7 and 5.2, respectively. However, it is likely that the higher pH of IF is due to more recent fertilisation than TF, which has not been treated in this way. Since the underlying bed rock and mineral composition of the soil is not likely to differ significantly over the relatively small area of these meadows (being a loamy/sand [IGER 1999]), the recorded variation in pH is likely to be a product of differential management.

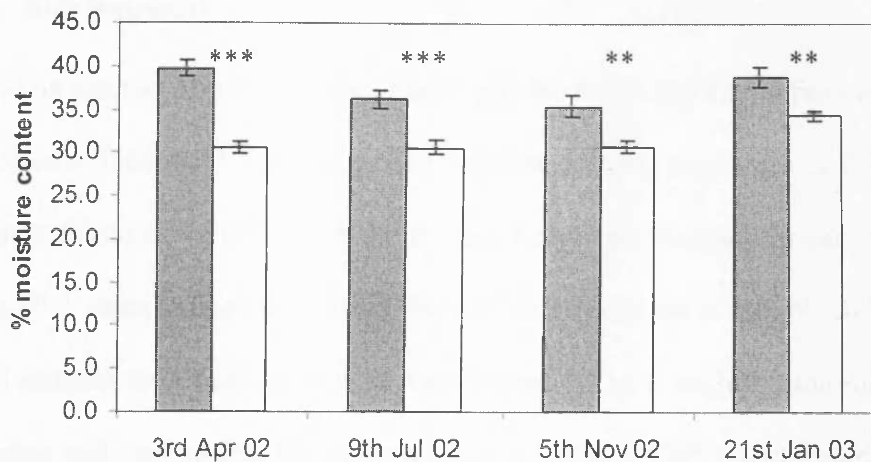

Figure 2.5 Soil moisture as % fresh weight. Shaded bars are TF soil, open bars IF soil from April 2002 to January 2003. Mean ± standard errors (n=12), levels of significant differences between fields at each time point are indicated *≤0.05, **≤0.01, ***≤0.001.

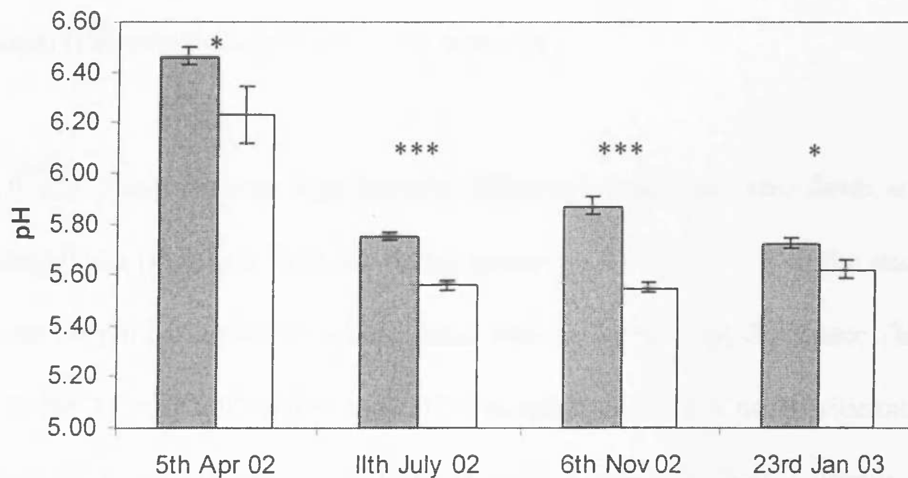

Figure 2.6 Seasonal pH of TF and IF soil. TF shaded bars, IF open bars. Standard errors are marked, levels of significant differences between fields at each time point are also displayed *≤0.05, **≤0.01, ***≤0.001.

2.4.3 Soil Nutrients

Single sub samples, from the bulked soil used for the phospholipid fatty acid and SCSU experiments (Chapter 3, Section 3.2.1), were oven dried over night at 105°C, sieved to 0.6mm to remove root debris. This provided 4 replicate samples for each of the 2 fields across all 4 seasons (Apr 02, Jul 02, Nov 02 & Jan 03) for C and N. Only July 02 and Jan 03 samples were analysed for P. Approximately 2g of each sub sample were used to determine soil carbon (C) by loss on ignition (Allen, 1989), and approximately 0.2g extracted to provide total organic and ammonium nitrogen (N) and total phosphorus (P). N and P were extracted from the same sample using sulphuric acid-hydrogen peroxide micro Kjeldahl digestion (Allen, 1989) and diluted to 25ml. N was then quantified by distillation (Allen 1989) using 5ml aliquots of the digest, while total P was determined by the molybdovanadate method (Hach Company, 1992) using 0.5ml of sample digest with Phosver3 reagent pillows (Hach) for 10ml samples (Appendix I.I), colour was read at 890nm (Unicam Heλios γ, spectrophotometer).

Both C and N content was significantly different between the two fields at each of the sampling times (Figure 2.7a & b), being greater in TF than IF, with the exception of N from the July 02 samples in which there was no significant difference (however, the mean N for TF was still higher than IF). Phosphorus showed no significant differences between fields at any sampling time (Figure 2.7c). All three nutrients showed no significant differences between seasons for each field type (One way ANOVA with Turkey's pairwise comparisons).

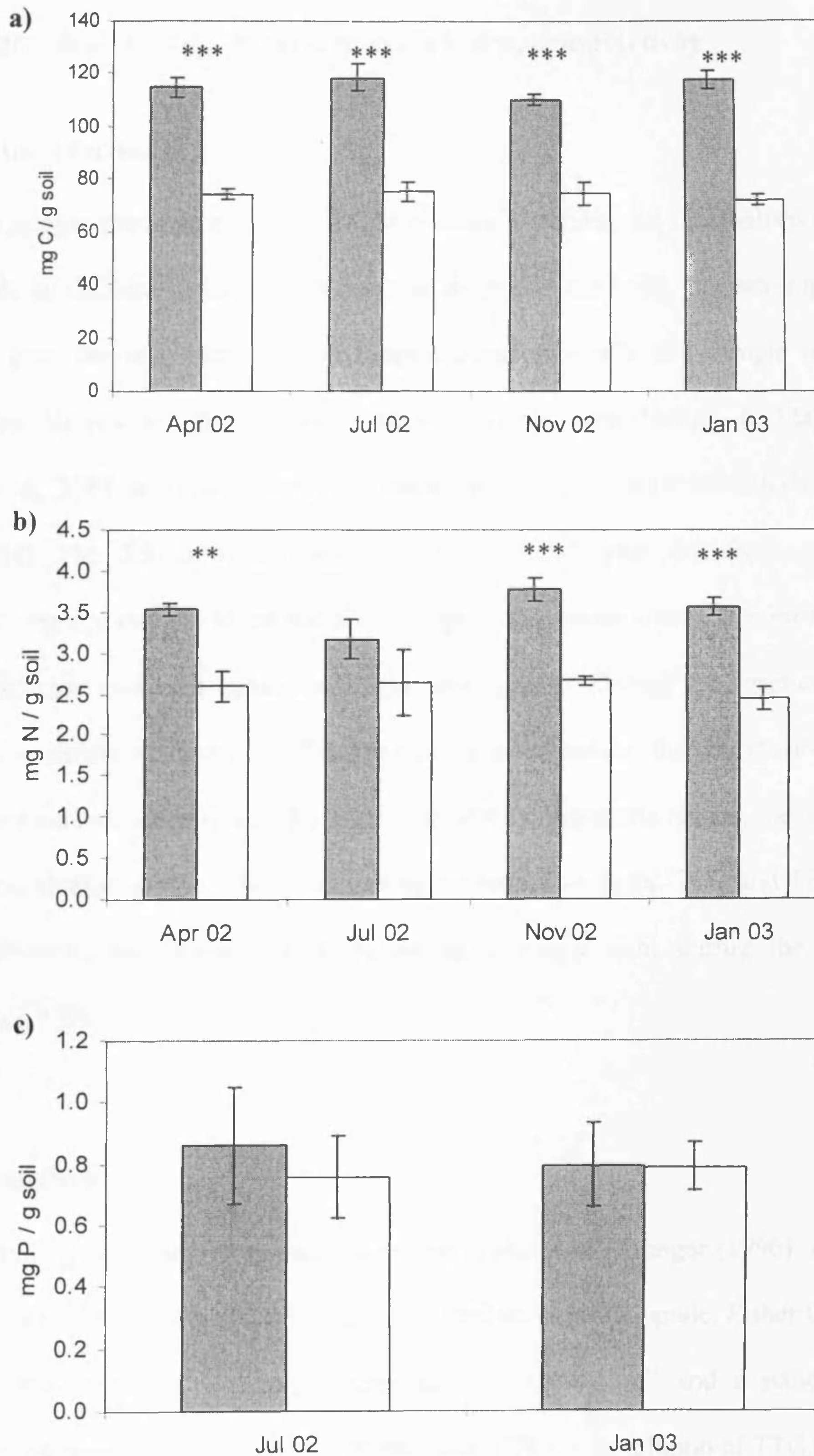

Figure 2.7 Soil nutrient content of TF and IF soil (n=4) taken from seasonal samples. Means and standard errors shown. Significant differences (2 sample t-tests) between fields at each time point are indicated *≤0.05, **≤0.01, ***≤0.001.

2.5 *Microbial Activity, Measured as Dehydrogenase Activity*

2.5.1 Introduction

Dehydrogenase enzymes catalyse various oxidation steps in the metabolism of organic materials by microbes. They do not accumulate in soil since they degrade rapidly upon release from the cell, thus the dehydrogenase activity of a soil sample reflects the oxidative activity of the microbes therein (Klimkowicz-Pawlas & Maliszewska-Kordybach, 2003) and is considered a reliable index of soil microbial activity (Casida *et al.*, 1964). The dehydrogenase assay employs 2,3,5-Triphenyltetrazolium formazan (TCC) to replace oxygen as the electron acceptor, to capture electrons released through dehydrogenase catalysed oxidation in the soil sample. During the reaction, TCC is reduced to Triphenyl formazan (TPF) producing a red colour, the absorbance of which is determined colourimetrically. In order to produce comparable results, the mass of soil and concentration of TCC should be optimised before analysis. TCC and TPF are also light sensitive, thus should be protected from bright light during the procedure (Ohlinger, 1996).

2.5.2 Method

The dehydrogenase assay was based upon the protocol of Ohlinger (1996). A standard stock solution (1mg ml^{-1}) of TPF (Sigma) in Methanol (HPLC grade, Fisher Chemicals) was diluted 1:10 to a working concentration of 0.1mg ml^{-1} and a standard curve produced (Appendix I.II). Trials determined that a 1% (w/v) solution of TTC (Sigma) in Tris buffer (0.1M; 12.11g of Tris(hydroxymethyl)aminomethane [Sigma] in 600ml sterile distilled water), pH7.8, with 3g samples of TF and IF soil provided optimal

measures of dehydrogenase activity, remaining within the linear part of the calibration curve.

Twelve soil cores (3 per field quadrant [Chapter 3, Section 3.2.1], 2.5cm x 10 cm) were collected from each field on 9th Sept 2003. The core positions were determined by pacing randomly generated co-ordinates from the Southwest corner of each field. Each core was divided into two 5cm lengths, providing a comparison of activity in the top 0-5cm of the soil with the lower 5-10cm depth. Core sections were placed in polythene bags in the dark and stored at 15°C until processing the following day.

Soil cores (field wet) were sieved to 2mm, 3g (field weight) transferred to 25ml autoclaved glass universal bottles. The remaining soil was oven dried (105°C over night) to determine soil moisture. To each 3g soil sample, 3ml TCC in Tris buffer (1% w/v) was added, a blank without soil was also included. The samples were tamped down, the bottles capped and incubated in darkness at 37°C for 24 hours (Casida *et al.*, 1964). Following incubation, the samples were transferred to 50ml screw capped centrifuge tubes using methanol washes, and the TPF extracted in a total of 45ml methanol (Casida *et al.*, 1964), incubating in darkness at room temperature for 1 hour (samples were shaken every 20 minutes to ensure complete extraction). Samples were then centrifuged (4000rpm) for 2 minutes to remove soil particles and the solution read against a methanol blank at 485nm (Perkin-Elma lambda 2UV/Vis spectrophotometer). Activity per g soil (dry weight) was calculated using Equation 2.1.

Equation 2.1 $$A = \frac{(T)(V)}{(W_d)(W_f)}$$

where: A = microbial activity measured as μg TPF g^{-1} soil (dry wt) 24 $hours^{-1}$

T is the concentration of TPF μg ml^{-1} in the sample calculated from the calibration curve.

V is the total volume (methanol + TCC sol^{n})

W_d = dry weight 1g soil

W_f = field wet weight of the sample

2.5.3 Results and Discussion

The microbial activity, as measured by dehydrogenase activity was significantly higher in TF than IF in the upper soil profile (0-5cm), on average being almost twice that found in IF (Figure 2.8). The lower soil profile (5-10cm) on average had a higher dehydrogenase activity in TF than IF, but the difference was not statistically significant (Figure 2.8). However, since this is a measurement of activity from only a single time point it only provides an indication of comparative activity between the two fields. If the experiment was replicated on more than one occasion throughout a year, the activities of each field might alter relative to one another.

There was also a distinct decline in activity with soil depth (Figure 2.8), the activity of the top layer (0-5cm) of each field being, on average, about twice that of the lower layer (5-10cm). This corresponds with analyses of microbial communities through soil depth profiles, which found a rapid decline in biomass, respiration and dehydrogenase activity from depths of 0-5, 5-10 and 10-15 cm (Bardgett *et al.*, 1997) and limited vertical distributions of specific microbial groups which was associated with the negative correlation of carbon availability with soil depth (Fierer *et al.*, 2003). Microbial distribution may also be attributed to the change in concentration of soil gases with

depth (Sheppard & Lloyd, 2002). The greater distinction between the depth profiles of TF may be a result of a greater amount of organic matter incorporation into the top layer of the TF soil.

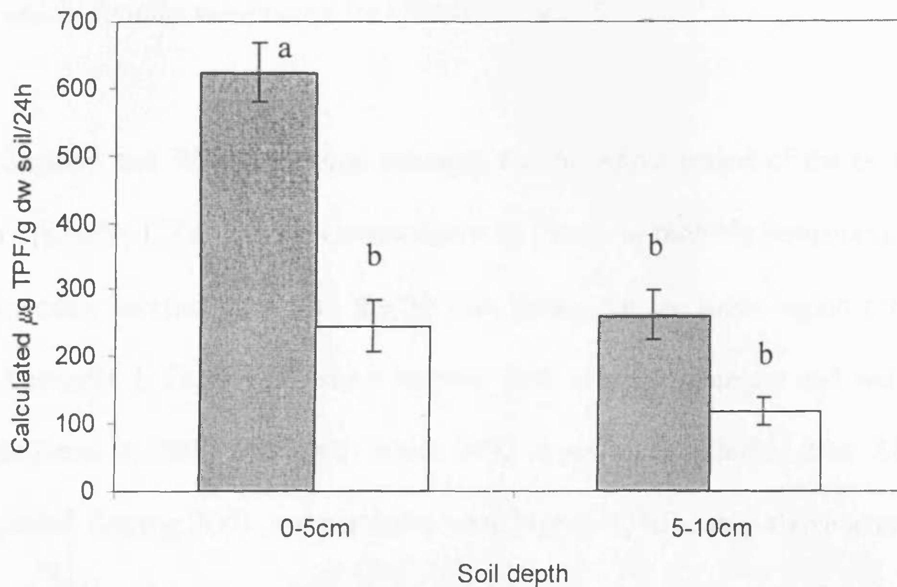

Figure 2.8 Dehydrogenase activity with soil depth. Shaded bars are from TF soil, open bars, IF soil. Means and standard errors shown (n=12). Bars marked with the same letter are not significantly different at $P \leq 0.05$ (One way ANOVA with Turkey's pairwise analysis).

2.6 Meteorological Data

Local min-max temperatures and rainfall data (Figure 2.9) were obtained from the nearest weather recording station which was The Hendre local weather station (Appendix I.III) 51:49:43N 02:47:44W, Height 78m AMSL. The local monthly averages were compared with Met. Office historical 30 year averages (1961-1990) for the England & Wales region (Appendix I, Table I.I) corresponding to the period over which samples were taken for Chapters 3, 4 & 5.

England and Wales regional averages for the whole period of the project are given in (Appendix I, Table I.II). Comparisons of the mean monthly temperatures and the mean monthly rainfall data with the 30 year means for the same region (one-sample t-tests Appendix I, Table I.II), show warmer than average summers and wetter than average autumns in 2000 and 2001, while 2002 experienced a hotter drier August-September period. During 2003, temperatures were highly significantly above average from March right through to September, much of the same period was also significantly drier. This was then followed by a significantly colder and drier October producing particularly poor fungal fruiting conditions.

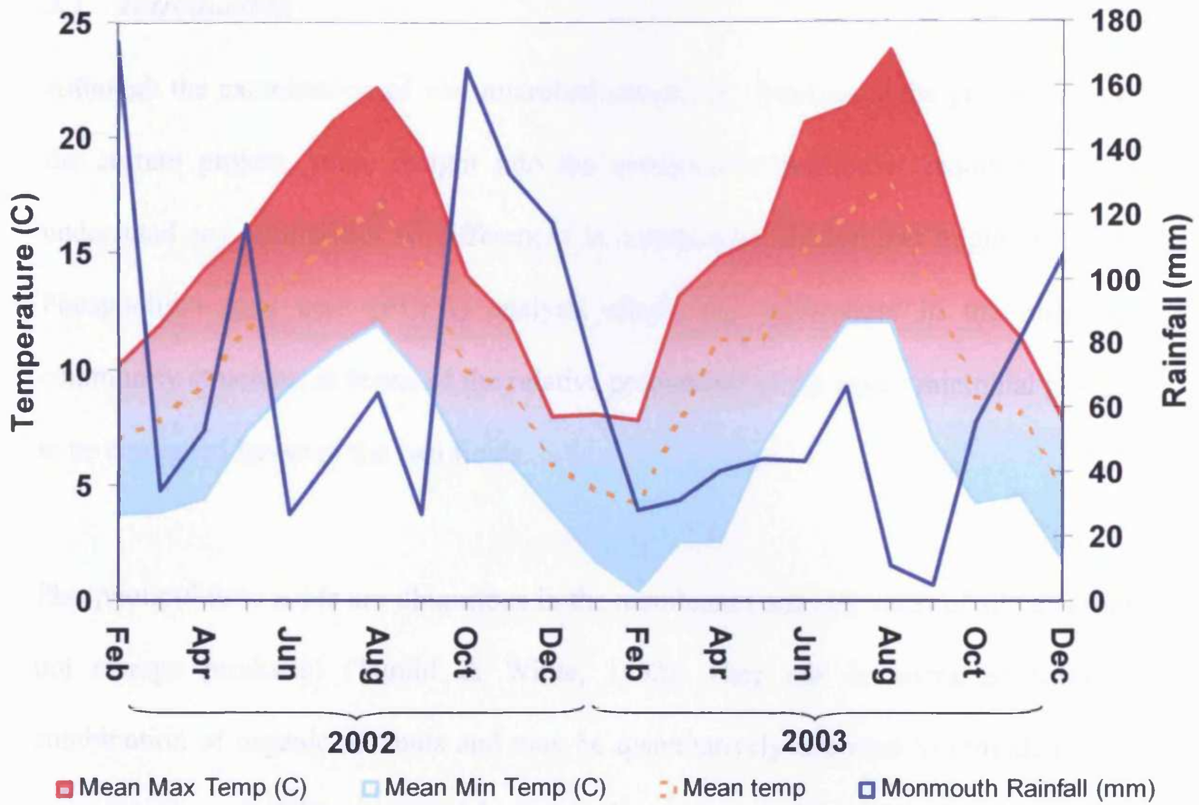

Figure 2.9 Local temperature and rainfall data for the sampling period 2002-2003, obtained from the closest local weather station to the field site (The Hendre, Appendix I.III).

Chapter 3. Community Structure: Phospholipid Fatty Acid Analysis

3.1 Introduction

Although the examination of soil microbial community function is the primary aim of the current project, some insight into the community structure is required to fully understand any similarities or differences in community function that might be found. Phospholipid fatty acid (PLFA) analysis allows any differences in the microbial community structure, in terms of the relative proportions of the major microbial groups, to be compared between the two fields.

Phospholipid fatty acids are ubiquitous in the membranes and cell walls of all cells (but not storage products) (Tunlid & White, 1992). They can be extracted using a combination of organic solvents and may be quantitatively analysed to provide (1) an approximation of viable microbial biomass, (2) an estimate of microbial physiological status through the identification of known stress indicators and (3) an outline of community structure (White & Ringelberg, 1998).

3.1.1 Viable Microbial Biomass

3.1.1.1 Viable Cells

There are various techniques available for the analysis of community structure, such as DNA extraction, cloning and sequencing, or less specific molecular methods like DGGE or RFLP, which can provide community fingerprints. However, these make use of total extractable DNA including that of the non-viable organisms, the DNA of which may remain in the soil long after their death. PLFAs are rapidly broken down by

enzymatic activity following rupture of the cell membrane, hydrolysis of the phosphate group occurring within minutes to hours of cell death (White, 1995). PLFA therefore provides a measure of the viable organisms in a sample (White, 1995).

3.1.1.2 Conversion Factors

Although PLFAs are used as indicators of biomass, within the cell they are correlated with membrane area. Therefore, variation in the amount of phospholipid per unit biomass occurs due to the different surface area to volume ratio of different organisms (Olsson, 1999). Various attempts at calculating suitable conversion factors have been made. Frostegård and Bååth (1996) suggest extracting a bacterial sample, counting them and then determining the PLFA concentration per cell, although this would be relatively time consuming. Kalmer and Bååth (2004) calculated conversion factors for 11 fungal species starting with the determination of linoleic acid (18:2ω6) concentration in pure fungal cultures. These were then used to calculate fungal biomass over time in compost. These conversion factors are relatively specific to the species/strains used in the studies, although, the Central Limit Theorem (Campbell, 1992) may apply (Kalmer & Bååth, 2004), whereby in a population with high variation, the mean at any one time will tend to remain constant given a large enough sample size (1g soil is suggested for a fungal population (Kalmer & Bååth, 2004)). Due to the difficulty in calculating valid conversion factors a biomass index may be used to compare relative changes between groups. For example, the ratio of fungal/bacterial biomass has been suggested using 18:1ω6 as an indicator of fungal biomass and 13 bacterial PLFAs as an estimate of bacterial biomass (Bååth & Anderson, 2003; Frostegård & Bååth, 1996). This is not a measure of actual fungal or bacterial biomass, but changes in the recorded mol% of the composite PLs will register providing a measure of relative abundance between sites.

3.1.1.3 Correlation with Biomass Determined by Other Techniques

Bailey *et al.* (2002) found a good linear correlation between biomass estimated by chloroform fumigation extraction and total PLFA, but only a weak correlation was identified between PLFA and substrate induced respiration (SIR) in this study. Comparisons of PLFA, intracellular ATP, cell wall muramic acid and acridine orange counts of bacteria showed equivalent determinations of viable biomass, except that the PLFA had a much smaller standard deviation (White, 1995).

The ergosterol content (a fungal cell membrane sterol) is an alternative measure for estimating fungal biomass in soil. However, ergosterol concentration can vary inter- and intra-specifically depending upon a number of factors including nutrient and oxygen availability, and mycelial age (Kalmer & Bååth, 2004). As with PLFAs this makes a realistic conversion factor difficult to estimate without knowing the composition of the community in advance. Nevertheless, a number of studies have found a good linear correlation between fungal biomass estimated by ergosterol and that estimated by the PLFA 18:2ω6 (Bååth & Anderson, 2003; Frostegård & Bååth, 1996; Kalmer & Bååth, 2004).

SIR with selective inhibition has previously been shown to produce concomitant results with that of the PLFAs (Bardgett *et al.*, 1996). A comparison of PLFA fungal/bacterial ratio with that determined by SIR with selective inhibition (using streptomycin as a bacterial and cyclohexamide as a fungal respiratory inhibitor) revealed no similarity between the results (Bååth & Anderson, 2003). The authors suggest that this lack of congruence between the fungal/bacteria ratios indicates that the methods pertain to

different aspects of fungal and bacterial metabolism and may be complimentary rather than either method being inadequate. For example, the PLFA regression line did not pass through the origin indicating the inclusion of some non-membrane bound PLFAs, while the selective inhibition method does not include dormant cells due to the requirement of an immediate response to the added glucose substrate. It was suggested that this may also be selective against slowly responding organisms, e.g. ectomycorrhizal fungi (Bååth & Anderson, 2003). Thus they conclude that selective inhibition might be an activity indicator rather than a biomass measure.

3.1.2 Indicators of Physiological Status

If structural modification of lipids occurs in response to specific environmental conditions, analysis of these may provide an insight into the nutritional/physiological status of the cell and the suitability of the local environment (White, 1995). In bacteria physiological stress can result in a relative increase in specific *trans* monoenoic PLFA compared to the *cis* isomer, while in the absence of essential nutrients, bacteria form poly β-hydroxyalkanoic acid (microeukaryotes form triglyceride). The relative proportion of this compound to PLFA gives a measure of nutritional status (White, 1995).

3.1.3 Community Structure

PLFAs may be referred to as signature lipid biomarkers (White & Ringelberg, 1998). However, the specificity of a signature fatty acid to a particular organism is questionable, since often less than 10 FAs make up the PLFA profile of an organism (Zelles, 1997). Zelles (1997) surveyed the FA profiles of 33 individual isolates of

culturable soil organisms belonging to Gram-positive bacteria, Gram-negative bacteria, fungi and plants. It was found that iso and anteiso branching and branching at other positions occurs primarily in Gram-positive bacteria, but also in some Gram-negative species. While 17:0 and 19:0 cyclopropane FA occur mainly in Gram-negative bacteria they are also found in other organisms, however, the occurrence of these FAs in plants may be due to bacteria on the tissues, since they are absent from sterile plants. Linoleic acid (18:2ω6) makes up a large proportion of the PLFAs in all plants and fungi, constituting on average 43% of the total fungal PLFA, and is also found in small quantities in some bacteria (Zelles, 1997). It is difficult to distinguish whether linoleic acid in a sample originates from plant or fungal material, however, if effort is made to remove all plant material from a soil sample (e.g., by careful sieving) the remaining linoleic acid signature should be mostly fungal.

Kalmer and Bååth (2004) tested 12 fungal species totalling 24 isolates including ascomycetes, zygomycetes and basidiomycetes. Between 77 and 79 mol% of the profile was made up of 3 PLFAs: 16:0, 18:2ω6 and 18:1ω9. Although the classic fungal indicator PLFA 18:2ω6 dominated the asco- and basidiomycete profiles (36-61 mol% and 45-57 mol% respectively) it was only 12-22 mol% in the zygomycetes tested. Conversely, 18:1ω9 dominated within the zygomycete order. Hence the use of 18:2ω6 as a fungal indicator is not necessarily proportionally representative of the whole fungal community and changes to the relative proportions of the fungal groups may be missed.

It has been suggested that arbuscular mycorrhizal (AM) fungi may be distinguished from saprotrophic fungi by the fatty acids 16:1ω5 and 18:1ω7, which make up a large proportion of the total FA profile in these species. These FAs are not common in other

fungi, but may occur in some bacteria. Hence, in open soil samples, there will be background levels of 16:1ω5 that are not of AM origin (Olsson, 1999). Where 16:1ω5 has been used to estimate AM fungal biomass (Hedlund, 2002; Madan *et al.*, 2002; Olsson, 1999), all studies concluded that the use of this FA as an AM indicator should be limited to controlled experimental situations.

Due to limited specificity, rather than identifying genus or species level groupings, PLFAs can be used to estimate the biomass or relative proportions of larger (possibly functional) groupings within a community (Zelles, 1997), such as, Gram positive and Gram negative bacteria, fungi (which may be further broken down to saprotrophs, AM or ectomycorrhizae under more controlled experimental conditions (Hedlund, 2002; Madan *et al.*, 2002; Olsson, 1999; Wallander *et al.*, 2001)), actinomycetes and methanotrophs. Aerobic and anaerobic bacteria may also be distinguished (Vestal & White, 1989). Also, pairing stable isotope techniques with PLFA analysis has enabled the identification of different functional groups decomposing more recalcitrant substrates, through the incorporation of ^{13}C from labelled substrate into PLFAs (Waldrop & Firestone, 2004).

In the present study PLFAs were used to estimate the fungal:bacterial ratio of the sites and to determine any seasonal changes to the relative biomass of the major groupings of Gram positive bacteria, Gram negative bacteria and fungi. Conversion factors were not used to calculate absolute biomass since the variability of cell surface area means that no appropriately realistic conversion factors have yet been established (Bååth & Anderson, 2003).

3.2 *Methods*

3.2.1 Soil Samples

The two fields were divided into fixed quadrants (A-D) (Figure 3.1), of approximately equal area, by the position of permanent landmarks around the perimeter to allow for soil sample replication (Bardgett *et al.*, 1999a) within each field. Due to the heterogeneous nature of the soil environment, it would not have been feasible to analyse the numbers of individual soil cores that would be required to realistically account for soil heterogeneity. Hence, 10 soil cores (1.5cm diam. x 10cm depth) per quadrant were randomly taken and subsequently bulked. Cores were taken from the top 10cm of the soil since the majority of the microbial biomass (78-90%) and microbial activity (63-99%) occurs in this surface fraction (Bardgett *et al.*, 1997). The positions from which the cores were taken were determined by producing co-ordinates from random numbers, starting at 1 so that a 2m buffer zone existed between the quadrants. These were paced out (2 paces being approximately 1m) from the quadrant intersection in the centre of the field. Co-ordinates were always paced first vertically, as orientated in Figure 3.1, then horizontally. Cores were stored in a cool box and, on return to the laboratory, bulked under sterile conditions and homogenised manually using a 4mm alcohol sterilised sieve. Any plant debris was removed. The bulked sample was then divided into 3 sub samples, for sole-carbon-source-utilisation (SCSU) analysis (Chapter 5) which was set up immediately, pH measurement that was carried out the following day and PLFA analysis. The PLFA samples were stored at -20°C (White & Ringelberg, 1998) until extraction. The air temperature and field soil temperature (3 random readings per quadrant) was also taken following collection of all the cores. Soil moisture was calculated as part of the SCSU study and is recorded in Chapter 2 (Section 2.4.1).

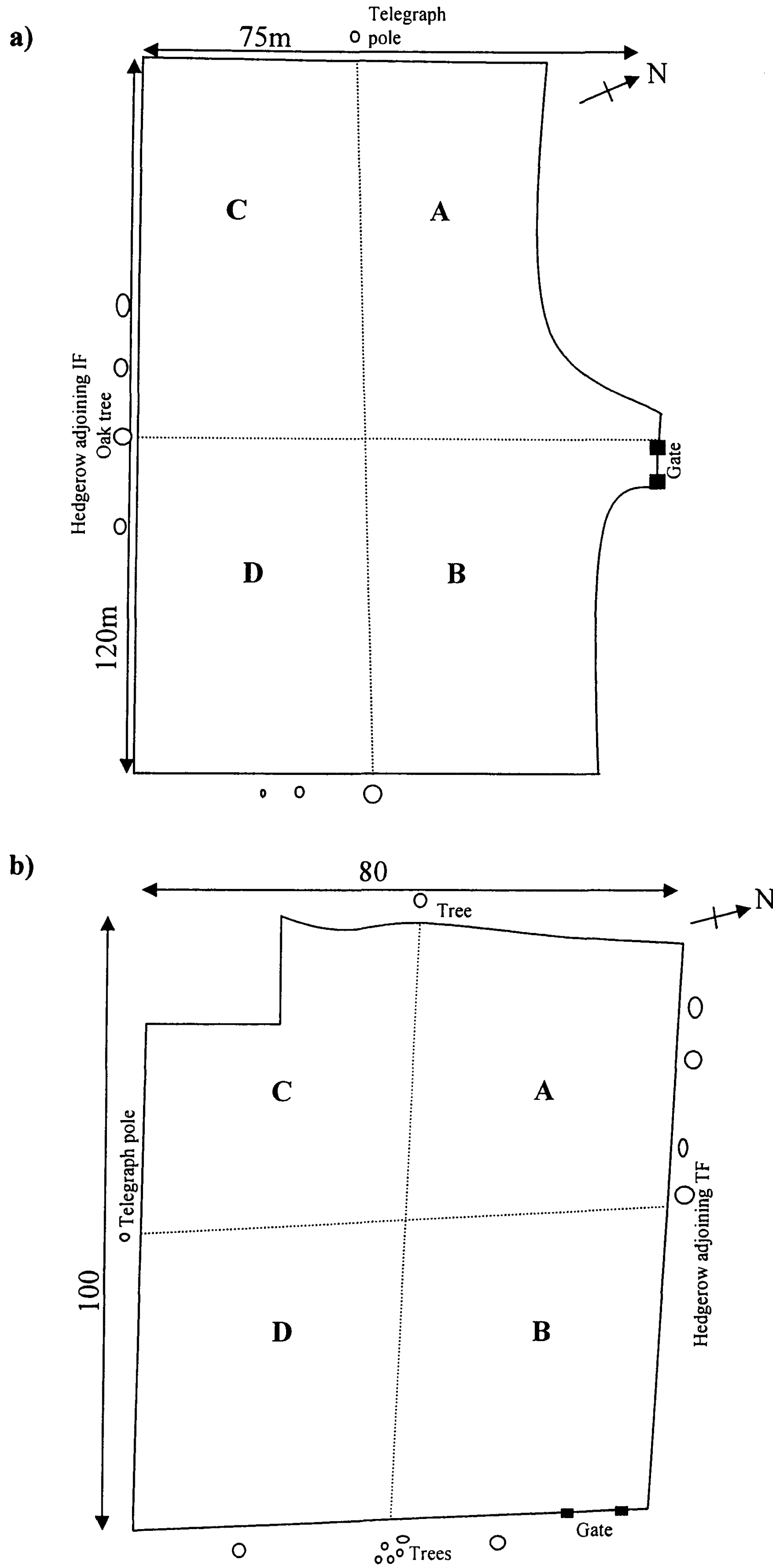

Figure 3.1 Layout of fixed sampling areas (A-D) for soil core samples, a) is the traditional field (TF) and b), the improved field (IF).

3.2.2 PLFA Procedure

All chemicals used were of an analytical grade (Fisher Chemicals, UK). To remove any lipid contaminants, glassware was acid-washed, rinsed with distilled water and then wrapped in aluminium foil and heated in a muffle furnace to at least 450°C for 4hours. Rinsed heat intolerant items were solvent rinsed with methanol followed by chloroform and allowed to dry. Ultra pure deionised water was extracted with chloroform to remove lipid contaminants, as were buffer solutions.

Lipid extractions followed the method of White and Ringelberg (1998), a modification of the original Bligh and Dyer (1959) method, which uses a single phase chloroform:methanol:water extraction system. Bulked soil samples (Section 3.2.1) from 10th July 2002 and 22nd January 2003 were lyophilised, sieved to 600μm to remove any plant debris and thoroughly homogenised by pestle and mortar. Three x 1g sub samples were weighed into glass centrifuge tubes with PTFE lined screw caps (Chromacol). Following the protocol in Appendix II, lipids were extracted by first phase extraction for 18 hours, separated by silicic acid column chromotography and the phospholipid fraction subject to mild alkaline methanolysis generating fatty acid methyl esters (FAMEs).

FAMEs were separated by gas chromatography (GC8000, Thermofinnigan) and identified by comparison of chromatographic retention times and mass spectral signatures (MD 800, Thermofinnigan) against FAME standards (19 FAME mix C8-C22 at 10, 5 and 1ng μl^{-1}, a qualitative bacterial FAME mix and an 18:2ω6 standard both at approximately 10ng μl^{-1} (Supelco)) (Chromatograms Appendix II, Figure II.I). A 50pmol cholestane internal standard (18.7mg ml^{-1}) was prepared in iso-octane and the

appropriate sample dilution, using the internal standard solution, determined by shooting range-finder with the first sample. The dried samples were redissolved in 200µl of the internal standard and measured in a mixed sequence, interspersed with the FAME standards, 1µl of samples and standards were injected (AS 800, Thermofinnigan) splitless, at 250°C and chromatographed over 30m (0.25mm internal diameter, 0.25µm film thickness) VF23ms (Varian) polar column, at 55kPa (8psi) helium. The temperature program was held at 50°C for 1 min, then ramped from 50°C to 130°C (15°C min^{-1}), then to 250°C at 5°C min^{-1} and held for 10 minutes. Mass spectra were recorded from m/z 40 to 600 in EI mode (source temperature 200°C, interface 280°C).

Following assessment of the literature (Appendix II, Table II.II), the fatty acids in Table 3.1, were chosen as being representative of the major soil microbial groups, due to minimal crossover of these FAs between groups. Chromatogram peak areas (Appendix II, Figure II.I) were resolved using Masslab verion 1.4. Relative abundance, as pmol PLFA g^{-1} soil (dry weight), of the PLFA were determined by calculating the peak area of the corresponding FAME, relative to that of the cholestane internal standard using Equation 3.1. The three replicate sub samples of each soil sample were averaged to account for variation during sample processing, leaving four replicates per field per date.

Equation 3.1 $C_x = ((A_x/A_{istd}) * C_{istd} * D) / W$

Where C_x is the calculated concentration, A is the GC peak area, of the PLFA (A_x) or internal standard (A_{itsd}), C_{istd} is the internal standard concentration (pmol μl^{-1}), D is the dilution factor (200µl) and W is the soil sample dry weight (g).

3.2.3 Fatty Acid Nomenclature

Standard fatty acid nomenclature, a:bωc where a is number of carbon atoms in the carbon backbone, b is the number of double bonds, and c is the position of the first double bond counted from the methyl end. Chemical names count double bond positions from the carboxyl and of the molecule. The prefix "i" (iso-branching) indicates a methyl branch on the second C from the methyl end and "a" (anti iso-branching) a methyl branch on the third C from the methyl end. "cy" or "cyc" indicates the presence of a cyclopropyl ring in the chain. The suffix "c" or "t" represents the cis and trans isomers of the molecule, cis being the more common natural form. Assume cis unless otherwise stated.

3.2.4 Statistical Analysis

One way ANOVA with Turkey's pairwise comparisons were used to compare responses to the four treatments (July TF, July IF, Jan TF and Jan IF) of individual PLFAs, microbial group totals and the fungal:bacterial ratio. The statistical significance of pairs of fields were also determined by two sample T-tests to the $P \leq 0.05$ level and below. Samples undergoing parametric tests fulfilled all the assumptions of the test. All statistical analyses were performed using MINITAB ver.13.

Table 3.1 Fatty acids chosen to represent the major soil microbial groups, with corresponding methyl ester (Me.) form and Me. molecular weight, used for identification from the chromatogram and mass spectral data. For fatty acid nomenclature see Section 3.2.3.

Microbial group	PLFA short form nomen-clature	PLFA chemical formula	PLFA mole-cular weight	Alternative nomen-clature from literature	FAME short form nomenclature	FAME chemical formula	FAME mole-cular weight	PLFA Common name	PLFA Chemical name
Gram negative bacteria	15:1ω4	$C_{15}H_{28}O_2$	240.387	15:1ω4c	Me. 15:1ω4	$C_{16}H_{30}O_2$	254.414		Cis-11-pentadecenoic acid
	16:1ω5	$C_{16}H_{30}O_2$	254.414	16:1ω5c	Me. 16:1ω5	$C_{17}H_{32}O_2$	268.441		Cis-11-hexadecenoic acid
	16:1ω7	$C_{16}H_{30}O_2$	254.414	16:1ω7c	Me. 16:1ω7	$C_{17}H_{32}O_2$	268.441		Cis-9-hexadecenoic acid
	17:0cyc	$C_{17}H_{34}O_2$	270.457	17:1ω9c	Me. 17:0	$C_{18}H_{36}O_2$	284.484		Cis-heptadecanoic acid
	17:1ω9	$C_{17}H_{32}O_2$	268.441	17:1ω9c	Me. 17:1ω9	$C_{18}H_{34}O_2$	282.468		Cis-8-heptadecenoic acid
	18:1ω5	$C_{18}H_{34}O_2$	282.468	18:1ω5c	Me. 18:1ω5	$C_{19}H_{36}O_2$	296.495		Cis-13-octadecenoic acid
	18:1ω7	$C_{18}H_{34}O_2$	282.468	18:1ω7c	Me. 18:1ω7	$C_{19}H_{36}O_2$	296.495		Cis-11-octadecenoic acid
	19:0cyc	$C_{19}H_{36}O_2$	296.503	cy19:0	Me. 19:0cyc	$C_{20}H_{38}O_2$	310.522		Cis-nonadecanoic acid
Gram positive bacteria	i14:0	$C_{14}H_{28}O_2$	228.376	i14:0c	Me. i14:0	$C_{15}H_{30}O_2$	242.403		Cis-tetradecanoic acid
	i15:0	$C_{15}H_{30}O_2$	242.403	i15:0c	Me. 15:0	$C_{16}H_{32}O_2$	256.430		Cis-13-methytetradecanoic acid
	a15:0	$C_{15}H_{30}O_2$	242.403	a15:0c	Me. 15:0	$C_{16}H_{32}O_2$	256.430		Cis-12-methytetradecanoic acid
	15:0	$C_{15}H_{30}O_2$	242.403	15:0c	Me. 15:0	$C_{16}H_{32}O_2$	256.430		Cis-pentadecanoic acid
	i16:0	$C_{16}H_{32}O_2$	256.430	i16:0c	Me. i16:0	$C_{17}H_{34}O_2$	270.457		Cis-14-methylpentadecanoate
	i17:0	$C_{17}H_{34}O_2$	270.457	i17:0c	Me. i17:0	$C_{18}H_{36}O_2$	284.484		Cis-15-methylhexadecanoate
	a17:0	$C_{17}H_{34}O_2$	270.457	a17:0c	Me. a17:0	$C_{18}H_{36}O_2$	284.484		Cis-14-heptadecanoate
	17:0	$C_{17}H_{34}O_2$	270.457	17:0c	Me. 17:0	$C_{18}H_{36}O_2$	284.484	Margaric acid	Cis-heptadecanoate
Fungi	18:2ω6	$C_{18}H_{32}O_2$	280.452	C18:2ω6, 9c	Me. 18:2ω6	$C_{19}H_{34}O_2$	294.479	Linoleic acid	Cis, 9,12-octadecadienoic acid.
Protozoa	20:3 (any)	$C_{20}H_{34}O_2$	306.490		Me. 20:3	$C_{21}H_{36}O_2$	320.517		Cis *,*,*-eicosatrienoic acid

3.3 Results

Differences in community structure, measured as PLFA concentration, between the traditionally managed and improved field were not clear-cut. The total PLFA showed a peak in TF in the January samples, however the total PLFA concentration of the July TF, IF and January IF samples were all approximately equal (Figure 3.2a). The total bacterial and the separate (Gram positive and Gram negative) bacterial groups also reflected this same pattern (Figure 3.2b-d), since around 93% of the total FAs measured in this study were made up from bacterial "signature" FAs.

In contrast to the bacterial FAs, the fungal signature FA, 18:2ω6, produced a more defined trend, being, on the whole, of greater concentration in the traditional field samples. This difference between fields was significant in January ($T_6 = 4.49$, $P = 0.004$), although the mean for July was higher in TF it was not statistically significant (July samples $T_6 = 2.17$, $P = 0.074$) (Figure 3.2e),. The fungal FA concentrations recorded for the individual fields did not differ significantly between sampling times (Figure 3.2e).

Microbial group total PLFA ratios can be used to indicate changes in the proportions of the community (i.e. changes to community structure), as a result of different management regimes (Bardgett *et al.*, 1996; Donnison *et al.*, 2000b; Grayston *et al.*, 2004; Grayston *et al.*, 2001). The fungal:bacterial ratios, recorded from the TF and IF samples, indicate that fungi constitute a greater proportion of the microbial community in TF compared with IF soil (Figure 3.2f). Again, however, the difference produced between the July samples was not significant ($T_6 = 2.19$, $P = 0.071$; $T_6 = 4.61$, $P = 0.004$ for July and January respectively) althought the TF mean was greater than IF.

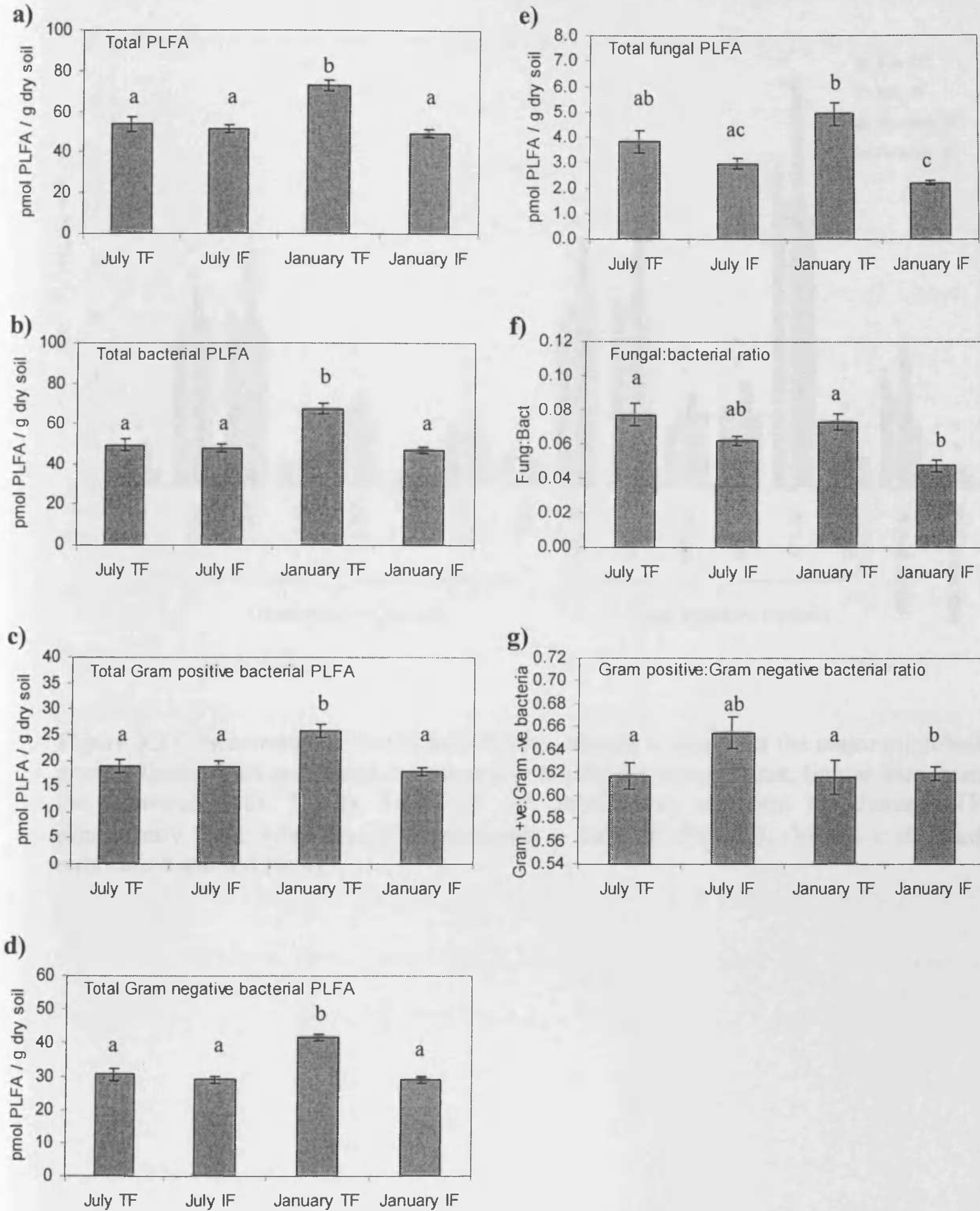

Figure 3.2 Concentrations (pmol PLFA g^{-1} dry soil) and ratios of the major groups of microorganisms forming the soil microbial community of grasslands under different management (TF, traditionally managed field; IF, improved field). Means and standard errors of the means are displayed (n=4). Bars marked with the same letter are not significantly different at $P \leq 0.05$).

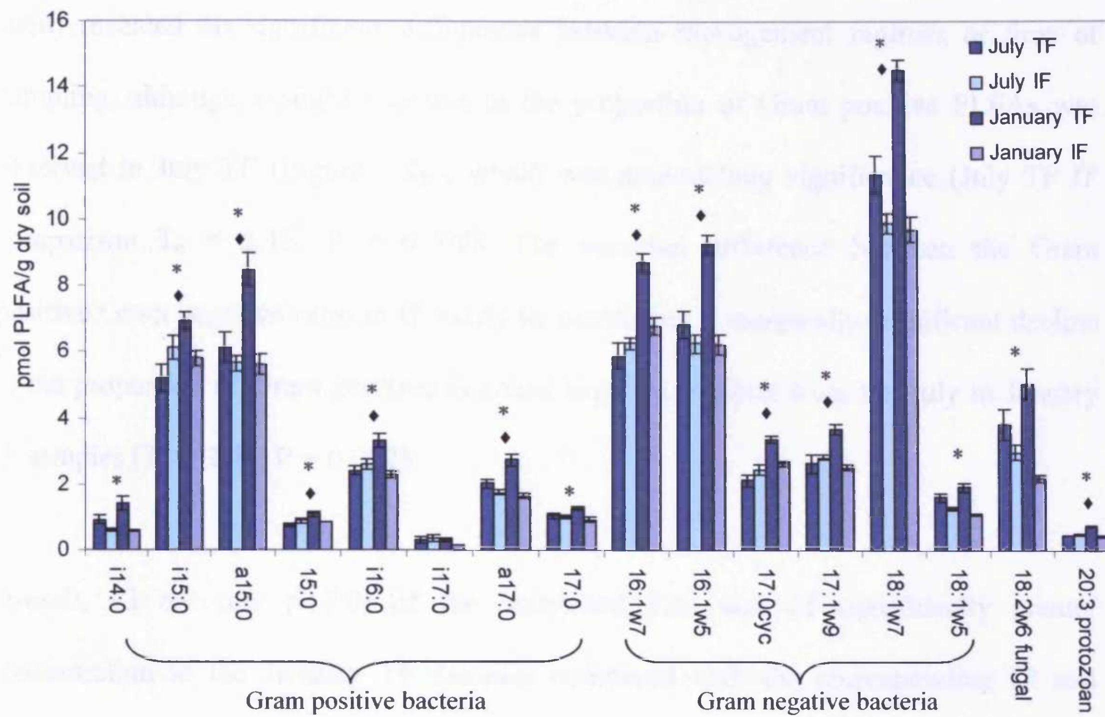

Figure 3.3 Concentration of individual PLFAs, chosen to represent the major microbial groups. Darker bars are samples from under traditional management, lighter bars from the improved field. * July TF & IF not significantly different but January TF significantly > IF; ♦ January TF significantly > July TF ($P \leq 0.05$). Means ± standard errors are displayed (n=4).

Comparison of the two main bacterial groups (Gram positive:Gram negative bacterial ratio) revealed no significant differences between management regimes or time of sampling, although a slight increase in the proportion of Gram positive PLFAs was observed in July TF (Figure 3.2g), which was approaching significance (July TF IF comparison $T_6 = 2.13$, $P = 0.078$). The seasonal difference between the Gram positive:Gram negative ratio in IF could be considered a marginally significant decline in the proportion of Gram positive to Gram negative bacteria from the July to January IF samples ($T_6 = 2.42$, $P = 0.052$).

Overall, all bar one (i17:0) of the individual FAs was of significantly greater concentration in the January TF samples compared with the corresponding IF soil (Figure 3.3). Also, higher mean concentrations in July TF than July IF samples were recorded for half of the individual FAs, although, these were not statistically significant (18:1ω7, 18:1ω5 and 18:2ω6 were close to significance at the $P \leq 0.05$ level) they were also divided almost equally between FAs representing Gram positive and Gram negative bacteria (4 Gram positive, 3 Gram negative and 1 fungal FA). In addition, the recorded concentrations for IF from the two sampling dates were not significantly different for any of the individual FAs measured.

3.4 Discussion

3.4.1 Sample Recovery

The concentration of almost all of the individual PLFAs investigated in this study was significantly higher in the January TF data set than any other sample set. One consideration as to the cause of this might be differences in extraction efficiency. Since

no internal standard was included from the start of the extraction process, the recovery efficiency cannot be determined. This appears to be a general flaw in the protocol used by the majority of studies, or at least they do not record any adjustment for, or assessment of extraction efficiency (which would not involve a lot of effort to control for). Thus, one improvement that could be made to the protocol used here would be the addition of an internal standard at the first extraction phase (chloroform:methanol:phosphate buffer extraction). The % recovery of this internal standard could then be used to adjust the final PLFA concentrations.

In theory, due to differential adsorption to soil particles, PLFA recovery might be affected by soil chemistry, although, Frostegård *et al.* (1991) found no difference in PLFA recovery from 13 soils of different organic matter content. If the high PLFA concentrations of the January TF samples were due to soil chemistry affecting recovery, then the July TF samples (being the same soil type) should also be significantly higher than the corresponding IF values. The experimental design of the extraction procedure (3 replicate batches each consisting of a full July TF and IF and January TF and IF sample set [Appendix II.I.I.I]) should have prevented any variability, in extraction between batches, from tracking through to the results. Therefore, it seems more likely that there are genuine differences between the structures of the two field microbial communities in the January samples.

3.4.2 Seasonal Variation

Assessment of microbial community structure through PLFA analysis on more than one sampling date over the year of this study, showed that although TF had a greater total PLFA concentration in January, the result from July were not corroborative, producing

no significant difference between the total PLFA concentrations of the two fields. Seasonal variation in total PLFA has been recorded previously, whereby total PLFA was highest in spring and lowest in autumn (Bardgett *et al.*, 1999a), suggesting that the higher total PLFA in January TF soil may be a result of this seasonal variation. The total fungal PLFA and fungal:bacterial ratio produced results that were similar to the total PLFA. However, the fungal and fungal:bacterial data sets showed a more defined trend in which TF was greater than IF, being statistically significant in January, but not quite significant in July.

The difference between the two seasonal results demonstrates the importance of multiple sampling dates if the aim is to produce a true picture of how the different management regimes affect microbial community structure. Some studies conclude that differences between communities were observed, based upon a single sampling date (Bardgett *et al.*, 1997; Waldrop *et al.*, 2000). While others combined the data from replicate sample dates, producing an over all significantly higher fungal:bacterial ratio associated with a traditional management regime compared with an improved system (Grayston *et al.*, 2004) or a particular soil salinity (Pankhurst *et al.*, 2001). If the data for each field from the two sampling dates of the current study are combined, the total PLFA concentration becomes significantly greater in TF than IF (Mann-Whitney test [due to unequal variances] $P = 0.031$), and the greater overall fungal:bacterial ratio in TF, is highly significant ($T_{14} = 4.05$, $P = 0.001$) in contrast with the separated July data. In fact, the fungal:bacterial PLFA measured by Donnison *et al.* (2000b) demonstrates how similar the fungal:bacterial ratios of traditional and intensively managed grasslands can be across four sampling dates from May to January, the results also differ between sites. A greater proportion of the variation in microbial community structure (measured

by PLFA), of differentially managed grasslands, has also been shown to be explained by date of sampling (39.7%) compared with grassland type (24.7%) (Grayston *et al.*, 2001). Therefore, combining the time points to give an overall comparison of management may provide misleading results and over simplifies a complex ecosystem. Since so may factors affecting the structure and function of the soil microbial community, such as microclimate, plant development and nutrient inputs, undergo seasonal variation, it seems important that microbial community structure is also compared across a comparative time scale.

3.4.3 Microbial Community Structure

Although both the group and individual PLFA results from IF soil in general showed no difference between sampling dates, the Gram positive:Gram negative bacterial ratio registered a decline in the improved field from July to January (Figure 3.2g), where the Gram negative portion of the community was slightly reduced in the July soil. This could suggest that the summer conditions in IF are less suitable for the Gram negative portion of the community. Whereas in TF, both bacterial groups respond equally well to the conditions, indicated by the equal ratios of the July and January TF soils even though there was an overall increase of PLFA in both bacterial groups in the January soils.

The fungal portion of the community remained equivalent within each field across the two sampling dates (Figure 3.2e). Therefore the fungal community appears less affected by seasonal variation of environmental factors than the bacterial community which is more ephemeral, by its very nature experiencing higher turnover of individuals due to the shorter lifecycle. In the comparison of TF and IF microbial community structure, the

greater fungal:bacterial ratio and total fungal PLFA produced by the January TF sampling, and the near significance of the July results suggests that there may be a consistently greater proportion of fungal PLFA in TF. Unfortunately, due to the lack of a suitable conversion factor, this cannot be converted directly to biomass. It does, however, at the very least indicate that the structure of the fungal communities in the two fields was consistently different. The lack of significance in the July samples may have been due to a similar community response to the relatively warm dry summer conditions, i.e., slow development of new hyphae. While the high significance of the January fungal PLFA concentration may indicate either an increased ability of the TF fungal community to maintain biomass during colder periods, or that greater buffering of the soil temperature occurs in TF due to the plant community type and organic horizon. Links between the microbial community structure, functional response, plant community and the various abiotic differences between the fields are discussed in greater detail in the general discussion (Chapter 6).

3.5 Conclusions

- There was a significant difference between fields, in all of the component parts of microbial community, in the January TF samples compared with the respective IF samples.
- The fungal communities differed between management type but are not affected significantly by sample date.
- The Gram positive:Gram negative bacterial ratio of the IF samples was significantly affected by sample date.

Chapter 4. Litterbag Decomposition Experiments

4.1 Introduction

Complex interrelated feedback mechanisms of decomposition and nutrient mineralisation processes operate between plants, which provide the main source of carbon input, and the soil decomposer community (including bacteria, fungi, protozoa and micro- and macro- invertebrates) that drive the mineralisation or immobilisation of nutrients (Knops *et al.*, 2001). Hence it is likely that the species richness and composition of a plant community exert a significant influence on the structure and activity of the concurrent soil community (Wardle *et al.*, 1997), not only in terms of nutrient inputs, including root/shoot senescence and root exudates, but also influences on the abiotic factors of soil temperature (affected by shading levels), structure and moisture (affected by root and leaf structure and leaf cover) (Chapin *et al.*, 1997; Hector *et al.*, 2000).

Experiments investigating the effect of litter species diversity on decomposition rates have generally found an idiosyncratic effect of plant species richness on decomposition, which depends on the individual species mixed into the litter and may result in both synergistic, antagonistic or non-additive effects in a complicated and unpredictable manner, when compared with expected results extrapolated from monoculture decomposition rates (Bardgett & Shine, 1999; Hector *et al.*, 2000; Knops *et al.*, 2001; Wardle *et al.*, 1997). It is suggested that litter quality influences decomposition rates (Bardgett *et al.*, 1999a; Wardle *et al.*, 1997). Litter quality (e.g. C:N ratio) differs depending upon species/functional groups, with, in general grasses having lower levels of nitrogen than herbs (Hector *et al.*, 2000; Wardle *et al.*, 1997). This has been found in

some cases to be reflected in decomposition rates, e.g. higher microbial activity on herb litter (measured by CO_2 evolution) rather than grass species (Bardgett & Shine, 1999) greater decomposition of legumes and herbs than grasses and a significant negative correlation between C:N ratio and decomposition rate of individual species litters (Hector *et al.*, 2000). However, not every study has found a significant correlation between single species C:N ratio and its decomposition rate (Bardgett & Shine, 1999). In litter mixing experiments it appears that the inclusion of functional groups rather than individual species has a greater affect on decomposition (Hector *et al.*, 2000; Wardle *et al.*, 1997). Particularly the inclusion of litter, with a high nitrogen content, into the mix which tends to enhance decomposition, while poor quality litters may inhibit the overall decomposition rate (Wardle *et al.*, 1997).

Not only have idiosyncratic effects been observed in the decomposition of mixed species litters, but also in the decomposition of test substrates in plots planted with varying species diversities. Knops *et al.* (2001) plant species richness gradient showed little effect on overall decomposition of *Schizachyrium scoparium* litter, although, greater species richness did result in lower available soil nitrate levels due to increased uptake or immobilisation. Decomposition of *Holcus mollis* litter (Hector *et al.*, 2000) along an experimental gradient of species richness produced a significant effect of diversity in the form of a complex and difficult to interpret interaction between species richness and functional group richness. On more recalcitrant substrates such as cotton strips (95% cellulose) and birch-wood strips (Spehn *et al.*, 2000; Stephan *et al.*, 2000), no effect of plot species richness was observed. However, like the litter-mixing experiments, it was the composition of the community rather than species numbers that produced an effect, legumes included in a plot, tending to increase decomposition rates

presumably as a result of increased nitrogen levels in the soil due to legume associated N fixation (Spehn *et al.*, 2000). In fact the presence of legumes in plant communities has resulted in significant increases in organic matter incorporation as well as elevated nitrogen demonstrating the stimulation of the microbial community by the inclusion of nitrogen fixing plant/bacterial associations (Girvan *et al.*, 2003).

These idiosyncratic effects seen in litter diversity approaches might be explained if the nutrient content of a plant is different when grown in polyculture compared with monoculture (Hector *et al.*, 2000), e.g. increased N due to fixation by legumes or increased phosphorus mobilisation due to mycorrhizal associations of neighbouring species. However, plants provide a variety of substrates that vary in the degree of difficulty of breakdown (Table 4.1) Thus, it should also be considered that microbial breakdown of more recalcitrant substrates, e.g. lignin, may require an additional, complementary supply of a more labile substrate to provide easily obtainable "power" (i.e. carbon source) to drive the process. In varying the species the balance of chemicals, including inhibitory substances (e.g. many secondary metabolites), is altered which may affect the rate at which decomposition progresses. Microenvironment, i.e. soil structure due to root habit and temperature/moisture may also contribute (Hector *et al.*, 2000).

If the species composition of plant communities influences decomposition rates and varying intensities of management (e.g. fertilisation, tillage and grazing) applied to grasslands produce plant communities of different species richness and composition, this would suggest that the function of microbial communities associated with grasslands under different management regimes could show different functional responses. For example, concomitant changes in the relative proportions of fungi and

bacteria within the soil community due to increased levels of fertilisation (Bardgett *et al.*, 1998; Donnison *et al.*, 2000b; Grayston *et al.*, 2001) or grazing (Bardgett *et al.*, 2001) have been recorded and are attributed to a combination of the direct effects of inorganic fertilisers and differences in litter type as a result of the altered plant community (Donnison *et al.*, 2000b).

Table 4.1 Breakdown of the main macromolecular components of plant tissues, compiled from Kögel-Knabner (2002).

Macromolecule	Main breakdown by:	Level of breakdown difficulty
Proteins (polypeptides, long chains of amino acids)	Multitude of microorganisms	High turnover rate, but some may be stabilised in soil over longer periods
Starch (polysaccharides)	Aerobic & anaerobic micro-organisms	Easily degraded
Fructan (polysaccharide)	Bacteria	Enzymes for degradation widespread among bacteria
Chlorophyll and other pigments	Unknown	Supposedly decomposed in soil, (Chlorophyll broken down within plant during senescence, carotenoids and anthocyanins accumulate
Cellulose (polysaccharide)	Fungi & eubacteria (aerobic). Certain bacterial groups (anaerobic)	Slow under aerobic or anaerobic conditions
Hemicelluloses, polyoses (non-cellulosic polysaccharides e.g. Xylans, Mannans,Glucomannans, Galactans, Pectins)	Many aerobic & anaerobic bacteria & fungi	Higher decomposition rate than cellulose
Lignin	Complete mineralisation by white rot fungi (others only induce structural changes). In soil may be a consortium of micro-organisms and fungi	Slow (aerobic only)
Tannins (polyphenols)	Unknown	Antifeedant and antimicrobial effect in plant cells, likely hydrolysable tannins decomposed more rapidly than condensed tannins.
Cutin & suberin (polyesters)	Unknown	Due to chemical structure should be relatively easy to decompose

Although plant species richness *per se,* does not necessarily affect soil microbial function, the presence or absence of specific functional groups or plant species can register an effect on decomposition rates. The traditionally managed and improved fields in the study provide plant communities of very different diversities. The species poor improved field, being mainly colonised by grasses, is likely to produce litter inputs of poorer quality, i.e. lower nitrogen, than the species rich, herb rich traditional meadow. Due to the difference in plant inputs (quality and quantity), and lack of fertiliser application for the last 10 years, it is plausible that the subtle effects of differences in litter type observed by Donnison *et al.* (2000a) might be more apparent in this system. Hence, the three main objectives of this study are to determine:

1. Whether there is any difference in decomposition rate between the two fields for a particular substrate (TF litter, IF litter, Cellulose, Oak (*Quercus*) leaves and Birch (*Betula*) wood).
2. Whether the two litter types (TF and IF) decompose at different rates, i.e. is the decomposer community tailored to the particular inputs provided by the plant community.
3. Real time changes in decomposition rate across the seasons using both a control substrate (cellulose) and seasonally changing substrates i.e. the TF and IF field litters, the quality of which will alter with time of collection.

4.2 Methods

4.2.1 Substrate Preparation

4.2.1.1 Litter Collection

Litter was collected 7-14 days prior to placing out an experiment in the field. A garden rake was used to collect primarily senescent rather than green litter from five 1m x 2m patches whose positions were determined by pacing out randomly generated pairs of numbers from the Southwest corner of each field. Any tree leaves were removed, and the litter was spread out to air dry at room temperature (Gray, 1990; Hector *et al.*, 2000; Wardle *et al.*, 1997) until constant weight was achieved (3 days). The litter was stored in this state in black plastic bags until use. Samples of undecomposed litter were also set aside for estimations of C content by combustion, total organic and ammonium N analysis using the micro Kjeldahl technique and total P (Allen, 1989).

4.2.1.2 Remazol Blue Cellulose Strips

Cellulose strips (2 x 5cm) were prepared based upon the procedure of Moore *et al.*, (1979) as follows. A sufficient number of strips (approx 6g) to last the entire experiment were boiled in 2 changes of distilled water to remove plasticizers. Having re-suspended them in a beaker containing 750ml fresh distilled water the strips were heated to 80°C in a water bath, stirring to keep them separated. At 80°C, 2.25g of Remazol Brilliant Blue R (R.A. Lamb, London) was added, followed by 150ml Sodium Sulphate solution (Sigma) (45g in 150ml distilled water; at 80°C), added gradually over a 15 minute period. Tri-Sodium Orthophosphate solution (Sigma) (3.96g in 22.5ml distilled water; at 80°C) was finally added and the mixture stirred at 80°C for 20 minutes. The strips were then rinsed with hot water until the water ran clear. This was followed by autoclaving

for 15 minutes in two changes (1.5 litres each) of distilled water to remove excess colour. The strips were air dried and stored in darkness to prevent fading.

Rate of cellulose metabolism was assessed by extracting the remazol blue dye from the cellulose strips. Upon cellulose hydrolysis, dye is lost proportionally to the rate of hydrolysis, hence extraction of the dye following decomposition provides an estimate of the level of cellulose metabolism. Control cellulose strips (undecomposed but from same dying session) and decomposed samples were placed in individual 100ml bottles containing 60ml 0.06M KOH (Sigma). All bottles were capped and autoclaved at 120°C for 15mins. The extracts were allowed to cool in darkness. The optical density was then read at 595nm (Unicam Heλios γ, spectrophotometer). The absorbance recorded at 595nm was converted to percentage of cellulose weight (W = % remaining of initial weight) (Equation 4.1)

Equation 4.1 $$W = 100 - \left(\frac{(\bar{c} - x)}{\bar{c}} \times 100 \right)$$

Where: $\bar{c}$ is the mean absorbance of the control strips

x is the absorbance of the sample strip

4.2.2 Experimental Design: Seasonal Experiments

Litterbags 10 x 10cm were constructed from 1mm nylon mesh (which excludes adult earthworms (Gray, 1990). The double length piece of mesh was folded and two open sides were stitched close with pure nylon thread, the final opening was stapled shut. Three separate types of litterbags were used:

1. Litter collected from the Traditional Field (TF) (1g per bag, 24 bags per field)
2. Litter collected from the Improved Field (IF) (1g per bag, 24 bags per field)

3. Remazol Blue stained cellulose strips 2 x 5 cm (1 strip per bag, 12 bags per field in the first Feb-Mar 2002 experiment and 24 bags per field subsequently)

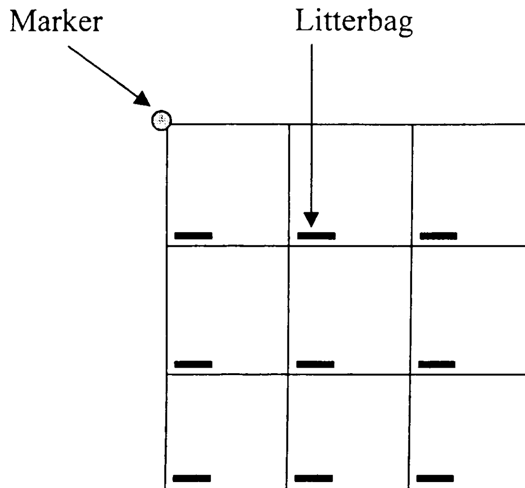

Figure 4.1 Layout of litterbags within $1m^2$ quadrats

Bags were placed in the fields at the beginning of four seasonal experimental periods, 4th Feb-3rd April 2002, 14th May-27th June 2002, 26th Aug- 18th Oct 2002 and 19th Dec 1002-18th Feb 2003. For each of the seasonal experiments the litterbags were laid out in eight randomly positioned $1m^2$ quadrats (positions determined as with litter collection above) per field, each quadrat being divided into 9 sections (3 x 3) (Figure 4.1). Each litter type was equally dispersed among the quadrats giving 3 x TF litter, 3 x IF litter and initially 1-2 cellulose but subsequently 3 x cellulose in each quadrat. This reciprocal design provided the opportunity to study the decomposition rates of each litter in the field from which it was collected and in the other field, the treatment combinations being: (1) TF litter decomposing in the traditional field, (2) IF litter decomposing in the traditional field, (3) TF litter decomposing in the improved field, (4) IF litter decomposing in the improved field. The field litters would obviously vary in quality

depending on the season of collection, thus cellulose provided a control substrate of constant quality, which could be compared between all four seasonal experiments.

The litterbags were inserted into the ground vertically at a depth of 0-10cm using a trowel to make a small slit in the soil, in order to cause minimal disturbance to the surrounding vegetation. Within the quadrats litterbags were randomly allocated to positions to avoid any interference from neighbouring substrates due to the direction of water drainage.

Time of retrieval varied depending upon the suspected effects of temperature and moisture on the decomposition rate, i.e. intervals between the retrieval of samples were longer during the winter than during warm, damp periods. Subsets of the litterbags were removed at three time points during each experimental period. At each litterbag harvest, one random bag of each substrate type was lifted from each of the quadrats, except for the first session where of the cellulose bags, only four were removed from each field at a time.

4.2.3 Analyses of Substrates

Upon retrieval, the litterbags were carefully washed to remove soil particles (Bardgett & Shine, 1999) and any plant roots extracted with forceps. Following this the bags were air dried at room temperature (covering the cellulose strips to avoid colour loss due to UV light). When constant weight was attained, the staples were removed and the bag plus litter weighed to avoid the loss of material on removal, the weight of the bag was then subtracted (Bardgett & Shine, 1999). Cellulose strips were removed from the litterbags and stored in foil wraps until remazol blue extraction. Rate of cellulose

metabolism was assessed by extracting the remazol blue dye from the cellulose strips (Section 4.2.1.2).

After the assessment of air-dried weight, litter substrates were removed from their bags and oven dried 105°C overnight to determine oven dry weights and moisture content (% oven dried weight). C:N ratios were calculated for each experimental period from TF and IF litter samples before decomposition. Carbon was determined through combustion (550°C, 5h) (Allen, 1989). Total N was extracted using the micro Kjeldahl technique (sulphuric acid digest mixture with selenium catalyst, (Allen, 1989) digest diluted to 50ml). N was quantified by distillation/titration (Allen, 1989) of 5ml aliquots of the digest.

4.2.4 Experimental Design: Extended Decomposition Experiment

Following the set of seasonal experiments carried out during 2002, a single much longer decomposition experiment was run for a 5 month period during 2003, from 15th May-15th Oct 2003. Field litter and cellulose strips were used as before, however, two substrates of greater recalcitrance, oak leaves (*Quercus*) cut perpendicular to the petiole into approximately 1cm strips and birch (*Betula*) wood veneers 20 x 20 x 2mm, were also used. The substrates were added to the litterbags as follows:

1) TF litter 1g air-dried weight, 60 bags per field.
2) IF litter 1g air-dried weight, 60 bags per field.
3) Remazol blue dyed cellulose strips, 36 bags per field.
4) Oak leaves, 1g air-dried weight, 30 bags per field.
5) Birch wood veneers, air-dried, 30 bags per field.

The bags were placed into the fields as detailed above (Section 4.2.2), except that 12 bags were placed in a 4 x 4 arrangement, making full use of the outer edge of the quadrat, rather than the 3 x 3 arrangement used previously. In each field, eight quadrats contained 6 x TF litter, 6 x IF litter and 4 x cellulose litterbags. One quadrat contained only the TF and IF litter, without the cellulose, in a 3 x 4 arrangement, and a further 5 quadrats contained 6 x oak leaf and 6 x birch wood veneer bags in a 3 x 4 arrangement. Quadrats and bags were positioned randomly as above. Upon removal from the field, 6 of each substrate type were removed from randomly drawn quadrats and positions therein. Field litters were removed after 7, 14, 22, 31, 44, 58, 75, 89, 104 and 132 days, and cellulose strips after 14, 31, 44, 58, 75 and 89 days. Oak leaf bags were left in the ground for 14, 44, 75, 104 and 132 days, while the wood veneers remained in the fields for slightly longer being collected after 44, 75, 104, 132 and 153 days.

Upon removal, bags were washed, any roots removed, air-dried and weighed as above. Wood veneers were removed from the bags for weighing since, unlike the leaf litters, there was no risk of losing any fragments. Cellulose weights were calculated following extraction of the remazol blue as above. Following assessment of the air-dried weight, natural litter substrates were removed from their bags and oven dried 105°C overnight to provide oven dry weights and moisture content (% oven dried weight). For each of the substrates, the content of alternate bags was homogenised with a coffee grinder (Braun) and divided into two samples of between 0.1-0.3g, one of which was used for the estimation of carbon (C) content and the other for total organic and ammonium nitrogen (N) and phosphate (P).

Carbon was determined by combustion (550°C, 5h) (Allen, 1989). Total N and P were extracted using the micro Kjeldahl technique with a sulphuric acid/peroxide digest mixture using selenium and lithium sulphate catalysts and diluted to 50ml (Allen 1989). N was quantified by the distillation/titration of 5ml aliquots of the digest (Allen 1989) and P determined using 1ml of digest with Phosver3 (Hach) molybdovanadate blue reagent pillows for 10ml samples (Hach Company, 1992), with colour development read at 890nm (protocol Appendix I.I) (Unicam Heλios γ, spectrophotometer).

4.2.5 Microclimate and Soil Chemistry

Local meteorological data providing information on temperature, precipitation and soil nutrient status and pH are recorded in Chapter 2.

4.2.6 Statistical Analysis

Weight loss was plotted, as % of initial weight, for each substrate in each field. Percentage data were arcsin transformed except for data series where some values were greater than 100% (cellulose and wood veneers). Linear or non-linear regressions were fitted to arcsin transformed or un-transformed data where appropriate to enable the comparison of decomposition rates. Linear regressions were compared by Analysis of Covariance (General Linear Model) ANCOVA (GLM), non-linear regression curves were compared as detailed below (Section 4.3.3). Linear regression and comparisons were performed using MINITAB™ version 13, non-linear regression models were fitted using GraphPad Prism ™ version 4 and comparisons made using a GraphPad protocol described below (Section 4.3.2).

4.3 Results

4.3.1 C:N Ratio of Field Leaf Litters: Seasonal Experiment

The C:N ratio of leaf litter from each field varied depending upon the time of year that it was originally collected (Figure 4.2). Litter from the traditional field (TF) had a significantly higher C:N ratio than litter collected from the improved field (IF) in February 2002 and December 2002, on the other hand IF recorded a significantly higher C:N ratio than TF litter when collected in August 2002 while there was no significant difference between litter from the two fields when collected in May 2002. C:N ratios were compared by One Way ANOVA ($F_{7,16}$=52.4, P<0.001) with the Turkey-Kramer *a posteriori* pairwise comparison of means. Therefore, in terms of C:N ratio, IF litter quality was better during the February and December decomposition experiment, while TF litter quality was higher during the August experiment.

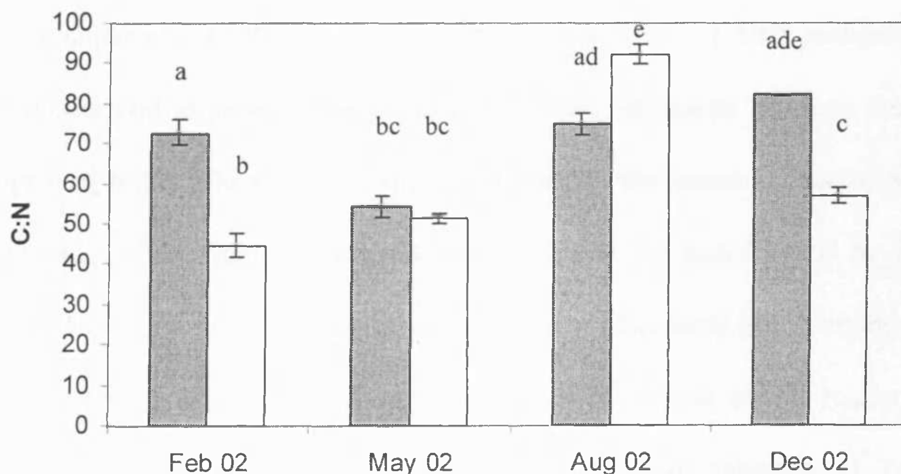

Figure 4.2 C:N ratios of field litter (prior to decomposition) collected for seasonal litter decomposition experiments. Means and standard errors shown, bars with the same letters are not significantly different at the P≤0.05 level as determined by Turkey-Kramer pairwise comparisons.

4.3.2 Field Litter Decomposition: Seasonal Experiment

Decomposition of field leaf litters appeared to be a two stage process of rapid initial decomposition followed by a considerably slower stage. Overall decomposition rates varied between seasons, being at their highest during the warm damp spring/early summer period of May-June 2002 (Figure 4.3). Total decomposition also varied with season, the remaining weight of litter in the litterbags falling to 60-85% during the Feb 2002 initiated experiment and 40-60%, 55-70% and 65-75% during the May, Aug and Dec 2002 initiated experiments respectively. Decay rates for all experiments are summarised in Table 4.4.

The reciprocal nature of the experimental design enabled litter decomposition to be compared both between litters within fields, and between fields within litter type, producing the following four comparisons of the arcsin transformed data (% of initial litter weight remaining): (1) TF litter compared with IF litter in the traditional field, (2) TF litter compared with IF litter in the improved field, (3) TF litter compared between the traditional and improved fields and (4) IF litter compared between the traditional and improved fields. The results of these analyses for the seasonal data are summarised in Table 4.2. Field litter decomposition could not be transformed to fit a linear regression model. Hence, exponential decay curves (Equation 4.2) (Graphpad Prism™ v4), were fitted to these data and comparisons of the whole curves made (Motulsky, 1998), regressions and comparison equations are recorded in Appendix III, Table III.I.

Equation 4.2 $Y = Span * e^{-KX} + Plateau$

Where the decay curve starts at *Span+Plateau* and decays to *Plateau* with rate constant *K*.

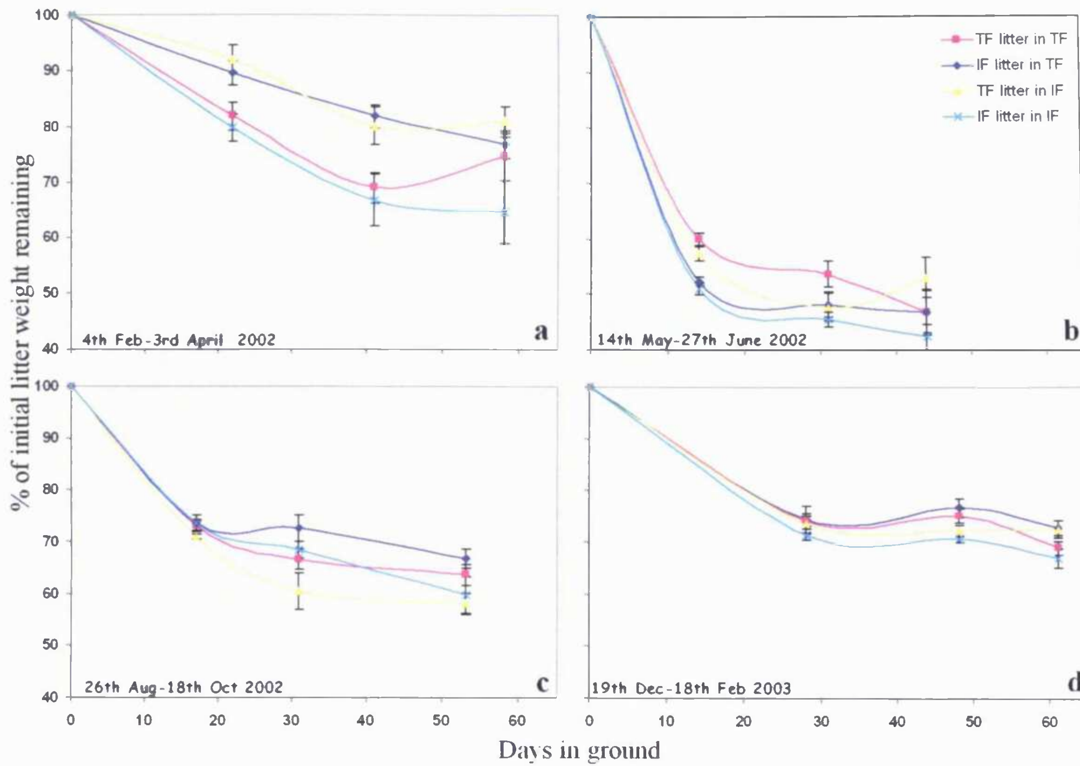

Figure 4.3 Decomposition of field litters in TF and IF in four seasonal experiments during a twelve month period. Means and standard errors are shown (n=8).

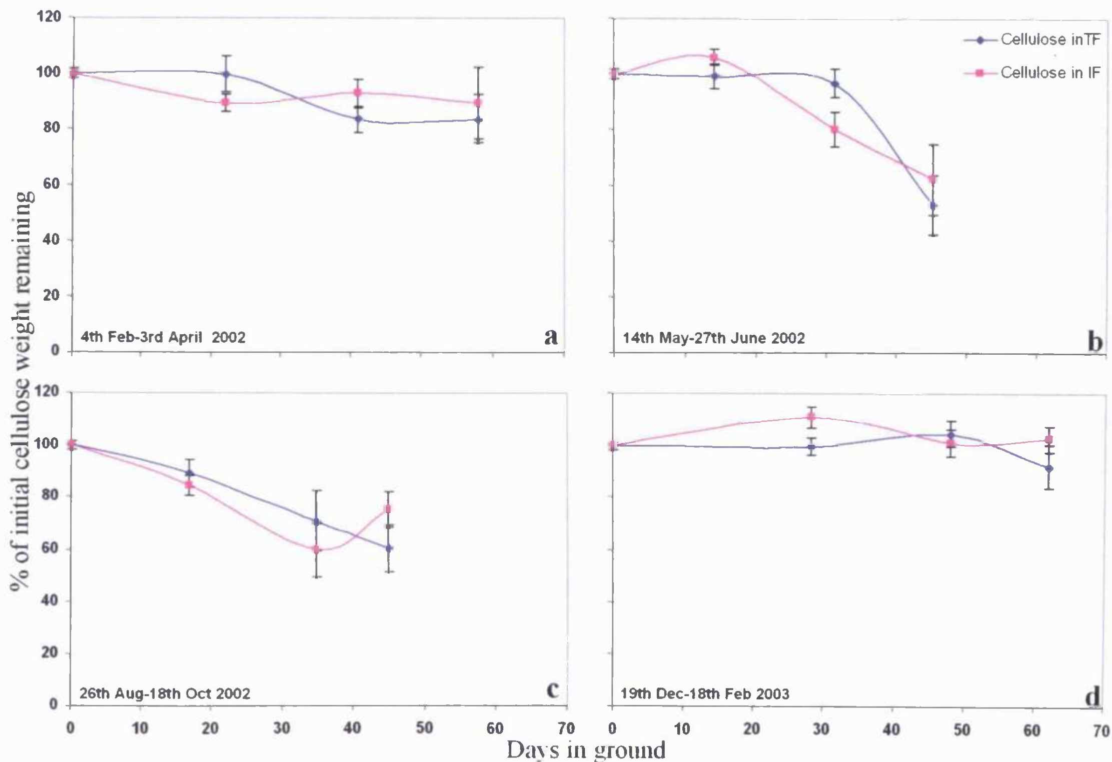

Figure 4.4 Cellulose decomposition in TF and IF over four seasonal experiments, means and standard errors shown (n=8).

Table 4.2 Comparison of field litter exponential decay curves in seasonal experiments. Letters in parenthesis indicate which litter type (comparisons 1 & 2) or which field type (comparisons 3 & 4) produced the faster and greatest overall decay.

Experimental period	**Comparison**			
	1) TF v IF litter in TF	2) TF v IF litter in IF	3) TF litter in TF v IF	4) IF litter in TF v IF
4th Feb-3rd April 2002	* (IF)	*** (IF)	ns	ns
14th May-27th June 2002	ns	* (IF)	ns	ns
26th Aug-18th Oct 2002	ns	ns	* (TF)	ns
19th Dec-18th Feb 2003	ns	ns	ns	ns

*P≤0.05, **P≤0.01, ***P≤0.001, ns = not significant

4.3.3 Cellulose Decomposition: Seasonal Experiments

Decomposition of the cellulose strips provided a substrate that was of constant quality that could be compared across all four seasonal experiments. Decomposition of cellulose was much slower than that of the field litters and was highly variable (Figure 4.4). Cellulose % weight remaining after decomposition was calculated from the amount of remazol blue extracted. Undecomposed control strips were also extracted to provide a pre-decomposition colour level. The mean colour of the control strips provided the 100% mark, however, some strips had a calculated weight remaining, of slightly greater than 100% due to slight variation in the remazol blue content of undecomposed strips. Where these strips experienced no decomposition, or fragments of soil remaining on them produced a slight discoloration of the extract their calculated weight was recorded as slightly over 100%, meaning that the data could not be arcsin transformed.

Linear regression models (Appendix II, Table III.II) were fitted to the decomposition of cellulose inserted into the fields in Feb, Aug and Dec-02, however, the slopes of both the Feb IF and Dec IF and TF data did not differ significantly from zero (Table 4.3). The slope of the Feb TF regression was significantly different from zero, however its slope and y intercept were not significantly different from the IF decomposition (Table 4.3). Linear regressions of Aug initiated decomposition required the removal of one extreme residual from the IF data to meet the requirements of normally distributed residuals, however, the removal of this data point did not alter the regression equation. It was thus considered that the regression was valid. Both regression lines were significantly different from zero, although they were not significantly different from each other (Table 4.3). The decomposition of cellulose during the May initiated experiment could not be transformed to fit a linear regression model since it underwent a lag prior to a period of comparatively rapid decay, instead the decay curve fitted a second order polynomial decline (Equation 4.3). The whole curves of the May initiated cellulose decomposition were compared (Motulsky, 1998) and were not significantly different (Table 4.3). The fit of the regressions to all of the cellulose decomposition data sets were fairly poor, ranging from R^2 of 29-54% for valid regressions, due to high variability in weight loss among cellulose strips removed at each time period. The results of the regressions, fits and comparisons of regressions are summarised in Appendix II, Table III.II.

Equation 4.3 $$Y = A + BX + CX^2$$

Table 4.3 Comparisons of cellulose decomposition rates in TF and IF, all linear regressions except May-June data fitted to non-linear regression. Linear regressions compared by ANCOVA (GLM), non-linear regression compared using Equation 4.2. NA = not applicable to non-linear model.

Experimental period	**Comparison**		
	Slope sig. diff. from zero		Regression for decomp. In each field sig. diff. from each other
	Decomposed in TF	Decomposed in IF	
4th Feb-3rd April 2002	**	ns	ns
14th May-27th June 2002	NA	NA	ns
26th Aug-18th Oct 2002	***	**	ns
19th Dec-18th Feb 2003	ns	ns	ns

*P≤0.05, **P≤0.01, ***P≤0.001, ns = not significant

4.3.4 Field Litter Decomposition: Extended Decomposition Experiment

Decomposition of the field litters over the extended period of 15th May – 24th Sep 2003 (Figure 4.5a) resulted in a similar initial pattern of fast decomposition as displayed in the 2002 seasonal experiments (Figure 4.3), 20-35% of the litter weight being lost within 14 days. However, this is less than that lost during the same initial period of the May 2002 experiment (30-55%). Following the initial burst of decomposition, the rate slowed as previously observed (Section 4.3.2) and continued at a similar rate until 89 days when the recorded weights became more variable and the decomposition rate more or less constant up to the end of the experiment at 132 days. From 89 days the infiltration of the litter with plant roots became a significant problem since there was probably as much root as litter remaining in each bag, thus variability of the litterbag weights, following root removal, becomes far more pronounced.

As with the seasonal litter decomposition, an exponential decay curve (Equation 4.2) was fitted to the arcsin transformed data (Appendix III, Table III.III). Comparisons of

the curves performed as above (Section 4.3.2), revealed no significant differences between the decomposition curves of either the two litters within fields or each litter between fields (Appendix III, Table III.III).

Since both the 2002 and 2003 May –June experimental periods were started on 14-15th May and removed up to 44 days, decomposition over this period for the two respective years could be directly compared. As described above, the initial weight loss differed between years, however the period of initial rapid weight loss was between 14-20 days for both years. Comparisons of the exponential decay curves up to 44 days decomposition (Appendix III, Table III.III) during May-June 2003, produced a significant difference for the TF litter between fields (overall decomposition being faster in IF) (Figure 4.5a). This is in contrast with May-June 2002 in which the only significant difference was found between litter types decomposing in IF (Table 4.2). On the other hand a similar result was produced by Aug-Oct 2002 litter decomposition (Table 4.2) in which the overall decomposition of TF litter between fields was again faster in IF.

4.3.5 Cellulose Decomposition: Extended Decomposition Experiment

Cellulose decomposition was gradual and registered very little weight loss for the first 14 days (Figure 4.5b). It was again not possible to arcsin transform the data since some values were greater than 100% due to the use of separate control strips for the determination of pre-decomposition remazol blue colour levels (see Section 4.3.3). The decline in weight could not be transformed to fit a linear regression model. Rather, it could only be fitted to a 3rd order polynomial model (Equation 4.4) (Appendix III, Table III.III), beginning with a lag period prior to the main decomposition stage, and ended

with a period of slow down due to an increase in variability (Figure 4.5). Variability of weight loss from bag to bag at individual time points was high and could range from 1.4-92.3% at a single time point (decomposition in IF at 75 days). The fit of the non-linear models to the TF and IF data was 59% and 50% respectively. Comparison of the curves produced no significant difference in cellulose decomposition (F = 0.4093, P = 0.8014, $DF_{separate}$70, $DF_{combined}$73) between the traditional and improved fields (Appendix III, Table III.III).

Equation 4.4 $Y = A + BX + CX^2 + DX^3$

4.3.6 Oak Leaf Decomposition: Extended Decomposition Experiment

The decomposition of oak leaf pieces was again gradual but declined much less than either the cellulose or field litters, retaining in the region of 62-88% of the initial litter weight after 132 days in the field (Figure 4.5c). Like the cellulose decomposition, weight loss at individual time points was quite variable, however weight loss appeared to be initiated marginally more rapidly than was recorded for cellulose, falling to a mean of 95% initial weight within 14 days as opposed to 98% (Figure 4.5b & c). Linear regressions of the arcsin transformed data explained 56% and 69% of the data in TF and IF respectively (Appendix III, Table III.III). ANCOVA (GLM) of the regression lines revealed no significant differences in oak leaf decomposition between the two fields (comparison of slopes, $F_{1,58} = 0.9302$, P = 0.3388; and y intercepts, $F_{1,59} = 1.9156$, P = 0.1716).

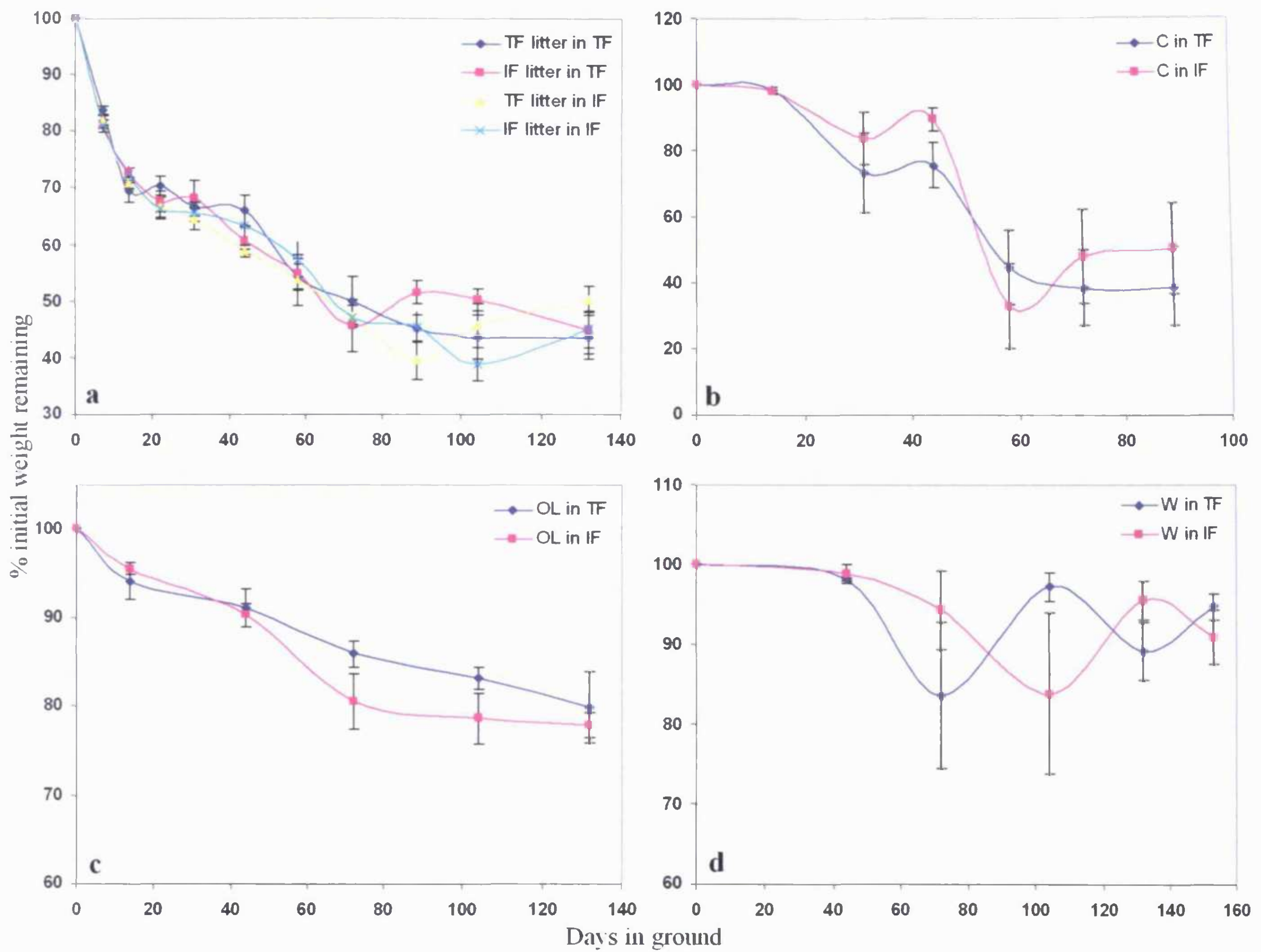

Figure 4.5 Extended decomposition of: a) field litters, b) remazol blue stained cellulose strips, c) oak leaf pieces and d) birch wood veneers, in the traditional and improved fields. Means and standard errors shown, n=6.

4.3.7 Wood Veneer Decomposition: Extended Decomposition Experiment

Decomposition of the birch wood veneers was in most cases minimal, the plot of mean % initial weight remaining (Figure 4.5d) not diverging significantly from zero (Appendix III, Table III.III). However, in the case of some individual bags, the weight loss of the veneers was as much as 59% (IF, 104 days). There was no significant difference between the rates of decomposition between the fields (comparison of slopes, $F_{58} = 0.0377$, $P = 0.8468$). Both fields produced similar numbers of veneers that had lost ≥10% of the initial weight, being 8 out of a total of 29 in TF and 8 out of a total of 30 in IF (one bag was missed from TF, during the collection at 132 days). In both fields, the first wood veneer to lose ≥10% of its initial weight was recorded at the second harvest following 75 days in the ground.

4.3.8 Nutrient Status of Field Litters and Oak Leaf Litterbags During the Decomposition Process

The C:N ratio, C:P ratio, total organic and ammonium N concentration and total P concentration of the reciprocal field litter extended decomposition experiment (May-Oct 2003) was determined from litter samples following 7, 14, 31, 75 and 104 days decomposition for C and N and 14, 75 and 104 days for P. Prior to decomposition the C:N ratio of IF litter was slightly but not significantly higher (two sample t-test, $T_4 = 1.57$, $P = 0.193$) than TF (Figure 4.6a). By 14 days, the mean C:N ratios of both litters in both fields had reached the same ratio and remained roughly constant for all subsequent time points analysed.

The N concentration in the litter samples prior to decomposition ranged from 1.3-2.1% (oven dry wt), however, there was no significant difference between the initial N

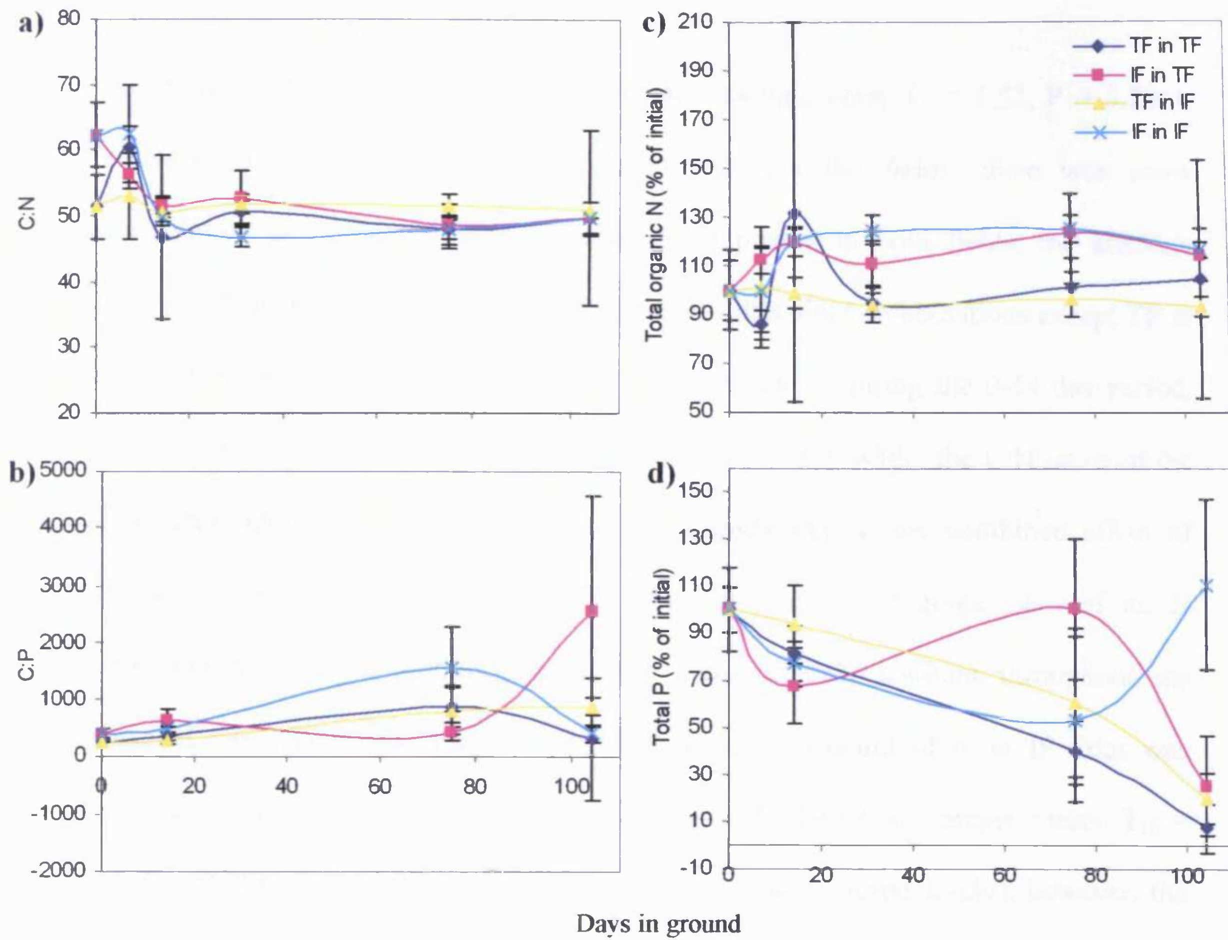

Figure 4.6 Nutrient concentrations in TF and IF litters during decomposition, a) C:N ratio, b) C:P ratio, c) total organic and ammonium N (% of initial concentration at t_0, in oven dried litter) and d) total P concentration (% of initial concentration at t_0, in oven dried litter). Means and standard errors displayed (n=3).

concentration of the litter from each field (two sample t-test $T_4 = 1.52$, $P = 0.204$). Following the placement of the litterbags out into the fields, there was some accumulation of nitrogen in the IF litter decomposing in both fields, the absolute amount of N increasing between 0-14 days for all litter-field combinations except TF in IF which declined slightly (Figure 4.6b). Concomitantly, during the 0-14 day period, the C:N ratio of TF in IF remained approximately constant, while, the C:N ratios of the other three litter-field combinations declined rapidly due to the combined effect of rapidly decreasing C and increasing N. The TF litter, on average, showed no N accumulation (absolute amount), remaining approximately constant throughout the course of the experiment (Figure 4.6b). The absolute amount of N in IF litter was significantly higher than that in TF litter at 31 and 75 days (two sample t-tests, $T_{10} = 4.85$, $P = 0.001$; $T_{10} = 4.38$, $P = 0.001$ for 31 and 75 days, respectively), however, the variability of TF litter replicates decomposing in TF was particularly high at 104 days resulting in no significant difference between litter types at this time point.

The oak leaf litterbags showed a much more distinct change to C:N ratio and absolute amount of N during decay (Figure 4.8a). The C:N ratio of the oak leaves, which was significantly higher than that of the field litters (Figure 4.7), followed a rapid exponential decay model, levelling off after about 25 days of decomposition. The comparison of the fitted exponential regression models showed that there was no significant difference between the decline of C:N ratio of oak leaf litterbags in the two fields (Appendix III, Table III.IV). Total N (absolute amount) increased rapidly towards an asymptote (Figure 4.8b). Following a natural log transformation of the x data (days), the N concentration data satisfied the constraints of a linear regression model. The

linear regression lines for oak leaf N decomposing in TF and IF were compared as above and no significant difference was found between the slopes or y intercepts (Appendix III, Table III.IV).

The P concentrations (mg P g^{-1} dry wt.) of two litter samples from 104 days of decomposition (one each from IF in TF, and TF in IF) were particularly low, about zero. P measurement by the molydovanadate blue method is particularly sensitive to acidity and it may have been that the acidity of these two samples was too high to allow colour development (possibly due to pipetting inaccuracy when adding the acid digest mixture to the litter samples). The inclusion of these two samples in the % initial P data set made very little difference to the overall pattern of the data. However, they made a considerable difference to the pattern of the C:P ratio, driving the data points for IF in TF and TF in IF right off the scale of all of the other measurements. Hence, it was considered that they should be removed from the C:P data set to enable realistic comparisons of the C:P ratios since their extreme values were most likely a result of high acidity.

The absolute amount of total P in the field litters before decomposition was in the range of 0.16-0.38% (oven dry wt). Following decomposition the P concentration tended to decline to almost zero in both litter types (Figure 4.6d), although the variability was quite large (particularly with IF litter in IF at 104 days). There was no comparable difference between litters in terms of P losses. The C:P ratio remained approximately constant throughout the period of the experiment (Figure 4.6d).

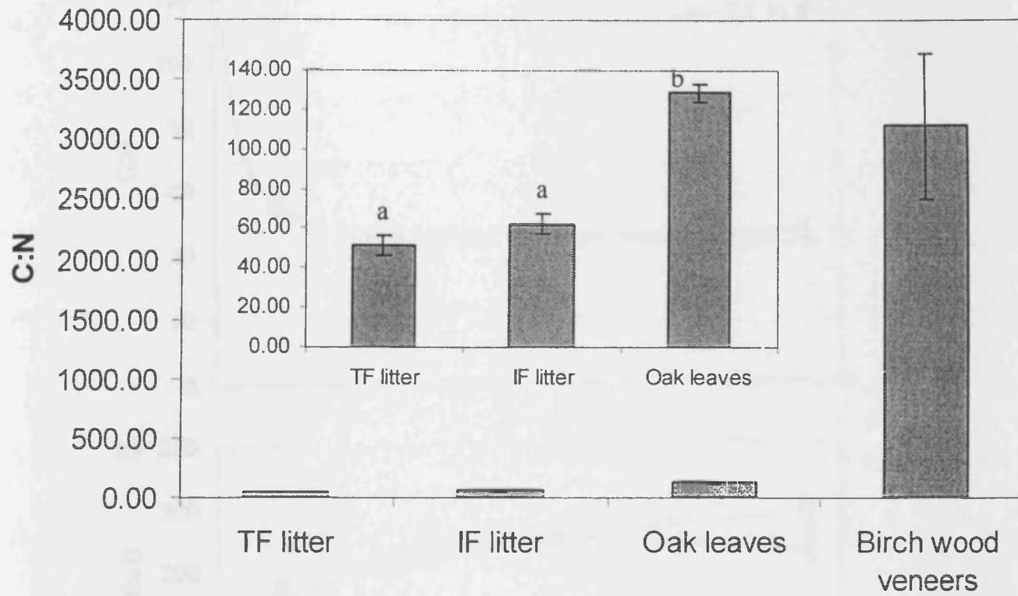

Figure 4.7 C:N ratios, prior to decomposition, of natural substrates used in the extended decomposition experiment. Expanded version of the first three substrates inset. Means and standard error indicated, n=3. Bars denoted with the same letter are not significantly different at P = ≤0.05 (one-way ANOVA with Turkey's pairwise comparisons).

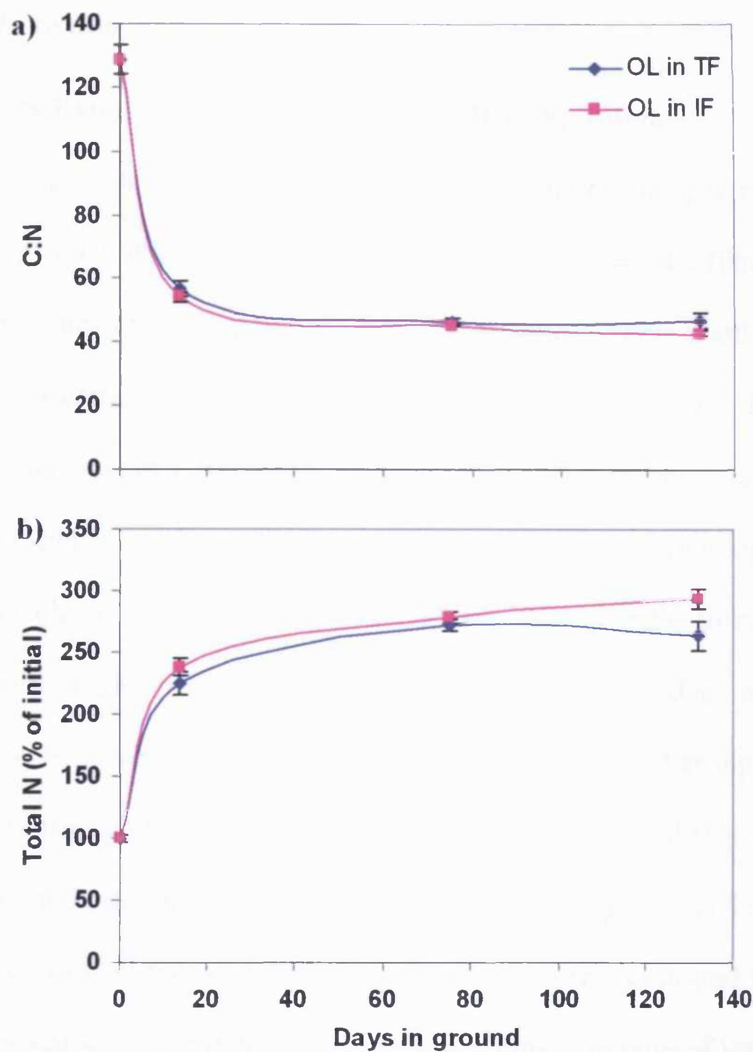

Figure 4.8 Change to C:N ratio and total organic and ammonium N (% of initial concentration at t_0, in oven dried litter) of oak leaves during decomposition in TF and IF. Means and standard errors are shown, n=3.

4.4 Discussion

4.4.1 C:N Ratios of Field Litters Prior to Decomposition

It is often suggested that plant communities of contrasting species richness, or more importantly functional group structure, produce litter inputs of different quality resulting in different rates of decomposition (Bardgett & Shine, 1999; Wardle *et al.*, 1997). For example, a significant negative correlation between C:N ratio and litter decomposition of plant monocultures has been observed (higher nitrogen = faster decomposition whereby, legumes > other herbs > grasses), i.e. grasses contain a higher C:N ratio than forbs (Wardle *et al.*, 1997). Therefore the litter inputs from grass dominated communities might be expected to decay more slowly than a mixed forb rich community. However, the seasonal affects on the quality of litter inputs appears to have undergone little scrutiny, although Wardle *et al.* (1997) noted that the effects of litter mixing varied depending upon the plant litter functional groups and the time of harvest. The analysis of C:N ratios in this study, from field litters collected from four seasonal time points across a year demonstrate how much the C:N ratio of litter inputs can vary, to the extent that the lower C:N ratio, better quality litter, changes from being the IF litter to TF litter, depending upon the time of year at which the samples were taken (Figure 4.2).

It is also interesting that in fact the pre-decomposition analysis of C:N ratio shows that the litter collected from the grass dominated IF is for much of the year either of better quality (in terms of C:N ratio) or the same as that from the forb rich TF. This was unexpected since, in general, grasses are considered to have a higher C:N ratio than forbs (Wardle *et al.*, 1997). However, Figure 4.2 demonstrates that litter collected from IF during August 2002 had a higher C:N ratio than that collected from TF. There was no

significant difference in C:N ratio between TF and IF litters collected in May 2002, this same result was repeated in May 2003 for the litter collected for the extended decomposition experiment. This together with the fact that the autumn/winter (Dec/Feb 2002) results both maintain higher C:N ratios in TF litter, suggest that there is likely to be a consistency in the results produced and that studies using litterbags should be aware of seasonal differences in grassland litter quality.

The IF litter was generally maintained at a constant C:N ratio during the year except for an increase in August 2002, this corresponds with the period shortly after the annual hay cut during which a large proportion of litter accumulation consists of hay debris. Hay is cut prior to any natural senescence processes, thus many compounds which may be translocated out of the tissues during natural senescence would remain in the cut material. During this seasonal period there would also be an increase in the use of C-compounds employed in structural strengthening of the flowering stems, such as lignin, which are of greater recalcitrance.

TF litter on the other hand, remained of constant C:N ratio throughout the year except for a decrease in May in which it fell to the level of the IF litter. In Britain, May is the peak time for regeneration of herbaceous perennials. During this period of rapid leaf generation/expansion there tends to be an investment of defensive secondary metabolites in young tissues produced from over-wintering buds (Zeleny *et al.*, 1997). Hence, these secondary metabolites might alter the C:N ratio from its normally steady state. Alternatively, the rapid expansion of young soft tissues may reduce the proportion of C prior to the accumulation of structural C in the toughening of the fully expanded leaves.

4.4.2 Seasonal Decomposition

Temperature and moisture are significant determinants of decomposition rates (Bardgett *et al.*, 1999b; Grayston *et al.*, 2001; Schimel *et al.*, 1999), thus, as expected, differences in overall decomposition rates of each substrate in each field occurred between the seasonal data (Figure 4.3 & Figure 4.4), being fastest and of greatest extent during the warm damp period of May-June 2002.

In the reciprocal comparisons of the decomposition of field litters (which is a comparison of both rate and extent of decomposition), the four possible treatment combinations (Section 4.2.2) mean that the decomposition of the two litters could be compared within each field to determine whether the microbial community was more able to decompose litter produced by its associated plant community. Litter decomposition could also be compared between fields, this determines whether one field has a decomposer community operating at a higher rate than the other when dealing with a particular substrate type. If this were the case then both litters would be undergoing significantly greater decomposition in one field than the other. In respect to Table 4.2 this would mean, for example, that for the comparisons (3) TF litter in TF & IF and (4) IF litter in TF & IF, both litters would undergo significantly faster decomposition in the same field.

In the case of the seasonal field litter data, and the first 44 day section of the extended field litter decomposition, there was no overall difference between fields; decomposition of either type of litter was not more rapid in one field than the other. However, in terms of comparing the two litters within each field some significant

differences in decomposition did occur (Table 4.2). Where there was a significant difference between litter types within a field, it was in all cases IF litter that decomposed faster and to a greater extent. This corresponds in part with the C:N ratios of the litter prior to decomposition particularly in the improved field and in this respect is in agreement with the findings of a number of studies in which a negative relationship was observed between C:N ratio and litter decomposition (Hector *et al.*, 2000; Spehn *et al.*, 2000; Wardle *et al.*, 1997). For example, during the experimental periods where the IF litter had an equivalent or lower C:N ratio than the TF litter, decomposition of the IF litter was significantly (or almost significantly, December 2002 $P = 0.059$) greater. Conversely, when the C:N ratio of IF litter increased in August, becoming greater than that of TF litter, there was no significant difference between the decomposition of the two litters in IF. Thus it appears that the microbial community in IF is more sensitive to decreasing field litter quality than that of the traditional field. This does not appear to be because a larger proportion of the microbial community in IF is suited to the rapid decomposition of more labile substrates, since the decomposition of the lower C:N ratio litter was not significantly different between fields. The fact that the decomposition of IF litter was greater than that of TF in the improved field during May-June 2002, even thought the C:N ratios were equivalent, suggests that although C:N ratio plays a part in determining the degree of decomposition, other aspects of the substrates chemical composition (such as the presence of secondary metabolites) are likely to affect the ability of the microbial community to decompose the substrate.

Although the statistically significant differences between litter decomposition primarily occurred in the improved field, there was generally no significant difference in the rate of decomposition of each litter type between fields. Only during the Aug-Oct 2002

period was there a significant difference observed and that was of greater TF litter decomposition in the traditional field than the improved field. One therefore might have expected to find a difference between the decomposition of TF and IF litters in TF. However, the lack of statistical significance may be a result of the inherent difficulties of patchiness of decomposition and the infiltration of the litterbags by plant roots, which are inevitably difficult to completely remove. The problem of patchy decomposition also occurs with the more recalcitrant substrates such as the low nitrogen high phenolic containing oak leaves and the extremely low nitrogen high lignin wood veneers. These are decomposed by a far more limited portion of the microbial community (Table 4.1) and thus a few of these bags were decomposed to a much greater extent, presumably where they were inserted in close proximity to patches which contain populations of these microbes.

4.4.3 Substrate Quality

Overall decomposition in each field appeared to be initially dependent on the quality (C:N ratio) of the substrate. For example, from the comparison of the four types of substrate (the field litters being counted as the same substrate type), in the extended decomposition experiment, which cover a range of substrate C:N ratios (Figure 4.8) (and correspondingly recalcitrance), the relative decomposition rates correspond closely with those found by previous studies of leaf litter (Gartner & Cardon, 2004), cellulose (Hu & vanBruggen, 1997) and wood (Tanesaka *et al.*, 1993). The average decay rates found in this study are summarised in Table 4.4.

Wood veneer decomposition was minimal on average due to its high lignin and low N content. Lignocellulose, despite being the most abundant terrestrial organic compound,

is primarily decomposed only by basidiomycete fungi, and of these, only the white-rot fungi are capable of complete break down, brown-rot fungi breaking down only the cellulose and hemicellulose, leaving behind the partly modified lignin (Tanesaka *et al.*, 1993). Other organisms decompose lignin only with difficulty, if at all (Kögel-Knabner, 2002; Tanesaka *et al.*, 1993). White-rot fungi can (after 80 days decomposition of beech wood blocks) cause 0-51% weight loss, and brown-rotters 0-30% weight loss, litter decomposing fungi and mycorrhizas may produce only 0-19 % and 0-1.5% weight loss respectively, the latter not including any lignin degradation (Tanesaka *et al.*, 1993). This is indicative of the range of extent and time scale over which lignin decomposition occurs. Hence, the birch wood veneers that experienced >30% weight loss (2 in each field) by 75 days and the individual veneer that lost 59% weight at 104 days, would have been placed within the foraging area of white-rot fungi. On the other hand, those that produced much lower weight losses were likely to have been located outside of the foraging area of white-rotters and would only have been exposed to decay by much slower white-rotters or litter fungi. The extent of white-rot fungal action on the wood veneers appeared to be equally spread between the two fields. No wood veneers showed any evidence of decomposition by brown-rot fungi (no brown coloration).

Table 4.4 Approximate overall decay rates of substrates in each experiment. With multiphasic decomposition, the rate corresponds to the most rapid phase.

Seasonal decomposition of substrates				
	Feb-April 2002	May-June 2002	Aug-Oct 2002	Dec-Feb 2003
Field Litter weight loss per day	0.5%	3.2%	1.6%	0.8%
Cellulose weight loss per day	0.2%	1.4%	0.8%	0%
Extended decomposition experiment				
	Field litters	Cellulose	Oak leaves	Birch wood veneers
% weight loss per day	2%	1%	0.2%	0%

Decomposition of oak leaves, which contain a comparatively high proportion of tannins and phenolics, was next slowest averaging 0.18% weight loss per day. Again, like wood, the decomposition of oak leaves was highly variable ranging from 12-38% weight loss by 132 days and decomposition was patchy, the contents of a few individual bags having been substantially bleached, indicating the localised action of particular microbial groups. The lignin content of 1 year old beech litter reportedly controlled the litter decomposition rates (Cortez, 1998) and the lignin content of the oak leaves used in this study is likely to have had a significant affect on their decomposition, although it is also likely that the phenolic content additionally influenced the decomposition rates (Kögel-Knabner, 2002; Sanger *et al.*, 1997). Unlike wood, both fungi and bacteria are involved in litter degradation, on beech leaves antagonistic results of fungal-bacterial interactions have been noted (Møller *et al.*, 1999) in which the more effective carbon processing of fungi was reduced. Thus, differential interactions between fungi and bacterial, during litter decomposition, may be observed in microbial communities which differ in their relative fungal bacterial proportions and composition, such as the communities of traditionally managed and improved fields (Donnison *et al.*, 2000b; Grayston *et al.*, 2004; Grayston *et al.*, 2001).

Cellulose decomposition in soil is a multiphasic process, controlled by the C and N availability which alters with microbial activity, and is generally composed of a slow initial phase followed by a period of more rapid decomposition (Hu & vanBruggen, 1997) as was observed in the May-June and Aug-Oct seasonal experiments (Figure 4.4) and the extended cellulose decomposition (Figure 4.5b). In the latter experiment, cellulose during its near linear decline over the 14-60 day period, lost about 1% of its initial weight per day. It has been suggested that the multiphasic decomposition is the

result of a succession from a bacterial to a fungal community (Hu & vanBruggen, 1997). Opportunistic bacteria are able to react rapidly to colonise new C-sources and utilise any easily available substrates therein. Although some bacteria produce cellulases, fungi primarily break down this material (Table 4.1). From the current study, the change from the first to the second phase of cellulose decomposition coincided extremely closely with the switch from rapid to slower decomposition of the field litters (Figure 4.3 & Figure 4.4). Since the initial rapid decomposition of the field litters is likely to be due to bacterial break down of the more labile portion of the substrate, this suggests that the concomitant slow phase of cellulose break down would be due to colonisation by the same group of opportunistic bacteria, with fungi, due to the nature of their growth, taking longer to colonise the substrate.

Comparing the substrates from the extended decomposition experiment, field litters showed the most rapid weight loss over all losing 2% per day during the first 14 days. Weight loss of field litter following the initial loss of the labile fraction of the litter averaged about 0.33% per day between 14 and 89 days (which was before the measurement of weight loss was affected too much by root infiltration of the litterbags). Therefore the decomposition rate of the more recalcitrant portion of the field litters is closer to that of the oak leaves, reflecting the decrease in substrate quality as the labile fraction of the litter is metabolised.

4.4.4 Litter C, N and P Content During Decomposition

Changes to the quality of the field litters during the extended decomposition experiment were measured by calculating the C:N ratio, C:P ration, and absolute amount of total N and P (% of initial N or P) over the period of the experiment. An increase or decrease

to the absolute amount of the nutrient reflects whether N or P is being accumulated or released from the substrate (Fioretto *et al.*, 2001; Frey *et al.*, 2000). Although no significant difference was seen between the decomposition of the two field litters within each field, there was a distinct tendency for N to be accumulated by IF litter in both fields, whereas the absolute amount of N in TF litter remained approximately the same throughout (Figure 4.6). The accumulation of N in the IF litter occurred even though the C:N ratios of the two litters were not significantly different prior to or following decomposition. This appears to be because the increase in IF litter N concentration and a very slight decrease in TF litter concentration is enough to separate the two litter types in terms of absolute amount of N in the litter. Accumulation of N by IF litter may also suggest that although the initial N concentrations were similar, the type of C compounds within the IF litter, following the initial rapid stage of decomposition, required more N source to be available to enable their breakdown. Generally the nutrient dynamics of decomposition predict that, following the accumulation of exogenous N by microbes, the absolute amount of N in the litter should decline due to mineralisation (Frey *et al.*, 2000), from 75 days, all but the TF in TF litter declines slightly suggesting the start of mineralisation. The oak leaves, on the other hand showed no real reduction in absolute N and having started with a much higher C:N ratio than the field litters, required the accumulation of far more N prior to the initiation of decomposition. Thus it is likely that the time taken to physically accumulate N and the relative availability of soil N influence decomposition rates.

Comparisons of the concentration of N in the oak leaf litterbag samples during decomposition again produced no significant difference between fields (Appendix III, Table III.IV). However, the accumulation of N in this high C:N substrate (Figure 4.7) is

clearly demonstrated (Figure 4.8). The concentration of N during decomposition more than doubling compared with the initial N concentration.

The absolute amount of total P in the field litters, although somewhat variable, seem to suggest a general net loss of P from the plant material. This may have been scavenged by fungal hyphae (Fioretto *et al.*, 2001). Overall there was little difference between fields in the losses of P from field litter samples. The C:P ratio on the other hand, remained the same throughout the course of the experiment. Therefore, C and P must have been declining at the same rate.

4.5 Conclusions

- The seasonal litterbag study demonstrated how the C:N ratio of plant litter could change from that which might normally be expected, and researchers should be aware of this factor when employing litterbags, containing natural substrates, in their experimental design.

- In the comparisons of field litters within and between fields there was no evidence to suggest that the microbial community was any better equipped to metabolise substrates that are native to that site compared with less commonly encountered plant substrates. This is further corroborated by the lack of difference between fields in the metabolism of a range of more recalcitrant substrates. Therefore the initial hypothesis that the two very different plant communities would have had an affect on the ability of each of the microbial communities to metabolise a range of substrates, due to their differential exposure to particular substrates, must be rejected.

- The legacy of the management system does not appear to have affected the decompositive ability of the microbial communities as far as can be determined by the resolution of this study. Differences between decomposition of the various substrates appear to be primarily based upon the substrate quality, i.e., C:N ratio, presence of secondary metabolites and structure of C compounds.

- The decomposition of field litter and cellulose appeared to be in synchrony, whereby the initial rapid phase of litter decomposition corresponded with the slow phase of cellulose decay and the initiation of the second slow phase of litter weight loss coincided with change to rapid decomposition of cellulose.

Chapter 5. Assessment of Community Functional Diversity through Sole-Carbon-Source-Utilisation Profiling

5.1 General Introduction

The functional diversity of a community is a measure of its potential activity, i.e., the capability of a community to cope with varying abiotic conditions, such as microclimate or substrates, by adapting metabolism and/or the relative proportions of constituent populations. Since the increase in interest of studying microbial community function, particularly that of soil communities, a number of techniques have been employed including classic litterbag substrate decomposition (e.g. Bardgett & Shine, 1999) substrate-induced-respiration (Degens & Harris, 1997) and enzymic profiling (Marx *et al.*, 2001; Vepsäläinen *et al.*, 2001). However, by far the most utilised method over the last decade has been sole-carbon-source-utilisation-profiling using commercially available Biolog Microplates™ (Oxoid, UK), which were originally developed for the identification of bacterial isolates.

These microtitre plates contain 95 different sole-carbon-sources and a water control in the 96 wells of a 12 x 8 matrix (1-12 x A-H), together with a redox dye that changes colour upon catabolism of the carbon substrate. Thus, inoculation with a bacterial isolate, results in the catabolism and colour change of those substrates that the microbe is capable of utilising, providing a colour 'fingerprint'. Various plates are available containing a selection of C-sources tailored to the identification of Gram negative bacteria (GN or the revised GN2 plate), Gram positive bacteria (GP plates), SF-N2 and SF-P2 plates which contain the same C-sources as GN2 and GP2 without the tetrazolium dye and Eco-plates which contain a selection of 31 more environmentally

realistic C-sources and a water control in triplicate for the analysis of microbial communities (e.g. from soil or water). However, the majority of studies have used GN or GN2 plates (Preston-Mafham *et al.*, 2002).

The sole-carbon-source-utilisation (SCSU) technique (alternatively referred to as the Biolog technique), however, is limited by the fact that it is to some extent a cultural method (Degens & Harris, 1997; Haack *et al.*, 1995; Konopka *et al.*, 1998) with the associated problems of exclusion of some species and domination by others (Smalla *et al.*, 1998). Hence, it only enables the comparison of the potential function of a portion of the microbial communities since from the moment a sample is taken from a soil environment, the proportions of the microbial community within it will be changing. An additional problem is created by the fact that the plates offer a relatively unrealistic set of substrates which, since they are sole carbon sources, do not necessarily provide favourable conditions for oligotrophic organisms. Despite its problems however, the technique does allow the direct comparison of the potential function (i.e., their ability to metabolise the replicate sets of carbon sources they are provided with) of the portion of sample communities able to survive within the plate environment.

Assessment of microbial community function, i.e., the actual catabolic activity expressed, remains a goal for the future. This is more likely to be achieved through profiling the enzymatic activity expressed in the environment. In the meantime however, the Biolog technique provides some level of information for the comparison of potential function. Interpretation of the results, however, should be cautious and only made alongside information collected from other community profiling techniques, including the analysis of community structure.

Although the majority of research using Biolog for community analysis has considered only the bacterial community, at least four studies (Buyer *et al.*, 2001; Classen *et al.*, 2003; Dobranic & Zak, 1999; Sobek & Zak, 2003) have now looked at the potential function of the fungal portion of the community by using SF-N2 plates. These contain the same set of substrates as GN2 plates, but do not contain the manufacturers tetrazolium dye which fungi are unable to metabolise (Dobranic & Zak, 1999; Heuer & Smalla, 1997). The first two fungal approaches (developed prior to this experiment) were similar, both using antibiotics to control bacterial growth, however, Dobranic and Zak (1999) favoured the inclusion of a tetrazolium dye, dimethylthiazolyl-diphenyl-tetrazolium bromide (MTT), selected specifically for metabolism by fungi in order to record levels of substrate metabolism rather than growth which is recorded by turbidity measurement (Buyer *et al.*, 2001). In the latter two approaches, Sobek and Zak (2003) continued to include MTT, while Classen *et al.* (2003) preferred to measure turbidity.

In the following study bacterial functional diversity is investigated using Biolog GN2 plates, while fungal functional diversity is assessed with SF-N2 plates. These plates were chosen over Eco-plates, which contain fewer more environmentally realistic substrates as a triplicate set per plate, because dye free Eco-plates (for assessing fungal function) were not commercially available.

5.2 Determination of Appropriate Inoculum Dilution

5.2.1 Introduction

Within species-rich soil communities, species losses through dilution occur more rapidly than in low diversity communities (Garland & Lehman, 1999) Hence, the

inoculum for Biolog plates should be diluted as little as possible. Problems with Biolog community profiling have occurred from the development of colour in the water-blank well. This was suggested to be caused by soluble carbon in the soil solution providing a substrate for metabolism by the micro-organisms (Haack *et al.*, 1995). To prevent this, further dilution was suggested (Haack *et al.*, 1995), however, this further confounds the problem of species losses.

Much of the Biolog literature reports the use of a method of inoculum preparation by which the soil sediment in the solution is allowed to settle allowing the particulate free supernatant to be used for inoculation. This potentially allows the loss of any organisms which remain attached to particulate material following shaking (Bossio & Scow, 1995), particularly mycelial fragments which have been shown to sediment out in soil solutions (Warcup, 1955). Reductions to the number of microbes in an inoculum following centrifugation at various speeds has also been demonstrated (Calbrix *et al.*, 2004). Hence, an experiment was run to determine; (1) the least dilute inoculum suitable for producing community colour profiles on Biolog GN2 plates; and (2) whether it was viable to inoculate with soil solution which still contained the soil particulate matter.

5.2.2 Methods

Soil suspensions were prepared from a sample of Pentwyn Meadow TF soil and a sample from the grounds of Cardiff University, in order to test two extremely different soils. The Cardiff soil was much darker and presumably contained more organic matter with concomitant higher microbial biomass. The suspensions were prepared using the method for GN2 plates as described below (Section 5.4.2.4.1), and were serially diluted to 1:10, 1:100 and 1:1000. Six GN2 plates were inoculated, one for each dilution level

of each soil. Following inoculation (150μl per well) the plates were read immediately and then incubated at 20°C. Readings were made regularly (Figure 5.1) over a 170h period for TF soil and 140h for Cardiff soil at 570nm (Emax plate reader, Molecular Devices, UK) (this being the closest to 590nm that was available at the time).

5.2.3 Results and Discussion

Both soil samples produced readable colour profiles that differed slightly between soil type and dilutions (Figure 5.1). All maximum readings were well within the Emax plate reader specifications for which the maximum readable optical density is 4 OD units. The optical density (OD) of the control well remained static no coloration greater than background concentration developing in either soil at any dilution. The variability of background OD for the field site soil at 1:10, 1:100, and 1:1000 was approximately ±0.18, 0.05 and 0.05, respectively, while the Cardiff soil was ±0.4, 0.1 and 0.05, respectively. Since the maximum endpoint OD of the samples was approximately 3 OD units, OD>0.1 is quite a large background level (Dobranic & Zak, 1999) and produced more variation around y_0 (prior to colour development), between 0.05 and 0.1 is perhaps more acceptable. Variation during the lag period of the 1:100 and 1:1000 samples is also less than that of the 1:10 dilution samples.

Colour development in the plate wells usually follow a logistic growth form, lag period followed by an exponential increase and an asymptote (Garland & Mills, 1991). Problems were apparent with the 1:10 dilution level, in which the rate of colour development was too rapid to produce reasonable sigmoidal logistic growth curves. Dilutions 1:10 and 1:100 produced similar end patterns (estimated by eye from +/- colour response chart (Appendix IV.III, Figure IV.I)), while the 1:1000 dilution did not

develop the same range of positive colour responses registered by the higher soil concentrations.

The particulate matter did not appear to cause any problems with the reading of the plates, and the background OD may be eliminated from resultant calculations by subtracting the initial OD at time zero (t_0) from subsequent readings.

In summary, a dilution level of 1:1000 did not produce a strong enough response, while 1:10 produced too rapid a rate of colour development, it therefore seems that for these soils, a dilution level of 1:100 would be the most suitable for inoculation of the Biolog plates.

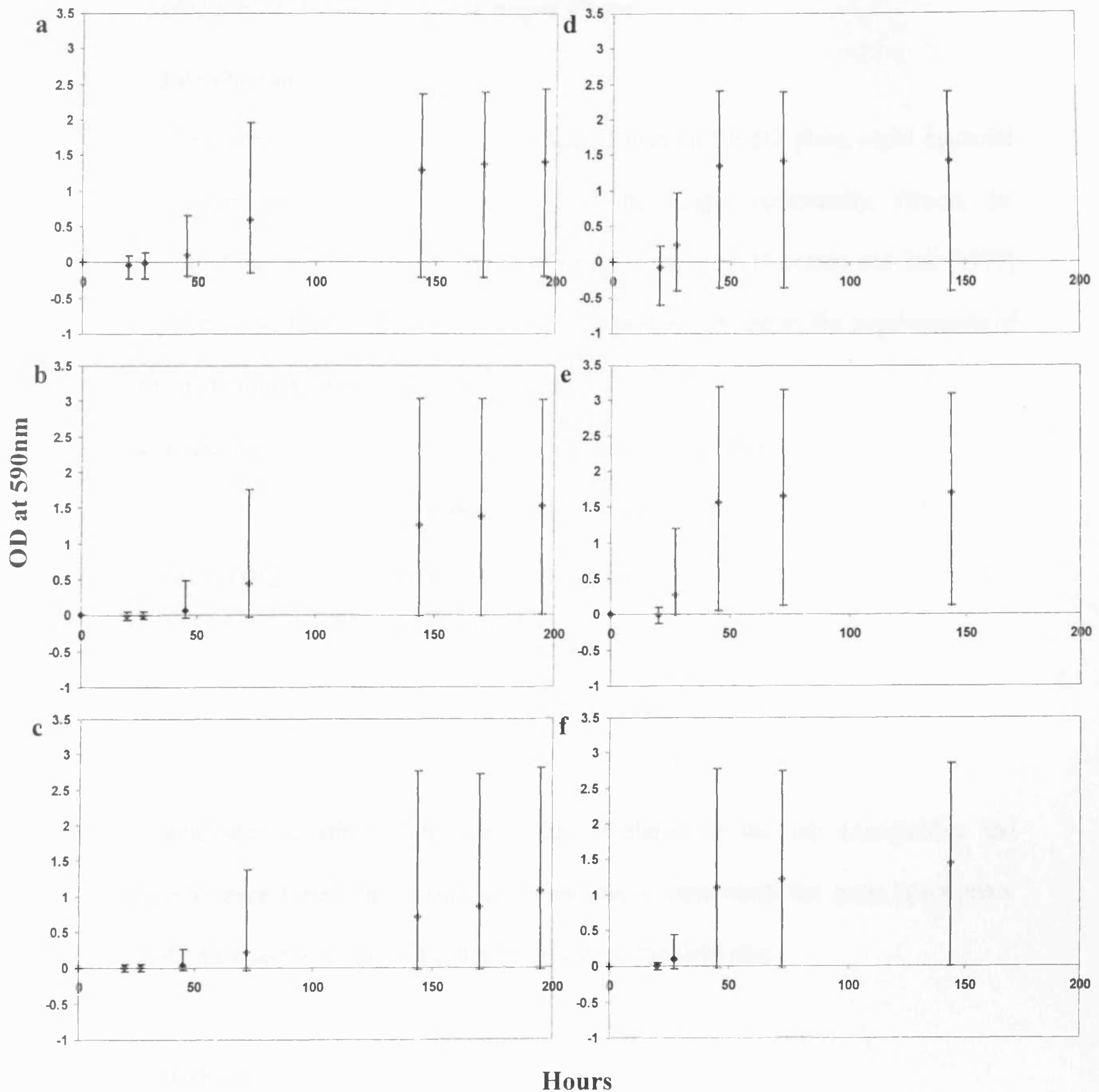

Figure 5.1 Colour development in all 96 wells of Biolog GN2 plates inoculated with TF soil (a-c) and Cardiff soil (d-f) at 1:10 dilution (a & d), 1:100 dilution (b & e) and 1:1000 dilution (c & f). Means and maximum-minimum spread of all 96 data points shown.

5.3 Selection of Antibiotics for Fungal Plates

5.3.1 Introduction

If a whole community soil sample is inoculated into an SF-N2 plate, rapid bacterial growth occurs preventing the assessment of the fungal community. Hence, the elimination of bacterial growth through antibiotics is required. Dobranic and Zak (1999) and Buyer *et al.* (2001) used different sets of antibiotics tailored to the requirements of the particular communities they were studying:

Dobranic and Zak (1999)	Streptomycin sulphate (100µg/ml)
	Chlortetracycline (50µg/ml)
Buyer *et al.* (2001)	Gentamycin (50µg/ml)
	Rifampicin (100µg/ml)
	Streptomycin (100µg/ml)

These antibiotics together with two others available in the lab (Ampicillin and Kanamycin) were tested in various combinations to determine the most appropriate combination for use with the soil communities from the field site.

5.3.2 Methods

For those in powder form the subsequent antibiotic solutions were filter sterilised to 0.22µm. Appropriate aliquots of the stock solutions (Table 5.1) were added in various combinations (Table 5.2) to 1g fresh soil (collected from field site Dec 2001) in 10ml of sterile ¼ strength Ringer's solution with 100µl of the tetrazolium dye, MTT (5mg ml^{-1}), in sterile universals. Controls were set up using autoclaved soil samples. The caps were screwed down tightly and the bottles shaken, on their sides on a flat bed shaker, for 30

minutes prior to spread plating 150µl onto triplicate soil agar plates (Appendix IV.II). Plates were then incubated at 16°C. Fungal colony forming units (cfu) and bacterial contamination were recorded after 4 and 21 days

Table 5.1 Dilutions of antibiotics tested for bacterial control. All antibiotic solutions made up in sterile deionised water except Rifampicin in DMSO.

Antibiotic	Stock soln	Working concentration	Vol. stock added to total volume of 10ml
Streptomycin sulphate	10mg/ml	50µg/ml	50µl
Kanamycin	10mg/ml	20µg/ml	20µl
Rifampicin	25mg/ml	100µg/ml	40µl
Gentamycin	10mg/ml	50µg/ml	50µl
Chlortetracycline	10mg/ml	10µg/ml	10µl
Ampicillin	10mg/ml	50µg/ml	50µl

Table 5.2 Antibiotic combinations tested

1/4 strength ringers soln (ml)	Total Antibiotic (ul)	Antibiotics (ul)					
10.00	0	Sterile soil control, 0					
9.95	50	Sterile soil + Strep sulph, 50					
9.98	20	Sterile soil + Kanamycin, 20					
9.96	40	Sterile soil + Rifampicin, 40					
9.95	50	Sterile soil + Gentamycin, 50					
9.99	10	Sterile soil + Chlortetracycline, 10					
9.95	50	sterile soil + Ampicillin, 50					
10.00	0	Live soil control					
9.95	50	Streptomycin sulphate, 50					
9.98	20	Kanamycin, 20					
9.96	40	Rifampicin, 40					
9.95	50	Gentamycin, 50					
9.99	10	Chlortetracycline, 10					
9.95	50	Ampicillin, 50					
9.93	70	Streptomycin sulphate, 50	Kanamycin, 20				
9.91	90	Streptomycin sulphate, 50	Rifampicin, 40				
9.90	100	Streptomycin sulphate, 50	Gentamycin, 50				
9.94	60	Streptomycin sulphate, 50	Chlortetracycline, 10				
9.90	100	Streptomycin sulphate, 50	Ampicillin, 50				
9.94	60	Kanamycin, 20	Rifampicin, 40				
9.93	70	Kanamycin, 20	Gentamycin, 50				
9.97	30	Kanamycin, 20	Chlortetracycline, 10				
9.93	70	Kanamycin, 20	Ampicillin, 50				
9.91	90	Rifampicin, 40	Gentamycin, 50				
9.95	50	Rifampicin, 40	Chlortetracycline, 10				
9.91	90	Rifampicin, 40	Ampicillin, 50				
9.94	60	Gentamycin, 50	Chlortetracycline, 10				
9.95	50	Gentamycin, 50	Ampicillin, 50				
9.94	60	Chlortetracycline, 10	Ampicillin, 50				
9.89	110	Streptomycin sulphate, 50	Kanamycin, 20	Rifampicin, 40			
9.88	120	Streptomycin sulphate, 50	Kanamycin, 20	Gentamycin, 50			
9.92	80	Streptomycin sulphate, 50	Kanamycin, 20	Chlortetracycline, 10			
9.88	120	Streptomycin sulphate, 50	Kanamycin, 20	Ampicillin, 50			
9.86	140	Streptomycin sulphate, 50	Rifampicin, 40	Gentamycin, 50			
9.90	100	Streptomycin sulphate, 50	Rifampicin, 40	Chlortetracycline, 10			
9.86	140	Streptomycin sulphate, 50	Rifampicin, 40	Ampicillin, 50			
9.89	110	Streptomycin sulphate, 50	Gentamycin, 50	Chlortetracycline, 10			
9.85	150	Streptomycin sulphate, 50	Gentamycin, 50	Ampicillin, 50			
9.89	110	Streptomycin sulphate, 50	Chlortetracycline, 10	Ampicillin, 50			
9.89	110	Kanamycin, 20	Rifampicin, 40	Gentamycin, 50			
9.93	70	Kanamycin, 20	Rifampicin, 40	Chlortetracycline, 10			
9.89	110	Kanamycin, 20	Rifampicin, 40	Ampicillin, 50			
9.90	100	Rifampicin, 40	Gentamycin, 50	Chlortetracycline, 10			
9.86	140	Rifampicin, 40	Gentamycin, 50	Ampicillin, 50			
9.89	110	Gentamycin, 50	Chlortetracycline, 10	Ampicillin, 50			
9.84	160	Streptomycin sulphate, 50	Kanamycin, 20	Rifampicin, 40	Gentamycin, 50		
9.88	120	Streptomycin sulphate, 50	Kanamycin, 20	Rifampicin, 40	Chlortetracycline, 10		
9.84	160	Streptomycin sulphate, 50	Kanamycin, 20	Rifampicin, 40	Ampicillin, 50		
9.85	150	Streptomycin sulphate, 50	Rifampicin, 40	Gentamycin, 50	Chlortetracycline, 10		
9.85	150	Streptomycin sulphate, 50	Rifampicin, 40	Chlortetracycline, 10	Ampicillin, 50		
9.88	120	Kanamycin, 20	Rifampicin, 40	Gentamycin, 50	Chlortetracycline, 10		
9.84	160	Kanamycin, 20	Rifampicin, 40	Gentamycin, 50	Ampicillin, 50		
9.87	130	Kanamycin, 20	Gentamycin, 50	Chlortetracycline, 10	Ampicillin, 50		
9.85	150	Rifampicin, 40	Gentamycin, 50	Chlortetracycline, 10	Ampicillin, 50		
9.83	170	Streptomycin sulphate, 50	Kanamycin, 20	Rifampicin, 40	Gentamycin, 50	Chlortetracycline, 10	
9.79	210	Streptomycin sulphate, 50	Kanamycin, 20	Rifampicin, 40	Gentamycin, 50	Ampicillin, 50	
9.83	170	Kanamycin, 20	Rifampicin, 40	Gentamycin, 50	Chlortetracycline, 10	Ampicillin, 50	
9.78	220	Streptomycin sulphate, 50	Kanamycin, 20	Rifampicin, 40	Gentamycin, 50	Chlortetracycline, 10	Ampicillin, 50

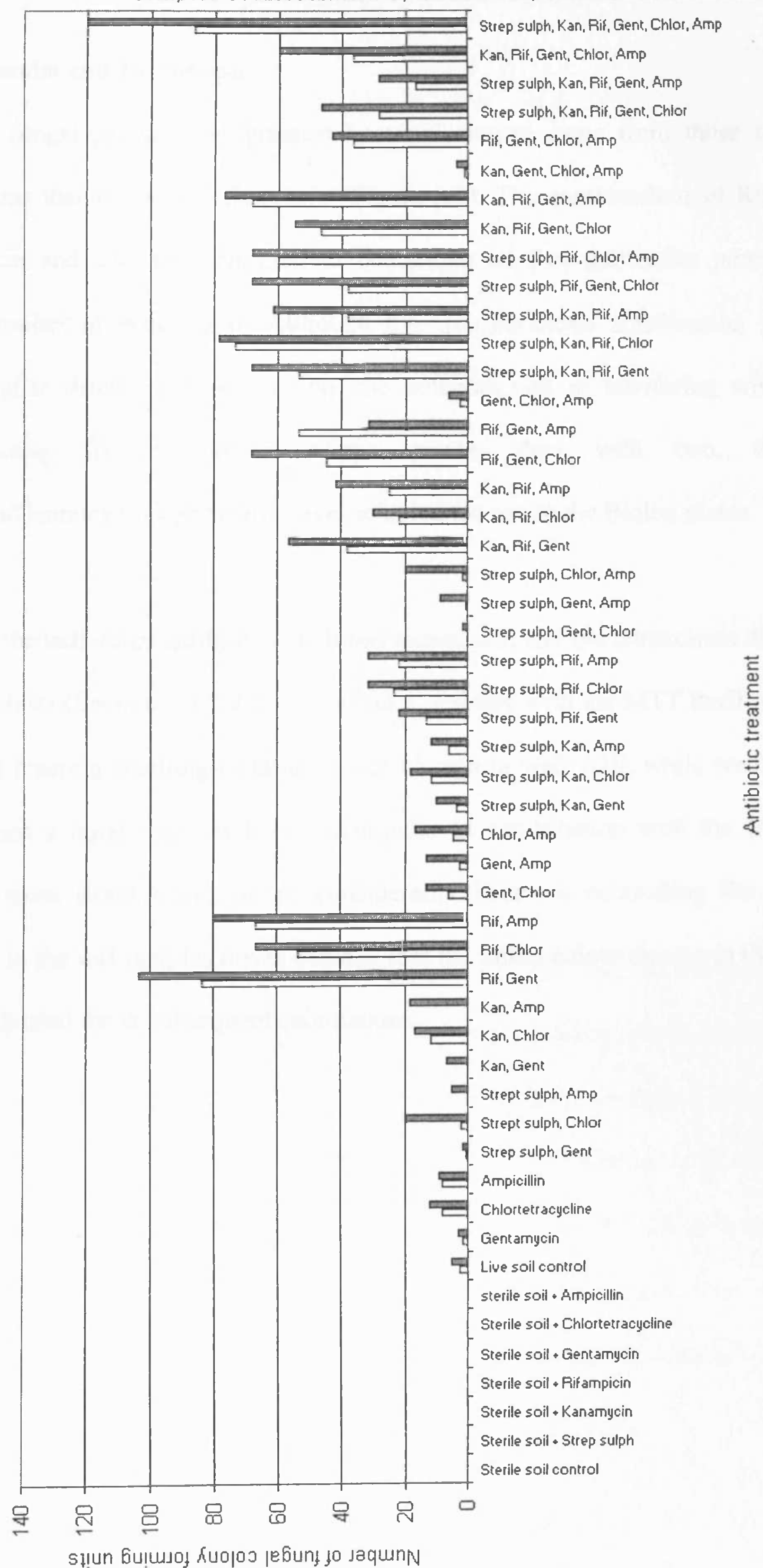

Figure 5.2 Fungal growth following addition of antibiotics combinations to control bacterial levels in the soil inoculum for SF-N2 plates.

5.3.3 Results and Discussion

The most fungal growth and greatest bacterial control came from those antibiotic combinations that included Rifampicin (Figure 5.2). The combinations of Rifampicin /Gentamycin and also the combination containing all five antibiotics produced the greatest number of fungal cfu. Although the five antibiotic combination produced slightly higher results in this situation, the potential risk of interfering with fungal growth using five antibiotics seems greater than with two, thus the Rifampicin/Gentamycin combination was selected for use in the Biolog plates.

Testing of the individual antibiotics with soil suspension and the tetrazolium dye, MTT, in SF-N2 plates (Section 5.4.2.4.2) did reveal a problem with the MTT itself producing a chemical reaction resulting in rapid colour change in well A10, while wells A5 and A6 produced a rapid reaction from Rifampicin in combination with the MTT dye. However, since Rifampicin was so considerably better at controlling the bacterial population in the soil samples it was decided that the initial colour change in these wells could be adjusted for in subsequent calculations.

5.4 Functional Diversity of the Traditional and Improved Field Soil Microbial Communities

5.4.1 Introduction

Comparison of the utilisation of the carbon sources in Biolog GN2 and SF-N2 plates by the bacterial and fungal portions of the soil community could provide an insight into the potential functional capabilities of these two microbial groups. However, changes are likely to occur, to the proportions of microbial strains/species, during incubation within the artificial environment of the Biolog plate (Smalla *et al.*, 1998). Thus, the results should only be interpreted as the community that is able to grow within the plates and not the original soil community.

5.4.2 Methods

5.4.2.1 Soil Moisture Assessment

To produce consistency between the amount of soil used to generate the inoculum for the Biolog plates, soil moisture was estimated by collecting soil cores the day before the samples for the Biolog plates were collected. Care was taken to ensure that sampling took place during a period of consistent weather conditions, whether it be wet or dry. These moisture cores were weighed, dried overnight at 105°C and re-weighed the following day. The average percentage moisture of soil from each field was then calculated and the soil samples used to produce the inocula were adjusted to 5g dry weight equivalent (Appendix IV.I).

5.4.2.2 Soil Temperature

Soil temperature was recorded at the end of each sampling session, using a digital thermometer, at a depth of 5cm from three random positions within each quartile of the fields.

5.4.2.3 Soil Sample Collection

Soil cores, for Biolog plate inoculation, were collected on 4th April 2002, 10th July 2002, 6th November 2002 and 22nd January 2003 as described in Chapter 3, Section 3.2.1.

5.4.2.4 Inoculum Preparation

On returning to the laboratory, the 10 soil cores for each individual field quadrant (Chapter 3, Section 3.2.1) were bulked under sterile conditions, any plant material was removed and the samples homogenised using a 4mm sieve (alcohol sterilised), producing a single bulked sample per field quadrant, eight samples in total: Traditional Field samples A-D and Improved Field samples A-D. From these bulked samples, the equivalent of 5g dry weight (Table 5.3) (adjusted from soil moisture calculations) was added to a 50ml polyethylene vial containing sterile ¼ strength Ringer's solution (Oxoid, UK, tablet form dissolved in deionised water and autoclaved). The total volume of liquid including the water content of the soil was made up to 45ml. After tightly capping the tubes they were vigorously shaken by hand and then placed horizontally on a flat bed shaker for 45min.

Table 5.3 Mean soil moisture content and fresh weights of soil (equivalent to 5g dry weight) used for Biolog plate inoculum.

Date	Field	Soil water content (%)	Fresh weight soil used for inoculum(g)
3-4 Apr 2002	TF	40	8.33
	IF	31	7.25
9-10 Jul 2002	TF	36	7.81
	IF	31	7.25
5-6 Nov 2002	TF	35	7.69
	IF	31	7.25
21-22 Jan 2003	TF	39	8.19
	IF	34	7.58

5.4.2.4.1 GN2 Plate Inocula

1:10 dilutions of the shaken samples were prepared by resuspending the soil in each tube and rapidly pipetting 5ml from the centre of the suspension into 45ml sterile ¼ strength Ringer's solution. 1:100 dilutions for the GN2 plates were prepared simply by repeating the process (5ml 1:10 suspension in 45ml Ringer's). The eight samples were then inoculated into triplicate GN2 plates (150μl per well) using a 12 tip multichannel pipette. To avoid the introduction of bubbles into the wells, the pipette was pushed beyond the first stop prior to the initial uptake of liquid. The samples were then dispensed to the first stop. GN2 plates were coded, field-quadrant-plate type-replicate, e.g. tfab2, where tf = traditional field, a = field quadrant A and b2 = bacterial (GN2) plate replicate 2. Fungal SF-N2 plates were coded similarly, except that f was used in place of b to represent fungal plates.

5.4.2.4.2 SF-N2 Plate Inocula

The SF-N2 1:100 dilutions were made up separately since they required additional constituents. MTT was made up aseptically to a concentration of 5mg ml^{-1} in sterile

deionised water. To eight sterile 50ml tubes the following were added to give a total volume of 50ml:

Tetrazolium dye (MTT)	1000μl
Rifampicin	200μl
Gentamycin	250μl
1:10 dilution soil suspension	5ml
¼ strength sterile Ringer's solution	43.55ml

As above, the resultant eight samples were inoculated into triplicate SF-N2 plates (150μl per well).

Samples of each constituent, Ringer's, antibiotics and MTT solutions, were streaked onto 2% malt agar to check for sterility and soil free GN2 and SF-N2 control plates were used to check for contamination during the extended plate reading period.

All Biolog plates were read immediately (590nm: E-max plate reader, Molecular Devices, UK) to produce readings for time zero (t_0). Subsequent readings were taken twice daily until total plate readings for GN2 plates reached an asymptote (Apr 2002, 252h; Jul 2002, 253h; Nov 2002, 259h; Jan 2003, 261h), SF-N2 plates were read daily following this until an asymptote was reached (Apr 2002, 589h; Jul 2002, 619.5h; Nov 2002, 643h; Jan 2003, 619.5h). Incubation of all plates was carried out at 17 (±1)°C in random block design.

5.4.2.5 Basic Investigation of community structure in SF-N2 plates

Triplicate SF-N2 plates were inoculated as above (Section 5.4.2.4.2) using TF soil, collected in Oct 2003, and incubated at 16°C until total plate optical density reached an asymptote. The whole of the contents of each well was plated onto individual water agar plates, incubated at 16°C and the subsequent fungal growth examined microscopically. The number of different morphotypes was noted.

5.4.2.6 Single Isolate cultures in SF-N2 plates

Cultures of *Lepista saeva* and *Stropharia pseudocyanea* were isolated from fruit body tissue collected from IF in autumn 2002. Cultures were grown on potato dextrose agar medium (Oxoid) at 20°C. Hyphae were aseptically removed from the surface of a culture using a sterile cotton wool bud and placed in 5ml sterile ¼ strength Ringer's solution. Sterile glass beads (3mm) were added and the suspension shaken to separate and break up the mycelium. The suspension was diluted (sterile ¼ strength Ringer's) to give 60% transmission at 630nm (Hobbie *et al.*, 2003) in a total volume of 44ml. MTT dye (1ml (5mg ml^{-1})) was added and the suspension plated into triplicate SF-N2 plates (150µl per well). Plates were incubated at 16°C and the colour development patterns of replicate plates compared.

5.4.2.7 Statistical Analysis

Biolog plate readings were corrected for background colour at t_0. Colour development in the plate due to the presence of dissolved carbon from the soil solution was corrected for by subtracting the value of the control well (A1) from all other wells on a plate at each time point. Kinetic data for each well (OD development over time) was analysed

by the method developed by Lindstrom *et al.* (1998) applying the logistic equation (Equation 5.1).

Equation 5.1 $$y = OD_{590nm} = \frac{K}{(1+e^{-r(t-s)})}$$

where: K = the asymtote

r = the exponential rate of OD change

s = time to the midpoint of the exponential region of the curve, i.e. $K/2$

t = time (corresponding value of x)

The total number of C-source wells for the whole experiment was 18240. In order to fit logistic curves to the time series data for all of these wells the 'Solver' facility, from Microsoft Excel ™, was employed. The running of Solver was then automated in Excel by a Visual Basic macro (Solver programmed by Ian Vaughan, macro programmed by Jeremy Hynes). Solver can be used to simultaneously estimate parameters for non-linear models, such as the logistic equation above, by using either Newton's or Conjugate search algorithms. In this case the Newton algorithm was used to determine the parameters, K, r and s. Cells were assigned to contain these parameters. Rows to contain the observed y-values, calculate y-values (cells containing the logistic equation) and residuals squared were also set up, as was a cell for the sum of the squared residuals (total error) (Appendix IV.IV, Figure IV.II).

Solver obtains the best-fit model by minimising the sum of squares (total error cell) through changing the unknown parameters (K, r and s). Details of the Solver options can be found in Appendix IV.IV, Figures IV.III & IV.IV. OD and x-axis (time) data are pasted directly into the Excel spreadsheet (Appendix IV.IV, Figure IV.II) and when the

macro is initiated, the data from each well are pasted into the observed y-value row, Solver is run, the calculated parameters and total error data is pasted to a results sheet and the process is repeated.

Principal component analysis (PCA) (MINITAB™ ver.13) was applied to all *r* (exponential rate of change) parameter data sets to detect differences between the traditional field (TF) and the improved field (IF). Analysis was performed using both the correlation and the covariance matrix to assess the value of each, in interpreting the portions of the microbial communities being tested for their potential functional abilities on the C-sources available in the Biolog plates. Ordination plots were compared with both the corresponding sets of *r*-parameter values and univariate testing of each individual C-source compared between fields (Mann-Whitney test, MINITAB).

In the comparison of individual C-sources (e.g. all data for well A2 from TF with all data for well A2 from IF, repeated for all wells) preliminary analysis indicated that a high number of the data sets were characterised by a lack of normality or inequality of variance, thus, the Mann-Whitney non-parametric test was applied to all of the data. The non-parametric method allows for simpler but more cautious significance testing, significance being harder to achieve, which still provides an indication of those C-sources contributing the most to any patterns in the ordination. This contribution can also be confirmed by cross-referencing with the colour formatted original *r*-parameter data (Appendix IV.VI.II, Figures IV.XIV – IV.XXI).

5.4.3 Results

5.4.3.1 Soil Temperature

The soil temperature of IF was consistently higher than that of TF, except during the Summer (July 2002) sampling session during which no significant difference was observed (Table 5.4).

Table 5.4 Mean Soil temperatures of TF and IF during Biolog sampling sessions. Significant differences between fields indicated, determined by Two-Sample T-Tests except Nov data which invalidated normality test and was subsequently analysed by non-parametric Mann-Whitney test (full results in Appendix IV.V, Table IV.I).

Date	Mean Temp. TF	Mean Temp. IF	Statistical Significance
3-4 Apr 2002	13.1	14.3	***
9-10 Jul 2002	15.0	14.5	NS
5-6 Nov 2002	11.4	11.6	***
21-22 Jan 2003	5.6	5.9	**

*=P≤0.05 **=P≤0.01, ***=P≤0.001, NS= not significant.

5.4.3.2 Soil pH

Across all time points the pH of TF was higher than that of IF see Chapter 2, Section 2.4.2.

5.4.3.3 Soil Moisture

The % water content of the soil from TF was significantly higher (statistics in Appendix IV.V, Table IV.II) than samples from IF across all four sampling seasons (Table 5.3).

5.4.3.4 Biolog Plate Results

5.4.3.4.1 Plate Readings

Total plate absorbance curves plotted against incubation time revealed that the fungal plates developed at a much slower rate than the bacterial plates, the majority of the

colour development occurring between 2-9 days in the bacterial plates compared with 4-20 days in fungal plates (e.g. Appendix IV.VI, Figure IV.V). Control plates were clear of contamination during both the bacterial and fungal plate reading time frames. Some problems of colour development in the control well (A1) occurred, particularly in the fungal plates. Data for each well was corrected against initial background colour at t_0 and colour in the control well each time the plate OD was measured. However, correction of the data against the colour in this well may result in false negatives and apparently lower levels of catabolism. Colour development in the control well appeared to be an inherent problem with the fungal plates possibly due to the inclusion of peptone and yeast extract in all wells. This allows growth without metabolism being linked to formazan production (Winding & Hendriksen, 1997) in the GN2 plates, although, it could be that the tetrazolium dye (MTT) added to the SF-N2 plates responds differently.

A simple preliminary analysis of Biolog data is to record whether a substrate (substrate list Table 5.5) can be catabolised (positive response) or not (negative response). A positive response can be subdivided into degree of response, and interpreted visually, by conditionally formatting cells in a table to correspond to arbitrary degrees of response. This procedure was carried out on bacterial and fungal data sets across all four sampling periods for both r-parameter values (Appendix IV.VI.II, Figures IV.XIV – IV.XXI) and endpoint colour (equivalent to observed K) (Appendix IV.VI.I, Figures IV.VI – IV.XIII). Endpoint positive responses were subdivided into three levels of colour intensity determined by dividing the maximum reading by 3. Individual well responses were classed as follows:

	Bacterial endpoint	Fungal endpoint
Negative	<0.1 OD units	<0.1 OD units
Low	0.1-1 OD units	0.1-0.7 OD units
Medium	1-2 OD units	0.7-1.3 OD units
High	>2 OD units	>1.3 OD units

Since *r* from the kinetic equation is an exponential value (the extent of rate of exponential change), the *r*-parameter data was subdivided into five classes on a log scale and was the same for both bacterial and fungal data sets (Appendix IV.VI.II, Figures IV.XIV – IV.XXI):

	r-parameter value
Negative	<0.001
Slow rate of formazan production	0.001-0.01
↓	0.01-0.1
↓	0.1-1
High rate of formazan production	>1

Endpoint responses showed that in the bacterial plates, catabolism of polymers and carbohydrates (wells A2-C12 [Appendix IV.VI.I, Figures IV.VI – IV.IX]) produced the greatest amounts of formazan (largest colour change). However this was not necessarily at the fastest rate (*r*-values), meaning that catabolism of carbohydrates and polymers began during the early stages of incubation but continued for much of the duration, whereas, other more complex substrates, e.g. E6 the carboxylic acid D, L-Lactic acid, produced only a low level of colour, but high exponential rate of colour development.

Table 5.5 Substrates and chemical guilds corresponding to the wells of Biolog GN2 and SF-N2 plates

Chemical Guild	Well	GN2/SF-N2	Chemical Guild	Well	GN2/SF-N2
	A1	Water	Carboxylic acid	E1	p-Hydroxy Phenylacetic Acid
polymer	A2	α-Cyclodextrin	Carboxylic acid	E2	Itaconic Acid
Polymer	A3	Dextrin	Carboxylic acid	E3	α-Keto Butyric Acid
Polymer	A4	Glycogen	Carboxylic acid	E4	α-Keto Glutaric Acid
Polymer	A5	Tween 40	Carboxylic acid	E5	α-Keto-Valeric Acid
Polymer	A6	Tween 80	Carboxylic acid	E6	D,L-Lactic Acid
Carboxylic acid	A7	N-Acetyl-D-galactosamine	Carboxylic acid	E7	Malonic Acid
Carboxylic acid	A8	D-Galactosamine	Carboxylic acid	E8	Propionic Acid
Carbohydrate	A9	Adonitol	Carboxylic acid	E9	Quinic Acid
Carbohydrate	A10	L-Arabinose	Carboxylic acid	E10	D-Saccharic Acid
Carbohydrate	A11	D-Arabitol	Carboxylic acid	E11	Sebacic Acid
Carbohydrate	A12	D-Cellobiose	Carboxylic acid	E12	Succinic Acid
Carbohydrate	B1	i-Erythritol	Carboxylic acid	F1	Bromo Succinic Acid
Carbohydrate	B2	D-Fructose	Carboxylic acid	F2	Succinamic Acid
Carbohydrate	B3	L-Fucose	Amines/Amides	F3	Glucuronamide
Carbohydrate	B4	D-Galactose	Amines/Amides	F4	L-Alaninamide
Carbohydrate	B5	Gentiobiose	Amino acid	F5	D-Alanine
Carbohydrate	B6	α-D-Glucose	Amino acid	F6	L-Alanine
Carbohydrate	B7	m-Inositol	Amino acid	F7	L-Alanylglycine
Carbohydrate	B8	α-D-Lactose	Amino acid	F8	L-Asparagine
Carbohydrate	B9	Lactulose	Amino acid	F9	L-Aspartic Acid
Carbohydrate	B10	Maltose	Amino acid	F10	L-Glutamic Acid
Carbohydrate	B11	D-Mannitol	Amino acid	F11	Glycyl-L-Aspartic Acid
Carbohydrate	B12	D-Mannose	Amino acid	F12	Glycyl-L-Glutamic Acid
Carbohydrate	C1	D-Melibiose	Amino acid	G1	L-Histidine
Carbohydrate	C2	β-Methyl-D-Glucoside	Amino acid	G2	Hydroxy-L-Proline
Carbohydrate	C3	D-Psicose	Amino acid	G3	L-Leucine
Carbohydrate	C4	D-Raffinose	Amino acid	G4	L-Ornithine
Carbohydrate	C5	L-Rhamnose	Amino acid	G5	L-Phenylalanine
Carbohydrate	C6	D-Sorbitol	Amino acid	G6	L-Proline
Carbohydrate	C7	Sucrose	Amino acid	G7	L-Pyroglutamic Acid
Carbohydrate	C8	D-Trehalose	Amino acid	G8	D-Serine
Carbohydrate	C9	Turanose	Amino acid	G9	L-Serine
Carbohydrate	C10	Xylitol	Amino acid	G10	L-Threonine
Carbohydrate	C11	Methyl Pyruvate	Amino acid	G11	D,L-Carnitine
Carbohydrate	C12	Mono-Methyl-Succinate	Amino acid	G12	γ-Amino Butyric Acid
Carboxylic acid	D1	Acetic Acid	Miscellaneous	H1	Urocanic Acid
Carboxylic acid	D2	Cis-Aconitic Acid	Miscellaneous	H2	Inosine
Carboxylic acid	D3	Citric Acid	Miscellaneous	H3	Uridine
Carboxylic acid	D4	Formic Acid	Miscellaneous	H4	Thymidine
Carboxylic acid	D5	D-Galactonic Acid Lactone	Amine/Amides	H5	Phenylethylamine
Carboxylic acid	D6	D-Galacturonic Acid	Amine/Amides	H6	Putrescine
Carboxylic acid	D7	D-Gluconic Acid	Amine/Amides	H7	2-Aminoethanol
Carboxylic acid	D8	D-Glucosaminic Acid	Amine/Amides	H8	2,3-Butanediol
Carboxylic acid	D9	D-Glucuronic Acid	Amine/Amides	H9	Glycerol
Carboxylic acid	D10	α-Hydroxy Butyric Acid	Amine/Amides	H10	D,L-α-Glycerol Phosphate
Carboxylic acid	D11	β-Hydroxy Butyric Acid	Amine/Amides	H11	Glucose-1-Phosphate
Carboxylic acid	D12	γ-Hydroxy Butyric Acid	Amine/Amides	H12	Glucose-6-Phosphate

The asymptote, *K*, of the logistic curve represents the carrying capacity of the well (Lindstrom *et al.*, 1998). This may be controlled by the finite amount of resource in the well, or could be restricted by the build up of inhibiting by-products of metabolism due to the physical restrictions of the well environment. Colour development in the wells, through formazan production, is controlled by the production of NADH during

glycolysis, which reduces the tetrazolium dye included in the well (Figure 5.3). The number of NADH molecules produced by the breakdown of an organic compound during glycolysis is dependent upon the structure of the compound (number of C and H atoms). Catabolism of glucose, for example, results in the production of two NADH molecules as a result of the reduction of NAD^+ by two hydrogen atoms which are cleaved from the organic compound by a dehydrogenase enzyme. To clarify further: rate of colour development is measured as formazan production (OD) per unit time, rate of formazan production depends on the rate of NADH production. NADH production is dependent on two things; (1) how quickly a molecule is catabolised; and (2) how many NADH are produced through the catabolism of each molecule of the C-source. Hence, if the different C-sources included in the wells produce different amounts of NADH per molecule, then the relative scale at which colour development occurs is not equivalent for all C-sources since it is not dependent purely on the rate of breakdown of the molecule. It is therefore not straightforward to compare rates of catabolism between different C-sources producing different numbers of NADH per molecule without some method of conversion to equivalence. Care should be taken when comparing any of the parameters from the kinetic colour development curve between different C-sources. Rather, comparisons should only be made between the same C-sources under the different treatment conditions (i.e. between plate comparison rather than within plate).

Bacterial plate endpoint colour readings (Appendix IV.VI.I, Figures IV.VI – IV.IX) were relatively similar between fields and across the four sampling times. In general, polymers, carbohydrates and amino acids achieved greater colour change than carboxylic acids, amines/amides and some miscellaneous compounds. A number of compounds were not catabolised in a large proportion of all of the plates (Table 5.6).

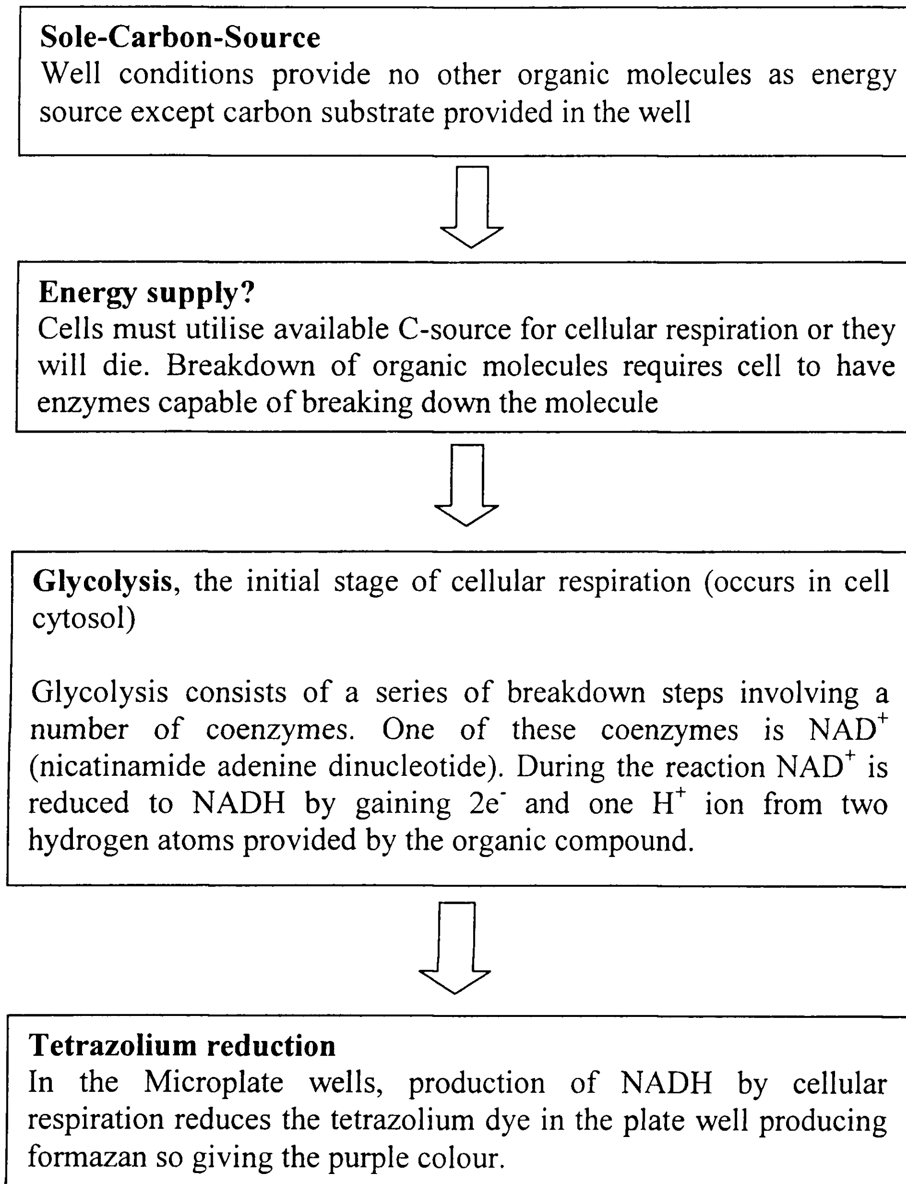

Figure 5.3 Chemistry of a Biolog plate well containing a tetrazolium dye.

The fungal endpoint readings were less defined due to the tendency for colour change in the control well, the response above the level of the control well being low for many C-sources following correction against the control well. However, the carbohydrate wells and some amino acid wells were darker than the other substrate wells. Negative responses in fungal plates occurred for a number of substrates, which differed from those recorded in the bacterial plates, although a number of substrates were consistently not catabolised by both fungal and bacterial plates (Table 5.6).

Table 5.6 Wells and corresponding C-sources in which catabolism was determined to be zero either by negative colour at the endpoint reading or the exponential rate of colour development being negligible. For endpoint response a tick indicates that 12 or more plates, out of the 24, were negative for that well (numbers indicate if fewer than 12 plates). Number of plates responding negatively is given for *r*-value data due to less definition because of the log scale of this parameter (i.e. slope can get very close to negative determination yet still be included in positive data).

C-source		Endpoint								*r*-values							
		Bacterial (GN2)				Fungal (SF-N2)				Bacterial (GN2)				Fungal (SF-N2)			
		Ap 02	Ju 02	No 02	Ja 03	Ap 02	Ju 02	No 02	Ja 03	Ap 02	Ju 02	No 02	Ja 03	Ap 02	Ju 02	No 02	Ja 03
A5	Tween 40					✓	✓	✓	✓					✓4	✓14	✓8	✓16
A6	Tween 80					✓	✓	✓	✓					✓5	✓15	✓9	✓13
A10	L-Arabinose						✓							✓2	✓15	✓10	✓13
D1	Acetic acid	✓	✓	✓	✓	✓	✓	✓	✓	✓4	✓6	✓4	✓1	✓5	✓5	✓8	✓11
D4	Formic acid	✓	✓	✓	✓	✓	✓	✓	✓	✓2	✓7	✓6	✓5	✓11	✓2	✓4	✓11
D10	α-Hydroxy Butyric acid									✓1	✓2	✓3	✓5				
E2	Itaconic acid													✓11	✓8	✓4	✓11
E3	α-keto Butyric acid									✓7			✓5		✓4	✓1	✓2
E5	α-Keto Valeric acid									✓7	✓2	✓5	✓8				
E7	Malonic acid	✓	6	6	7					✓10	✓3	✓1	✓2				
E8	Propionic acid	✓	✓	✓	✓	9	9	9	3	✓5	✓9	✓3	✓7	✓2	✓2	✓5	✓3
E12	Succinic acid									✓6	✓4	✓1	✓4				
F1	Bromo Sucinic acid	9	✓	10	9					✓8	✓6	✓6	✓6				
F3	Glucuronamide	11	✓	8	✓	✓	✓	✓	✓	✓3	✓3	✓3	✓3	✓8	✓5	✓5	✓6
F4															✓6	✓5	
G1	L-Histidine					✓	✓	✓	✓					✓3	✓5	✓3	✓3
G11	D,L-Carnitine					✓	✓	✓	✓					✓4	✓6	✓10	✓5
H1	Urocanic acid					✓	✓	✓	✓					✓3	✓1		✓2
H4	Thymidine					✓	9	10	✓					✓4	✓6	✓9	✓7
H5														✓4	✓7	✓4	✓1
H8	2,3-Butanediol	✓	✓	✓	✓	✓	10	7	✓	✓7	✓4	✓6	✓2	✓4	✓3	✓2	✓3
H10	D,L-α-Glycerol Phosphate	11	✓	5	9	✓	✓	✓	✓	✓7	✓1	✓1	✓6	✓10	✓5	✓7	✓5
H11	Glucose-1-Phosphate					✓	✓	✓	✓					✓8	✓6	✓10	✓6
H12	Glucose-6-Phosphate					✓	✓	✓	✓					✓7	✓7	✓10	✓9

5.4.3.4.2 Logistic Curve

Kinetic analysis of the colour development in individual plate wells produced logistic curves of relatively good fit which did not break the constraints of the model, thus, no curves were removed from the data set. Plate readings that were negative following adjustment were not set to zero as some studies have done (Classen *et al.*, 2003; Lindstrom *et al.*, 1998) since it was considered that these indicated inhibition by a substrate preventing even the metabolism of carbon included in the inoculum.

5.4.3.4.3 Principal Component Analysis

Principal component analysis (PCA) has been used to interpret the data produced by Biolog plates in a number of studies (e.g. Glimm *et al.*, 1997; Gomez *et al.*, 2004; Haack *et al.*, 1995; Lawley & Bell, 1998). However, using PCA for these data sets does not completely satisfy all of the assumptions of the analysis, in that there are considerably more variables than replicates. Due to the high number of variables, any slight change to the data set can greatly influence the arrangement of the data points on the PC axes. Therefore PCA must only be used to broadly interpret the data by looking at the influence of substrate group (e.g. carbohydrates or amino acids etc. [substrates chemical guilds and corresponding wells listed in Table 5.5]) rather than trying to take the focus down to the influence of individual substrates over the arrangement of the samples on the PC score-plots.

5.4.3.4.3.1 Covariance or Correlation

When using PCA it should be considered whether the data should be standardised or not by choosing one of two similarity coefficients. In this scenario, the correlation

coefficient increases the importance/influence of C-sources with a less variable rate of colour development. Individuals are re-scaled in terms of relative proportions. On the other hand the covariance coefficient compares communities without re-scaling so that more variable C-sources, those used at the extreme ends of the scale, exert the greatest influence over the analysis, while the more evenly catabolised substrates have little influence.

Covariance is usually used for variables that are measured on a common scale, and while it may initially appear that the catabolism of a substrate based upon the rate of colour change (OD unit increase per unit time) would be interpreted as a common scale. In reality, the rate and amount of NADH produced, which reduces the tetrazolium dye, can differ depending upon the C-source available for cellular respiration. Therefore colour development in wells containing a different C-source does not operate on a common scale.

The important part of the analysis is to compare all of the individual C-sources between fields, therefore we are not necessarily interested in which C-sources are always rapidly catabolised because this is not what would define the community. For example, it might always be expected that simple carbohydrates would be rapidly catabolised by all organisms. The more significant factor is how well the more complex recalcitrant substrates are catabolised by the communities from each field. Thus, comparison between fields is more important than comparisons between substrates, i.e., relative rates between fields are more important than actual rates between carbon sources. Under this assumption it is likely that the correlation coefficient which re-scales the variables to even out the variation in the data is more appropriate than the covariance matrix.

However, to test whether this assumption was correct, PC scores were plotted for both the correlation and covariance based analyses.

5.4.3.4.3.2 The Covariance Matrix

Running PCA with the covariance matrix tended to result in tight clustering of a large proportion of the samples from a data set, with a few outliers. This applied to both bacterial and fungal plates at all four sampling times. Usually, PCA using the covariance matrix, explains the variation in the majority of the data in fewer PCs than the correlation matrix due to the analysis being based upon the most variable populations. This is true of the analyses for these data sets (Table 5.7). The correlation matrix produced a greater spread of the data along the PCs of the bacterial score-plots. The fungal score-plots however, were tightly clustered with a few outliers (Appendix IV.VIII, Figure IV.XXIII). These fungal data will be discussed later.

Closer inspection of bacterial data from the April 2002 covariance PCA (Appendix IV.VII, Figures IV.XXIIa & b) showed tight clustering in the score-plot of PC1 against PC2, with a few outliers on PC1 and 2: plates tfab1, tfab3, tfdb2, ifcb3 and ifdb2. The corresponding plot of the component loadings (Appendix IV.VII, Figures IV.XXIIc & d) suggests that wells D1, E5, E7, E8, F3, H8 and H10 were responsible for these outliers. When checked against the original *r* data set (Appendix IV.VI.II, Figure IV.XIV), these C-sources had an extreme degree of variation between plates. All other C-sources were tightly clustered around zero on both PCs. Spread of samples along PC3 was explained by the same reasoning. Similar patterns were repeated with the other bacterial and fungal data sets, the PCA providing little evidence of any grouping of the

samples even though overall differences between groups of plates could be seen on the *r* data chart. Therefore the correlation matrix was used for all further analyses.

Table 5.7 Percentage of variation in data explained by principal component analysis using both the correlation and covariance coefficients for Biolog plate data.

	Bacterial plates								Fungal plates							
	correlation				covariance				correlation				covariance			
	Apr-02	Jul-02	Nov-02	Jan-03	Apr-02	Jul-02	Nov-02	Jan-03	Apr-02	Jul-02	Nov-02	Jan-03	Apr-02	Jul-02	Nov-02	Jan-03
%PC1	19.0	17.6	13.2	19.0	24.3	28.1	24.5	31.9	23.7	26.0	15.0	19.0	27.5	22.7	27.9	32.7
%PC2	12.4	9.9	10.1	10.6	16.4	19.2	21.5	19.3	16.9	18.3	13.3	13.7	18.6	15.6	19.4	21.3
%PC3	7.6	8.5	8.4	7.9	14.9	15.5	13.2	15.1	8.2	13.1	10.3	8.5	15.0	10.2	11.9	11.3
%PC4	6.7	7.0	7.0	6.9	9.8	8.9	11.0	10.6	7.3	8.3	7.3	7.3	11.7	8.7	11.6	9.3
PC by which cumu-latively >50%	5th	6th	6th	5th	3rd	3rd	3rd	2nd	4th	3rd	5th	5th	3rd	4th	3rd	2nd

5.4.3.4.3.3 Bacterial Plate PCA (Correlation Matrix)

April 2002 bacterial plates analysed with the correlation matrix appeaed to cluster into three groupings (Figure 5.4a). A separation of plates along PC1 occured due to a lower rate of catabolism of substrates F1-H12 (amines/amides, amino acids and miscellaneous compounds) in Group 3, than in Groups 1 and 2. While along PC2, Group 1 utilised polymers and carbohydrates (wells A2-C12) more rapidly than Group 2. The substrates responsible for these separations could be determined from the position of substrates in the corresponding plot of component loadings (Figure 5.4e). Substrates in F, G and H wells being concentrated in the lower region of the plot, while A-C wells were predominantly in the upper portion. The chart of relative *r*-parameter values (Appendix IV.VI.II, Figure IV.XIV) also clearly showed that tfa and tfb plate replicates had similar rates of carbohydrate and polymer catabolism as the IF plates. Plates ifab1-3 also had similar rates of colour development in wells F-H as tfa and tfb plates causing them to

cluster together on the right of the score-plot. While tfc-d and ifb-c-d plates separated out based on the carbohydrate and polymer use but had similar F-H well values. There was however, no separation of the two fields in terms of potential function. No further separation of the data occurred along PC3. Since PC1 and 2 explained the largest proportion of the variation in the data and PC3 revealed no further separation, no more PCs were plotted.

Correlation PCA of July 2002 bacterial plates (Figure 5.4b) revealed no distinct separations of the data along PC1, 2 or 3. The majority of the data were fairly closely clustered, only plates ifab1, ifab2 and ifbb2 being negatively displaced from the group, while tfdb3 was positively displaced along PC1. Component loading plots corresponding to PC1 and 2 (Figure 5.4f) indicated that E12 largely influenced the position of tfdb3 at the more positive end of PC1. This C-source produced an extremely variable response, in terms of *r*-parameter values, either not being catabolised or catabolism occurring at a high rate (*r*-parameter range 0.1-1). The three negative outliers were responsible for three of the four negative *r*-values from well E12, in contrast, tfdb3 produced the highest rate of colour production in E12 of 0.806, which was considerably faster than the next highest rate at 0.329. Hence the negative rates in E12 from the negative outliers pushed them to the opposite end of PC1, while the majority of plates were positioned closer to the positive end. The three negative outliers were also distinguished from the rest of the plates by higher catabolic rates in F and H wells than the rest of the plates. The data was generally more evenly spread along PC2 and 3 where no groupings were visible.

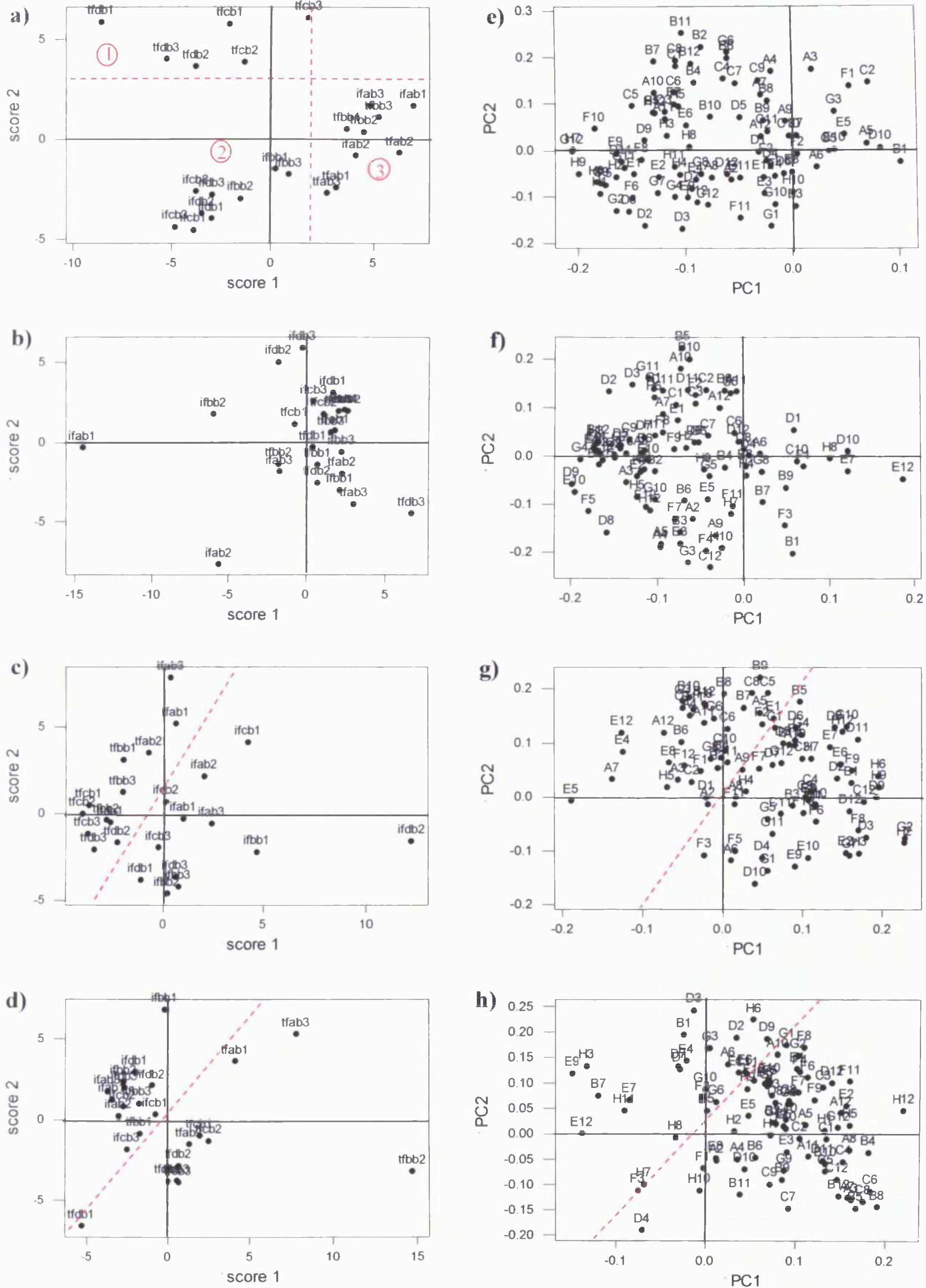

Figure 5.4 Biolog GN2 bacterial plates, PCA (correlation matrix) score-plots for PC1 & PC2, a) Apr-02, b) Jul-02, c) Nov-02, d) Jan-03; and corresponding component loadings plots, e) Apr-02, f) Jul-02, g) Nov-02, h) Jan-03, dashes indicate separation of apparent clusters.

Samples from Nov 2003 and Jan 2003 suggested that some separation of the field communities utilising the Biolog plates may occur (Figure 5.4c & d). In both cases the separation was concentrated along PC1. While the improved field plates were more positive along PC1 than the traditional field in the November samples, the fields switched orientation in the January data set. Consulting the *r*-values (Appendix IV.VI.II, Figure IV.XVI) indicated that group separation was again due to catabolism of F, G and H carbon-sources during November, IF plates tending to catabolise these compounds at higher rates than TF plates. A corresponding dividing line could be drawn across the component loadings plot (Figure 5.4g), those F, G and H compounds matching the TF region of the plot generally showed similar catabolic rates in both TF and IF plates, whereas those lying in the IF region experienced higher rates of catabolism.

On the Nov 2003 score-plot (Figure 5.4c), TF plate replicates tended to cluster together more closely than IF replicates. Crossover of tfa plates with IF plates on PC1, corresponded with the faster catabolism of D well substrates (carboxylic acids) in those compared with the other TF plates. No further separation of fields occurred along PC2 or 3.

Carbohydrates and polymers (A, B & C wells) appeared to be responsible for the majority of the separation between January 2003 TF and IF plates along PC1 (Figure 5.4h). Most IF plates were closely clustered on the slightly negative side of PC1, TF plates being more positive (Figure 5.4d). TF plates formed a main cluster but with several outliers. tfab1, tfab3 and tfbb2 had a more positive tendency while tfdb1 was more negative than all other plates. Plate tfbb1 was clustered with the IF data. Both

tfbb1 and tfdb1 had fewer carbohydrate wells undergoing catabolism at the faster rates of the other TF plates and were similar in range to the IF plates, thus bringing them to the negative end of PC1. Plate ifbb1 was the only IF plate that sat in line with the TF cluster and accordingly had the greatest number of rapidly catabolised carbohydrate substrates of the IF samples.

Along PC2, tfdb1 was extremely negative (Figure 5.4d). Well D4, which matched its position on the corresponding component loading plot, had an extremely high catabolic rate in this plate. On the other hand in ifbb1, which was positioned at the opposite end of PC2, D4 was not catabolised. However, other undetermined C-sources also must have contributed to the position of these plates on the score-plot. Along PC3 (Appendix IV.VII, Figure IV.XXIV), separation of plates ifdb3, tfdb1 and tfab3 away from the main mixed group to the more positive region of PC3, was due in part to higher rates in well B2 than in all the other plates.

Although no separation of any groups occurred in the July sampling (Figure 5.4b) and no distinction could be made between fields in the April sampling (Figure 5.4a). The separation of groups of plates that did occur in April, November and January PCAs was largely based upon differential rates of utilisation of carbohydrates and polymers (A, B & C wells), and amines/amides, amino acids and miscellaneous substrates (F, G & H wells). Carboxylic acids (D & E wells) apparently exerted little influence over plate groupings. The identity of C-sources exerting the most influence over community separation for each data set, was confirmed by Mann-Whitney testing of the replicate data sets for individual wells (Table 5.8).

Differences in extent and orientation of separation between times of sampling could suggest that the communities might separate out in PCA on a seasonal basis. However, the PCA (correlation) of this multi-seasonal data set (Figure 5.5a) revealed no distinction between communities sampled at the four different times of year. Most of the plates clustered between 0-5 on PC1. However, about half of the April plates were pulled to the more negative region of PC1, although these were mixed TF and IF plates. If the full year PCA plot is compared with the April plot, the group of April outliers corresponds to Group 1 and 2 of the April plot. Although it was not absolutely defined there was a tendency for TF plates to be positively correlated with PC2, while IF plates were more negatively correlated along this axis. The component loadings plot elucidated the C-sources causing this division (Figure 5.5b). Carbohydrates and polymers were concentrated at the positive side of PC2 corresponding to more rapid catabolism in the TF plates, with most other C-sources being negatively correlated with PC2 indicated slower catabolism of carbohydrates and polymers, and more rapid catabolism of the other substrates. The spread along PC1 indicated the particularly significant use of carbohydrates by April Group 1, and amino acids, carboxylic acids etc. by April Group 2 (Figure 5.4e). A few mixed C-sources collected on the positive side of PC1.

5.4.3.4.3.4 Fungal Plate PCA

The similarity of replicate fungal plates was quite poor (Appendix IV.VI.II, Figures IV.XVIII – IV.XXI), and fungal plates overall showed little separation of community function between fields (Figure 5.6), neither at any single sampling time nor the multi-seasonal plot (Appendix IV.VII, Figure IV.XXV). In general the correlation plots closely resembled covariance plots (Appendix IV.VII, Figure IV.XXIII), having a main

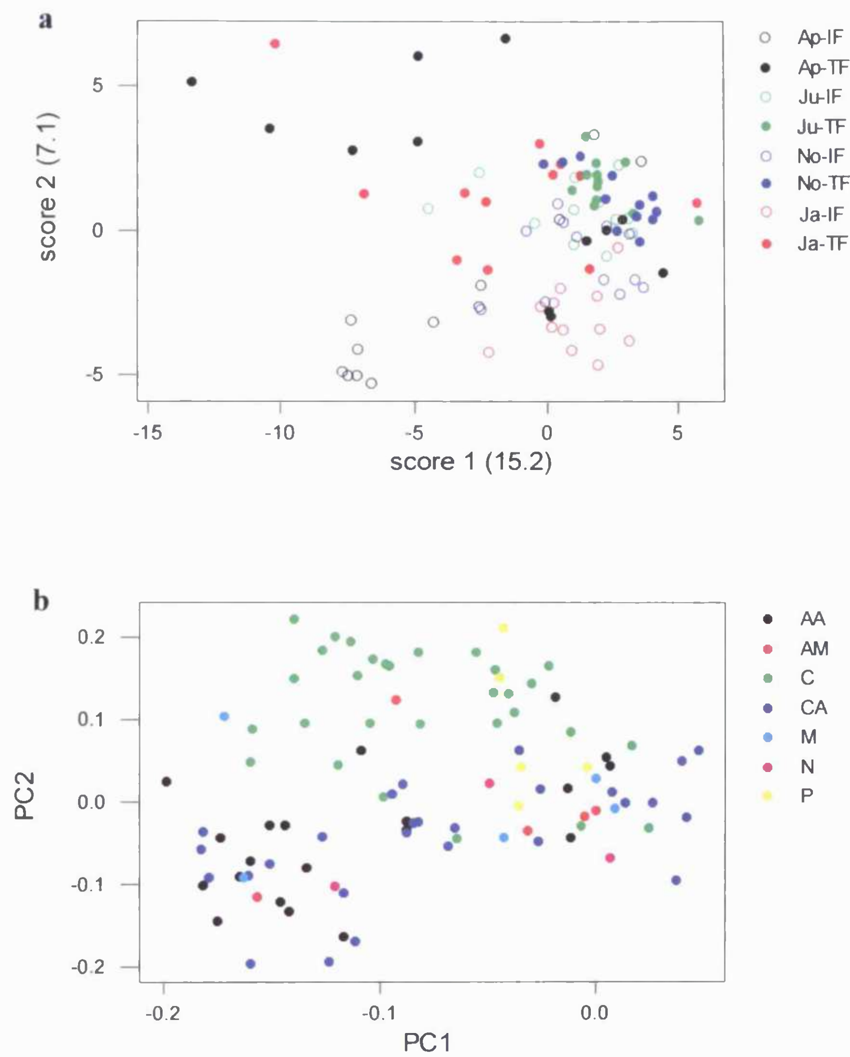

Figure 5.5 PCA (Correlation Matrix), bacterial plates PC1 & 2 for all four seasonal data sets *r*-parameter data, proportion of variation explained by each PC in parenthesis. a) score-plot, solid circles TF data, open circles IF data. b) component loadings plot, AA, amino acids; AM, amines/amides; C, carbohydrates; CA, carboxylic acids; M, miscellaneous; N, nucleosides; P, polymers. Substrate list Table 5.5.

cluster of points with a few outliers. The component loading plots (Figure 5.6e-f) were similar in general appearance to those of the bacterial plates such that correlation plots were more evenly scattered, while covariance plots consist of a main cluster with a few very extreme C-sources as expected.

The April correlation PCA score-plot of PC1 and 2 (Figure 5.6a) produced a main cluster around zero, with two extreme outliers, plate ifcf1 on PC1 at about –19 and tfcf3 at about +18 on PC2. Ifcf1 contained several very high rates of colour change, while tfcf3 had considerably more zero responses than the other plates (22 wells out of 95 registering no colour development). Overall the other plates from this data set exhibited *r*-values in the 0.01-0.1 range.

Samples from July, November and January (Figure 5.6c & d) all appeared to be more variable than April, but as before showed no separation except a few outliers. The three outliers from July's PC1 and 2 score-plot (Figure 5.6b) were all tfc replicates. Plate tfcf3 being offset from all other data points to the extreme negative of PC1 (about -23), whereas tfcf1 and tfcf2 were displaced negatively along PC2.

Fungal plates from the November sampling (Figure 5.6c) produced outliers of a less extreme nature along PC1 and 2, making the main cluster seem more open than the previous two data sets. Plates tfbf3, tfdf3 and ifcf1 were displaced to the positive side of the cluster on PC1, tfaf2, tfaf3 and tfcf2 were more negative and tfaf2 was also extremely negative on PC2 compared with the rest of the data set. Low *r*-values (Appendix IV.VI.II, Figure IV.XX) from wells E5, G2, G10 and H3 which clustered at the top of PC2 on the component loadings plot appeared to influence the position of

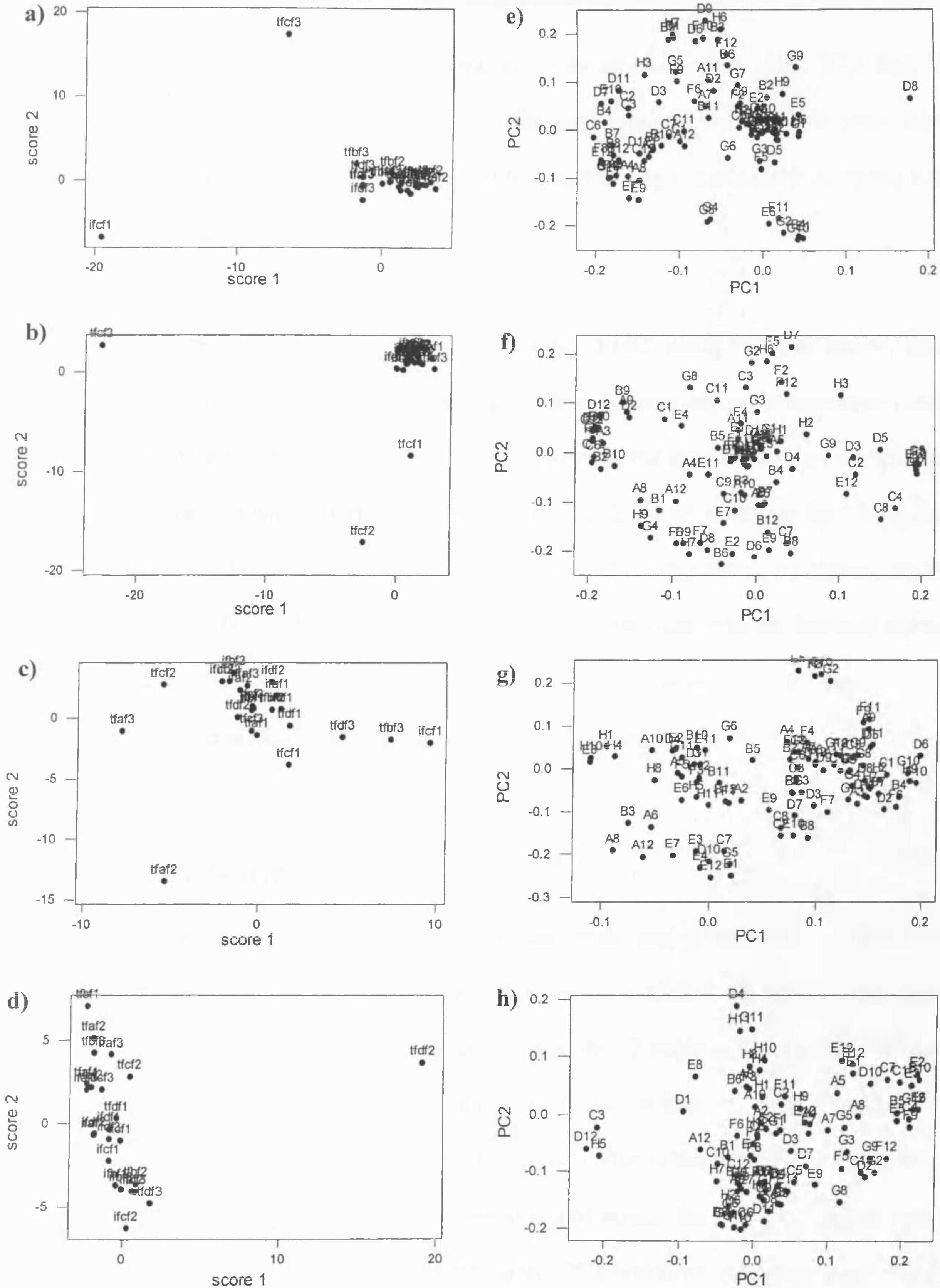

Figure 5.6 Biolog SF-N2 fungal plates, PCA (correlation matrix) score-plots for PC1 & PC2, a) Apr-02, b) Jul-02, c) Nov-02, d) Jan-03; and corresponding component loadings plots, e) Apr-02, f) Jul-02, g) Nov-02, h) Jan-03.

tfaf2. There was a slight tendency for TF to be more negative along PC2 than IF although some mixing occurred, however the plate replicate variation was quite large particularly for the TF plates so it is unlikely that this is a true separation of the two field communities.

Although there was one extremely positive outlier (tfdf2) along PC1, the January data was otherwise much less scattered and again showed some degree of separation along PC2, TF being more positive (Figure 5.6d). However, the division was not completely clean, three TF plates crossing into the IF region and one IF plate into the TF region. The C-sources on the component loadings plot (Figure 5.6h) were very mixed and no patterns of carbon guild use were apparent such as those seen with the bacterial plates. It appears that with the fungal plates, individual C-sources cause Biolog plate community separations rather than groups of similar compounds.

5.4.3.5 The Lag Period

The end of the lag period in the bacterial plate wells was determined to be the time reading at which the well colour was >0.1. Comparisons of the mean lag period for each set of field data at each sampling data showed that the TF community, in the Apr 02 and Jan 03 Biolog plates, was responding more rapidly to the substrate since it produced a shorter lag period (Figure 5.7). Corresponding PCA plots of the full data sets (all plates, all wells except A1) agreed with the comparison of means, the April and January data sets generally separating into field types along PC1 which explained the majority of variation in the data (Figure 5.8a & c), although there was some slight mixing of the April data. The July and November data, on the other hand tended to separate into fields on the PC2 and 3 plots (Figure 5.8f & g).

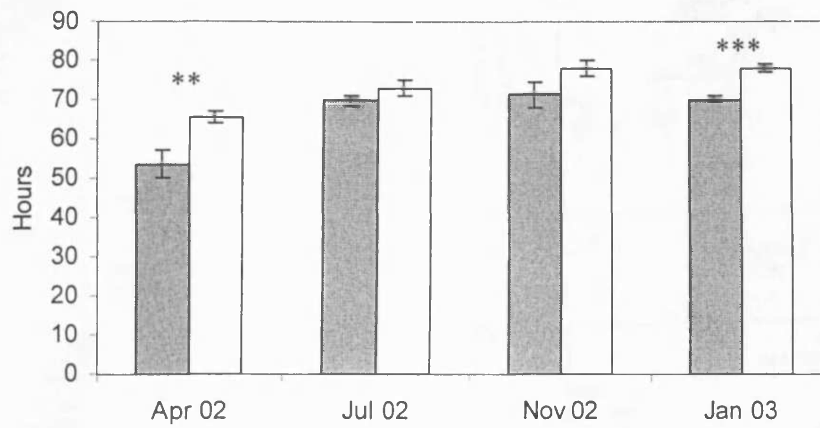

Figure 5.7 Comparison of mean bacterial plate lag period ± standard errors of the mean (n=12). Shaded bars = TF, clear bars = IF. Significant differences indicated *=P≤0.05 **=P≤0.01, ***=P≤0.001 (two sample t-tests).

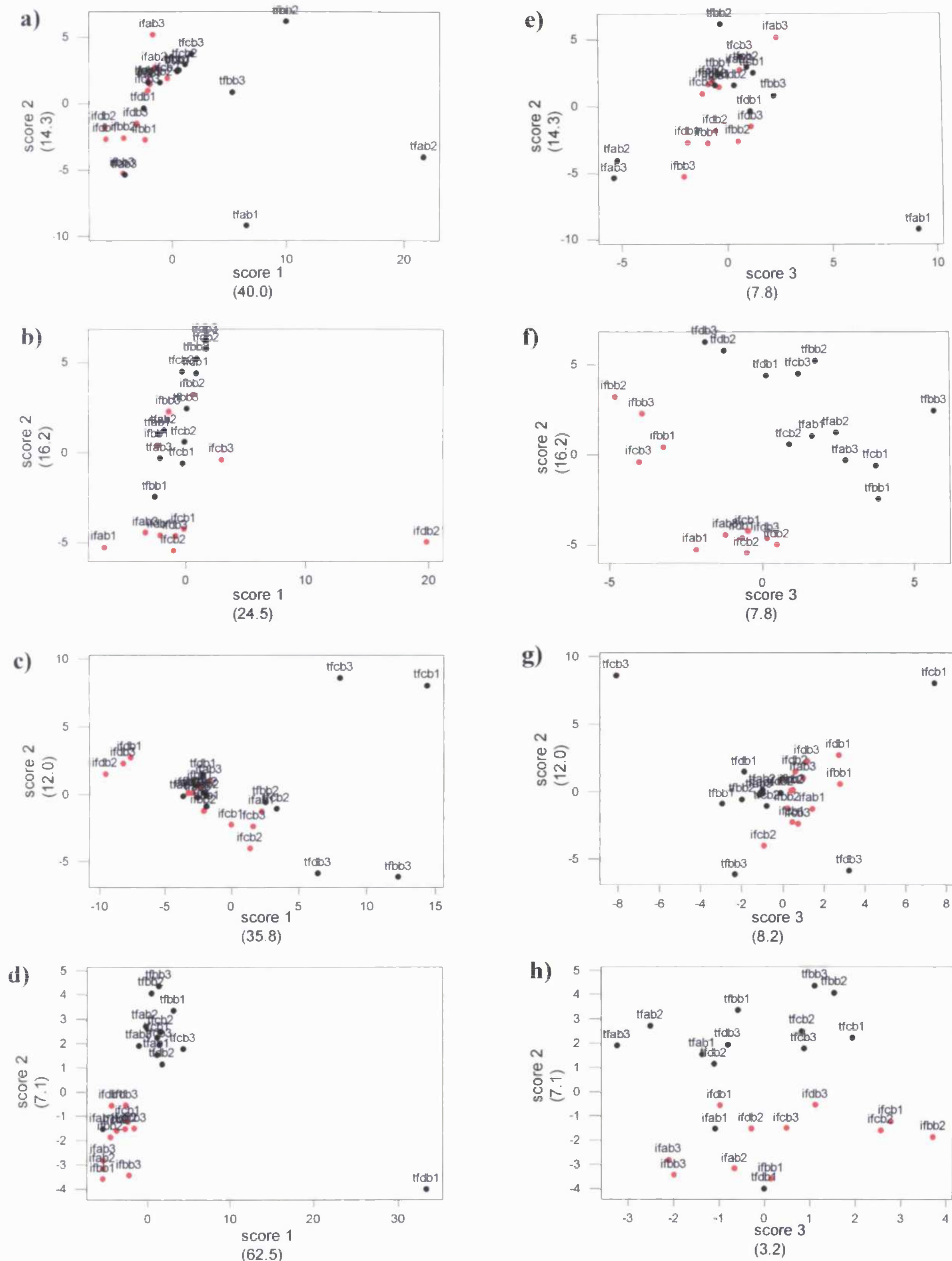

Figure 5.8 PCA (correlation matrix) of the lag period of all wells in all bacterial plates (except well A1). TF plates black dots, IF plates red dots. a) Apr 02, PC1 & 2; b) Jul 02, PC1 & 2; c) Nov 02, PC1 & 2; d) Jan 03, PC1 & 2; e) Apr 02, PC2 & 3; f) Jul 02, PC2 & 3; g) Nov 02, PC2 & 3; h) Jan 03, PC2 & 3. Proportion of the variation in the data explained by each PC in parenthesis.

5.4.4 Discussion

5.4.4.1 Soil Moisture and Temperature

TF experienced consistently greater soil moisture content and lower soil temperatures than IF. This was due to a combination of greater organic matter content in the soil and differences in the levels of shading provided by the two vegetation types since both fields lie on similar aspects with the same underlying soil structure.

5.4.4.2 Correction for Background and Control Well Colour

Data correction for background well colour at t_0 was necessary to account for slight differences between the colour of the well substrates and slightly variable colour added to the wells by the soil suspension. Thus correction of the background colour normalises the data to zero at the start of the experiment. However, a further complication is added by the common occurrence of slight colour development in the control well (A1).

A degree of colour change in the control well has been documented as a problem ever since Biolog Microplates were first applied to environmental community analysis. Colour change occurs in the control well due to carbon added to the well with the inoculum. Reduction in A1 well colour was observed with dilution of the inoculum proving that this was the source of the carbon (Garland & Mills, 1991). A large number of studies have corrected for control well colour by subtraction of A1 from the rest of the data set (e.g. Belete *et al.*, 2001; Classen *et al.*, 2003; Ellis *et al.*, 2002; Garland & Mills, 1991; Glimm *et al.*, 1997). In fact, Classen *et al.* (2003) justified A1 correction by referencing Biolog Inc. personal communication. However it should be noted that

across plate replicates, colour does not always develop in the control well of every plate to the same extent.

Carbon from the inoculum may exist either as soluble carbon or small particulate matter. If only soluble carbon was transferred to the wells from the inoculum one might expect all A1 replicates from a sample to develop colour to a similar extent, and the influence of the soluble carbon on all other wells would be equivalent. However, if the carbon is added as a result of small particulate matter, it might not be dispersed quite so evenly giving the more variable results in A1 replicates that were observed particularly in the fungal plates of this study.

Correction of the data set for background colour at t_0, together with A1 correction across all wells each time the plate is read, may well result in false negatives where carbon is particulate rather than soluble. PCA comparing the same data set adjusted either for background only, or both background and A1 colour, indicate that background only data is more likely to show field separation (Appendix IV.VII, Figure IV.XXVI). However, when univariate testing is employed to indicate which C-sources exert a significant influence over the separation, background-only corrected data is separated by scattered individual C-sources while A1-and-background data are separated by C-sources that tend to cluster into substrate groupings, e.g. carbohydrates or amino acids etc. (Table 5.8). Adjusting for background-and-A1 also accentuates outlying plates due to the possible inequalities in inoculum carbon distribution throughout a plate resulting in greater well colour variability. Nonetheless, it is better to be more cautious in interpreting community separations (since the Biolog plate represents only the functional diversity of a subset of the community) by correcting the data set for both

background-and-A1. Otherwise, a false separation may be interpreted in the data, which is just an artefact of the less than perfect PC analysis. Just because the situation the samples are taken from is different, we should not automatically expect to see a difference between community responses. This is a functional assessment and different species would be capable of performing the same function even in distinctly different environments.

5.4.4.3 A Sub-Set of the Community?

The Biolog assay is a cultural method in that growth occurs within the wells. Changes to the community are effected by the disturbance to the community and the selective pressures of the Biolog well environment. These selective pressures are likely to favour the fast growing adaptive species (Grayston *et al.*, 2004; Winding & Hendriksen, 1997). Changes to model communities have been observed where patterns were dominated by the fastest growing strains which could mask slower strains even when they were inoculated at a very low proportion (10%) of the whole community (Verschuere *et al.*, 1997). Molecular analysis has also confirmed changes to the community structure following inoculation into GN plates (Smalla *et al.*, 1998).

Analysis of the resultant community in SF-N2 plates inoculated with TF soil, following plating of the whole well content onto water agar revealed only 12 morphotypes from all 96 wells, with the number of individual morphotypes per well ranging between 1 and 6 (5 being the modal value). The original inoculum suspension when plated directly onto 0.5% malt extract agar revealed a number of different morphotypes which were not found in the Biolog wells. No basidiomycetes (with clamp connections) were observed. This is obviously a much reduced sub-set of the original soil fungal community as

identified by traditional culturing (32 morphotypes) or direct rDNA extraction from soil (at least 21 species) (Hunt, 2003).

Table 5.8 Carbon source significance determined by Mann-Whitney test. The *r*-parameter data for an individual C-source from all TF plates was compared with the IF data for the same C-source. The effect of correcting for background colour only and well A1 & background colour was compared. Ticks indicate significance in both background and background & A1 corrected data, TF or IF indicate which field samples the greater colour development was produced by.

	Bacterial			Bacterial			Bacterial			Bacterial			Fungal		
	Apr A1 & Backg	Apr Backgr		July A1 & Backg	July Backgr		Nov A1 & Backg	Nov Backgr		Jan A1 & Backg	Jan Backgr		Jan A1 & Backg	Jan Backgr	
A1					* TF			** TF							
A2															
A3							* TF	* TF	✓	** TF	** TF	✓			
A4										* TF					
A5	* IF														
A6															
A7							* TF	* TF	✓	** TF	*** TF	✓		* TF	
A8	** IF	** IF	✓												
A9															
A10															
A11								* TF		* TF	** TF	✓	** IF		
A12				*** IF	*** IF	✓	* TF	** TF	✓	* TF	* TF	✓			
B1													* IF		
B2		* TF								* TF					
B3													* IF		
B4										** TF	* TF	✓			
B5										*** TF	*** TF	✓			
B6															
B7															
B8										*** TF	*** TF	✓	** IF		
B9				* TF	** TF	✓				* TF	* TF	✓	* IF		
B10								* TF		** TF	* TF	✓		** TF	
B11	** TF	** TF	✓					** TF		*** TF	** TF	✓	** IF		
B12		* TF								*** TF	*** TF	✓	* IF		
C1															
C2	* TF	** TF	✓												
C3							* IF								
C4										** TF	** TF	✓			
C5	* IF							* TF		** TF	** TF	✓			
C6				* TF	* TF	✓		** TF			*** TF		** IF		
C7															
C8								* TF		** TF	* TF	✓			
C9										* TF					
C10					* TF									** TF	
C11				* IF											
C12					* TF										
D1											* IF			* IF	
D2				** IF	*** IF	✓									
D3	* IF	** IF	✓	** IF	* IF	✓				*** IF	* IF	✓		* IF	
D4	** IF	* IF	✓				* IF				** IF			*** TF	
D5															
D6	* IF														
D7														** TF	
D8														*** TF	
D9							* IF				* IF				
D10		* IF									* TF				
D11							* IF			* TF					
D12				* IF									* TF	* TF	✓
E1													*** TF	** TF	✓

E2							** IF	* IF	✓						
E3													* TF	* TF	✓
E4															
E5		** IF												* TF	
E6															
E7										* IF					
E8											* IF				
E9							* IF								
E10							* IF			* IF	* IF	✓		** TF	
E11															
E12					** IF								** TF	*** TF	✓
F1	* TF													* TF	
F2														*** TF	
F3													* TF		
F4															
F5				* IF											
F6				* IF	* IF	✓									
F7		* TF													
F8							** IF	* IF							
F9							* IF								
F10															
F11	* IF	* IF	✓												
F12															
G1	** IF														
G2							*** IF	** IF	✓						
G3														* IF	
G4	* IF			*** IF	** IF	✓							* IF		
G5	* IF						** IF	* IF	✓				* TF		
G6	* TF	* TF	✓										** IF		
G7							*** IF	** IF	✓					* TF	
G8	* IF							* TF			* TF				
G9															
G10	* IF														
G11													* TF	** TF	✓
G12				* IF						** TF	** TF	✓			
H1														** IF	
H2							*** IF	*** IF	✓						
H3															
H4	* IF	* IF	✓							* TF	* TF	✓	* TF	** TF	✓
H5															
H6							* IF								
H7													* IF		
H8															
H9					* TF		*** IF	* IF	✓		* IF				
H10											** TF				
H11										* TF	* TF	✓	* TF		
H12										** TF	** TF	✓			

*=P≤0.05 **=P≤0.01, ***=P≤0.001, NS= not significant.

Traditional fungal culturing methods also experience the inherent problem of plates being rapidly overrun with fast growing opportunistic species. Many saprotrophic fungi occupy a niche in which they degrade many of the more recalcitrant compounds not dealt with in the most part by other organisms. Experience of culturing soil fungi or attempting isolations from environmental samples demonstrates how sensitive some species can be to the nutrient conditions of the growth media. Nutrient enrichment

inhibits or prevents growth in some cases, and it may be that this occurs in the Biolog plate wells where nutrient conditions are quite high (Konopka *et al.*, 1998).

Although careful selection of media can result in successful culturing of some basidiomycetes, the proportions of these fungi that would be cultured or contribute to a Biolog fingerprint, is unlikely to come close to the 41% of sequences that were derived from basidiomycetes from the TF and IF soil communities (Hunt *et al.*, 2004). However, both traditional culturing and Biolog Microplates would fail to detect the contribution of arbuscular mycorrhizal species, which are associated with 80-85% of terrestrial plant species (van der Heijden *et al.*, 1998) but cannot be cultured without the host plant. Basidiomycete isolates in sole culture are able to form a relatively reproducible fingerprint on SF-N2 plates as tested by *Stropharia pseudocyanea* and *Lepista saeva* both isolated and cultured from fruit-bodies collected from IF. So it is not that basidiomycetes are incapable of utilising the Biolog plates, rather that they are out competed and so do not contribute to the pattern of C-source utilisation.

5.4.4.4 The Use of SF-N/SF-N2 Plates to Assess Fungal Functional Diversity

The application of Fungal SF-N or SF-N2 plates to the analysis of fungal functional potential from environmental samples has apparently been used successfully by Dobranic and Zak (1999), Buyer *et al.* (2001), Classen *et al.* (2003) and Sobek and Zak (2003) to differentiate between communities from different soils or leaf samples. Dobranic and Zak (1999) who developed the original fungal Biolog protocol made no mention of problems with the distribution of colour within the wells. In the present study, however, it has become apparent that because tetrazolium reduction occurs in the cytosol, colour in the fungal plates is mainly restricted to the mycelium and not

dispersed more generally throughout the well as occurs with the more uniform dispersal of bacterial cells. This obviously increases the level of variability in repeated readings of a single well. Continuous shaking during incubation might improve this situation, but that also presents increased risks of spillage and cross contamination and due to the small volume of liquid, may still not evenly suspend the mycelia since shaking often induces a ball like form of growth in larger broth culture. The two latest studies that have used SF-N2 plates (Classen *et al.*, 2003; Sobek & Zak, 2003) improved the suspension of hyphae in the wells by including a thickening agent in the inoculum, 0.2% water agar and 0.01% Gellan gum respectively. This may help to improve the accuracy of replicate readings by enabling mycelia to distribute more evenly throughout the well.

An additional problem created by the use of the tetrazolium dye is that some mycelia do not appear to generate formazan from the MTT within the wells, although their own biomass will still contribute to the optical density readings (increased turbidity). In turn this complicates the interpretation of the well OD readings (as do dark melanised hyphae which limit the transmission of light through the well and may mask the contribution of additional paler species). If the inclusion of MTT was supposed to measure metabolism rather than simply growth (Dobranic & Zak, 1999), then the failure to produce formazan results in unregistered metabolism and a plate that does not fully represent the functional potential of the contributing community. These problems together with the limited range of fungal species/strains that contribute to colour development (considering the range and types of species identified even through just the fruit-body survey) strongly suggest that SF-N2 plates are of little use in the assessment of mixed fungal community functional potential.

5.4.4.5 The Value of Kinetic Analysis

Initial studies that developed the use of Biolog Microplates for the analysis of community function required the choice a single time point for the analysis of the data, which was determined arbitrarily by the researcher and could be the point of maximal activity (Zak *et al.*, 1994), 24h following the start of colour development in a significant number of the wells (Garland & Mills, 1991) or the time to reach midpoint in average well colour development (AWCD, the sum of differences between control (water) and substrate wells divided by 95) (Garland, 1996a). The latter approach can mean comparing plates from different time readings. It is apparent from the total plate colour development curves (Appendix IV.VI, Figure IV.V) that the colour development, even in the bacterial plates, had barely begun after 24 hours. While, comparisons involving the midpoint of exponential increase would become increasingly difficult when comparing samples taken from different times of the year such as in the current study, and such analysis requires the colour development to be recorded until the asymptotic phase is reached anyway. Without taking the whole sigmoidal colour development curve for data analysis, an intrinsic problem occurs with inoculum density, where a well containing more cells would reach the stage of colour development more rapidly than the same well with a smaller inoculated population of cells. For example, only 25-30% of substrates responsible for separating communities under differing treatments were stable when the analysis was undertaken at different time points (Bossio & Scow, 1995). To account for this problem of variable inoculum density, the suggestion of normalisation, by dividing each cell by the AWCD, was made (Garland & Mills, 1991) and subsequently commonly used (Preston-Mafham *et al.*, 2002). With average or total (Classen *et al.*, 2003) well colour normalisation of a plate one assumes that the total

plate or final well colour is linked directly to the initial inoculum density. This is unlikely to be true since microbial degradation of macromolecular carbon substrates is commonly the result of interactions within a community (Waldrop *et al.*, 2000). These synergistic or antagonistic interactions also occur within the Biolog well (Haack *et al.*, 1995).

Kinetic analysis of the data (Lindstrom *et al.*, 1998) removes the requirement for a decision to be made about which time point to use for analysis, since plate colour is recorded to the cessation of colour development. The parameters K and r are independent of inoculum density, although s is correlated with inoculum density since it includes the lag phase, and is also correlated with r and K (Lindstrom *et al.*, 1998; Lindstrom *et al.*, 1999). The parameter r is dependent on the rate of MTT reduction, which is initiated upon the community reaching sufficient population density, around 10^8 cells per ml has been suggested (Konopka *et al.*, 1998; Winding & Hendriksen, 1997). The analytical value of r-parameter data in PCA (correlation matrix) has been demonstrated by the differentiation of functional diversity in oil contaminated and spill free Alaskan soil samples (Lindstrom *et al.*, 1999). Although, the separation became less distinct when the dilution factor was increased to 10^5 presumably due to increased species losses with dilution.

Although it has not yet been widely used due to the complexity of the data handling, kinetic analysis should provide the most robust and detailed analysis of substrate use (Haack *et al.*, 1995; Hackett & Griffiths, 1997; Lindstrom *et al.*, 1999) since it removes the qualitative assessment of at which time reading the plates should be compared. However, in the current study, the use of PCA to interpret the kinetic data suffers from

the inherent PCA problem of using more variables than replicates. This means that the orientation of the plates on the PCA plots changes with the removal of a single datum point. Even though the PCA creates instability in the analysis, any community separations that are observed must be relatively strong in order to show through. From the bacterial community data, robust separations were observed in the November and January samples (Figure 5.4c & d), and a trend was seen in the combined seasonal data (Figure 5.5).

5.4.4.6 Field Community Separations

A number of substrates are not utilised by bacteria, fungi or both (Table 5.6). Of these, C-sources which are intermediates in the cellular respiration pathway, such as Glucose-6-phosphate, may not be utilised in the Biolog wells because the cellular membrane transport mechanisms are designed to keep them inside the cytosol where they are produced and further catabolised during the respiration process. Therefore there may be no, or only very low transport of this molecule across the cell membrane from outside where it would not normally be found. C-sources such as these, which are not likely to be found in the environment, are redundant to the analysis of environmental samples, thus, the use of Eco-plates or specifically tailored plated designed for the particular requirements of a study would be more appropriate (and less wasteful).

Although the analyses of bacterial plates from the separate seasons are not consistent in their differentiation of the TF and IF soil communities, ultimately the multi-seasonal plot indicates that there may be underlying differences in the potential functional ability of the two meadows. Patterns of field (Figure 5.4c & d) or other group (Figure 5.4a) separation that do occur in the bacterial plates tend to be influenced by clusters of C-

sources belonging to substrate guilds rather than a selection of C-sources scattered across all of the chemical guilds. This grouping of C-sources suggests a more reliable separation since it is more likely that community separation would occur due to the presence or absence of enzymes for particular groups of substrates such as aminotransferases which catalyse the deamination of amino acids.

The community separation which occurs between TF and IF is largely influenced by the more rapid catabolism of polymers and carbohydrates (wells A-C) in TF and D, E, F, G and H wells (carboxylic acids, amines/amides, amino acids, nucleosides and other miscellaneous compounds) in IF across all seasons. It is possible that the colour change in F, G and H wells of the IF plates is more complicated than simply one main species/strain per well being capable of more rapid catabolism of the substrate. Most of the substrates in these wells are linked by the fact that their catabolism during cellular respiration requires the removal of the nitrogen containing amino group. This is cleaved by the aminotransferase enzymes and results in the formation of ammonia, which must be excreted. In the confined environment of the Biolog well it might be expected that deamination would fairly rapidly result in the accumulation of ammonia to inhibiting levels. However, nitrifying bacteria are able to use ammonia as an energy source and if present in a well this oxidation reaction may also contribute to the development of well colour if it is coupled to formazan formation. It is possible that IF soil might contain more nitrifying bacteria than TF soil as a legacy of the previously more intensive grassland management system, in which it underwent regular nitrogen fertiliser additions. Increases in inorganic fertiliser application have been shown to increase nitrification potential, it can also alter on a seasonal basis, peaking from late August-October in a study in the USA (Fortuna *et al.*, 2003). This peak corresponded with crop

maturation and up to one month beyond. Rapid catabolism of amino acids occurs during a similar stage in the IF field and may indicate a similar nitrification cycle.

The traditional field contains a plant community which is not only more species rich but also consists of a wide range of plant types that contribute litters and root exudates of variable quality and complexity in terms of microbial decomposition and metabolism. It has been suggested that more variable inputs to a system would produce a microbial community capable of metabolising a wider range of substrates, i.e. have a greater functional diversity, than a system that inputs a limited range of substrate types (Myers *et al.*, 2001). The more diverse range of substrates produced by the plant community in the traditional field are likely to contain a good mixture of labile and more recalcitrant compounds. The availability of labile compounds may be reflected by the more rapid metabolism of carbohydrates and polymers observed in the Biolog GN2 plates.

The length of lag phase, of the well colour response, should reflect the rapidity of the initiation of C-substrate catabolism. Primarily, two thing may affect this, firstly the inoculum density (Garland & Mills, 1991) and secondly the type of microorganism, i.e. r-selected organism should respond more rapidly to high nutrient levels than K-selected species. The Biolog plates are likely to support mainly r-selected species (Haack *et al.*, 1995). The comparison of the lag periods, both mean plate lag (Figure 5.7) and PCA of all individual plate-well responses (Figure 5.8) shows that during the colder months, January and April, the response of the TF plates was more rapid than that of the IF plates (Figure 5.7). Since the plates were inoculated at equivalent dry weights (Section 5.4.2.4), the difference observed should be a result of the relative differences in community structure. Even if this was purely due to cell inoculum density it remains a

valid difference in community structure, indicating that TF, during the colder months, either maintains a higher cell density per g soil, or has a greater proportion of r-selected bacteria, in its microbial community, than IF.

Bossio and Scow (1995) suggested that substrate utilisation patterns inferred from the Biolog data should be used to generate hypotheses for testing in microcosm situations rather than as conclusions in themselves. As the procedure stands at the moment this seems a more sensible use of the results produced by the plates which are so restricted by growth and community changes within the wells.

Perhaps the resolution of the technique could be improved if we consider the scale at which we are working. By bulking soil we mix up the populations within a community so that differentially selective species that normally exist on separate spatial scales are thrown together. Inevitably the faster growing more opportunistic species will take over. One of the restrictive factors affecting the number of replicates that can be undertaken, as part of an investigation, is the cost per pre-prepared microplate. If the researcher prepared their own substrate plates, perhaps with fewer substrates and more replicates per plate (as found in Eco-plates) then it would be possible to run far more replicates. Much smaller non-bulked samples could then be used, for example 0.1-0.5g (although the optimum soil sample size would need investigation), these could then be taken along a transect from points 10cm apart (the transects could also be replicated). Samples from different depths could also be taken from each core to build up a more complete picture of microbial function. Ultimately the aim is to reduce the extent of population interactions (which are currently increased by the homogenisation of comparatively large soil samples) within the plates and increase the number of replicates to cover a fair

representation of the soil heterogeneity, remembering that we are looking for function not species, and the distribution of functional groups should reflect the distribution of substrates. Patchy distribution of plant types may produce a concomitant patchy distribution of microbial functional processes. While more evenly mixed vegetation could either produce a greater level of heterogeneity over a smaller scale, or reduce observed heterogeneity by providing a more evenly spread homogenised substrate.

Due to the large amount of heterogeneity experienced in the soil environment and the many variables that influence the system, no single measure is capable of fully describing the functional diversity and structure of the microbial community. A number of different techniques should be used together to produce a more coherent picture. Consequently, the contribution of plant diversity to the functional diversity of the microbial community will be discussed further and placed in context with the other aspects of this study in the general synthesis at the end of this thesis.

Chapter 6. Synthesis

6.1 Site Conditions

The traditionally managed and improved fields used in this study had, over the last 8 years (conditions permitting), both been maintained by the same traditional hay meadow management system (Chapter 2). However, a large difference in plant diversity was still evident, the traditional field being plant species rich (a mixture of around 53 species of forbs and grasses) while the improved field remained species poor, being dominated by vigorous grasses (e.g. *Holcus mollis* and *Festuca rubra*) which typically replace the sown fodder species *Lolium perenne* and *Trifolium repens* after the abandonment of an intensive management system (Marriott *et al.*, 2003). The two fields also sported a wide range of fungal fruiting bodies, and were both classified as sites of regional importance on the CHEG classification system (Rotheroe, 1999 and Rald, 1985), although TF scored slightly higher (Section 2.3.3). In terms of the range of fruit bodies recorded, TF was more species rich and the species tended to be larger fruiting types.

The management legacy observed above-ground was also found below-ground both in the abiotic conditions and microbial community structure. The fields are neighbouring, sit on the same bedrock and are of the same soil type, slope and aspect, therefore, any differences are likely to be due to differences in historical management. The TF soil had a significantly greater moisture content (Chapter 2) than IF at all times of the year (Figure 6.1a). This may be due to a combination of factors including the plant architecture (shading of soil), which resulted in TF having a significantly lower soil temperature in all but the summer months (Figure 6.1b), and the different root structures

of forbs and grasses, some forbs being deep rooted so drawing water from deeper in the ground, while grasses, which tend to be mainly fibrous rooted, take water from the surface layers. Also, moisture retention would be influenced by the higher organic content (Chapter 2) of the TF soil (had a significantly higher % C at all times of year [Figure 6.1d]), which would have built up undisturbed over at least the last 80 years, unlike IF which was ploughed and heavily grazed during this time.

The higher organic content was also reflected in the amount of total organic and ammonium N (Chapter 2), which was again significantly higher in TF (Figure 6.1e). Records of higher N in improved grasslands compared with unimproved grasslands are usually measures of inorganic nitrate (Bardgett *et al.*, 1999a; Donnison *et al.*, 2000b), while total organic nitrogen records higher concentrations in unimproved soils (Grayston *et al.*, 2004). The technique used for extracting N did not extract nitrate and therefore, the amount of inorganic fertiliser residue that may have remained in IF was not measured, however, due to the high solubility of nitrate it is likely that much of it would have leached away, it being over 8 years since the last application of inorganic fertiliser.

Phosphorus concentrations (Chapter 2) were the same in both field soils, and did not change between seasons (Figure 6.1f), suggesting that the inorganic fertilisers used in IF in the past had dissipated, since, total P is generally not affected by the plant community (Grayston *et al.*, 2004). Finally, the last of the abiotic parameters measured, soil pH (Chapter 2), was significantly higher in TF at all times of the year (Figure 6.1c). This may have been a result of the previous fertilisation of IF, since N fertilisation decreases pH (Bardgett *et al.*, 1999b) due to the activity of nitrifying bacteria

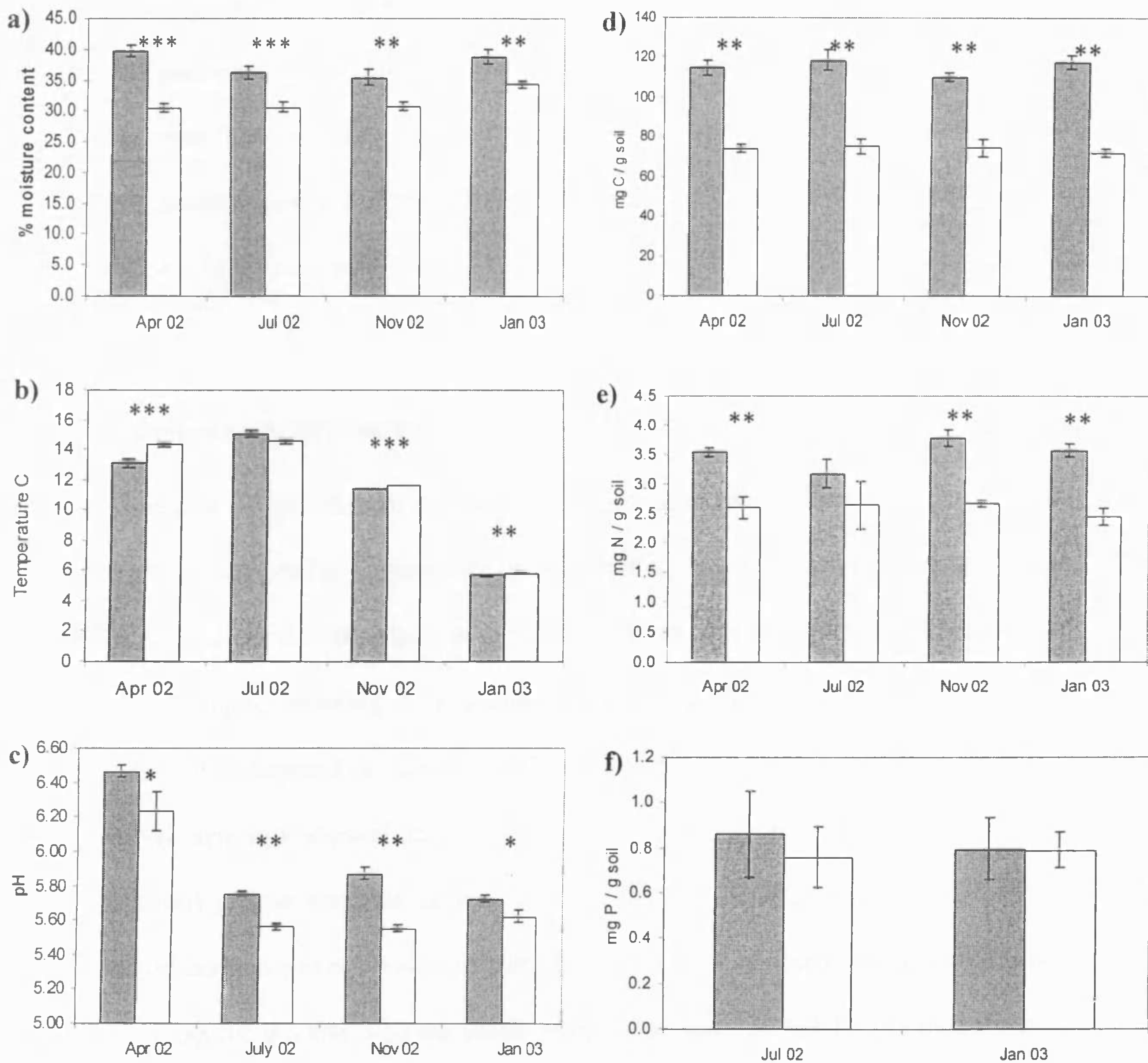

Figure 6.1 a) soil moisture content, soil temperature, c) soil pH, d) loss on ignition soil of organic content (C), e) soil total organic and ammonium nitrogen, f) total soil phosphorus. Shade bars are TF results, open bars IF. Means and standard errors shown. Significant difference between fields indicated, *≤0.05, **≤0.01, ***≤0.001. Some of the Figures above can also be found in previous chapters, a) Figure 2.5, c) Figure 2.6, d, e & f) Figure 2.7.

(Fortuna *et al.*, 2003). In addition, plant species can affect soil pH, *Agrostis capillaris, Lolium perenne, Holcus lanatus* and *Festuca rubra* showing a trend of increasing acidity when grown in pot monoculture (Bardgett *et al.*, 1999b). *Festuca rubra* and *Holcus lanatus* were the dominating species in IF, so may have contributed to the increased acidity found in IF soil.

6.2 Community Structure

The size and composition of the soil microbial community is known to change with depth of the soil profile, the majority of microbial activity and biomass occurring in the upper 5cm of the soil (Bardgett *et al.*, 1997), due to oxygen depletion and increasing CO_2 with depth, resulting in a change from an aerobic to increasingly anaerobic environment (Sheppard & Lloyd, 2002). Dehydrogenase activity (as a measure of microbial activity) showed that, in the upper 0-5cm of the soil profile, TF had significantly greater microbial activity than IF (Figure 6.2). The activity in the lower 5-10cm soil horizon was equivalent in both IF and IF soil. A constant level of activity was found across the 0-5 and 5-10 cm depth profile of IF soil. Overall TF registered greater microbial activity than IF (two sample t-test, $t_{34} = 4.87$ $P<0.001$). This was probably a result of the higher organic content, available for microbial metabolism, and a combination of increased moisture holding capacity (due to the organic material) and soil temperatures.

A large number of studies investigating the microbial community structure of grasslands under traditional and intensive/improved management regimes, have found that traditional management results in a community structure that is less dominated by bacteria, fungi contributing more to the community structure (measured by PLFA

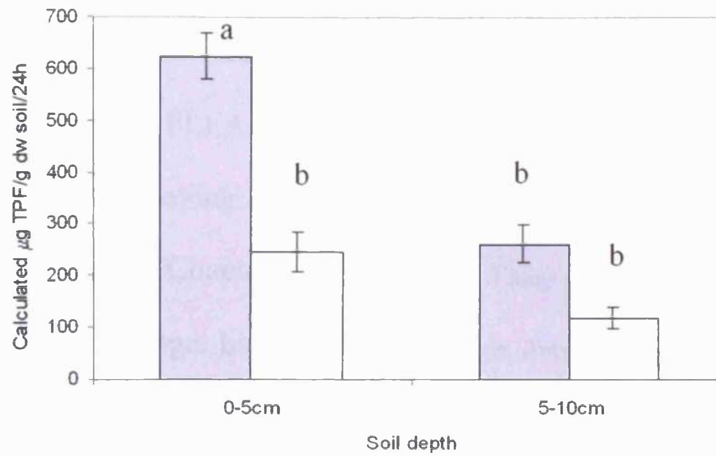

Figure 6.2 Microbial activity in soil samples taken on 9th Sept 2003, measured by dehydrogenase activity in TF (shaded bars) and IF (open bars) soil at 0-5cm and 5-10cm depths. Bars with the same letters are not significantly different at P≤0.05. Means and standard errors shown (n=12). Also can be found in Chapter 2, Figure 2.8.

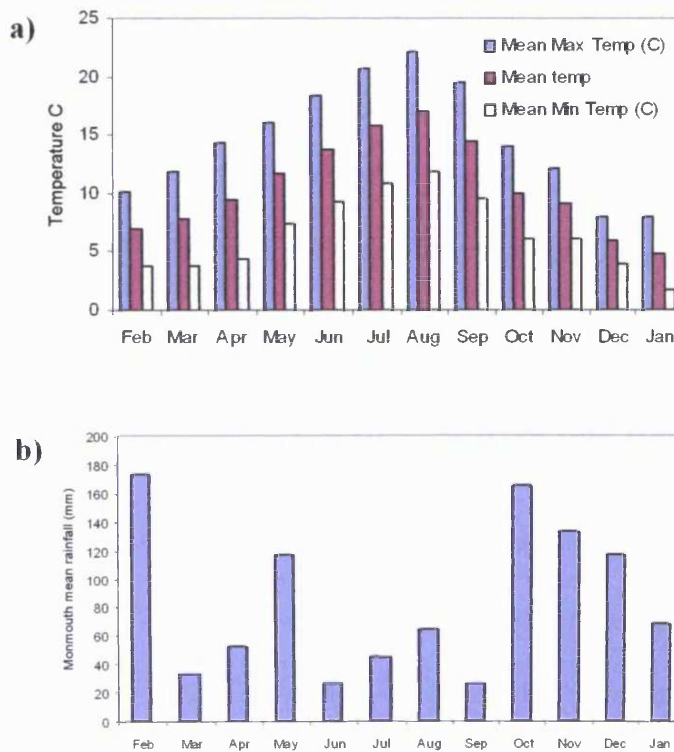

Figure 6.3 Local temperature (a) and rainfall (b) for the period Feb 2002 to Jan 2003.

analysis) than is found under improved management systems (Bardgett *et al.*, 1999a; Bardgett & McAlister, 1999; Grayston *et al.*, 2004; Grayston *et al.*, 2001) and that heavily grazed sites also have a more bacterially dominated decomposition system (Bardgett *et al.*, 2001). PLFA results from the current study, in general, agree with these findings, TF overall having a higher fungal:bacterial ratio and higher fungal PLFA concentration than IF (Chapter 3, Figure 3.2). This, on average, should equate to TF containing a greater fungal biomass than IF since, even though different fungal species contain variable amounts of PLFA per unit biomass, a 1g soil sample should be large enough for the central limit theorem (Section 3.1.1.2) to apply (Kalmer & Bååth, 2004). However, the difference between fields was only statistically significant in the January 2003 soil samples, being not significant in July 2002 although the mean fungal:bacterial PLFA ratio was grater for TF than IF.

Seasonal variation in PLFA measures have also been noted in other studies, (Bardgett *et al.*, 1999a). Bardgett *et al.* (1999a) found seasonal differences in PLFA (being highest in spring and lowest in autumn) to be related to soil moisture and soil mineral N. TF soil, in the present study, showed January maxima and July minima in total and bacterial PLFA, IF remaining approximately constant over the two sampling dates. However, soil moisture (Figure 6.1a) did not differ significantly between July and January (although rainfall was higher in January [Figure 6.3b]). The only other factor that was slightly different between these two months was soil N concentration, although, it was again not a significant difference (Figure 6.1e). Thus, it is difficult to explain the seasonal difference between TF total PLFA without data from more sampling dates to contribute to the picture. It may be that a combination of soil moisture and temperature allowed a bacterial flush of growth in January (bacteria dominating the

total PLFA measurement), bacteria being able to respond more rapidly to favourable conditions than fungi, while the IF soil was not as favourable (having had a significantly higher moisture content in January, combined with colder temperatures, than July [Figure 6.1b & Figure 6.3a]). Site (Grayston *et al.*, 2004) or seasonal (Bardgett *et al.*, 1999a) differences often explain more of the variation in total PLFA then management type, indicating that moisture and temperature (microclimate) are determining factors in soil microbial community structure.

Higher pH and greater soil fertility are generally conditions thought to favour a bacterially dominated community (Grayston *et al.*, 2001). However, the TF, which had both a higher pH and N concentration, supported a potentially greater fungal biomass than IF. Therefore, soil pH does not appear to affect the microbial community structure, in terms of large-scale microbial groups, in a predictable way (also noted by Brodie *et al.* (2003)). Thus, the effect of acidification limiting plant community diversity (Bakker & Berendes, 1999) could be a more specific affect of individual microbial species, or a more direct affect of the pH tolerance of individual plant species.

6.3 Community Function

Two different aspects of community function were measured in the current study. Litterbag experiments (Chapter 4) provide an insight into in-situ community function, through the decomposition of a relatively realistic set of recalcitrant substrates, although in a completely natural system invertebrates would have started mechanical breakdown processes prior to the incorporation of litter into the soil profile. Sole-carbon-source-utilisation (SCSU) (Chapter 5), on the other hand, tested the functional potential (on a range of relatively labile sole-C-sources) of only the r-selected bacteria that dominate

the Biolog plate environment (the fungal plates having been deemed of no informative value).

The rate of decomposition of litterbag substrates was determined by seasonal conditions (temperature and moisture), which are known to affect microbial abundance and activity (Robinson, 2002), and the substrate type. The decomposition rates of each substrate type (during its fastest phase of decomposition) were in the order: field litter > cellulose > oak leaves > wood. However, following the metabolism of the more labile components of the field litters, the decomposition of this substrate slowed to the equivalent of the oak leaves, indicating the importance of cellulose and lignin content in determining decomposition rates (Bardgett & Shine, 1999). In addition, reciprocal comparisons of the decomposition of field litters also confirmed the importance of initial C:N ratio (Knops *et al.*, 2001). However, the accumulation of N in IF litter, even though initial C:N content was not significantly different from TF litter (Section 4.4.8), suggests that IF litter was more recalcitrant and required a smaller C:N ratio before decomposition could proceed. This did not however, result in any difference in overall decomposition rate of TF and IF litters in this particular experiment. There were also no discernible differences, between fields, in the ability of the respective microbial communities to break down each substrate type, even though the community structure and recorded levels of dehydrogenase activity suggested that differences between the communities did exist. Thus this may indicate functional redundancy, or functional equivalence (Setälä & McLean, 2004) between the two communities.

The SCSU, on the other hand, suggested that community differences between C-source utilisation were apparent. Overall, TF inoculated plates appeared to catabolise the

simple carbohydrates more rapidly than the IF community which catabolised carboxylic acids, and N containing amines/amides, amino acids and nucleosides more rapidly, although there was some overlap between the communities (Chapter 5, Figure 5.5). This may mean that TF contained a greater proportion, or higher total numbers of r-selected bacteria, which respond very rapidly to exposure to labile compounds, than IF particularly in November 2002 and January 2003 when the community separations were more apparent. Community separation in the Biolog plates also seems to correlate with the increased bacterial PLFA recorded in January 2003, and supports the hypothesis stated above, that the TF bacterial community was more able to respond rapidly to favourable conditions, perhaps due to the more insulative soil environment (increased organic layer) in TF preventing frost damage to cells during the colder months. However, the same set of substrates was catabolised by both microbial communities and the separation of fields, in terms of catabolic rates, was not apparent in the individual PCA plots for April 2002 or July 2002 (Chapter 5, Figure 5.4a & b).

Results from SCSU for both July 2002 and January 2003 corresponded with the PLFA analysis in which July total bacterial PLFA was equivalent in both fields and no difference in SCSU was recorded, whereas, January bacterial PLFA was significantly higher in TF and distinction in the rates of SCSU occurred. Rapid increases in bacterial biomass in response to favourable conditions are likely to be led primarily by the r-selected proportion of the community, resulting in the January 2003 Biolog response from the TF samples and corresponding to the increase in TF bacterial PLFA observed in January 2003. In the decomposition of more natural complex substrates, however, no significant differences between the microbial communities were found, indicating that

the bacterial community had less influence over the decomposition of recalcitrant substrates.

In the current study, Biolog plates were inoculated with fresh soil of equivalent dry weights, with no centrifugation, since centrifugation results in the loss of a proportion of the organisms in a sample (Calbrix *et al.*, 2004). As a result, the SCSU results correspond well with the PLFA results. Previous studies have found that PLFA and SCSU did not correlate well, having accounted for differential inoculum densities by AWCD transformation of the Biolog data (Buyer & Drinkwater, 1997; Pennanen, 2001). Thus, the data from the current study shows the importance of maintaining soil communities at their natural relative densities if the responses are to be more equivalent to the field communities (in terms of the portion of the community that contributes to Biolog well catabolism), since the relative density of the various groups, within and between communities (i.e. community structure), is primarily important in comparisons of community function. If the full functional potential of the community (i.e. the functional potential of each component species/strain) is required, then the mixed community inoculation of Biolog plates is not viable in the form taken by most studies to date, since it brings organisms into competition that would not normally spatially coexist due to the heterogeneity of the soil environment. Ideally, many more replicates of much smaller soil samples would be required, thus, a smaller set of the most discriminating substrates, replicated several times per plate, would be of more use.

6.4 Structure and Functional Status of the Microbial Communities in Relation to Plant Diversity and Management History

The effects of fertilisation from an intensive management regime are thought to affect the microbial community both directly and through resultant changes in plant productivity and input quality (Bardgett & McAlister, 1999; Donnison *et al.*, 2000b). However, direct affects of fertilisers on fungal growth appear to be more significant than input quality (Donnison *et al.*, 2000a). The total microbial biomass in grasslands under intensive management systems has consistently been found to be lower than in traditional systems (see Bardgett & McAlister (1999) for references). These studies compare long term fertilised with unfertilised systems, however, the improved grassland in the present study (although it had pre-1996 been under intensive management) is no longer fertilised and since 1996 has been managed in the same way as the traditional field. This may explain why there was not a consistent significant difference in total microbial PLFA, since the accumulation of inorganic nutrients due to fertilisation seems to have dissipated (equivalent soil P and higher total N in TF). In other studies, the affects of fertilisation on the microbial community, were still apparent after 6 years of no inputs, and residual inorganic nutrients were still discernible (Bardgett & McAlister, 1999), whereas IF had not been fertilised for at least 8 years.

Fungal PLFA in TF was greater than that in IF, but the lack of difference in the decomposition of litterbag substrates corresponds with the functional redundancy recorded in the fungal community of forest soils, in which functional diversity increases with increasing fungal diversity, but only at the lower end of the species diversity gradient (Setälä & McLean, 2004). In fungal terms both TF and IF were quite species rich (certainly as indicated by the numbers of fruiting fungi). Donnison *et al.* (2000b)

suggest that, although management might result in differing sizes and compositions of fungal communities, no concomitant change in function is necessarily observed. Thus, although differing in fungal community structure, both the TF and IF communities were able to decompose even the more recalcitrant substrates to an equivalent extent.

There is evidence that functional characteristics (e.g. resource utilisation and capture), particularly of the dominant plants, have a greater effect on ecosystem properties than diversity levels. Chapin *et al.* (1997) states that species traits that are most likely to affect ecosystem processes are those that "(1) modify the availability, capture and use of soil resources such as water and nutrients (e.g. root structure, protection shading of soil from leaves), (2) affect the feeding relationship (trophic structure) within a community, and (3) influence the frequency, severity and extent of disturbances such as fire". The first statement certainly corresponds with the data collected from the current study in which the main influences on the composition and activity of the microbial community seem to be related to the affect of the plant community and management on soil moisture, temperature and organic content. However, in terms of carbon metabolism, the functional ability of the two microbial communities appears to be equivalent.

Thus it is likely that the specific structure rather than the combined functional diversity of the microbial community has prevented the diversification of the TF plant community. A particularly important group of grassland microbes is the arbuscular mycorrhizal (AM) fungi (Chapter 1, Section 1.5). Plant community competition and resultant diversity has been shown to be affected by the abundance and diversity of AM fungi (Johnson *et al.*, 2003; Smith *et al.*, 1999; van der Heijden *et al.*, 1998). Whereby the interaction between a particular plant species and AM fungus gives it a strong

enough competitive advantage to completely dominate a plant community. Hence, suppression of the AM-plant interaction can result in competitive release enabling sub-dominant plant species to enter the system (Smith *et al.*, 1999; van der Heijden *et al.*, 1998). Since, IF is closely bordered by more diverse grasslands, so should not be limited by propagule dispersal, the stability of the system of highly competitive grasses may be a result of interactions between a limited number of plant species and AM fungi, creating an almost locked system which prevents the establishment of less competitive species. Only by disrupting the dominant plant-AM association could less competitive species gain a foothold. However, grassland restoration studies have shown that reseeding together with various intensities of disturbance could not successfully reintroduce all plant species. Thus the microbial community may be lacking particular species which are essential for the establishment of particular plants. For example, certain plant species, such as *Rhinanthus minor,* are important in enabling the successional colonisation of other seeded species following the establishment of suitable conditions (Smith *et al.*, 2003). Such amelioration may be the action of the pioneer plant on the local abiotic conditions (Smith *et al.*, 2003) (which would alter the conditions for microbes as well as plants), or microbial community, particular plant species having a positive effect on soil microbial biomass (Bardgett *et al.*, 1999b).

The management implications for IF really depend upon what the Wildlife Trust aims to achieve with the set of meadows (Pentwyn and Bush) as a whole. The fields are not isolated from each other and with small patch disturbance and reseeding from the neighbouring meadows, the plant diversity of IF would gradually increase (as long as the traditional management system is maintained). The transplantation of plants (with attached rhizosphere community might also enhance re-establishment (but the efficacy

of this would require further study). However, increasing the complexity of the plant community risks the loss of some of the fungal diversity. In terms of fruit bodies, TF and IF as a whole would currently register higher on the CHEG scale then they do individually (Section 2.3.3), because species such as *Hygrocybe psitticina* and the clavaroid fungi currently favour the conditions in IF and were not found fruiting in TF. Thus, management needs to be tailored to the diversity status that one wishes to achieve.

6.5 Future Work

One of the limitations in this study was the lack of site replication, therefore, the continuation of the project would benefit greatly from comparisons of the microbial community structure and function at Pentwyn with those found at other similar sites such as the waxcap grasslands at The National Botanic Gardens of Wales, Carmarthenshire.

From the work undertaken at Pentwyn, it was apparent that although the two meadows were functionally equivalent on an ecosystem level, the community structures of the microbial communities differed. The specific effects of individual components of the community on the corresponding plant community, would be an interesting area into which the research could be expanded. Specific interactions, such as those between AM fungi and the plant communities may be particularly important in holding the IF plant community in its low diversity state.

The implications of patch disturbance of the establishment of reseeded plant species, and particularly the established fungal community, requires further investigation. Some

fungi are particularly slow growing and can only survive in undisturbed ground, therefore disturbance of the ground to help plant species establish may actually destroy some potential plant/microbe association that ultimately assist plant survival. Therefore, the optimal size of patch disturbance would need to be established, providing a balance between reducing the competition from vigorous grasses and enabling the survival of the beneficial microbial community.

In terms of analysis of the functional diversity of the microbial community, techniques need to be developed or adjusted to enable far greater replication of much smaller soil samples so that a less homogenised picture of the structure and functional ability can be built up. The process of litter breakdown also needs further examination, such as monitoring the chemical composition of the substrate during breakdown, and examining which microbes are associated with the substrate during each stage.

6.6 Conclusions

6.6.1 Techniques for the Investigation of Soil Microbial Community Function

Nutrient cycling and decomposition by microbes is a long-term successional process in which many interactions including facilitation and competition and replacement, such that, the enzyme production and decompositional ability of individual species depends upon microclimate and interactions with other species (Setälä & McLean, 2004). Interactions during decomposition may occur over a prolonged period, for example, grass litter may decompose fully over a year while beech leaves may take 2-3 years to decompose, on the other hand, root exudates are available for immediate metabolism. This makes it difficult to glean meaningful information from a short temporal snapshot of a community. Perhaps rather than concentrating on quick relatively simple methods,

such as Biolog Microplates from which we can gain only a small amount of information about a subset of the community (while not even knowing what the subset we are studying actually is), we should employ more long term methods that might be considered "old fashioned" such as baiting with realistic substrates. The removal of substrate replicates and assessment of the chemical state of the substrate (stage of breakdown), together with molecular identification of the immediate (attached) and adjacent (substrate cascade, whereby the by-product of substrate metabolism by one organism is utilised by another organism) microbial community, might provide a better ecological insight into the structure and associated function of soil microbial communities. This should ideally be conducted on a much smaller scale to begin with, until we develop truly valuable methodologies, since our small experimental scale is actually large to the microbial world. In this manner we could attempt to tease apart and identify functional groups within the community and how these interact. Focus on developing techniques that do not disturb the community prior to assessment or if extraction is necessary then extract and test immediately for enzyme activity rather than allowing growth and change in the community structure to occur.

6.6.2 Conclusions for the Management of Plant and Microbial Diversity in the Pentwyn and Bush Meadows

- The traditional management regime (Section 1.1), must be strictly maintained if the current plant diversity of the species rich meadows is to be maintained and the diversity of the species poor meadows is to be increased.

- If more rapid acceleration of plant diversity is required, then patch disturbance of the sward and reseeding with locally collected seed may encourage plant establishment.

- The size of the patch disturbance may require more research, but should be large enough to reduce competition from the current vigorous grass community, while remaining small enough to prevent the destruction of the established saprotrophic fungal community. Strips of plant removal and soil disturbance might provide a balance between the two.

- However, if the Pentwyn/Bush site as a whole is to be maintained as an area of fungal conservational value, in addition to the plant diversity status, patch disturbance of IF should not be used and the field should be maintained with the traditional management regime and twice yearly grazing. Sheep grazing may be of particular importance since fruiting in IF was prolific, following close cropping of the turf by sheep grazing during the foot and mouth crisis. This may indicate that grazing had either, simply triggered fruiting, or that extra mycelial growth had also occurred.

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Appendix I. Field Site Data Corresponding to Chapter 2.

I.I Phosphorus Concentration of Soil or Plant Litter Samples

Phosphate standard curve Figure I.I, dissolved 0.4393g of oven dried KH_2PO_4 in ultra-pure double deionised water, made up to 1 litre in an acid washed volumetric flask (100mg P litre^{-1}). The equivalent amount of acid as contained in the aliquot of sample digest (0.5ml soil, 1ml plant litter) was added to each calibration solution. Standards containing 0, 0.1, 0.2 and 0.5mg P litre^{-1} plus acid, plus one Phosver 3 pillow, made up to 10ml with double deionised water in 10ml volumetric flasks, the flask was shaken and the colour allowed to develop for 2 minutes prior to reading the absorbance at 890nm. Samples of the digests, 0.5ml if soil sample, 1ml if plant litter, were made up to 10ml with a Phosver3 pillow and double deionised water in 10 ml volumetric flasks, the flasks were shaken and left for 2 minutes for the colour to develop before reading at 890nm.

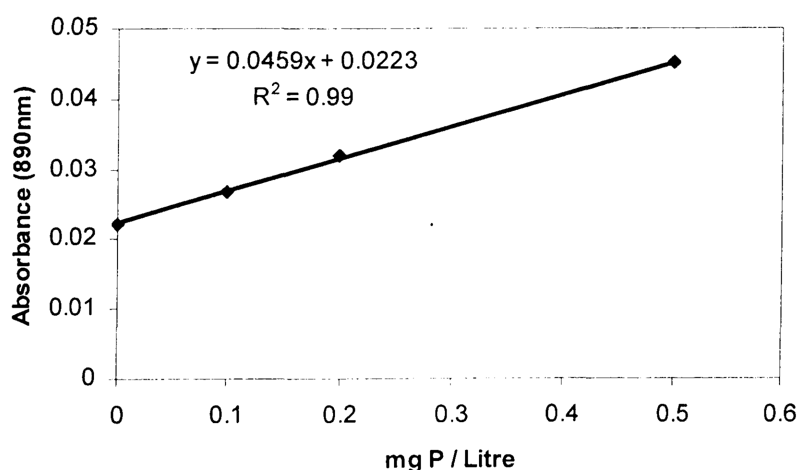


Figure I.I Phosphorus calibration curve

I.II Dehydrogenase Activity

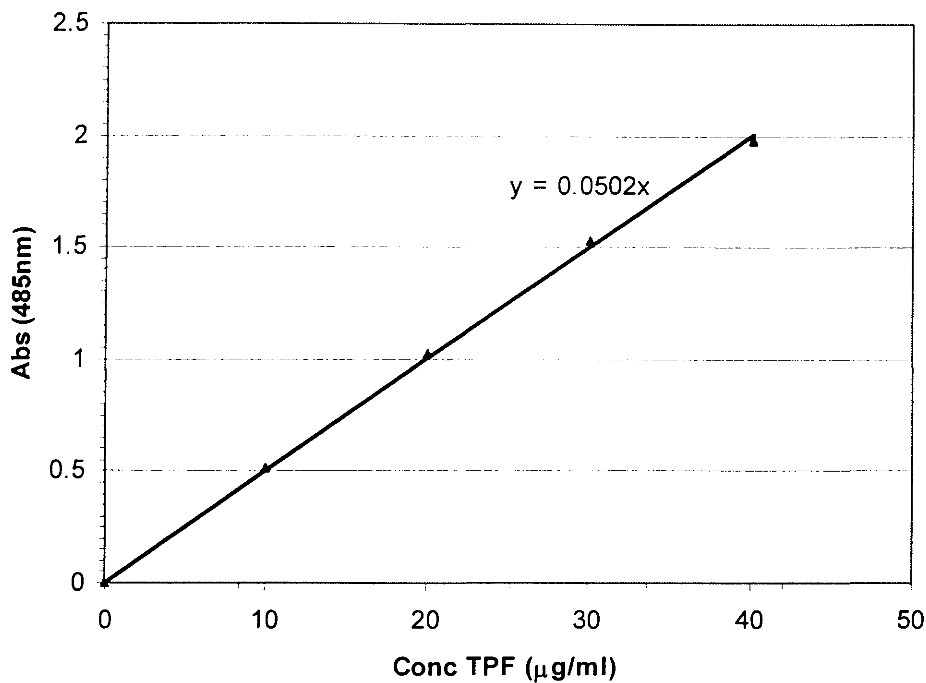


Figure I.II Trimethyl formazan calibration curve, dehydrogenase assay.

I.III Meteorological Data

Meteorological data was obtained from the following public sites:
The Hendre weather station **http://www.thehendre.com**
Met Office weather data **http://www.metoffice.co.uk**

Table I.I Mean temperature and rainfall data for the local Monmouth area compared with England & Wales area average for a 30 year period (1961-1990). The difference from the 30 year mean is marked as more or less than (+ or -) the 30 year mean. The statistical significance is graded significant to highly significant: *≤0.05, **≤0.01, ***≤0.001.

Year	Month	local Mean temp (°C)	England & Wales region 30 yr mean temp (°C)	Difference from 30 yr average (°C)	Monmouth Rainfall (mm)	England & Wales region 30 yr mean rainfall (mm)	Difference from 30 yr average (mm)
2002	Jan	-	3.4	-	-	90.2	-
	Feb	6.9	3.4	+ 3.5 ***	173.7	64.5	+ 109.2 ***
	Mar	7.8	5.2	+ 2.6 ***	34.1	73.1	- 39.0 ***
	Apr	9.4	7.3	+ 2.1 ***	53	60.1	- 7.1
	May	11.7	10.5	+ 1.2 ***	116.9	63.4	+ 53.5 ***
	Jun	13.8	13.5	+ 0.3	26.9	63.6	- 36.7 ***
	Jul	15.7	15.4	+ 0.3	45.4	62.3	- 16.9 **
	Aug	17	15.2	+ 1.8 ***	64.5	76.6	- 12.1 *
	Sep	14.5	13.1	+ 1.4 ***	26.7	78.2	- 51.5 ***
	Oct	10	10.2	- 0.2	165.6	86.4	+ 79.2 ***
	Nov	9.1	6.2	+ 2.9 ***	132.5	91.7	+ 40.8 ***
	Dec	5.9	4.3	+ 1.6 ***	117.4	95.5	+ 21.9 ***
2003	Jan	4.8	3.4	+ 1.4 ***	67.9	90.2	- 22.3 **
	Feb	4.1	3.4	+ 0.7	27.8	64.5	- 36.7 ***
	Mar	7.8	5.2	+ 2.6 ***	31.3	73.1	- 41.8 ***
	Apr	11.2	7.3	+ 3.9 ***	40.1	60.1	- 20.0 **
	May	11.2	10.5	+ 0.7 ***	43.7	63.4	- 19.7 ***
	Jun	15	13.5	+ 1.5 ***	43.3	63.6	- 20.3 ***
	Jul	16.6	15.4	+ 1.2 ***	66.3	62.3	+ 4.0
	Aug	17.8	15.2	+ 2.6 ***	10.8	76.6	- 65.8 ***
	Sep	13.3	13.1	+ 0.2	4.7	78.2	- 73.5 ***
	Oct	8.8	10.2	- 1.4 ***	55.1	86.4	- 31.3 ***
	Nov	8	6.2	+ 1.8 ***	81.3	91.7	- 10.4
	Dec	4.7	4.3	+ 0.4	107.4	95.5	+ 11.9 *

Table I.II Monthly average temperature and rainfall data 2000-2003 for the England & Wales region, compared with the 30 year (1961-1990) averages for the same region. The difference from the 30 year mean is marked as more or less than (+ or -) the 30 year mean. The statistical significance is graded significant to highly significant: *≤0.05, **≤0.01, ***≤0.001.

Year	Month	England & Wales region mean temp (°C)	England & Wales 30 yr mean temp (°C)	Difference from 30 yr average (°C)	England & Wales mean rainfall (mm)	England & Wales 30 yr mean rainfall (mm)	Difference from 30 yr average (mm)
2000	Jan	4.6	3.4	+ 1.2 **	54.5	90.2	- 35.7 ***
	Feb	5.8	3.4	+ 2.4 ***	96.1	64.5	+ 31.6 ***
	Mar	7.0	5.2	+ 1.8 ***	38	73.1	- 35.1 ***
	Apr	7.5	7.3	+ 0.2	132.5	60.1	+ 72.4 ***
	May	11.6	10.5	+ 1.1 ***	83.8	63.4	+ 20.4 ***
	Jun	14.4	13.5	+ 0.9 ***	47.6	63.6	- 16.0 **
	Jul	14.9	15.4	- 0.5 *	62.3	62.3	0.0
	Aug	16.2	15.2	+ 1.0 ***	66.4	76.6	- 10.2
	Sep	14.4	13.1	+ 1.3 ***	121.5	78.2	+ 43.3 ***
	Oct	10.0	10.2	- 0.2	180.1	86.4	+ 93.7 ***
	Nov	6.6	6.2	+ 0.4 *	174.8	91.7	+ 83.1 ***
	Dec	5.3	4.3	+ 1.0 **	136.6	95.5	+ 41.1 ***
2001	Jan	3.2	3.4	- 0.2	74.2	90.2	- 16.0 *
	Feb	4.2	3.4	+ 0.8 *	96.3	64.5	+ 31.8 ***
	Mar	4.9	5.2	- 0.3	91.3	73.1	+ 18.2 ***
	Apr	7.3	7.3	0.0	98.1	60.1	+ 38.0 ***
	May	12.0	10.5	+ 1.5 ***	42.5	63.4	- 20.9 ***
	Jun	13.7	13.5	+ 0.2	41.9	63.6	- 21.7 ***
	Jul	16.5	15.4	+ 1.1 ***	74.0	62.3	+ 11.7 **
	Aug	16.4	15.2	+ 1.2 ***	86.1	76.6	+ 9.5 **
	Sep	13.0	13.1	- 0.1	81.3	78.2	+ 3.1 *
	Oct	13.0	10.2	+ 2.8 ***	131.6	86.4	+ 45.2 ***
	Nov	7.1	6.2	+ 0.9 ***	67.7	91.7	- 24.0 ***
	Dec	3.4	4.3	- 0.9 **	44.3	95.5	- 51.2 ***
2002	Jan	5.3	3.4	+ 1.9 ***	95.1	90.2	+ 4.9
	Feb	6.6	3.4	+ 3.2 ***	140.7	64.5	+ 76.2 ***
	Mar	7.2	5.2	+ 2.0 ***	49.3	73.1	- 23.8 ***
	Apr	8.8	7.3	+ 1.5 ***	53.7	60.1	- 6.4
	May	11.5	10.5	+ 1.0 ***	93.7	63.4	+ 30.3 ***
	Jun	13.9	13.5	+ 0.4	57.4	63.6	- 6.2
	Jul	15.4	15.4	0.0	83.3	62.3	+ 21.0 ***
	Aug	16.6	15.2	+ 1.4 ***	67.3	76.6	- 9.3
	Sep	13.9	13.1	+ 0.8 ***	34.8	78.2	- 43.4 ***
	Oct	9.7	10.2	- 0.5 *	133.3	86.4	+ 46.9 ***
	Nov	8.2	6.2	+ 2.0 ***	154.1	91.7	+ 62.4 ***
	Dec	5.4	4.3	+ 1.1 ***	128.5	95.5	+ 33.0 ***
2003	Jan	4.2	3.4	+ 0.8 *	86.1	90.2	- 4.1
	Feb	3.7	3.4	+ 0.3	38.8	64.5	- 25.7 ***
	Mar	7.2	5.2	+ 2.0 ***	38.9	73.1	- 34.2 ***
	Apr	9.3	7.3	+ 2.0 ***	45.4	60.1	- 14.7 **
	May	11.6	10.5	+ 1.1 ***	74.6	63.4	+ 11.2 *
	Jun	15.5	13.5	+ 2.0 ***	67.2	63.6	+ 3.6
	Jul	17.0	15.4	+ 1.6 ***	75.6	62.3	+ 13.3 ***
	Aug	17.7	15.2	+ 2.5 ***	17.6	76.6	- 59.0 ***
	Sep	14.0	13.1	+ 0.9 ***	36.7	78.2	- 41.5 ***
	Oct	8.9	10.2	- 1.3 ***	57.7	86.4	- 28.7 ***
	Nov	7.9	6.2	+ 1.7 ***	100.1	91.7	+ 8.4 **
	Dec	4.7	4.3	+ 0.4	100.5	95.5	+ 5.0

Appendix II. Phospholipid Fatty Acid Analysis

II.I Extraction and Analysis

PLFA extraction based on White and Ringelberg (1998), adapted for 1g (dry wt) soil samples.

II.I.I Soil Samples

Lyopholised soil samples were extracted in three batches:

1) July TFA1 to July IFD1 and January TFA1 to January IFD1, plus no soil control
2) July TFA2 to July IFD2 and January TFA2 to January IFD2, plus no soil control
3) July TFA3 to July IFD3 and January TFA3 to January IFD3, plus no soil control

Where TF or IF is the tradition or improved field, A-D is the quadrant from which the bulked soil sample originated (see Chapter 3, Figure 3.1) and the number indicates the replicate sub sample 1-3.

II.I.II First-Phase Extraction

- First-phase extractions add in the following order: 0.8ml phosphate buffer, 1ml chloroform, 2ml methanol.
- Sonicate for no more than 2 mins.
- Extract for 2-18h at room temp.
- Centrifuge 30mins at 2000rpm to separate sediment from solvent.
- Decant solvent extract into clean test tubes with lids.

II.I.III Modified Bligh/Dyer

- Add 1ml Chloroform and 1ml water to split the phase (to produce final solvent volume ratio of 1:1:0.9 for chloroform:methanol:water/buffer).
- Shake tube vigorously and vent it, allow to separate (or centrifuge 1500rpm) until upper aqueous phase no longer cloudy.
- Remove all of upper aqueous phase by pipette.
- Filter lower organic phase through Whatman no. 2 paper into round bottomed flask making sure no aqueous phase is left in it.
- Remove the solvents from organic fraction in the round bottomed flask under vacuum (<37°C) with rotary evaporator. *Take care not to exceed 37°C as heat breaks down the fatty acids. Also never expose the lipids to air as oxygen will react with double bonds further breaking down the unsaturation. Avoid exposure to light as much as possible.*
- Transfer the dried total lipid to test tubes with 3 x 2ml chloroform washes.
- Sample cloudiness upon the addition of chloroform indicates the presence of water. Add methanol (approx 0.5ml) until cloudiness disappears, and redry on rotovap, may need to repeat again.
- Once transferred, remove the solvent from the test tube under constant nitrogen flow.
- Store the total lipid under nitrogen at -20°C until proceeding to lipid class separation.

II.I.IV Silicic Acid Column Chromatography

- Silicic acid must be activated at 100°C for a minimum of 1h in a fired test tube or flask and subsequently stored in a desiccator.
- Construct columns from Pasteur pipettes with a small glass wool plug in the bottom (oven fire to ensure no contamination).
- Wet glass wool in the bottom of the glass Pasteur pipette with chloroform and transferring a silicic acid slurry (0.5g silicic acid suspended in 5ml chloroform in a 10ml beaker) by Pasteur pipette. *There should be no sign of air pockets within the bed. If there are, add additional chloroform and agitate the bed with a Pasteur pipette until the bubbles rise to the surface. Do not allow to dry, or disturb the surface of the bed once the procedure has begun.*
- Allow tubes to warm slowly to room temp before opening.
- Resuspend the total lipid in a minimal volume of chloroform (100-200µl) and load onto the top of the silicic acid bed with a Pasteur pipette. Repeat 3 times for quantitative transfer. Take care not to disturb the surface of the bed.
- Once column is loaded, use a series of three solvents of increasing polarity to separate the lipid classes: Neutral lipids, 5ml chloroform Glycolipids, 5ml acetone, Phospholipids, 5ml methanol.
- Collect the lipid classes in separate test tubes set up below the column.
- Once each fraction is collected, remove the solvent under constant nitrogen flow and store the lipid under nitrogen at -20°C for further analysis.

II.I.V Preparation of Fatty Methyl Esters from Esterified Lipids

- Make up fresh 0.2M methanolic potassium hydroxide, 0.28g KOH per 25ml methanol.
- Redissolve the dried lipid in 1ml toluene:methanol (1:1 vol/vol) and 1ml methanolic KOH.
- Vortex the mixture briefly and incubate the samples for at least 15mins at no greater than 37°C.
- After samples have cooled to room temp. add 2ml hexane:chloroform (4:1 vol/vol), and mix the sample.
- Neutralise the sample (pH 6-7) with approximately 200µl 1N acetic acid and check the pH with litmus paper.
- Add 2ml nanopure distilled water to break phase and vortex mix the sample for at least 30secs. Separate phases by centrifugation (5mins at 2000rpm) upper organic phase contains fatty acid methyl esters.
- Transfer the upper phase to clean test tube without any of the aqueous phase.
- Re-extract the lower phase with 2ml hexane:chloroform (4:1 vol/vol), centrifuge and transfer as above twice more.
- Remove the solvent with nitrogen gas blow down, and store the FAME under nitrogen at -20°C until separation and quantification.

II.I.VI Separation, Quantification and Identification of Organic Compounds

- A suitable concentration of sample was determined by using one sample as a range-finder with cholestane in iso-octane as an internal standard (18.7µg ml^{-1}, 50pmol ml^{-1}). Small volume vials were used with 125µl of the following samples:

1. 1ml iso-octane (solvent blank).
2. 1ml cholestane in iso-octane (internal standard blank).
3. FAME sample dissolved in 250μl internal standard solution.
4. FAME sample dissolved in 500μl internal standard solution (take 125μl of 3. Add 125μl internal standard sol^n).
5. FAME sample dissolved in 1000μl internal standard solution (take 125μl 4. Add 125μl internal standard sol^n).

- Range-finder determined that dissolving the dried samples in 200μl of internal standard solution provided a suitable concentration of the samples for loading for GC/MS.

Table II.I Preparation of buffers and solvents required for PLFA extraction and analysis.

Buffers and solvents etc.	Preparation	Vol. required per 1g sample	Vol. required per 8 sample run
Water	nanopure-filtered organic-free deionized distilled water. All water should be chloroform extracted using approx. 200ml chloroform per 4 litres distilled water	3.0ml	24.0ml
Chloroform		18.6ml	148.8ml
Methanol		8.0ml	64.0ml
50mM phosphate buffer	Dissolve 8.7g K_2HPO (dibasic) in 1 litre nanopure distilled water, adjust to pH 7.4 with approx 3.5ml 6N HCL. Should be chloroform extracted using 50ml chloroform per 1 litre buffer	0.8ml	6.4ml
Acetone		5.0ml	40.0ml
Methanolic potassium hydroxide	0.2M KOH in methanol, made fresh before each use; 2.8g KOH per 25ml methanol (1.12g per 10ml)	1.0ml	8.0ml
Toluene:methanol 1:1 vol/vol		1.0ml	8.0ml
Hexane:chloroform 4:1 vol/vol		8.0ml	64.0ml
1N Acetic acid	5.72ml conc. (17.5N) glacial acetic acid per 100ml nanopure distilled water	0.2ml (200µl)	1.6ml
Silicic acid, 100-200 mesh powder		0.5g	4.0g
Cholestane internal standard in iso-octane, working conc. 18.7mg/litre	Stock sol^n, dissolve 0.0187g cholestane in 100ml, dilute 1:10 to give working conc. of 18.7mg/litre		

Table II.II Literature and corresponding fatty acids used to assess soil microbial community structure. Colouring of the microbial groups indicates crossover of fatty acids between microbial groups. Those fatty acids which were referred to as being present in more than one microbial or organism group were not chosen to represent the microbial groups in this study, since they were not considered to be group specific indicators.

Organisms	Fatty acids	Vestal & White 1989	Tunlid & White 1992	Frostegard & Baath 1996	Zelles 1997	Pennanen 1998	Bardgett & McAlister 1999	Bardgett et al 1999	Ohtonen et al 1999	Olsson 1999	Andersson et al 2000	Bull et al 2000	Hill et al 2000	Olsson & Johansen 2001	Olsson & Wilhelmsson 2000	Pietikainen et al 2000	Bardgett et al 2001	Enami et al 2001	Feng 2001	Grayston et al 2001	Pankhurst et al 2001	Pennanen 2001	Wilkinson & Anderson 2001	Baath E 2002	Hedlund 2002	Madan et al 2002	Ponder & Tadros 2002	Ruess et al 2002	Fierer et al 2002	Marschner et al 2003	Grayston et al 2004
General Bacterial	14:0																							✓							
	15:0		✓	✓		✓	✓	✓	✓				✓			✓	✓			✓	✓		✓		✓					✓	✓
	i15:0		✓	✓		✓	✓	✓	✓		✓		✓			✓	✓			✓	✓		✓	✓	✓		✓			✓	✓
	a15:0		✓	✓		✓	✓	✓	✓		✓		✓			✓	✓			✓	✓		✓	✓	✓		✓			✓	✓
	16:0												✓																		
	i16:0			✓		✓	✓	✓	✓		✓					✓	✓			✓	✓		✓	✓	✓		✓			✓	✓
	16:1w5		✓										✓																		
	16:1w7c																							✓							
	16:1w7t					✓			✓														✓				✓				
	16:1w9			✓		✓			✓		✓		✓			✓							✓		✓						
	10Me16:0																							✓							
	17:0		✓	✓		✓	✓	✓	✓				✓			✓	✓			✓	✓		✓				✓			✓	✓
	i17:0		✓	✓		✓	✓	✓	✓		✓		✓			✓					✓		✓	✓	✓		✓			✓	
	a17:0			✓		✓			✓				✓			✓					✓		✓	✓	✓		✓				
	cy17:0			✓		✓	✓	✓	✓		✓					✓	✓			✓	✓			✓	✓		✓			✓	✓
	10Me17:0																							✓							
	br18																							✓							
	18:1w7		✓	✓		✓	✓	✓	✓							✓	✓			✓			✓	✓	✓		✓				✓
	18:1w7t												✓																		
	10Me18:0																							✓							
	19:0																										✓				
	i19:0												✓																		
	a19:0												✓																		
	cy19:0			✓		✓	✓	✓	✓		✓					✓	✓			✓	✓		✓	✓	✓					✓	✓
Gram neg bacteria	15:1w4c																												✓		
	16:1w5c																														✓
	16:0																			✓											✓
	16:1w7t																			✓											✓
	16:1w9c/a																			✓									✓		✓
	16:1w7c								✓									✓		✓											✓
	17:0cyc																	✓													
	cy17:0								✓											✓	✓								✓	✓	✓
	17:1w9c																												✓		
	18:1w5c																			✓											
	18:1w7c								✓									✓		✓									✓		✓
	cy19:0/19:1w6c																			✓											✓
	cy19:0								✓									✓			✓								✓		
Gram pos bacteria	i14:0								✓																				✓		
	15:0																				✓										
	i15:0																			✓	✓								✓	✓	✓
	a15:0								✓												✓								✓	✓	✓
	i16:0								✓											✓	✓			✓					✓	✓	✓
	10Me16:0																			✓											✓
	17:0																				✓										
	i17:0																			✓	✓								✓	✓	✓
	a17:0																			✓	✓								✓		✓
	10Me18:0								✓																						
Fungi	14:0 (myristic acid)																											✓			
	C16 palmitic acid widely distributed in other organisms)				✓																										
	16:1w7 (palmitoleic acid)																											✓			
	18:0 (stearic acid)																											✓			
	18:1w6 (for eukaryotic)																										✓				
	18:1w7 (vaccenic acid)																											✓			
18:2 and 18:3 almost exclusively plants and fungi	18:1w5c (for eukaryotic)																										✓				
	18:1w9 (oleic acid)												✓											✓				✓			
	18:2w6 (linoleic acid mainly used as fungal identifier if all plant material excluded)		✓	✓	✓	✓	✓		✓	✓	✓		✓			✓	✓	✓		✓	✓		✓	✓	✓			✓	✓	✓	✓
	18:3w3												✓																		
	18:3w6		✓										✓																		
	18:2 nonspecific bond position				✓																										
	18:3 nonspecific bond position				✓																										
Arbuscular Mycorrhizae	16:1w5 (not in other fungi but some in bact)									✓				✓	✓							✓			✓	Gigaspora spp. only					
	18:1w7 (not in other fungi but some in bact)									✓																					
Actinomycetes	10Me16:0																												✓		
	10Me17:0																												✓		
	10Me18:0		✓																										✓		
Plants/Animals	16:0																														
	16:2 (only in plants)				✓																										
	18:2 almost exclusively plants and fungi				✓																										
	18:3 almost exclusively plants and fungi				✓																										
	>20C																		✓												
Aerobic bacteria	16:1w7	✓											✓																		
	16:1w7t	✓											✓																		
	18:1w7t	✓											✓																		
	18:1w11c (suggested for aerobic bacteria)				✓																										
Anaerobic bacteria	cy17:0 (also occur widely through many bacterial genera)	✓			✓								✓																		
	cy19:0 (also occur widely through many bacterial genera)	✓			✓								✓																		
Sulphate reducing bacteria	10Me16:0		✓										✓																		
	br17:1		✓																												
	17:1w6		✓										✓																		
	i17:1w7												✓																		
Methane oxidising bacteria	type I methanotrophes																														
	16:1w5c		✓										✓																		
	16:1w8t		✓										✓																		
	16:1w5c		✓										✓																		
	type II methanotrophes																														
	18:1w8c		✓									✓	✓																		
	18:1w8t		✓										✓																		
	18:1w6c		✓										✓																		
Barophilic/psychrophilic bacteria	20:5												✓																		
	22:6												✓																		
Cyanobacteria	18:2w6												✓																		
Protozoa	20:3w6												✓																✓		
	20:4w6												✓																✓		
	20:2w6																												✓		
Actinobacteria	10Me18:0												✓																		
Microalgae	18:3w3												✓																		
Flavobacterium balustinum	i17:1w7		✓										✓																		
	Br2OH-15:0		✓										✓																		

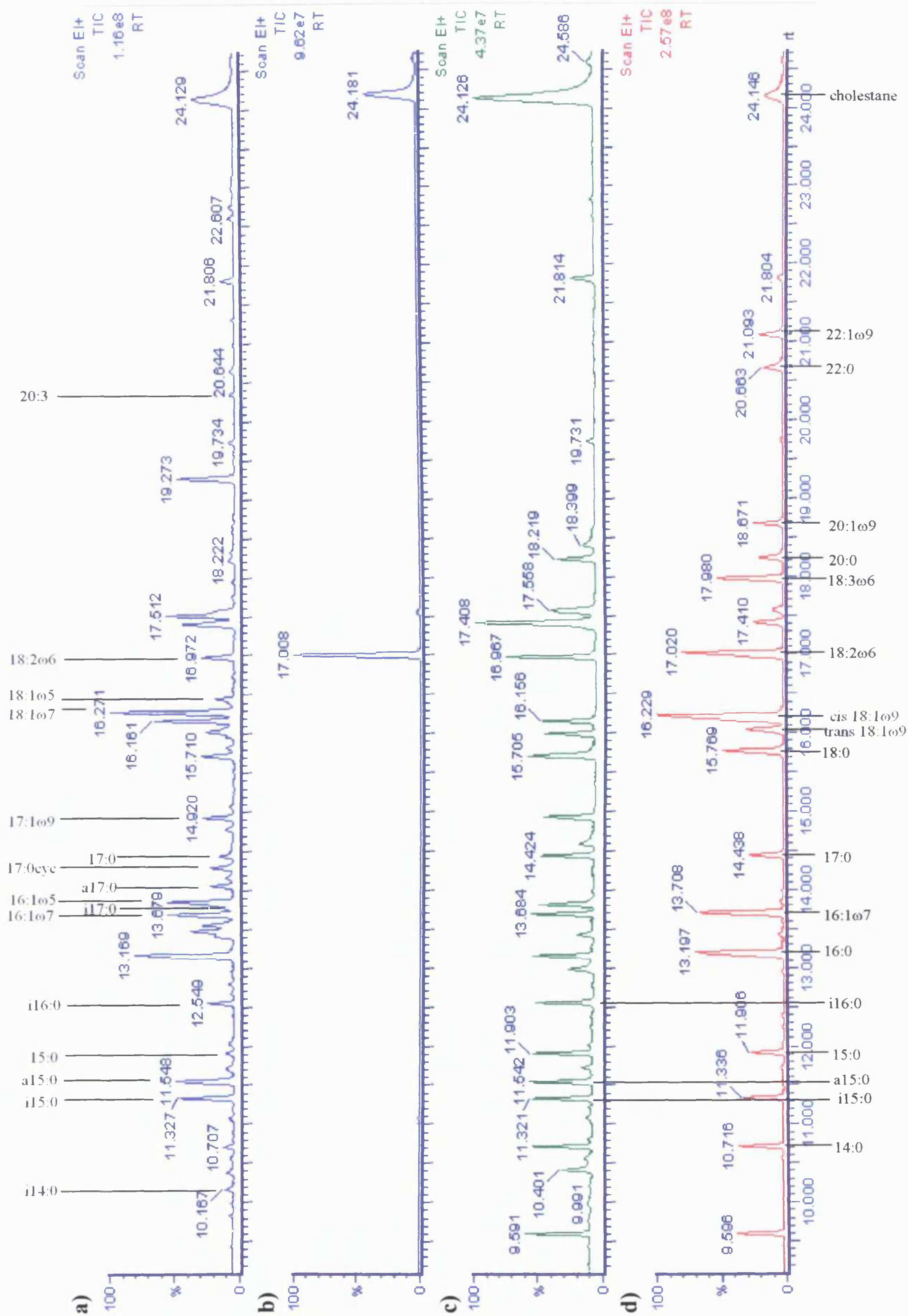


Figure II.I Chromatogram of FAME sample July TFA1 (a) and standards: b) Fungal (18:2ω6), c) Bacterial standards and d) FAME C8-C22 mix.

Appendix III. Litterbags

III.I Litterbag Decomposition Statistical Results Tables

Table III.I Seasonal litterbag experiments, fitted curves for field litter decomposition data.

		Curve fitting			Curve comparison						
Experimental period	**Individual curves**	Regression Equation	R^2 (%)	DF	**Comparison**	Sum of squares separate	Sum of squares combined	DF separate	DF combined	F ratio	P value
4th Feb-3rd April 02	TF litter in TF	$Y=0.553e^{(0.038X)}+1.015$	65	22	1) TF-IF litter in TF	0.5129	0.6308	44	47	3.3714	0.0267
	IF litter in TF	$Y=0.572e^{(0.074X)}+1.011$	47	22	2) TF-IF litter in IF	0.7395	1.1040	42	44	6.9006	0.0007
	TF litter in IF	$Y=0.556e^{(0.038X)}+1.044$	48	21	3) TF litter in TF & IF	0.4774	0.4879	43	46	0.3152	0.8143
	IF litter in IF	$Y=0.665e^{(0.056X)}+0.910$	50	21	4) IF litter in TF & IF	0.7750	0.8203	43	46	0.8378	0.4806
14th May-27th June 02	TF litter in TF	$Y=0.789e^{(0.139X)}+0.779$	81	22	1) TF-IF litter in TF	0.2708	0.3028	44	47	1.7331	0.1741
	IF litter in TF	$Y=0.812e^{(0.199X)}+0.759$	82	22	2) TF-IF litter in IF	0.1878	0.2392	44	47	4.0182	0.0130
	TF litter in IF	$Y=0.783e^{(0.175X)}+0.789$	81	22	3) TF litter in TF & IF	0.2754	0.2806	44	47	0.2769	0.4528
	IF litter in IF	$Y=0.849e^{(0.174X)}+0.721$	93	22	4) IF litter in TF & IF	0.1832	0.1943	44	47	0.8920	0.4528
26th Aug-18th Oct 02	TF litter in TF	$Y=0.643e^{(0.108X)}+0.017$	87	22	1) TF-IF litter in TF	0.1539	0.1724	44	47	1.7684	0.1671
	IF litter in TF	$Y=0.586e^{(0.138X)}+0.982$	77	22	2) TF-IF litter in IF	0.2039	0.2271	43	46	1.6316	0.1961
	TF litter in IF	$Y=0.714e^{(0.095X)}+0.858$	86	22	3) TF litter in TF & IF	0.1349	0.1658	44	47	3.3649	0.0269
	IF litter in IF	$Y=0.663e^{(0.087X)}+0.897$	77	21	4) IF litter in TF & IF	0.2229	0.2555	43	46	2.0978	0.1446
19th Dec-18th Feb 03	TF litter in TF	$Y=0.527e^{(0.292X)}+1.044$	74	21	1) TF-IF litter in TF	0.1470	0.1541	43	46	0.6943	0.5606
	IF litter in TF	$Y=0.561e^{(0.099X)}+1.009$	85	22	2) TF-IF litter in IF	0.0932	0.1101	44	47	2.6614	0.0597
	TF litter in IF	$Y=0.552e^{(0.126X)}+1.019$	84	22	3) TF litter in TF & IF	0.1491	0.1540	43	46	0.4691	0.7054
	IF litter in IF	$Y=0.597e^{(0.099X)}+0.974$	89	22	4) IF litter in TF & IF	0.0911	0.1058	44	47	2.3760	0.0828

Table III.II Regression models fitted to cellulose seasonal decomposition data and comparison of regressions.

Linear regression model fitting							Regression comparison					
Experimental period	Field	Linear regression equation	R^2 (%)	DF	F ratio	P value, sig. diff. of slope from zero	Slope F ratio	DF	Slope P value	Y intercept F ratio	DF	Y intercept P value
4th Feb-3rd April 02	TF	y= -0.3184x +101.1	34	1, 18	9.241	0.007	0.7775	1, 36	0.3837	0.0692	1, 37	0.7939
	IF	y= -0.1773x +98.6	11	1, 18	2.147	0.1601						
26th Aug-18th Oct 02	TF	y= -0.8954x +101.8	36	1, 30	16.53	0.0003	0.4083	1, 60	0.5253	0.0025	1, 61	0.9607
	IF	y= -0.7048x +96.88	29	1, 30	12.29	0.0015						
19th Dec-18th Feb 03	TF	y= -0.06961x +101.5	1	1, 30	0.3879	0.5381	0.2859	1, 60	0.5949	1.8270	1, 61	0.1815
	IF	y= -0.008445x +103.4	0	1, 30	0.0081	0.929						

Non-linear regression model fitting					Curve comparison					
Experimental period	Field	Non-linear regression equation	R^2 (%)	DF	Sum of squares separate	Sum of squares combined	DF separate	DF combined	F ratio	P value
14th May-27th June 02	TF	$y=97.5+1.291x-0.04939x^2$	54	29	22328	22685	58	61	0.3091	0.8187
	IF	$y=101.9+0.3173x-0.02766x^2$	42	29						

Table III.III Extended decomposition experiment results of regressions from field litter, cellulose, oak leaves and wood veneer decomposition.

		Curve fitting			Curve comparison						
Substrate	**Individual curves**	Exponential decay	R^2 (%)	DF	**Comparison**	Sum of squares separate	Sum of squares combined	DF separate	DF combined	F ratio	P value
Field litters 0-132 days	TF litter in TF	$Y=0.555e^{(0.030X)}+0.716$	77	57	1) TF v IF litter in TF	0.7842	0.7957	114	117	0.5573	0.6443
	IF litter in TF	$Y=0.537e^{(0.042X)}+0.767$	77	57	2) TF v IF litter in IF	0.8172	0.8348	116	119	0.8328	0.4785
	TF litter in IF	$Y=0.585e^{(0.047X)}+0.752$	78	58	3) TF litter in TF v IF	0.8050	0.8258	115	118	0.9905	0.4000
	IF litter in IF	$Y=0.550e^{(0.029X)}+0.703$	77	58	4) IF litter in TF v IF	0.7964	0.8149	115	118	0.8905	0.4484
Field litters 0-44 days	TF litter in TF	$Y=0.623e^{(0.170X)}+0.955$	89	27	1) TF v IF litter in TF	0.1483	0.1512	55	58	0.3547	0.7859
	IF litter in TF	$Y=0.615e^{(0.158X)}+0.937$	83	28	2) TF v IF litter in IF	0.1408	0.1456	56	59	0.6405	0.5922
	TF litter in IF	$Y=0.646e^{(0.143X)}+0.907$	92	28	3) TF litter in TF v IF	0.1030	0.1197	55	58	2.9787	0.0392
	IF litter in IF	$Y=0.625e^{(0.163X)}+0.935$	84	28	4) IF litter in TF v IF	0.1861	0.1865	56	59	0.0371	0.9903

		Non-linear regression model fitting			Curve comparison					
Substrate	Field	Polynomial non-linear regression equation	R^2 (%)	DF	Sum of squares separate	Sum of squares combined	DF separate	DF combined	F ratio	P value
Cellulose	TF	$y=101+0.0475x-0.0311x^2+0.00026x^3$	59	39	42802	43689	79	83	0.4093	0.8014
	IF	$y=98.96+0.9802x-0.0572x^2+0.00045x^3$	50	40						

		Linear regression model fitting					Regression comparison					
Substrate	Field	Linear regression equation	R^2 (%)	DF	F ratio	P value, sig. diff. of slope from zero	Slope F ratio	DF	Slope P value	Y intercept F ratio	DF	Y intercept P value
Oak leaves	TF	$y=-0.0022x+1.383$	56	1, 29	36.9	<0.0001	0.9302	1, 58	0.3388	1.9156	1, 59	0.1716
	IF	$y=-0.0027x+1.387$	69	1, 29	63.35	<0.0001						
Birch wood veneers	TF	$y=-0.0438x+96.33$	2	1, 28	0.6885	0.4132	0.0377	1, 57	0.8468	0.0744	1, 58	0.7859
	IF	$y=-0.0586x+98.66$	4	1, 29	1.1190	0.2989						

Table III.IV Regressions and comparisons of C:N and absolute total N in oak leaves decomposing in TF and IF

		Non-linear regression model fitting			Curve comparison					
Nutrient	Field	Exponential decay	R^2 (%)	DF	Sum of squares separate	Sum of squares combined	DF separate	DF combined	F ratio	P value
C:N	TF	$Y=81.86e^{(0.150X)}+46.71$	99	9	345.2	380.8	18	21	0.6188	0.6118
	IF	$Y=84.64e^{(0.153X)}+43.93$	99	9						

		Linear regression model fitting					Regression comparison					
Nutrient	Field	Linear regression equation	R^2 (%)	DF	F ratio	P value, sig. diff. of slope from zero	Slope F ratio	DF	Slope P value	Y intercept F ratio	DF	Y intercept P value
Absolute N	TF	y= 35.24x +109.8	92	1, 10	122.5	<0.0001	1.1614	1, 20	0.2940	2.6086	1, 21	0.1212
	IF	y=39.62x +109.2	96	1, 10	246.3	<0.0001						

Appendix IV. Potential Function: Biolog Analysis.

IV.I Biolog Soil Moisture Compensation Calculation

$$Fr = \frac{D}{(100 - W)} \times 100$$

Fr = required fresh weight of soil.

D = dry weight of soil for which an equivalent fresh weight is required, i.e. 5g.

W = calculated % soil water content from the sample soil.

IV.II Soil Extract Agar Medium

- 100g soil suspended in 1 litre distilled water.
- Stir for 40 minutes.
- Centrifuge at 8000rpm for 20 minutes to sediment soil particles, pour off and retain the solution.
- Add 15g Agar No. 2 (Lab M) to the 1 soil solution and make up to 1 litre with distilled water.
- Autoclave at 120°C for 15 minutes.

IV.III Determination of Soil Background OD

Field soil	1:10	1:100	1:1000
A1	[illegible]	0.092	-0.018
A2	2.072	2.315	0.481
A3	1.569	2.570	1.419
A4	1.645	2.119	0.425
A5	1.341	1.576	1.105
A6	2.267	2.184	[illegible]
A7	1.368	0.627	0.283
A8	1.421	1.719	[illegible]
A9	1.222	1.151	0.118
A10	2.057	2.536	[illegible]
A11	1.839	2.474	[illegible]
A12	1.508	1.929	[illegible]
B1	0.725	1.554	0.251
B2	2.088	2.729	2.136
B3	2.229	2.725	2.222
B4	2.128	2.658	2.062
B5	2.028	1.495	1.231
B6	1.950	2.749	1.870
B7	2.187	2.782	[illegible]
B8	1.507	1.285	0.543
B9	0.929	1.760	0.804
B10	[illegible]	2.422	[illegible]
B11	1.854	2.321	2.018
B12	1.613	2.216	1.294
C1	2.176	2.248	0.677
C2	1.670	1.149	1.622
C3	1.678	1.517	0.081
C4	2.108	2.314	0.787
C5	2.101	[illegible]	[illegible]
C6	2.311	2.685	[illegible]
C7	1.772	2.140	2.412
C8	2.145	2.157	[illegible]
C9	1.105	1.603	0.695
C10	1.380	1.802	1.274
C11	1.640	1.802	1.042
C12	1.724	1.785	1.176
D1	-0.016	0.156	0.154
D2	1.809	1.894	1.731
D3	1.379	1.250	1.310
D4	0.105	0.000	0.081
D5	1.481	1.370	1.044
D6	1.789	1.424	1.560
D7	1.105	1.063	1.466
D8	1.473	1.443	1.134
D9	1.216	1.443	1.294
D10	0.958	0.245	0.052
D11	1.069	1.681	0.870
D12	1.312	0.473	0.119
E1	1.691	2.066	1.842
E2	1.524	1.235	0.611
E3	0.969	1.022	0.076
E4	1.186	1.681	0.619
E5	0.158	0.248	-0.013
E6	0.696	0.736	0.360
E7	0.153	0.378	0.483
E8	0.571	0.535	0.479
E9	1.750	1.387	0.981
E10	1.081	1.188	1.014
E11	1.495	1.742	0.117
E12	0.524	0.577	0.857
F1	0.067	0.334	0.179
F2	1.206	1.313	1.157
F3	0.580	0.153	0.225
F4	1.060	0.610	0.673
F5	2.027	1.408	1.944
F6	1.755	1.864	1.916
F7	1.272	1.106	0.510
F8	2.227	2.671	1.478
F9	1.681	2.043	2.068
F10	2.436	2.517	1.752
F11	0.680	0.237	0.717
F12	1.347	0.680	0.847
G1	1.214	1.675	1.117
G2	1.997	2.488	0.869
G3	2.161	2.873	0.680
G4	[illegible]	[illegible]	[illegible]
G5	1.716	0.943	0.143
G6	2.265	2.020	2.186
G7	2.251	1.764	1.658
G8	1.138	1.115	0.017
G9	1.677	1.705	1.791
G10	0.491	1.116	0.402
G11	0.949	0.827	0.480
G12	1.864	2.443	2.641
H1	1.955	1.569	0.972
H2	0.992	0.674	1.285
H3	0.426	1.087	0.786
H4	0.620	0.236	-0.006
H5	1.875	2.062	1.990
H6	0.725	1.288	1.505
H7	1.318	1.810	0.470
H8	-0.197	0.085	0.061
H9	1.930	1.768	1.742
H10	0.234	0.108	0.178
H11	1.021	0.467	0.256
H12	0.957	0.129	0.022

Cardiff soil	1:10	1:100	1:1000
A1	-0.005	0.150	0.077
A2	1.521	2.149	2.120
A3	1.915	2.174	2.250
A4	1.597	1.825	1.863
A5	1.362	2.184	1.486
A6	2.008	2.629	2.399
A7	1.048	1.443	1.001
A8	1.487	1.547	1.866
A9	1.034	1.109	1.100
A10	2.061	2.438	2.410
A11	1.940	2.355	2.342
A12	1.152	2.152	2.341
B1	0.823	1.817	0.520
B2	1.810	2.511	2.186
B3	2.356	2.583	2.174
B4	2.222	2.524	2.420
B5	2.186	2.688	2.424
B6	1.644	2.655	2.318
B7	1.947	2.715	2.342
B8	1.981	[illegible]	[illegible]
B9	1.020	1.724	0.785
B10	2.001	2.265	2.357
B11	1.709	2.420	2.544
B12	1.925	2.002	2.246
C1	1.879	2.487	2.265
C2	1.882	2.665	1.877
C3	1.908	2.636	2.333
C4	2.166	2.597	0.836
C5	2.008	2.787	2.486
C6	1.893	2.432	2.430
C7	2.009	2.401	2.106
C8	1.881	2.590	2.115
C9	1.988	1.943	1.916
C10	1.130	1.992	0.521
C11	1.522	1.810	1.531
C12	1.904	2.222	0.595
D1	-0.113	0.124	0.044
D2	1.893	2.342	1.725
D3	1.434	1.671	1.748
D4	0.302	0.165	0.288
D5	1.322	1.789	1.712
D6	1.010	1.559	2.158
D7	1.424	1.813	1.559
D8	1.782	1.413	1.733
D9	1.349	1.877	1.890
D10	0.220	0.548	0.455
D11	1.183	1.741	1.421
D12	1.061	0.526	1.045
E1	1.666	1.879	0.965
E2	1.998	1.706	0.770
E3	0.428	0.518	0.001
E4	1.682	1.431	1.171
E5	0.432	0.526	0.148
E6	0.736	0.909	0.991
E7	0.488	0.514	0.709
E8	-0.219	0.382	0.760
E9	1.482	1.513	1.162
E10	1.582	1.476	1.306
E11	1.163	1.485	0.230
E12	0.379	0.751	0.580
F1	-0.329	0.396	0.175
F2	1.483	1.945	1.264
F3	0.264	0.237	0.102
F4	1.098	0.893	0.767
F5	1.751	1.545	1.555
F6	2.009	2.294	1.528
F7	1.772	1.811	1.244
F8	2.113	2.608	2.040
F9	1.568	2.312	2.210
F10	[illegible]	2.653	2.370
F11	0.762	0.819	0.592
F12	0.997	0.649	0.805
G1	1.498	1.587	1.357
G2	2.147	2.549	2.179
G3	2.297	2.346	2.210
G4	1.930	1.424	1.264
G5	2.164	1.184	0.828
G6	2.291	2.589	2.790
G7	2.287	2.574	1.429
G8	1.535	1.462	1.360
G9	1.883	1.876	1.421
G10	0.950	0.944	1.266
G11	0.879	0.821	1.147
G12	2.268	2.185	1.836
H1	2.193	2.115	1.829
H2	1.477	0.941	1.009
H3	0.736	0.945	0.887
H4	1.449	0.255	0.117
H5	1.950	2.077	2.287
H6	1.205	1.192	0.814
H7	1.330	1.330	1.230
H8	-0.400	0.184	0.123
H9	1.593	1.498	1.596
H10	0.313	0.365	0.189
H11	0.925	0.831	0.654
H12	1.179	0.720	0.529

Figure IV.I End response utilisation of Biolog GN2 plates Coded arbitrarily from light to dark with colourless being negative utilisation ≤0.1, through light >0.1 ≤1.0, medium >1.0 ≤2.0 and dark being >2.0.

IV.IV Biolog Data Processing

Microsoft Excel - original Biolog data processing [Read-Only]

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	Obs. y =		0	0.013	0.017	0.066	0.191	0.536	1.002	1.454	1.794	1.897	2.007	2.184	2.264
2	Calc. y =		0.083	0.142	0.191	0.321	0.421	0.657	0.815	1.183	1.418	1.785	1.952	2.215	2.324
3	Error² =		0.007	0.017	0.030	0.065	0.053	0.015	0.035	0.074	0.142	0.013	0.003	0.001	0.004
4	Total error	0.47													
5															
6	Coeffs from Lindstrom et al. Eq. (2)														
7	K	2.68													
8	r	0.04													
9	s	92.88													
10–19															
20	date		22/01/03	23/01/03	23/01/03	24/01/03	24/01/03	25/01/03	25/01/03	26/01/03	26/01/03	27/01/03	27/01/03	28/01/03	28/01/03
21	t=		0	1	2	3	4	5	6	7	8	9	10	11	12
22	time		19.30	10.30	19.00	10.30	19.00	10.00	18.00	10.00	19.30	11.00	19.00	10.30	19.00
23	hours		0.0	15.0	23.5	39.0	47.5	62.5	70.5	86.5	96.0	111.5	119.5	135.0	143.5
24		A1	0	0.019	0.025	0.048	0.051	0.055	0.062	0.085	0.107	0.164	0.178	0.18	0.193
25		A2	0	0.016	0.015	0.035	0.04	0.071	0.188	0.439	0.555	0.794	1.071	1.666	1.869
26		A3	0	0.005	0.006	0.044	0.085	0.269	0.452	0.68	0.938	1.238	1.432	1.754	1.896
27		A4	0	0.014	0.017	0.08	0.12	0.224	0.339	0.52	0.683	0.9	1.047	1.356	1.54
28		A5	0	0.051	0.039	0.097	0.268	0.416	0.505	0.652	0.765	0.874	0.947	1.088	1.17
29		A6	0	-0.003	-0.003	0.111	0.276	0.422	0.531	0.679	0.814	0.97	1.071	1.266	1.384
30		A7	0	0.012	0.013	0.028	0.032	0.045	0.109	0.325	0.84	1.182	1.324	1.624	1.723
31		A8	0	0.008	0.012	0.069	0.221	0.887	1.392	1.516	1.551	1.486	1.468	1.51	1.626
32		A9	0	0.016	0.018	0.031	0.035	0.045	0.08	0.22	0.394	0.6	0.767	1.297	1.563

Answer Report 1 / Sensitivity Report 1 / Limits Report 1 / ResultsSheet / Sheet1 / Bacterial / R

Ready NUM

Figure IV.II Solver data entry spread sheet

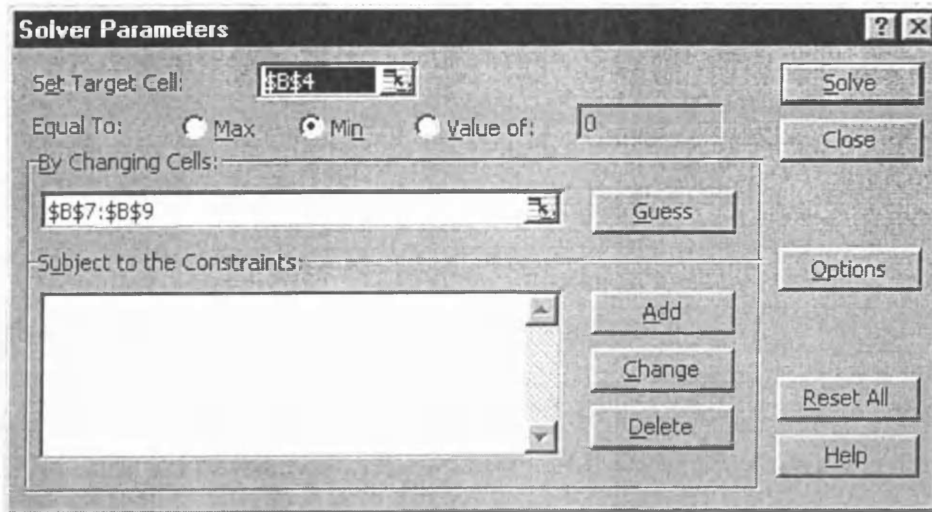


Figure IV.III Solver constraints

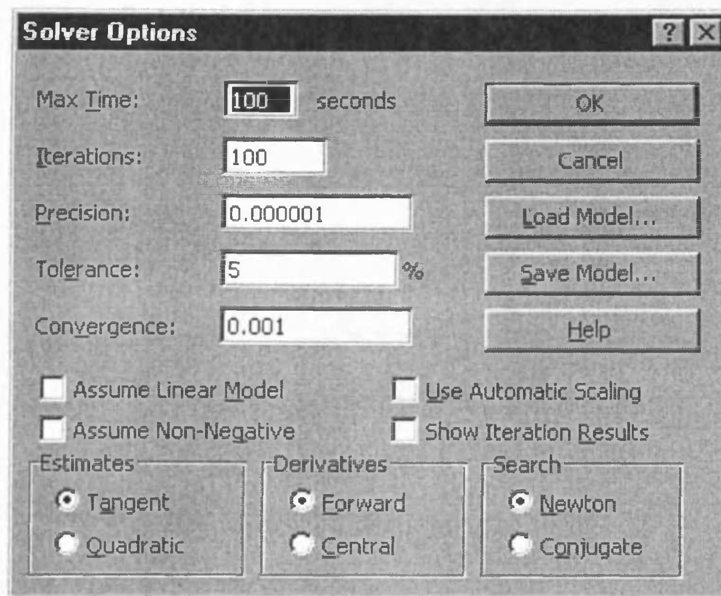


Figure IV.IV Solver constraints

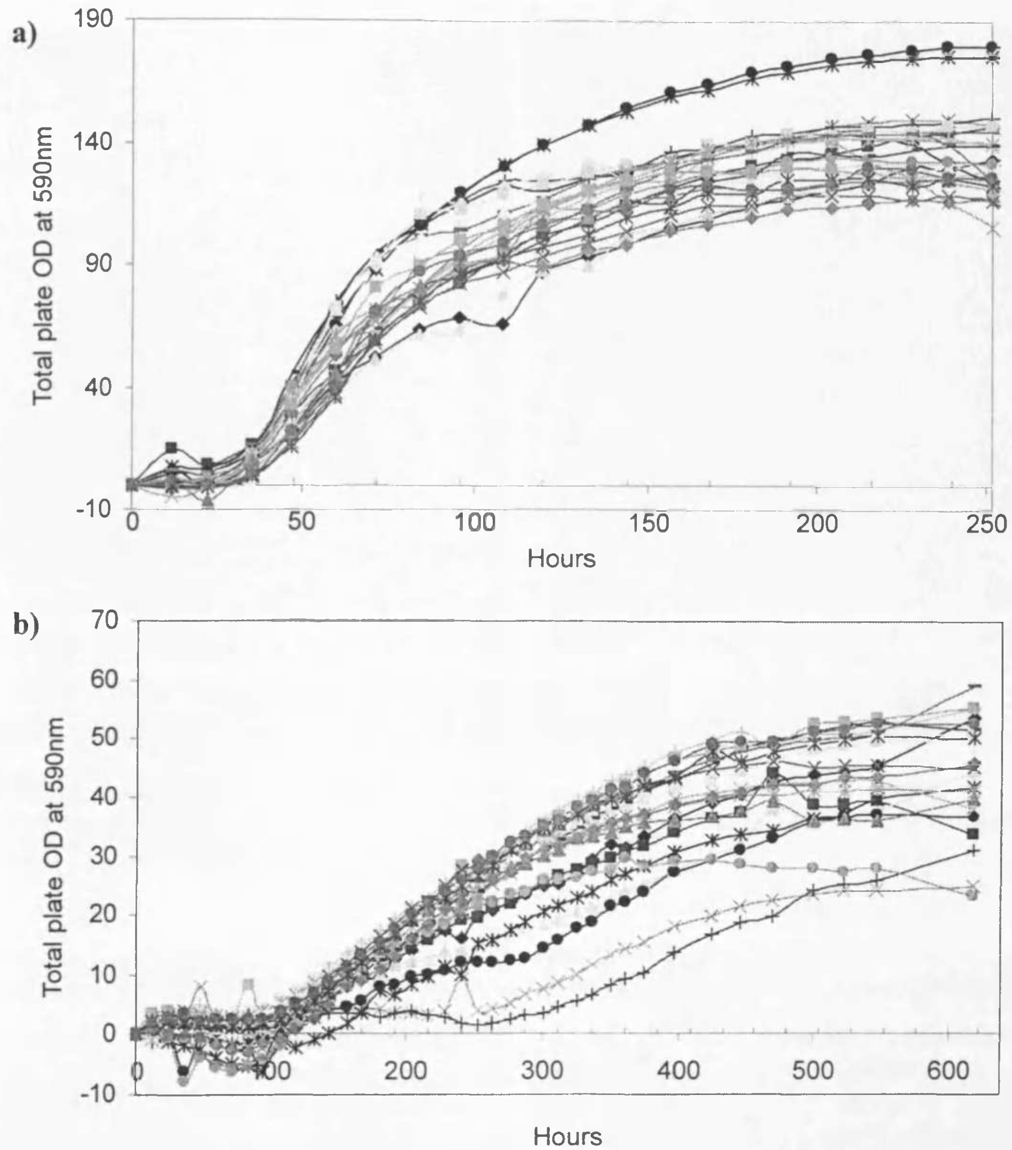
IV.V Soil Microclimate

Table IV.I Soil temperature compared between TF and IF

Results from 2 sample t-test					
	T	P	DF	TF mean	IF mean
Apr	-4.3	0.000	22	13.063	14.313
Aug	1.6	0.125	22	15.042	14.542
Nov	-6.55	0.000	22	11.3667	11.5917
Nov non parametric Mann-Whitney test	W=82.0	0.0001		median 11.4	median 11.6
Jan	-2.99	0.007	22	5.64	5.85

Table IV.II Soil moisture compared between TF and IF

Results from 2 sample t-test					
	T	P	DF	TF mean	IF mean
Apr-02	7.78	0.000	22	39.7	30.52
Jul-02	4.28	0.000	18	36.2	30.62
Nov-02	3.38	0.003	22	35.47	30.84
Jan-03	3.47	0.002	22	38.86	34.33



IV.VI Bacterial Biolog Plate Colour development

Figure IV.V Example of total plate colour development from, a) a full set of bacterial plates and b) a full set of fungal plates.

IV.VI.I Endpoint Well Colour of Bacterial and Fungal Biolog Plates

Figure IV.VI April 2002 bacterial plates Endpoint OD at 252 hours, colour represents extent of colour development from white (no development) to dark (high OD).

Figure IV.VII July 2002 bacterial plates Endpoint OD at 253 hours, colour represents extent of colour development from white (no development) to dark (high OD).

Figure IV.VIII November 2002 bacterial plates Endpoint OD at 259 hours, colour represents extent of colour development from white (no development) to dark (high OD).

Figure IV.IX January 2003 bacterial plates Endpoint OD at 261 hours, colour represents extent of colour development from white (no development) to dark (high OD).

	rfa1	rfa2	rfa3	rfb1	rfb2	rfb3	rfc1	rfc2	rfc3	rfd1	rfd2	rfd3	Fa1	Fa2	Fa3	Fb1	Fb2	Fb3	Fc1	Fc2	Fc3	Fd1	Fd2	Fd3
A1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A2	-0.012	0.26	0.287	0.004	0.111	0.068	0.155	0.266	-0.289	0.111	-0.05	0.126	-0.013	0.162	-0.121	0.294	0.033	0.083	-0.344	0.216	0.029	0.062	0.07	0.15
A3	0.326	[illegible]	0.373	0.298	0.587	0.391	[illegible]	[illegible]	0.617	0.446	0.403	0.593	0.341	0.321	0.625	[illegible]	0.518	0.282	0.017	0.506	0.22	0.634	0.305	0.372
A4	0.106	0.187	0.607	0.421	0.546	0.11	0.561	0.649	0.357	0.44	0.658	0.597	0.366	0.329	0.168	0.542	0.529	[illegible]	0.005	0.316	0.126	0.596	0.377	0.135
A5	-0.369	-0.378	-0.293	0.155	0.355	-0.291	-0.337	-0.39	-0.658	-0.153	-0.145	-0.109	-0.129	-0.363	-0.346	-0.589	-1.013	-0.459	-0.87	-0.431	-0.782	-0.484	-0.304	-0.671
A6	-0.085	-0.69	-0.332	-0.195	-0.039	0.035	-0.261	-0.493	-0.78	-0.096	-0.081	-0.131	[illegible]	-0.275	0.518	-0.516	-0.568	-0.338	-0.037	-0.695	-0.549	-0.565	-0.477	-0.763
A7	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A8	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A9	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A10	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A11	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A12	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
B1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
B2	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
B3	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
B4	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
B5	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
B6	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
B7	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
B8	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
B9	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
B10	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
B11	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
B12	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C2	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C3	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C4	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C5	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C6	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C7	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C8	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C9	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C10	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C11	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C12	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
D1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
D2	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
D3	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
D4	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
D5	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
D6	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
D7	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
D8	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
D9	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
D10	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
D11	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
D12	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
E1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
E2	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
E3	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
E4	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
E5	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
E6	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
E7	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
E8	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
E9	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
E10	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
E11	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
E12	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
F1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
F2	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
F3	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
F4	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
F5	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
F6	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
F7	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
F8	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
F9	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
F10	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
F11	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
F12	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
G1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
G2	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
G3	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
G4	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
G5	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
G6	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
G7	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
G8	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
G9	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
G10	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
G11	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
G12	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
H1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
H2	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
H3	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
H4	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
H5	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
H6	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
H7	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
H8	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
H9	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
H10	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
H11	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
H12	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Figure IV.X April 2002 Fungal plates Endpoint OD at 589 hours, colour represents extent of colour development from white (no development) to dark (high OD).

	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A2	0.174	-0.009	0.156	0.002	[illegible]	0.207	0.177	[illegible]	-0.179	0.25	0.185	0.424	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A3	0.646	0.917	[illegible]	[illegible]	[illegible]	0.473	0.144	[illegible]	[illegible]	[illegible]	0.677	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A4	0.604	[illegible]	0.61	0.261	0.297	0.367	0.279	0.141	0.029	0.671	0.566	0.424	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A5	-0.232	-0.484	[illegible]	-0.33	[illegible]	-0.384	[illegible]	[illegible]	-0.78	-0.17	[illegible]	-0.178	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A6	-0.451	-0.589	-0.004	-0.459	[illegible]	[illegible]	-0.417	[illegible]	[illegible]	[illegible]	-0.167	-0.401	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A7	0.511	0.004	0.516	0.154	[illegible]	0.336	0.081	[illegible]	[illegible]	[illegible]	0.573	0.559	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A8	[illegible]	0.481	0.611	[illegible]	[illegible]	[illegible]	0.449	[illegible]	[illegible]	[illegible]	0.629	0.472	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A9	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A10	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A11	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A12	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Figure IV.XI July 2002 Fungal plates Endpoint OD at 619.5 hours, colour represents extent of colour development from white (no development) to dark (high OD).

Figure IV.XII November 2002 Fungal plates Endpoint OD at 643 hours, colour represents extent of colour development from white (no development) to dark (high OD).

	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A2	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A3	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A4	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A5	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A6	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	-0.518	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A7	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A8	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A9	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A10	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A11	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A12	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
B1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
B2	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
B3	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
B4	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
B5	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
B6	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
B7	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
B8	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
B9	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
B10	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
B11	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
B12	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C2	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C3	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C4	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C5	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C6	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C7	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C8	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C9	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C10	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C11	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C12	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
D1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
D2	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
D3	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
D4	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
D5	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
D6	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
D7	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
D8	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
D9	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
D10	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
D11	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
D12	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
E1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
E2	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
E3	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
E4	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
E5	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
E6	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
E7	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
E8	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
E9	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
E10	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
E11	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
E12	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
F1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
F2	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
F3	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
F4	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
F5	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
F6	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
F7	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
F8	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
F9	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
F10	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
F11	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
F12	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
G1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
G2	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
G3	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
G4	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
G5	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
G6	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
G7	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
G8	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
G9	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
G10	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
G11	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
G12	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
H1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
H2	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
H3	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
H4	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
H5	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
H6	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
H7	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
H8	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
H9	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
H10	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
H11	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
H12	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Figure IV.XIII January 2003 Fungal plates Endpoint OD at 619.5 hours, colour represents extent of colour development from white (no development) to dark (high OD).

IV.VI.II Categorised r-Parameter Data

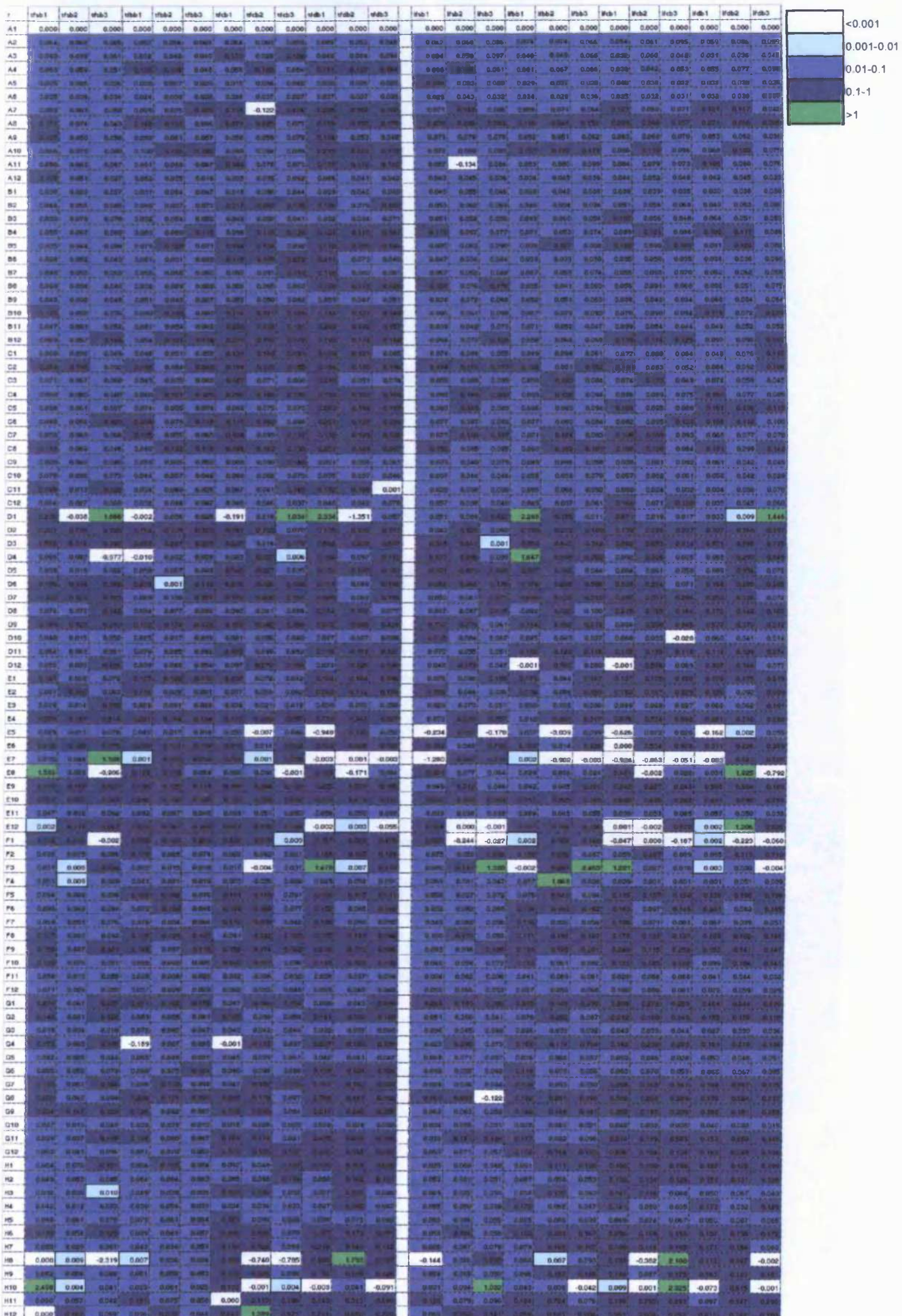


Figure IV.XIV April 2002 r-parameter categorised bacterial plates.

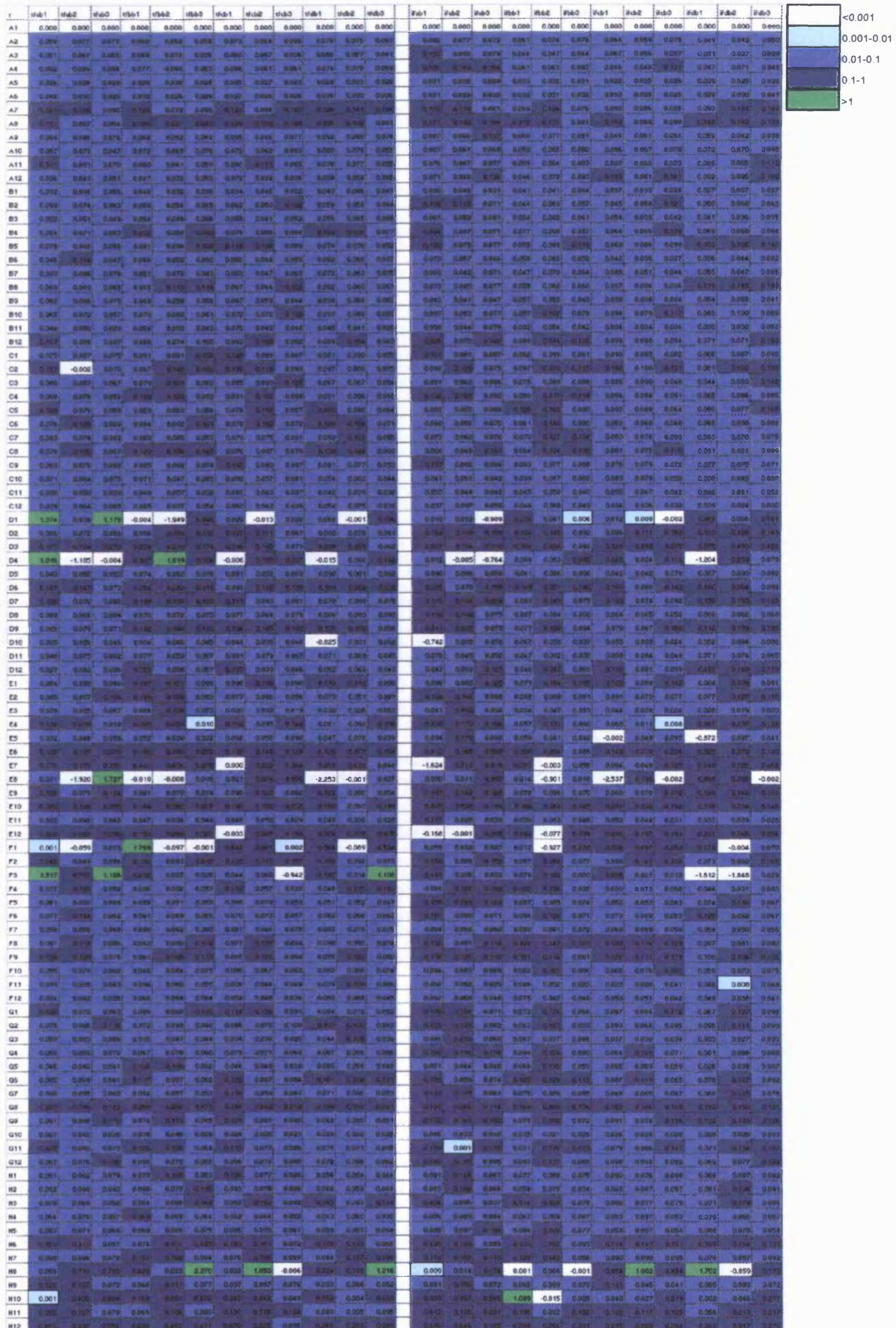


Figure IV.XV July 2002 r-parameter categorised bacterial plates.

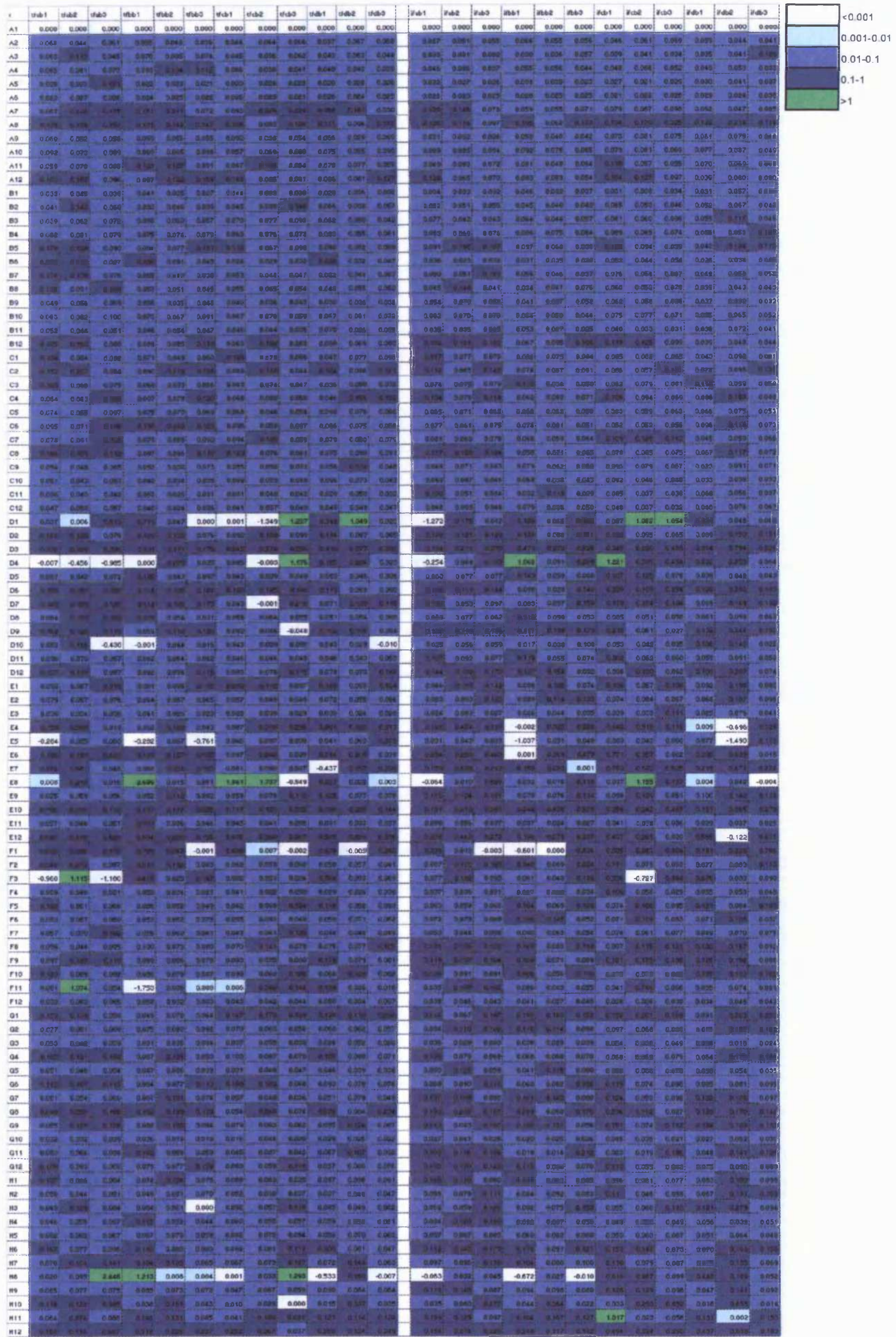


Figure IV.XVI November 2002 r-parameter categorised bacterial plates

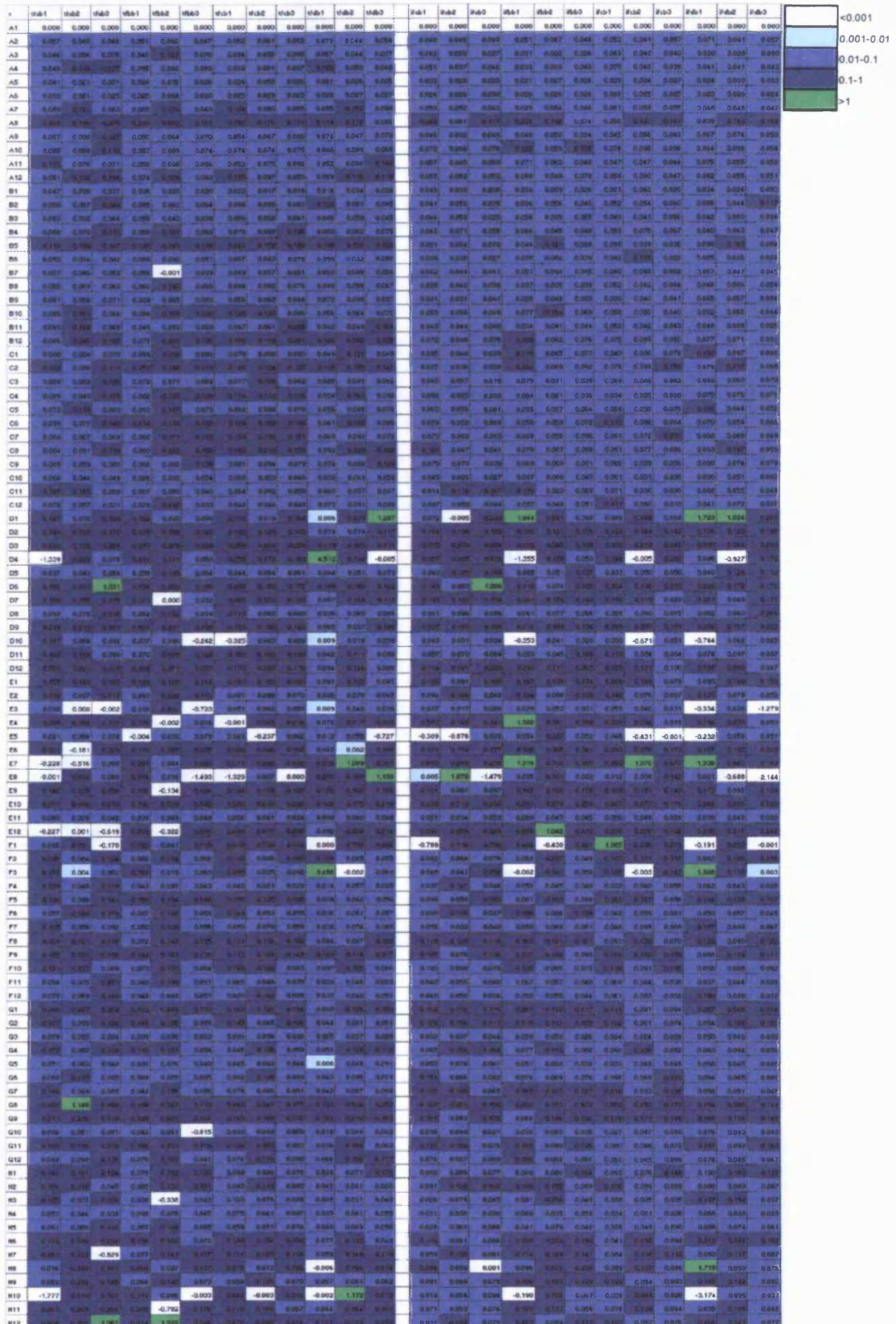


Figure IV.XVII January 2003 r-parameter categorised bacterial plates

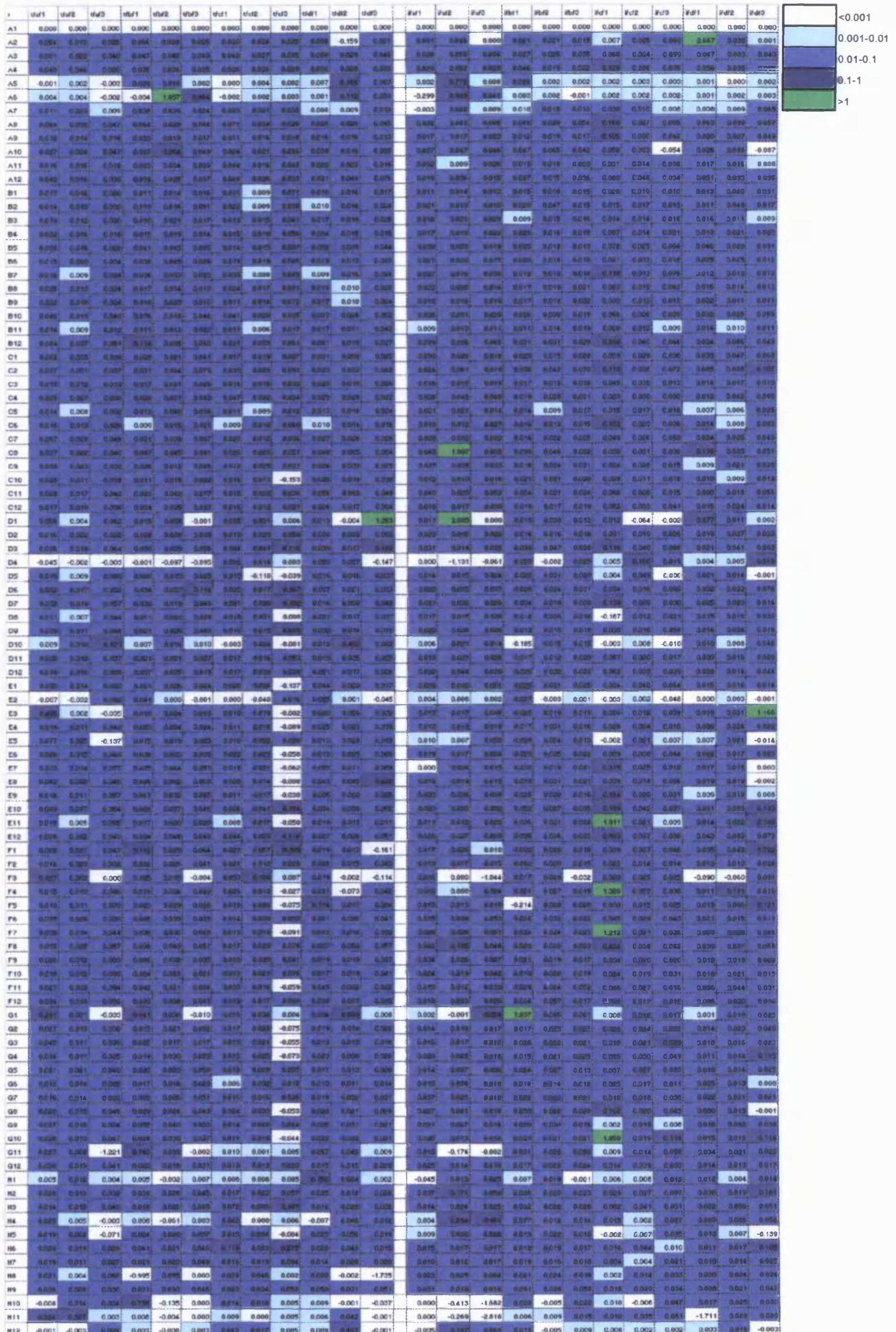


Figure IV.XVIII April 2002 r-parameter categorised fungal plates.

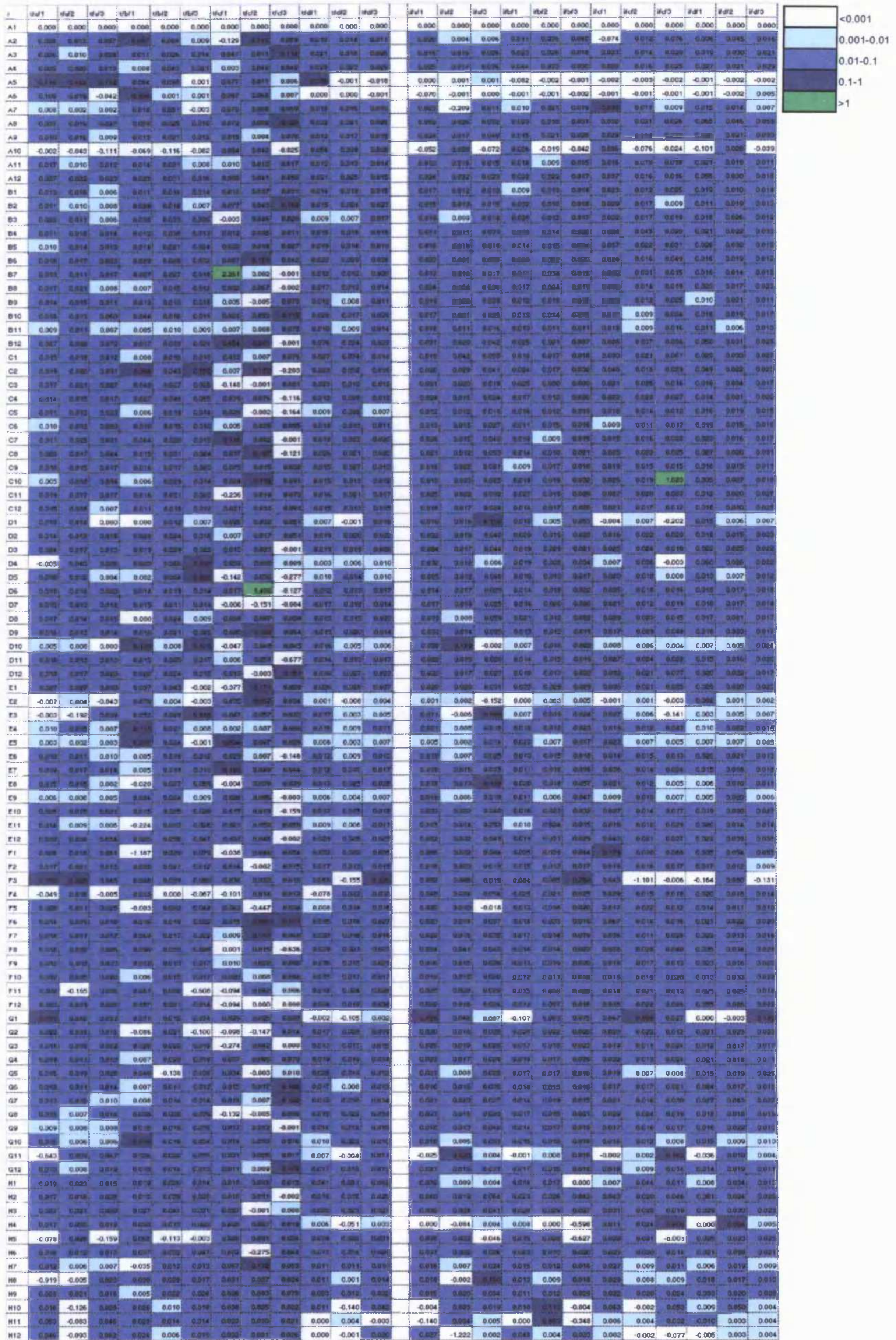


Figure IV.XIX July 2002 r-parameter categorised fungal plates.

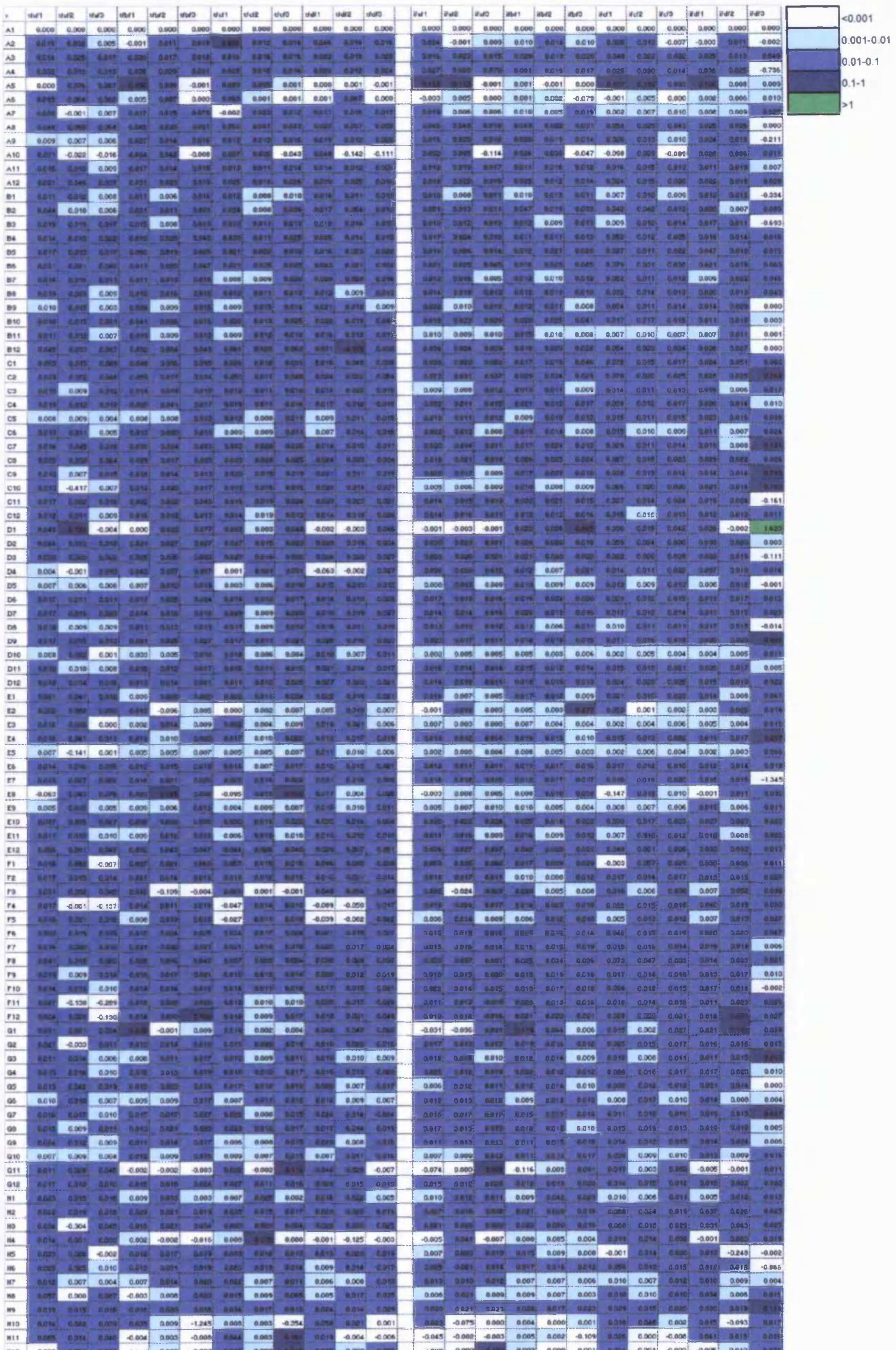


Figure IV.XX November 2002 r-parameter categorised fungal plates.

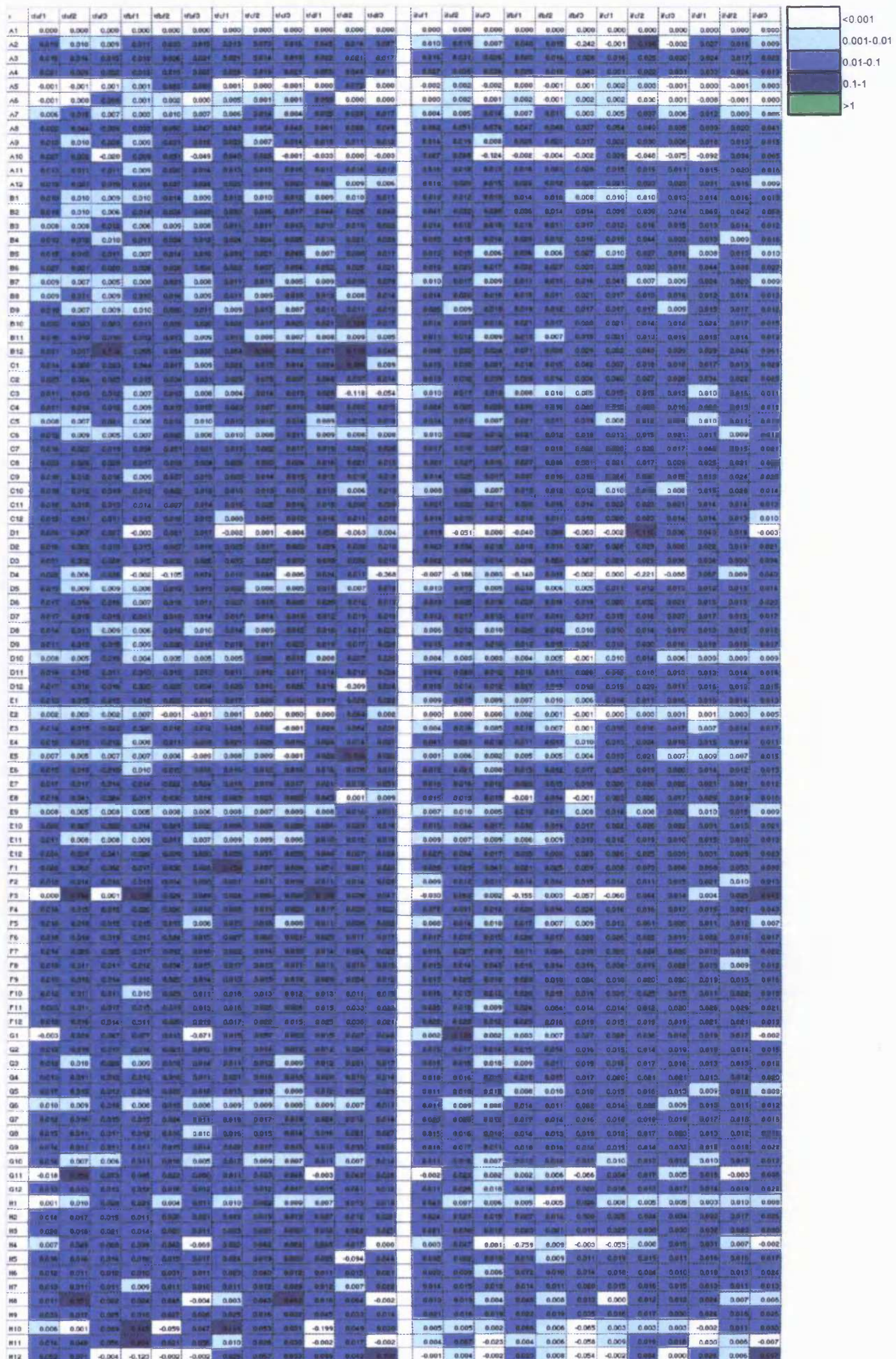


Figure IV.XXI January 2003 r-parameter categorised fungal plates.

IV.VII Principal Component Analysis (PCA) of Biolog r-Parameter Data Sets

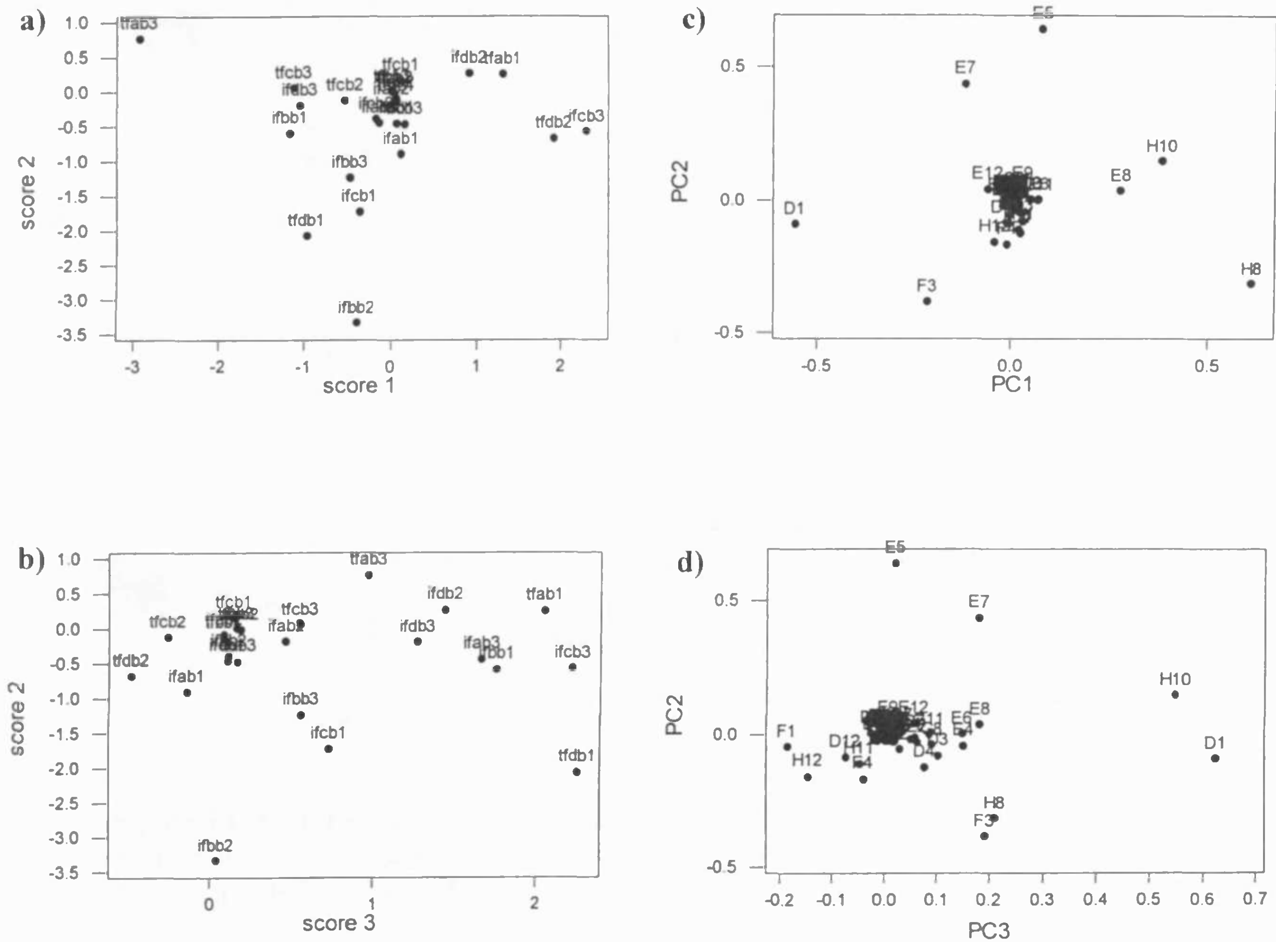
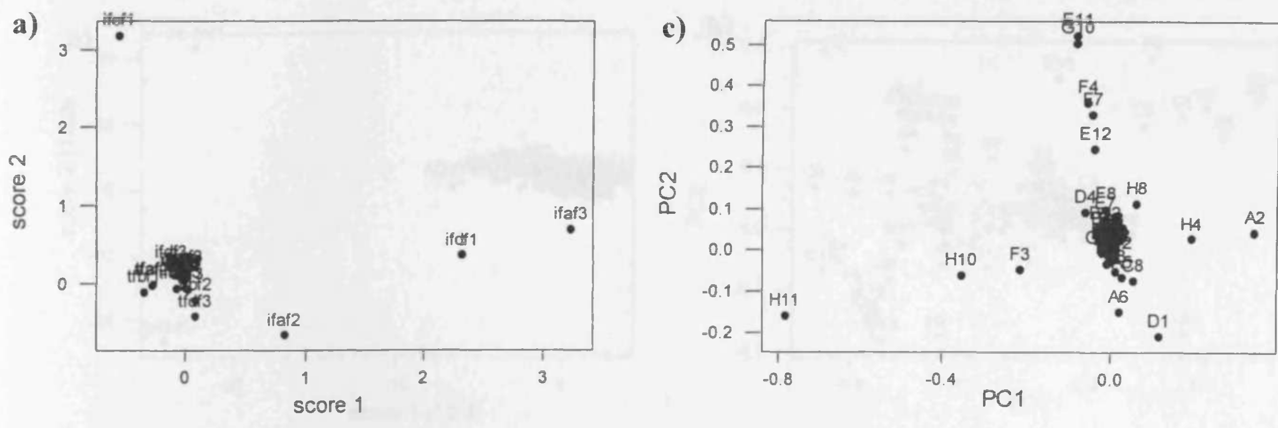


Figure IV.XXII Biolog GN2 April 2002 bacterial plates, PCA with covariance matrix. Score plots for PC1 & PC2 (a), PC2 & PC3 (b). Component loadings plots for PC1 & PC2 (c) and PC2 & PC3 (d).



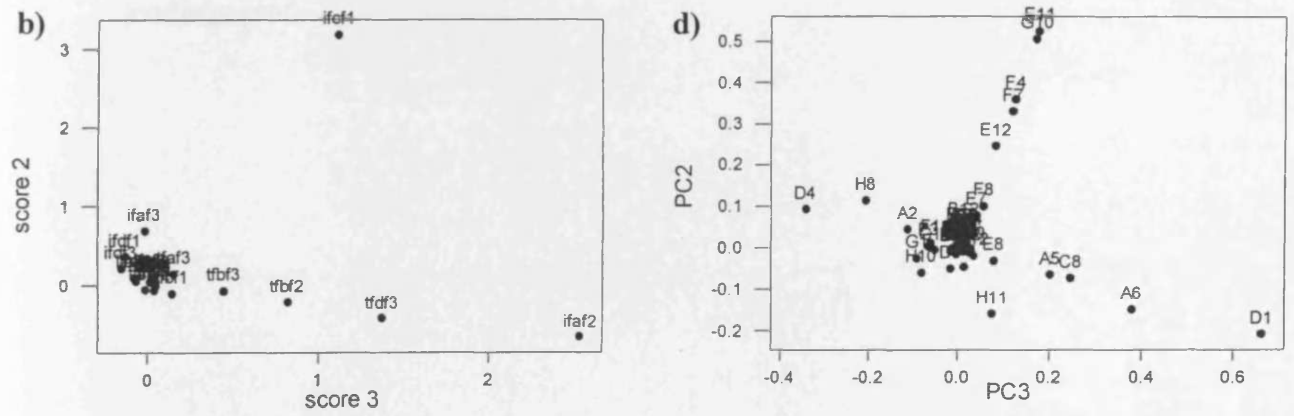


Figure IV.XXIII Biolog SF-N2 April 2002 fungal plates, PCA with covariance matrix. Score plots for PC1 & PC2 (a), PC2 & PC3 (b). Component loadings plots for PC1 & PC2 (c) and PC2 & PC3 (d).

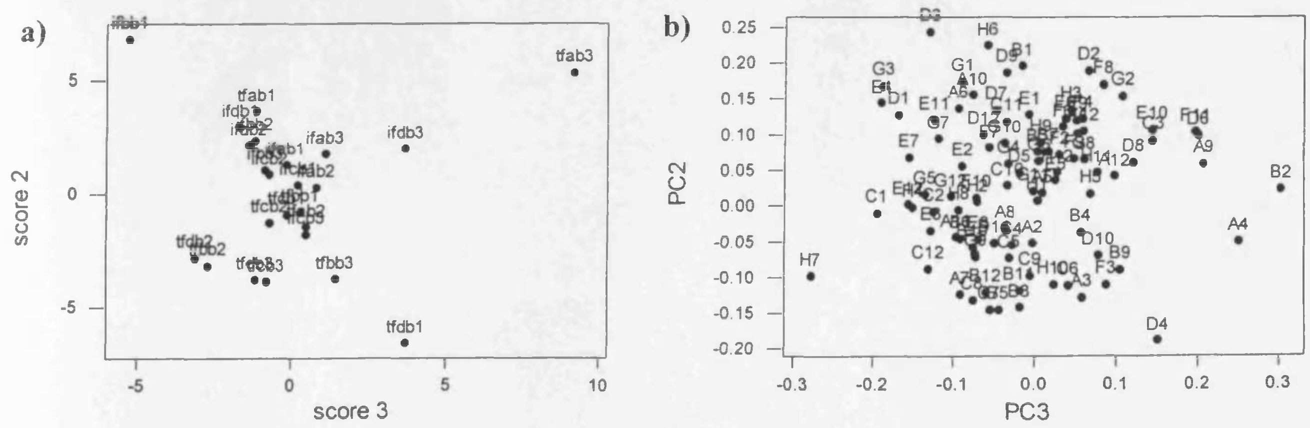


Figure IV.XXIV Biolog GN2 Jan 2003 bacterial plates, PCA with correlation matrix. Score plots for PC1 & PC2 (a). Component loadings plots for PC1 & PC2 (b).

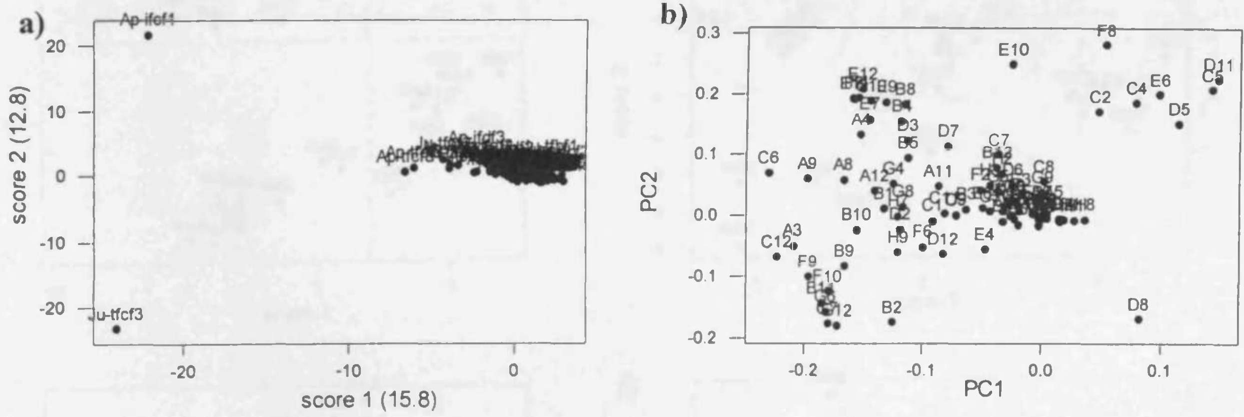


Figure IV.XXV Fungal plates PC1 & 2 for all four seasonal data sets r-parameter data, proportion of variation explained by each PC in parenthesis. a) score-plot, b) component loadings plot.

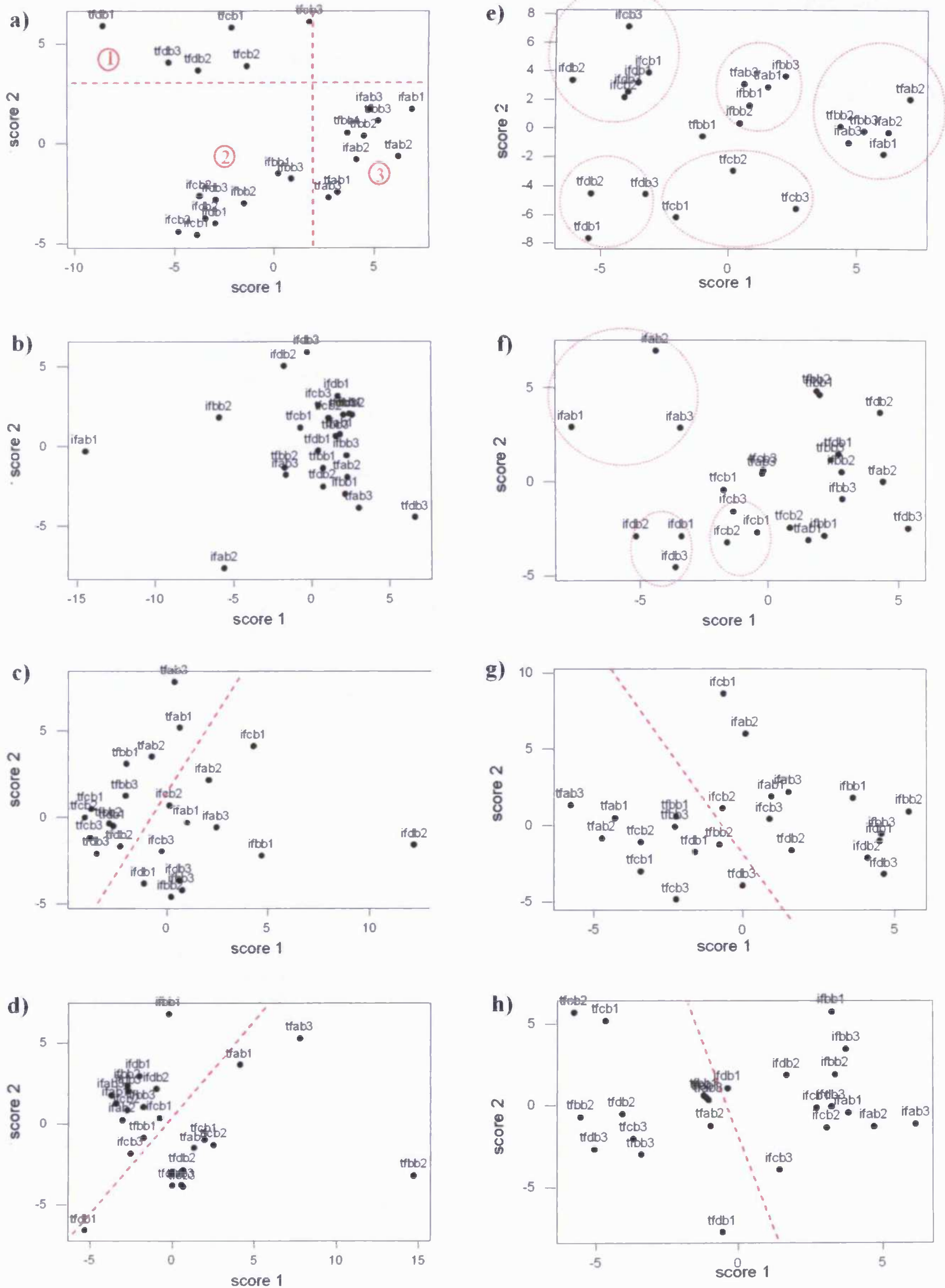


Figure IV.XXVI PCA with correlation matrix of bacterial plate *r*-parameters, from data adjusted for both well A1 and background; a) Apr-02, b) Jul-02, c) Nov-02, d) Jan-03, and data adjusted only for background colour; e) Apr-02, f) Jul-02, g) Nov-02, h) Jan-03. Dashed lines show separations of apparent clusters or groups of plates.

Appendix V. Publication

ELSEVIER

FEMS Microbiology Ecology 42 (2002) 1-14

FEMS MICROBIOLOGY Ecology

www.fems-microbiology.org

Minireview

Analysis of microbial community functional diversity using sole-carbon-source utilisation profiles – a critique

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Received 22 January 2002; received in revised form 26 June 2002; accepted 27 June 2002

First published online 3 August 2002

Abstract

Information on functional diversity (metabolic potential) is essential for understanding the role of microbial communities in different environments. Variations of the commercially available BIOLOG bacterial identification system plates are now widely used to assess functional diversity of microorganisms from environmental samples, based on utilisation patterns of a wide range (up to 95) of single carbon sources. There are many problems as well as benefits of using the approach, but the former are often disregarded. Here the basis of the approach is summarised, including type of plate to use, treatment of samples, replication, incubation conditions, monitoring of plates, and statistical analysis. The pros and cons of its use are critically assessed, inherent biases and limitations are pointed out and methodological difficulties are considered. Possible ways of overcoming some of the difficulties are suggested. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Metabolic diversity; Substrate induced respiration (SIR); Microtitre plate; Community level physiological profile (CLPP); BIOLOG

1. Introduction

There is now a plethora of information on microbial diversity in a vast range of environments. Most of this concerns genetic and taxonomic diversity, but to understand the role of microbial communities in different environments it is essential to have knowledge of microbial community function and functional diversity. Microbial community function implies *actual* catabolic activity expressed. In contrast, functional diversity indicates its *potential* activity, i.e. the capability of the community to adapt metabolism (catabolism) and/or the relative composition and size of constituent populations to varying abiotic conditions (microclimate and added substrates). Information on both functional aspects is essential. To this end Garland and Mills [1] introduced the use of the commercially available BIOLOG MicroPlate™ bacterial identification system to assess functional diversity of microorganisms from environmental samples, based on utilisation patterns of 95 single carbon sources.

To date over 120 scientific papers have been published on the use of this method for characterising bacterial communities from a range of environments, though only a few for fungal communities (Table 1). However, although the approach has obviously attracted a lot of attention, perhaps largely because data are fairly easily generated, it is not without problems, especially in terms of interpretation. If researchers are to continue using this approach for analysis of microbial community function and functional diversity it is essential that the limitations are fully appreciated. Here we explain the basis of the approach, review the pros and cons of using it, outline ways of overcoming some of the difficulties, and briefly indicate where the approach might be valuable. Firstly, we summarise the methodology and approach and consider procedural problems (Section 2). We then highlight inherent problems in interpretation, and discuss possible ways of overcoming or minimising these where possible (Sections 3 and 4). Exploratory data analysis is considered in Section 5, and finally recommendations are made for using the approach and for further work on development of the approach (Section 6).

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PII: S0168-6496(02)00324-0

2. Using BIOLOG plates for analysis of microbial community function

BIOLOG MicroPlates were originally developed for the rapid identification of bacterial isolates by sole-carbon-source utilisation, through the inoculation of 95 individual carbon sources plus a water control on a 96 well plate. The plates are read between 24 and 72 h following inoculation with a pre-grown isolate. Metabolism of the substrate in particular wells results in formazan production, producing colour change in the tetrazolium dye. Individual species may be identified by the specific pattern of colour change on the plate, providing an identifiable metabolic fingerprint. There is a BIOLOG database enabling the rapid identification of 1449 bacterial and yeast species/taxa (BIOLOG sales information) mainly of medical importance. However, the tetrazolium dye immediately introduces some bias since not all bacteria are able to reduce it [2] nor are fungi (Section 2.2), hence the plates do not necessarily give a complete picture. A range of different types of BIOLOG MicroPlates are available containing different substrates for which we have compiled a single table to allow direct and rapid comparison of the substrates available (Table 2).

2.1. *BIOLOG plates for assessment of bacterial functional diversity in environmental samples*

GN MicroPlates™ were developed specifically for the identification of Gram-negative bacteria and contain carbon sources appropriate to that group, whereas GP MicroPlates™ are available for Gram-positive bacteria (Table 2). Selective elimination of Gram-positive bacteria and fungi, by antibiotic addition, indicates that these microorganisms contribute little to the colour profile on GN plates [2,3]. SF-N and SF-P MicroPlates™ are available as alternatives, providing the same range of substrates, but without the tetrazolium dye (Table 2). EcoPlates™ are now produced, which are tailored to ecological applications and comprise three replicate sets of 31 environmentally applicable substrates at least nine of which are considered as constituents of plant root exudates [4] (Table 2). MT Microplates™ contain the redox chemicals but no substrates, allowing researchers to produce customised plates.

The selection of carbon sources in GN plates is biased towards simple carbohydrates [5]; only a few of the carbon sources in GN plates actually seem to contribute to community separation of environmental samples and many of them are redundant to the analysis [4]. EcoPlates, using more ecologically relevant structurally diverse compounds, are likely to provide a more useful test for microbial analysis and to pick up those organisms usually missed through being swamped by faster growing *r*-selected species on GN plates [4]. Combinations of substrates would be appropriate, where some substrates can not be used as sole carbon sources by some microorganisms, and using complex natural substrates, e.g. plant root exudates, might be of value [4]. For bacteria this could be achieved using MT plates.

Around 75% of papers reporting community analysis by BIOLOG plates over the last 10 years have used GN plates (Table 3). Initially this would have been because they were the only appropriate type available. Yet even now, with the availability of EcoPlates, MT plates or fungal plates (Section 2.2), GN plates are favoured, even though there is evidence to suggest that greater relevance and analytical power might be achieved with the alternatives.

2.2. *BIOLOG plates for assessment of fungal functional diversity*

Fungi do not contribute to the community colour development profile of GN [2], GP, MT and EcoPlates, because they are incapable of utilising the tetrazolium dye incorporated into them [3,6]. Though there are currently few reports of their use (Table 1), BIOLOG SF-N and SF-P plates, which contain the same carbon sources as the corresponding GN or GP plates but lack the usual tetrazo-

Table 1
Reported use of BIOLOG community catabolic profiling from 122 papers published prior to June 2001, selected from the ISI database under search terms BIOLOG and COMMUNITY

Application	Number of papers	%	Example references
Terrestrial (soil)	102	83.6	[36,37]
Methodology (development, comparisons or analysis)	31	24.6	[17,38]
Pollution	24	19.7	[12,39]
Management impacts (incl. fertilisers)	19	15.6	[40,41]
Aquatic	10	8.2	[22,42]
Rhizosphere	10	8.2	[43,44]
GM or bacterial introductions	7	5.7	[45,46]
Terrestrial (plant tissues)	6	4.9	[3,47]
Plant interactions (diversity, resource competition)	3	2.5	[43,48]
Effects of elevated CO_2 levels	2	1.6	[49,50]
Fungal	2	1.6	[6,7]

More than one category may be covered in each paper.

lium dye, can be used for assessment of fungal activity. They can be read through changes in turbidity rather than colour [7], or the tetrazolium dye dimethylthiozolyl-diphenyltetrazolium bromide (MTT) can be incorporated with the fungal preparation into the current dye-free BIOLOG plates as an indicator of fungal activity [6]. The use of MTT gives a colour response, allowing functional diversity to be assessed through metabolism (as with the bacterial plates) rather than purely growth assessment by turbidity measurement, and it reduces errors due to the presence of melanised hyphae and pigment production [6]. However, well A10 may colour rapidly (within 2 h) due to a chemical reaction under certain inoculum conditions [personal observation], but the colour change is not strong, and may be corrected for by adjusting the background colour reading.

BIOLOG FF plates are now available specifically for fungi, and contain some different carbon substrates from GN and GP plates (Table 2), plus a different tetrazolium dye that can be metabolised by fungi. To prevent interference of turbidity or colour development by bacteria an appropriate combination of antibiotics (e.g. 50μg ml^{-1} gentamycin, 100μg ml^{-1} rifampicin, 50μg ml^{-1} streptomycin), which do not affect fungal growth, should be included [6,7]. In choosing the antibiotics when also using a tetrazolium dye, care should be taken to ensure that the selection does not produce a colour change due to a chemical reaction, such as occurs with rifampicin and the Tween 40 and Tween 80 substrates [personal observation].

2.3. Sample treatment from collection to inoculation

For comparison it is important that samples are of equivalent size, in terms of volume and/or weight. It is straightforward to obtain samples of similar volumes, and this might be appropriate when comparing different sites. Samples of equivalent dry weight may be more appropriate if comparisons of soils are being made on the same site but at different times, since soil volume may alter due to wetting/drying, but are difficult to obtain without first knowing the water content. Samples for determining water content could be taken prior to those for inoculation of BIOLOG plates to reduce time between collection and inoculation, but then water content may change between these samplings.

Collecting environmental samples inevitably causes disturbance of the physical, chemical and biotic components. Thus, from the moment a sample is taken, the community within it is likely to be changing, due to death of some individuals or populations, the expansion of certain opportunistic species, and the use of phenotypic or genotypic mechanisms to adapt to the altered environment [8]. Time from sample collection to inoculation of BIOLOG plates should, therefore, be kept to a minimum, preferably within the same day. Soil storage, even in cold store or frozen, is not recommended, as bacterial, actinomycete and fungal populations can be dramatically reduced and differentially affected [9]. Pretreatment of soils before inoculation can also alter communities: sieving for homogenisation of bulked samples can often only be achieved after some drying, which together with the breaking up of aggregates, inevitable oxygenation and carbon dioxide evolution etc. can result in community change and growth/activity spikes.

For inoculating BIOLOG plates water samples can be used directly but soil samples are usually suspended in phosphate buffer or 1/4 strength Ringer's etc. The highest possible inoculum density would provide the most realistic observation window for the BIOLOG system. However, preliminary investigation is required to determine suitable dilution levels to reduce additional colour development that would be caused by soluble carbon sources in the sample. In the majority of soil studies, the soil has been agitated in suspension for up to 1 h, and then either left to settle or centrifuged and the clear supernatant used to inoculate the plates. However, this may cause problems through losses of bacteria and fungal hyphae that remain attached to soil particles. Plate cultures from the liquid portion of a soil suspension produce far fewer species than those taken from the sediment or direct soil inoculation [10,11]. Hence it is more sensible to maintain the suspension of the soil particulates and inoculate with these. A correction can be made for background colour by subtracting the initial well reading at time zero from subsequent readings [4,12].

2.4. Replication

To properly address community differences replicates must be used to account for within-community variation. Within a methodology various levels of replication should be considered. Community replication may be difficult to achieve due to obtaining access to equivalent habitat sites, thus requiring careful handling/interpretation of any results to avoid pseudo-replication. Sample replication is more easily achieved: often in published studies sites have been divided into different replicate areas/treatments and multiple samples (up to 20) are bulked together within each replicate area/treatment to cover site heterogeneity. Separate subsamples have then been taken from the bulked samples to inoculate three replicate plates to cover any variability inherent in the sample processing and data generation methods. Alternatively, to cover solely variation in the method of data generation, one subsample can be taken from which three plates can be inoculated.

In practical terms, the use of a limited number of plates is understandable due to cost; however, whether these minimum levels of replication are sufficient is doubtful, since the heterogeneity of microbial communities over the proportionally large area of even a small site will be considerable. Further, effects of rare members of communities may not be detected with low replication: the pres-

Table 2
Carbon substrates found in the different microtitre plates from BIOLOG, divided into the substrate guilds suggested by Dobranic and Zak [6]

Chemical guild	Substrate	Chemical formula	GN/SF-N	GP/SF-P	Eco	FF
Amines/amides	2-Amino ethanol	C_2O_7NO	√			√
	D-Glucosamine	$C_6H_{13}NO_5$				√
	Glucuronamide	$C_6H_{11}NO_6$	√			√
	Lactamide	$C_3H_7NO_2$		√		
	L-Alaninamide	$C_3H_8N_2O$	√	√		√
	Phenylethylamine	$C_8H_{11}N$	√		√	
	Putrescine	$C_4H_{12}N_2$	√	√	√	√
	Succinamic acid	$C_4H_7NO_3$	√	√		√
Amino acids	γ-Amino butyric acid	$C_4H_9N0_2$	√			√
	D,L-Carnitine	$C_7H_{15}NO_3$	√			
	D-Alanine	$C_3H_7NO_2$	√	√		
	D-Serine	$C_3H_7NO_3$	√			
	Glycyl-L-aspartic acid	$C_6H_{10}N_2O_5$	√			
	Glycyl-L-glutamic acid	$C_7H_{12}N_2O_5$	√	√	√	√
	Hydroxy-L-proline	$C_5H_9NO_3$	√			
	L-Alanine	$C_3H_7NO_2$	√	√		√
	L-Alanyl-glycine	$C_5H_{10}N_2O_3$	√	√		√
	L-Arginine	$C_6H_{14}N_4O_2$			√	
	L-Asparagine	$C_4H_8N_2O_3$	√	√	√	√
	L-Aspartic acid	$C_4H_7NO_4$	√			√
	L-Glutamic acid	$C_5H_9NO_4$	√	√		√
	L-Histidine	$C_6H_9N_3O_2$	√			
	L-Leucine	$C_6H_{13}NO_2$	√			
	L-Ornithine	$C_5H_{12}N_2O_2$	√			√
	L-Phenylalanine	$C_9H_{11}NO_2$	√		√	√
	L-Proline	$C_5H_9NO_2$	√			√
	L-Pyroglutamic acid	$C_5H_7NO_3$	√	√		√
	L-Serine	$C_3H_7NO_3$	√	√	√	√
	L-Threonine	$C_4H_9NO_3$	√		√	√
Carbohydrates	α-D-Glucose	$C_6H_{12}O_6$	√	√		√
	α-D-Lactose	$C_{12}H_{22}O_{11}$	√	√	√	√
	α-Methyl-D-galactoside	$C_7H_{14}O_6$		√		√
	β-Methyl-D-galactoside	$C_7H_{14}O_6$		√		√
	α-Methyl-D-glucoside	$C_7H_{14}O_6$		√		√
	β-Methyl-D-glucoside	$C_7H_{14}O_6$	√	√	√	√
	α-Methyl-D-mannoside	$C_7H_{14}O_6$		√		
	Adonitol	$C_5H_{12}O_5$	√			√
	Arbutin	$C_{12}H_6O_7$		√		√
	3-Methyl glucose	$C_7H_{14}O_6$		√		
	D-Arabinose	$C_5H_{10}O_5$				√
	D-Arabitol	$C_5H_{12}O_5$	√	√		√
	D-Cellobiose	$C_{12}H_{22}O_{11}$	√	√	√	√
	D-Fructose	$C_6H_{12}O_6$	√	√		√
	D-Galactose	$C_6H_{12}O_6$	√	√		√
	D-Mannitol	$C_6H_{14}O_6$	√	√	√	√
	D-Mannose	$C_6H_{12}O_6$	√	√		√
	D-Melezitose	$C_{18}H_{36}O_{16}$		√		√
	D-Melibiose	$C_{12}H_{22}O_{11}$	√	√		√
	D-Psicose	$C_6H_{12}O_6$	√	√		√
	D-Raffinose	$C_{18}H_{32}O_{16}$	√	√		√
	D-Ribose	$C_5H_{10}O_5$		√		√
	D-Sorbitol	$C_6H_{14}O_6$	√	√		√
	D-Tagatose	$C_6H_{12}O_6$		√		√
	D-Trehalose	$C_{12}H_{22}O_{11}$	√	√		√
	D-Xylose	$C_5H_{10}O_5$		√	√	√
	Gentiobiose	$C_{12}H_{22}O_{11}$	√	√		√
	i-Erythritol	$C_4H_{10}O_4$	√		√	√
	Lactulose	$C_{12}H_{22}O_{11}$	√	√		√
	L-Arabinose	$C_5H_{10}O_5$	√	√		√
	L-Fucose	$C_6H_{12}O_5$	√	√		√
	L-Rhamnose	$C_6H_{12}O_5$	√	√		√
	L-Sorbose	$C_6H_{12}O_6$				√
	Maltitol	$C_{12}H_{24}O_{11}$				√

Table 2 (*Continued*).

Chemical guild	Substrate	Chemical formula	GN/SF-N	GP/SF-P	Eco	FF
	Maltose	$C_{12}H_{22}O_{11}$	√	√		√
	Maltotriose	$C_{18}H_{32}O_{16}$		√		√
	Mannan	$2(C_6H_{10}O_5)_n$		√		
	Methyl pyruvate	$C_4H_6O_3$	√	√		
	m-Inositol	$C_6H_{12}O_6$	√	√		√
	Mono-methyl-succinate	$C_5H_8O_4$	√	√		
	N-Acetyl-D-galactosamine	$C_8H_{15}NO_6$	√			√
	N-Acetyl-D-glucosamine	$C_8H_{15}NO_6$	√	√	√	√
	N-Acetyl-D-mannosamine	$C_8H_{15}NO_6$		√		√
	Palatinose	$C_{12}H_{22}O_{11}$		√		√
	Sedoheptulosan	$C_7H_{12}O_6$		√		√
	Stachyose	$C_{24}H_{42}O_{21}$		√		√
	Sucrose	$C_{12}H_{22}O_{11}$	√	√		√
	Turanose	$C_{12}H_{22}O_{11}$	√	√		√
	Xylitol	$C_5H_{12}O_5$	√	√		√
Carboxylic acids	α-Hydroxy butyric acid	$C_4H_8O_3$	√	√		
	β-Hydroxy butyric acid	$C_4H_8O_3$	√	√		√
	γ-Hydroxy butyric acid	$C_4H_8O_3$	√	√	√	√
	α-Keto butyric acid	$C_4H_6O_3$	√		√	
	α-Keto glutaric acid	$C_5H_8O_5$	√	√		√
	α-Keto valeric acid	$C_5H_8O_3$	√	√		
	Acetic acid	$C_2H_4O_2$	√	√		
	2-Keto-D-gluconic acid	$C_6H_9O_7$				√
	2-Hydroxy benzoic acid	$C_7H_6O_3$			√	
	4-Hydroxy benzoic acid	$C_7H_6O_3$			√	
	Citric acid	$C_6H_8O_7$	√			
	Cis-aconitic acid	$C_6H_6O_6$	√			
	D,L-Lactic acid	$C_3H_6O_3$	√			
	D-Galactonic acid γ-lactone	$C_6H_{10}O_6$	√		√	
	D-Galacturonic acid	$C_6H_{10}O_7$	√	√	√	√
	D-Gluconic acid	$C_6H_{12}O_7$	√	√		√
	D-Glucosaminic acid	$C_6H_{13}NO_6$	√		√	
	D-Glucuronic acid	$C_6H_{10}O_7$	√			√
	D-Malic acid	$C_4H_6O_5$		√	√	√
	D-Saccharic acid	$C_6H_{10}O_8$	√			√
	Formic acid	CH_2O_2	√			
	Fumaric acid	$C_4H_4O_4$				√
	Itaconic acid	$C_5H_6O_4$	√		√	
	L-Lactic acid	$C_3H_6O_3$		√		√
	L-Malic acid	$C_4H_6O_5$		√		√
	Malonic acid	$C_3H_4O_4$	√			
	N-Acetyl-L-glutamic acid	$C_7H_{11}NO_5$		√		√
	p-Hydroxy phenylacetic acid	$C_8H_8O_3$	√	√		√
	Propionic acid	$C_6H_6O_2$	√	√		
	Pyruvic acid	$C_3H_4O_3$		√		
	Quinic acid	$C_7H_{12}O_6$	√			√
	Sebacic acid	$C_{10}H_{18}O_4$	√			√
	Succinic acid	$C_4H_6O_4$	√	√		√
Miscellaneous	2,3-Butanediol	$C_4H_{10}O_2$	√	√		
	Adenosine	$C_{10}H_{13}N_5O_4$		√		√
	Amygdalin	$C_{20}H_{27}NO_{11}$		√		√
	2′-Deoxy adenosine	$C_{10}H_{13}N_5O_3$		√		
	Adenosine-5′-monophosphate	$C_{10}H_{14}N_5O_7P$		√		√
	Bromo succinic acid	$C_4H_5O_4Br$	√			√
	D,L-α-Glycerol phosphate	$C_3H_9O_6P$	√	√	√	
	D-Lactic acid methyl ester	$C_4H_8O_3$		√		√
	Fructose-6-phosphate	$C_6H_{13}O_9P$		√		
	Glucose-1-phosphate	$C_6H_{13}O_9P$	√	√	√	√
	Glucose-6-phosphate	$C_6H_{13}O_9P$	√	√		
	Glycerol	$C_3H_8O_3$	√	√		√
	Inosine	$C_{10}H_{12}N_4O_5$	√	√		
	Pyruvic acid methyl ester	$C_4H_6O_3$			√	
	Salicin	$C_{13}H_{18}O_7$		√		√
	Succinic acid mono-methyl ester	$C_5H_8O_4$				√

Table 2 (*Continued*).

Chemical guild	Substrate	Chemical formula	GN/SF-N	GP/SF-P	Eco	FF
	Thymidine	$C_{10}H_{14}N_2O_5$	√	√		
	Thymidine-5′-monophosphate	$C_{10}H_{15}N_2O_8P$		√		
	Uridine	$C_9H_{12}N_2O_6$	√	√		√
	Uridine-5′-monophosphate	$C_9H_{13}N_2O_9P$		√		
	Urocanic acid	$C_6H_6N_2O_2$	√			
Polymers	α-Cyclodextrin	$C_{36}H_{60}O_{30}$	√	√	√	√
	β-Cyclodextrin	$C_{42}H_{70}O_{35}$		√		√
	Dextrin	$C_6H_{10}O_5$	√	√		√
	Glycogen	$(C_6H_{10}O_5)n$	√	√	√	√
	Inulin	$(C_6H_{12}O_6)n$		√		
	Tween 40		√	√	√	
	Tween 80		√	√	√	√

ence of a rare member of the community might be indicated by a substrate response that is negative in most replicates but strongly positive in one [13].

2.5. Incubation conditions

Most bacterial BIOLOG investigations have used fixed incubation temperatures between 15 and 28°C [4,14]. However, when seasonal changes to a community are being investigated, the appropriate environmental temperature for that time of year may be key to determining the functional response of the community. Replicating plates at different temperatures may provide interesting information on the functional diversity of a site and on the functional response of a community to temperature fluctuations reflecting seasonal changes [6].

The BIOLOG approach is sensitive to O_2 concentration [2,15]. This is indicated by the fact that under anaerobic conditions no formazan is produced [2] and that since the density of actively respiring cells is better correlated with rate of colour development than is total cell density, indicating that the physiological state of the cell (i.e. rate of O_2 consumption) can influence rate of colour development [15]. Thus, the even distribution of O_2 throughout the wells is important to limit errors.

Potentially the main abiotic limit to the maximum value obtained is if the tetrazolium dye is fully reduced ahead of nutrient limitation; however, potential reducing power can be estimated so that enough dye is added to avoid this situation. Also, it has been suggested that due to the dependence of colour change on the production of reducing power, any microbial energy-releasing reactions other than those ascribed to ATP production, cell growth and maintenance may result in rate of colour production or the maximum value obtained greater than is directly connected to microbial growth [16].

2.6. Monitoring colour development in BIOLOG plates

Quantifying colour development in the individual wells of the plates can be achieved rapidly by employing any microtitre plate reader with an appropriate filter (590 nm). Data can then be expressed either as individual well optical densities or average well colour development (AWCD, i.e. the sum of differences between control (water) and substrate wells divided by 95) [1].

However, the length of incubation time before readings are made can markedly affect results, due to several causes [1,13,15]. Colour development in individual wells and AWCD follow an asymptotic sigmoidal curve with time, and response of individual wells varies with respect to time, with some having a longer lag than others (Fig. 1). Thus if time before readings is too short, colour development in some wells may be missed, but if time is too long, saturation levels will be reached in some wells. Further, since colour development–time relationships may vary between wells there may not be a single optimum time for reading, between the extremes of too short and too long, hence repeated monitoring is essential (see Section 3.4).

Data are corrected against the control well or the initial readings at time zero, and corrected optical densities equivalent to the background level are omitted (since these have no positive optical density response above background noise).

Table 3
Reported use of different types of BIOLOG plates for community catabolic profiling from 122 papers published prior to June 2001, selected from the ISI database under search terms BIOLOG and COMMUNITY

Plate type	Number of papers	%
GN	107	87.7
GP	12	10.8
MT	11	9.0
Eco	7	5.7
SF-N	2	1.6
SF-P	0	0
Made own	2	1.6
Mixed	23	18.9
Unknown	4	3.3

More than one type was used in some papers.

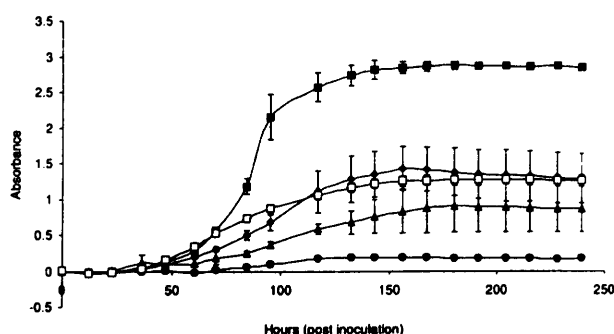

Fig. 1. Colour development during a 10-day incubation period (18°C) of meadow soil sample in BIOLOG GN MicroPlates with standard errors of the mean from three replicate plates: ♦ = well A4 (glycogen), ■ = well A6 (Tween 80), σ = well D4 (formic acid), ● = well F12 (glycyl-L-glutamic acid), □ = AWCD of the three plates. Data corrected for initial optical density at time zero, plates read at 590 nm.

3. Problems of interpretation: variation in inoculum density and incubation time

3.1. The issue of inoculum density

Microbial communities at different sites and at different times at the same site frequently differ in terms of species composition and therefore in terms of relative abundance of actively respiring cells (potentially BIOLOG dye reducing) that can utilise substrates in the different BIOLOG wells. The number/biomass and physiological state of those cells inoculated into the BIOLOG wells will inevitably affect the rate of colour development. The cells inoculated may interact with each other, giving rise to synergistic or antagonistic effects, and these effects may depend on density. Further, with time of incubation the absolute and relative number/biomass of cells and their interactive effects will change, and this may vary between wells/substrates. Not surprisingly, therefore, inoculum density and incubation time have become major issues in the debate on how to interpret and what is the real value of BIOLOG as an assay of community function.

With regard to density, though it was initially noted that colour development was correlated with cell density [1], it is now evident that it depends on both the number/biomass of cells and their activity. This was highlighted by Haack et al. [13], who found that although dilution increased the lag time prior to colour development, producing a correlation between inoculum density and rate of colour development, cell growth did not appear to be correlated with rate or degree of colouration. Rather, colour development was linked with cellular respiration. Garland [15] also found that colour development was better correlated with density of *actively* respiring cells (70%) than with total cell density (53%), i.e. rate of O_2 consumption not just cell density influenced rate of colour development. That the degree of colour change is not entirely dependent on the cell densities is further indicated by synergistic and antagonistic effects; mixtures of species do not necessarily result in the colour development that would be predicted based on the species growing alone [13].

A further density issue revolves around effects on time to formazan production. It has been proposed that a critical cell density (10^5 cells ml^{-1} [17] or 10^8 cells ml^{-1} [2,18]) is required before formazan production is initiated, although the actual density may be species-dependent [19]. If this is the case then concerns over inoculum density are less relevant since only time to reach critical density would be inoculum density-dependent and this could be accounted for by doing a kinetic analysis (Section 3.4). After reaching the critical density, rate of colour development would reflect species/strains.

Incubation time is an important issue. Comparing community patterns on BIOLOG plates at a single fixed time may largely reflect differences due to total number of microorganisms rather than substrate utilisation differences or whether all members or only part of the community are capable of utilising each carbon source [1,13]. This is because sampling at a single fixed time might be too early for some of the wells to have changed colour. Thus in studies where samples/sites were separated by ordination analysis (e.g. principal component analysis (PCA); Section 5.1) and interpreted to imply that functional differences exist between the communities, these may simply be the result of variation in the abundance of bacteria in samples, i.e. faster rate of colour development due to greater abundance [1,13]. For example, a canonical variate analysis (CVA) of single time point readings (day 5) revealed the domination of inoculum density differences (measured by total plate optical density, equivalent to AWCD) over the differing carbon-source utilisation due to soil and plant type [7]. When inoculum differences were compensated for (by taking plate readings at the same AWCD (0.5 in this case) irrespective of incubation time – see Section 3.2), plant and soil types separated out into distinct communities, plant type exerting a greater influence over the clustering than soil.

Conversely, in a study of compost piles, the communities of two differently aged composts separated due to distinctly different total plate optical densities when single time point readings were used. However, when corrections were made for inoculum density/time (again by taking plate readings at the same AWCD (0.5)) separation was much reduced, indicating similar patterns of carbon-source utilisation [7] but different inoculum densities (hence community densities), causing different total optical densities.

3.2. Overcoming possible effects of inoculum density and time by transforming data

To reduce effects of differential rates of colour development (due to inoculum density) on the classification of samples it was originally suggested that the data be transformed by dividing the raw difference (substrate well–con-

trol well value) of each well by the AWCD of the plate, giving greater indication of difference in sole-carbon-source utilisation patterns between samples [1]. Although this method is still widely applied (Table 4), its validity has been seriously questioned [20,21].

This transformation would only be valid if differences between the AWCD of plates are caused solely by differences in bacterial numbers (inoculum density) and that if the bacterial density of plates was the same they would produce the same AWCD independent of the fact that the communities might be constructed from populations with different physiological activities (i.e. that microbial populations of equal biomass have equal levels of respiratory activity) [20]. However, even for standard cell densities and incubation times there can be a 10–20% variation in AWCD [15]. Further, different communities of the same cell density will often not have the same respiratory activity (or AWCD) [20], reflecting synergistic interactions [13,22] and preferences for different carbon sources: in communities comprising greater numbers of multi-carbon-source using generalists a greater rate of AWCD would be expected than for a community of specialist single-source utilisers [15]. Garland [23] suggested that AWCD transformation should only be used on plates of similar AWCD since unequal numbers of positive responses makes normalising the data difficult. Another problem of this transformation is that amounts of carbon in the wells differ and thus when metabolised result in different colour endpoints [21].

A second means of compensating for differences in inoculum density is to use a fixed level of AWCD to determine the reading times for plate comparisons [15,23]. This has mostly been achieved by taking a series of readings across a prolonged incubation period, then selecting each plate reading closest to the reference AWCD [17]. However, again the normalisation of samples of very different cell densities, by this approach, would be less successful than for more similar samples. This is because a sample of lower inoculum density is likely to have fewer positive responses due to losses of rarer species through dilution, requiring the colour of the positive responses to be stronger in order to reach the same level of AWCD as a more dense inoculum [23].

A third solution to remove the confounding effects of differing starting densities is to adjust inoculum concentration to similar values prior to inoculation [5,15,23]. However, this is not a practical solution when comparing field sites, since this requires cell number counts or estimates of microbial carbon to be done prior to the inoculation of the BIOLOG plates, delaying the process and allowing further alteration of the community structure during this time. Further, when employing fungal plates, which do not involve single cells, the concept of equal inoculum density is meaningless, unless biomass is used. Also, dilution can result in extinction of rare species [24].

3.3. The relevance of accounting for inoculum density by transforming data

It is not appropriate to blindly transform the data to suppress the influence of inoculum density, without first recognising whether density effects might be an important aspect of differences between the microbial communities under consideration. For example, differences in inoculum density might be a major distinguishing feature between communities which otherwise show no significant differences in the carbon sources which can be used. In functional terms, a community containing the same species in the same proportions but double the total numbers per gram soil would be different because of faster breakdown and cycling effects. As another example, one community may contain five species at equal proportions while another may contain the same five species with four maintaining the same population numbers as the first community, but the fifth species being at double the previous population level, so increasing the total number of individuals in the community. The same positive and negative responses on BIOLOG plates might be expected from these two samples, yet the rates of colour development may be different in particular wells due to the relative proportions of microbes in the two communities, hence the overall plate optical densities would differ.

Table 4
Reported use of different types of data analysis approaches for interpreting BIOLOG community catabolic profiles from 122 papers published prior to June 2001, selected from the ISI database under search terms BIOLOG and COMMUNITY

Data analysis approach	Number of papers	%
Cluster analysis	25	20.5
AWCD transformed	32	26.2
Principal component analysis (PCA)	74	60.7
Canonical variate analysis (CVA)	8	6.6
Canonical discriminate analysis (CDA)	5	4.1
Canonical correspondence analysis (CCA)	4	3.3
Detrended correspondence analysis (DCA)	2	1.6
Area under curve	12	9.8
Kinetic equation parameters	8	6.6

More than one was used in some papers.

The effect of inoculum density is an important factor to recognise when comparing communities and this demonstrates the importance of analysing not only the total optical density but also the patterns of substrate utilisation that may be disguised by density-dependent effects. If data are transformed without looking for density effects, or the inoculum is adjusted to equivalent densities, much information could be lost in community comparisons. What is important is that multiple time point readings are taken to allow enough data for a full analysis (Section 3.4). However, it is important to be aware that the same AWCD does not necessarily result from the same inoculum density if the communities are composed of species/strains of differing physiological activities and the same arguments as with AWCD transformations may be applied [20].

To conclude, where the research question is focused purely on comparison of actual microbial community function at different sites then the question of inoculum density should not be a problem so long as equivalently sized (e.g. in terms of soil volume or oven dry weight) samples are taken for inoculation of the plates. If functional diversity, i.e. potential function as opposed to actual function, is being examined then it is important to take into account inoculum density.

3.4. Overcoming possible effects of inoculum density and time: the kinetic approach

Evaluating the kinetics of colour development is important to enable the comparison of multiple reference points. Being independent of incubation time, kinetic analysis removes the need for a decision on the optimum incubation time for analysis [1,13,15,19]. Several kinetic approaches have been tested [2,16,19,22] and all involve measuring colour density of each well repeatedly over a time course of several days (Section 2.6). Due to the sigmoidal shape of the colour development curves (Fig. 1), logistic curves have tended to be used as the basis for curve-fitting models, varying from the adaptation of a general logistic growth curve or modified logistic function [16,22] to the Gompertz logistic equation [2,17,19]. All estimate the logistic parameters of lag, slope (r) and asymptote (K), under varying names, which can be plotted out in PCA. Slightly conflicting results have been produced by these curve-fitting analyses.

With dilution series of a single bacterial species or a single species added to river water, there tended to be little correlation between inoculum density and model parameters [2,16,22], though sometimes a correlation or a trend was observed with the lag parameter. Thus, time to the mid-point of the linear part of the curve ($K/2$, also called s) showed a negative correlation with *Escherichia coli* [16], and no correlation with *Pseudomonas fluorescens* MM6, modelled by a modified Gompertz equation, although there was some tendency for higher densities to have shorter lag phases [2]. There was no evidence of significant effects on any of the kinetic parameters due to increasing concentrations of *E. coli* [22]. However, in this experiment, *E. coli* was added to river water with the original community still intact (although samples were frozen for storage, so likely to have lost some proportion of the community). It was suggested that BIOLOG might be insensitive to alterations in the density of a single species, but thought to be unusual that there was no effect on any of the parameters [22]. However, if *E. coli* is completely dominating the community it may be responding in the same way as a single-species experiment.

In contrast, in some studies on mixed community samples, the kinetic curve parameters were affected by inoculum concentration. With extracts from agricultural soil or meadow soil, the lag phase was significantly reduced and r (rate of colour development) significantly higher with increased inoculum concentration. Not only did the overall plate intensity increase, but also the number of wells exhibiting a positive response increased from 12 to 71 in a dilution range from 4 cells ml^{-1} to 4.1×10^6 cells ml^{-1}, indicating that very low dilutions confound BIOLOG results [2].

Kinetic parameters can be more sensitive than other BIOLOG measures, allowing finer separation of communities using ordinations. Thus, model communities consisting of three species, but in differing proportions, were much more easily separable by PCA using parameters derived from the Gompertz equation than with single time point readings at late (96 h) stages of incubation [19]. Likewise, communities of differently aged composts were separable by kinetic parameters [25]. K and r correlated with compost age, corresponding to more rapid well colour development by younger composts, while s as in single-species studies [16] was correlated with microbial biomass (density) [25].

A major difference between single-species and community dilution experiments is that in the former, when concentration is reduced, functional extinction will not occur and therefore kinetic parameters are less likely to alter. On the other hand, mixed communities may experience functional losses due to extinctions, which could alter the kinetics of wells where mixed species contribute to colour development [24], though effects on the kinetic parameters are seemingly impossible to predict. It is therefore important to dilute environmental samples as little as possible. Sensitivity to dilution extinctions should be considered during the interpretation of results and may be particularly important if high functional diversity is indicative of high species diversity.

Kinetic parameters may be the most useful format for BIOLOG data, since they are only very weakly correlated with each other. Since these parameters are based on different information about the carbon-source utilisation, each may produce different classifications when subject to ordinations [17]. If the lag phase correlates with inoculum density it is likely that an ordination of the lag-based

parameter would supply information about the initial inoculum density and relative growth rates of the species able to utilise the carbon source within each well. On the other hand, the rate parameter provides information on how rapidly a carbon source can be metabolised by a community once the density has reached the level at which colour production begins and may therefore be the most useful parameter for comparing the relative functional responses of different communities. Asymptote, being limited by the exhaustion of the carbon source, is perhaps less useful. The weak correlation between lag and rate may be due to the contribution of growth rate to both these parameters [17].

3.5. *Area under the kinetic curve*

Since the area under the colour development curve responds to changes in rate and asymptote, it has been suggested as a valid alternative to measure the relative utilisation of single carbon sources [26]. This may be particularly useful when the data do not fit the logarithmic form. The colour development profile can be broken down into four summary statistics [26], at least two (K and $K/2$) of which are the same as used in some kinetic equations [16]:

1. maximum value attained (i.e. asymptote if reached (K)),
2. time at which maximum value is attained,
3. time at which colour development is half maximum value ($K/2$ or s),
4. area under the profile.

The area under the curve is correlated with the kinetic parameters, longer lags result in smaller areas, higher asymptote or faster rates produce larger area [17].

For each individual substrate, the optical densities read at successive time points are joined by straight lines (frequent readings being important for accuracy). The area under each time segment is summed using the trapezium rule, producing an estimate of the total area under the curve [26–29]. Having determined the area under each curve, several different analyses have been used to interpret the data, including PCA [26,27], stepwise discriminant analysis [28] and canonical discriminant analysis [29]. Which of the four summary statistics is used affects interpretation; however, area was considered the most useful, since the same substrates showing high PCA loadings for statistics 1 and 3 (above) also showed high loadings for the area under the curve results [26]. Since area combines all aspects of colour development, the resultant ordination plots might be more difficult to interpret than the individual parameters. Another analytical approach is to sum the areas under all curves for each microtitre plate sample and then express the data for individual substrates as a percentage of the plate total, which has been suggested to measure 'proportional substrate utilisation efficiency' unaffected by inoculum density [29].

4. Problems of interpretation: growth of microorganisms in BIOLOG plates

4.1. *Is BIOLOG a culture method?*

Several authors have questioned whether the BIOLOG environment is at all dissimilar from standard culturing techniques [13,18,30]. If not, it is unlikely that any more than the 1% of presently culturable bacteria will grow in the wells [30]. Likewise, the fungi that will grow will reflect the limits of culturability. Additionally, since conditions in BIOLOG plates are so unlike the natural environment and because cells/communities are affected by collection and pre-processing procedures, it is likely that the BIOLOG response provides a very biased representation of the functional/metabolic capabilities of the soil community [21].

Due to the length of the incubation period (several days) required for a colour response in the plates, it is inevitable that growth occurs in the plates [1,13], even in the control well (due to peptin) where growth is not coupled to formazan formation [2]. Sustained growth only occurs under carbon-source utilisation [19], but the relative proportions of taxa have been shown to change during growth in GN plates [31]. There will be a tendency for fast-growing ruderals to predominate. For example, the well contents of triplicate BIOLOG SF-N plates in which a fungal community from a grassland soil sample had been allowed to grow for 10 days, when plated onto 2% water agar revealed growth in all wells (including the control) [personal observation]. The same limited set of around 11 species was observed from across the whole BIOLOG plate. Rapidly growing opportunistic species such as from the genus *Penicillium* dominated.

The wide range of single carbon sources, unlike in typical media, results in a variety of selection pressures, allowing species not normally able to be cultured to survive and contribute to colour formation [31]. Also, unlike enrichment culture, the microorganisms in a single well do not appear to be reduced to just one isolate [2]. This is not to suggest that the BIOLOG environment does not inhibit the growth of some species. Species which are unable to use some carbon sources as a sole carbon source will be eliminated. Molecular methods indicate that wells contain a less diverse community than originally inoculated [23]: normally dominant phyllosphere bacterial species were not detected in a recent study [32] and limitations were noted with *Bacillus* and *Arthrobacter* species failing to produce formazan consistently [13].

Although it appears that both culturable and non-culturable bacteria contribute to formazan formation, the conditions of the BIOLOG plate environment may be biased towards fast growing bacteria suited to growth in high nutrient conditions [2,3,18], hence the BIOLOG profile may be dominated by these species. For example, the faster growing marine bacterium *Vibrio alginolyticus* dominated the response pattern of two-species model commu-

nities with the slower growing *Cytophaga marinoflava*. *V. alginolyticus* still had a major, though reduced, influence on the profile when a species with intermediate growth rate (*P. fluorescens*) was included in the community [19]. Communities were separable by PCA even where only the proportions of the species differed. Even in wells where only *Cyt. marinoflava* affected colour production, growth of the more vigorous species seemed to interfere, competition presumably slowing *Cyt. marinoflava*'s metabolism of the tetrazolium dye. Growth rates may affect colour production such that the initial population size, together with the specific growth rate (doubling time) of a species within a multi-species well, determines the lag time prior to formazan production and thus, which of the species initiates colour development [18]. Not only are high-nutrient-tolerant, fast growing species likely to dominate the colour profile, but high concentrations of the carbon sources in BIOLOG plates may actually inhibit some species or result in substrate-accelerated cell death [18].

BIOLOG plates clearly have many of the problems associated with assessing species diversity by culturing techniques. Indeed, they have been described as measuring 'the diversity of organisms capable of rapidly growing on substrates under culture conditions' [30].

4.2. An alternative assay for functional diversity, which avoids growth problems: the SIR method

Since the BIOLOG procedure can be considered a cultural method in which the originally inoculated community is altered, monitoring substrate induced respiration (SIR) (the magnitude of which is (over 0–6 h) characteristic of the original soil community) has been suggested as an alternative means of assessing functional diversity of microbial communities [30]. With the SIR method, catabolic diversity is assessed by adding substrate solutions (similar to those in BIOLOG plates) to soil samples and incubating in sealed containers at 25°C for 4 h [30]. The amount of CO_2 released into the headspace through respiratory activity is then assessed. Thus it has been suggested that, unlike BIOLOG, the SIR approach measures in situ catabolic diversity, thereby producing greater accuracy in measurement of functional diversity, since the short (4 h) incubation period enables the characterisation of substrate response from the initial soil community [30]. This approach has yet to be critically examined.

5. Interpreting BIOLOG data: exploratory data analysis

BIOLOG community analysis is probably most useful for comparing the similarity or difference (in terms of the set of substrates provided) of the microbial communities under investigation. If differences are detected it is useful to identify what is causing the community divergence, whether it is use or not of particular carbon sources, which may then be interpreted through site differences in treatments or conditions. Finding and using appropriate methodology to process and interpret the profiles from community-inoculated BIOLOG plates is a significant hurdle, which is yet to be entirely overcome. The most significant problem, as with much ecological data, is the large number of variables compared with the number of samples.

As in classical ecology, a number of multivariate techniques have been applied, most popular being PCA (an ordination technique) and second, clustering (Table 4).

5.1. Ordinations

Ordination methods allow samples to be represented on scatter plots of two or more axes, the most similar samples being closest together [33,34]. PCA reduces a multivariate data set (here comprising the numerical responses in the 95 substrates, i.e. variables, of the environmental samples, i.e. individuals) into a small number of principal components that in turn account for a diminishing proportion of the variation in the data. These components are extracted from correlation or covariance similarity matrices, the software default tending to be the correlation matrix. The correlation coefficient is weighted to increase the contribution of those variables with relatively low variance in the data set (or numerically small) while covariance does not rescale their relative value, hence when covariance is used these variables will have little effect on the ordination [33]. Which method is best is a moot point and has been argued about for over 20 years in the plant community ecology literature [33]. It should be noted that the two approaches result in different ordinations, and which is most appropriate depends on the questions being asked. It has been suggested that interpretation of PCA that has used the covariance matrix is simpler, and that with BIOLOG data it is not necessary to sacrifice data by using the correlation matrix [14].

Although PCA is the most commonly used ordination procedure, each research question should be individually assessed before deciding on the most appropriate ordination method (e.g. PCA, CVA, CDA, CCA, DCA, see Table 4). Whichever method is used, following ordination, the substrates contributing the most to community separation can be assessed by univariate testing.

5.2. Substrate utilisation indices and cluster analysis

Indices of substrate richness, diversity and evenness can be calculated in the same way as is commonly done for species [5]. This does not, however, explain which types of substrate are being utilised, and for this purpose cluster analysis can be performed on the presence or absence of utilisation of each substrate. Cluster analysis (classification) extracts natural groupings among individuals (substrates), identifying units whose members are more similar to each other than they are to members of another unit.

However, the approach is very sensitive to the way in which clustering is achieved and the measure of distance used [14,34].

Instead of using data for each well separately, the substrates can be assigned to guilds [5] of carbohydrates, carboxylic acids, polymers, amines/amides, amino acids and miscellaneous (Table 2). Qualitative measures of functional diversity can then be calculated for each guild in the same way as for the whole plate [5,6]. To produce results comparable with those of substrate richness, guilds should be equally weighted to the largest guild (carbohydrates) [6]. The number of compounds in the largest guild is divided by the number of compounds in each of the other guilds, giving a correction factor for each guild by which it can be multiplied.

An alternative method, which has been employed in various studies of plant biodiversity and change, is the Lorenz curve, which provides a graphical representation of the information contained in the Shannon index [35]. Derived from this is the Gini coefficient – a measure of inequality. It has been suggested that the Lorenz curve and Gini coefficient could be used to investigate changes to soil communities through changes in carbon-source utilisation and to enable a comparison of functional diversity of microbial communities by quantifying the inequality of carbon-source utilisation [35]. An even optical density across all wells would be total equality, producing a line of positive slope of 45° from the origin. The Gini coefficient is the expected difference between pairs of carbon sources divided by AWCD, or twice the area between the plotted Lorenz curve and the line of total equality [35]. Hence a low value for the Gini coefficient represents a high level of functional diversity, suggesting that similar metabolic activities occur across the majority of the 95 well plate [35].

The value of the Gini coefficient does not change if the optical density of all wells is raised by the same proportion, e.g. when blanking against the control well [35], which possibly means it is not affected by changes to inoculum density because that would effectively alter all wells on a plate by the same proportion. However, different Lorenz curves can produce the same Gini coefficient value and no information is provided relating to individual carbon sources [35]. Data can be partitioned to reveal within and between-plate variability by treating a plate as a plot and wells as subplots, and performing split-plot analysis [35].

6. Conclusion

It appears that what was initially thought of as a quick and easy method for investigating functional diversity of microbial communities has a range of technical and more general problems which make valid interpretation very difficult and which may mean that the BIOLOG method will always be less suitable for characterisation of communities than it is for identifying single-species isolates. There are several aspects of the approach that need detailed further research.

Firstly, it is essential to determine how best to ensure that the approach adequately represents the function of all members of the community. Biases in community representation derive from: (1) technical problems in extracting all microorganisms from environmental samples to inoculate onto plates; and (2) the fact that the contributions of particular populations to the BIOLOG pattern do not necessarily reflect the relative proportion of that population within the original inoculum [31].

In terms of extracting microbes from environmental samples, current approaches simply do not achieve a representative sample. Since microbes are located on and within soil particles, litter components etc., simply shaking up a soil/litter sample with a buffer, allowing it to settle and inoculating with the supernatant results in a significant amount (both in terms of numbers and species) of the community being left in the sediment (Section 2.3). Other issues include the length of time the sample is left prior to inoculation; further information on this is necessary, as it can have differential effects on different members of the community. In addition, if current functional activity as opposed to potential is being examined then means of negating effects of dormant spores must be developed.

Poor reflection of the catabolic ability of the community has various causes: some inoculated populations fail to contribute to the colour profile due to a lack of culturability or as the result of unsuitable carbon sources in the BIOLOG environment. Populations change in the plates, relative to the inoculated community, as a result of differential growth and competition, resulting in changes to catabolism/formazan production arising from interactions between species in the wells. Hence, a better understanding of interspecific interactions within the BIOLOG environment is important because the reactions are complex and non-additive – two species together do not necessarily produce a simple additive combination of the individual patterns produced by the species ([13]; Section 3.1). Indications of how microbial communities change within BIOLOG plates and which unculturables are not contributing to the profile could be unravelled by a combination of isolation and modern molecular identification procedures on samples taken prior to inoculation of plates and then over a time course during plate incubation.

A second major area for further research is exploration of kinetic analyses and data analytical approaches. While the forgoing has demonstrated that using a kinetic approach to extract data on the plates is essential, it is not yet entirely clear which statistics or combination of statistics are most appropriate, nor necessarily what they mean (Sections 3.4 and 3.5). Further to this, which is the best approach(es) to exploratory analysis of these data has not

been reviewed and if data generated by the plates are to be used to make valid ecological comparisons and to test hypotheses, both aspects must be elucidated.

So, bearing in mind all of the forgoing caveats, in what situations might it be suitable to use BIOLOG functional community profiling? BIOLOG is more useful for the comparison of communities rather than community characterisation, due to the arbitrary concentrations and selection of carbon sources used in the plates which do not reflect the natural environment [14]. Thus, for straight comparisons of, say, the communities of two sites, it provides a potential insight into the functional ability of the community, and any differences may be apparent even if it is only from the contribution of a limited portion of that community. Due to the number of caveats associated with community profiling importance should be focussed on using BIOLOG observations in conjunction with other techniques, such as phospholipid fatty acid profiles (as done by, for example, [8,36]) and/or more traditional ecological methods. Perhaps the most erudite proposal is that 'the patterns in substrate utilisation suggested by the BIOLOG data should be considered as hypotheses worth testing, e.g. in microcosm studies, rather than conclusions in themselves' [21].

At the current stage of research it is still difficult to give a full set of recommendations, but the following should certainly be considered:

1. Keep time between sample collection and plate inoculation to a minimum. Avoid storage, even cold store or frozen.
2. Ensure equivalence of sample size, in terms of weight or volume, depending on comparisons being made.
3. For soil samples, inoculate plates with a suspension of soil, not supernatant or washings – though this needs more research.
4. Take multiple time point readings to allow full kinetic analysis.
5. If potential functional diversity, as opposed to actual function, is being examined then it is important to take into account inoculum density.

The widespread interest in this system should perhaps be used as a catalyst to trigger more research to develop a similar simple approach that is more specifically designed for ecological applications. Assay plates should contain a more suitable range of substrates that might be graded into levels of recalcitrance. In a terrestrial system, derivatives from the different stages of plant tissue decomposition, e.g. cellulose, holo/hemicelluloses and lignin or plant root exudates, could be appropriate and also importantly combinations of substrates should be used. For example, in order to degrade lignin fungi usually need simultaneous access to an additional more easily accessible energy source. More appropriate substrates would provide a simple assay to assess the presence and relative contribution of microbial functional groups within a habitat.

Acknowledgements

This work was carried out during the tenure of a Ph.D. studentship (J.P.-M.) funded by Cardiff University and Gwent Wildlife Trust. Thanks to Dr Bill Symondson for use of his plate reader. We also thank two anonymous reviewers for comments that have helped to improve the paper.

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