Assessment of the Potential Functional Diversity of the Bacterial Community in a Biofilter

by

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I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

A biofilter removes biodegradable contaminants from air by passing it through a biologically-active packed bed. The microorganism community is of fundamental interest but has been the focus of few studies. This work is an investigation of the bacterial community based on the potential functional diversity of the community.

A number of experiments were performed in laboratory-scale biofilters using ethanol as a model contaminant. All biofilters were able to remove the ethanol with elimination capacities in the range 80 to 200 g VOC m^{-3} h^{-1}; these values are comparable with published literature. Natural organic media (peat or compost) was used as packing.

The potential functional diversity of the community was assessed by Community-Level Physiological Profiling (CLPP) using sole-Carbon Source Utilisation Profile (CSUP). Community samples were used to inoculate Biolog EcoPlates™: microplates containing a selection of 31 different carbon-substrates and an indicator dye responding to bacterial growth. This technique was found to be sensitive to changes in the community structure over time and location.

Results showed that the community in samples taken close together (over a scale of a few centimetres) are similar and that relatively small media samples (0.5 to 1 g) provide reproducible information. A study of a single biofilter indicated stratification of the community occurring with the community near the inlet diverging from that near the middle and outlet of the unit; this is attributed to the ethanol being degraded in the upper part of the column and the lower part of the column not being subjected to ethanol loading. In a study of two units at a higher loading rate, stratification was not observed over a period of weeks; it is suggested that the stratification may develop over this timescale as a result of the presence or absence of the Volatile Organic Compound (VOC) and not due to differences in concentration.

An acclimation period of 7 to 10 days was observed before near-complete removal of ethanol was attained. Monitoring of the community suggested a subsequent shift in diversity. It is suggested that the initial acclimation period is due to biofilm formation and the subsequent shift in community diversity is due to re-organisation of the community as species specialise. In a portion of the biofilter with minimal ethanol exposure, a sudden shift in community is observed after a period of some weeks. This may reflect changes as a result of starvation and indicates that periods of shut-down (when the biofilter is not loaded) may affect the community.

Two studies of biofilters operating in parallel were carried out. The first provided evidence of a divergence in the communities over a period of two weeks. In the second, communities in the two units underwent changes over time but observations from both units at any one time were similar. This demonstrates that biofilters set-up and operated in a similar manner may maintain similar communities but that this is not necessarily the case. This has implications for the reproducibility of laboratory experiments and for the variation of community structure with horizontal position in industrial units.

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To my parents

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Nomenclature

The following symbols are used in the text. These have been divided into those used in the statistical analysis of CLPP data and those used in the modelling section. For reasons of convention, the same symbol may be defined differently in the two sections; the correct meaning should be obvious from the context. A reference to the definition of each symbol (usually the first usage) is given.

Modelling Symbols

δ Biofilm thickness ($\delta \geq \delta^*$) [m], see equation (2.4), page 16.

δ^* Effective biofilm thickness [m], see equation (2.2), page 16.

μ Biodegradation rate [g m^{-3}], see equation (2.2), page 16.

μ_{max} Maximum specific growth rate [h^{-1}], see equation (2.3), page 16.

ϕ_n Thiele modulus for nth order kinetics [dimensionless], page 17.

${}^n\chi$ nth order connectivity index [dimensionless], page 19.

A_s Specific surface area of packing [m^2 m^{-3}], see equation (2.1), page 15.

c Gas phase concentration [g m^{-3}], see equation (2.1), page 15.

D_{eff} Effective diffusivity [m^2 s^{-1}], see equation (2.1), page 15.

h Distance from inlet [m], see equation (2.1), page 15.

i Species (oxygen or carbon dioxide), see equation (2.1), page 15.

$K_{s,voc}$ Specific growth rate constant (VOC) [g m^{-3}], see equation (2.3), page 16.

m Distribution coefficient [g m^{-3} (biofilm) / g m^{-3} (gas)], see equation (2.2), page 16.

s Biofilm phase concentration [g m^{-3}], see equation (2.1), page 15.

u Superficial gas velocity [m s^{-1}], see equation (2.1), page 15.

X Biofilm density [g m^{-3}], see equation (2.2), page 16.

x Distance from surface into biofilm [m], see equation (2.1), page 15.

$Y_{X|i}$ Yield coefficient for biomass on component i [g g^{-1}], see equation (2.2), page 16.

Statistical Symbols

$\mathbf{\Lambda}$ Matrix containing eigenvalues corresponding to $\mathbf{P}$, see equation (A.5), page 133.

λ_j Eigenvalue corresponding to Principal Component (PC) j, see equation (A.7), page 134.

μ True population vector mean, see equation (A.11), page 135.

$\mathbf{\Sigma}$ True population covariance matrix, see equation (A.11), page 135.

σ^2 Variance due to replication in Experiment 1, see equation (3.3), page 54.

σ_1^2 Variance due to media sample size in Experiment 1, see equation (3.3), page 54.

σ_2^2 Variance due to the media sub-samples in Experiment 1, see equation (3.3), page 54.

σ_T^2 Total variance in the PCs in Experiment 1, see equation (3.3), page 54.

OD Optical Density (OD), see equation (3.1), page 52.

$\overline{\text{OD}}$ Normalised OD, see equation (3.2), page 52.

AWCD Average Well Colour Development (AWCD), see equation (3.1), page 52.

$\tilde{N}$ Number of observations in the training set, see equation (A.19), page 136.

A Number of PCs retained in the model, see equation (A.9), page 134.

$B(\alpha; a, b)$ Beta-distribution at a and b degrees of freedom and significance level α, see equation (A.11), page 135.

$\mathbf{E}$ Residual matrix, see equation (A.9), page 134.

$\mathbf{e_i}$ Residual vector for observation i, see equation (A.14), page 135.

e_{ik}^2 x_k residual for observation i, see equation (A.16), page 136.

$F(\alpha; a, b)$ F-distribution at a and b degrees of freedom and significance level α, see equation (A.12), page 135.

H_0 Null hypothesis, see equation (3.3), page 54.

H_1 Alternative hypothesis, see equation (3.3), page 54.

K Number of observed variables, see equation (A.3), page 133.

N Number of observations in the data set, see equation (A.3), page 133.

$N(\mu, \sigma^2)$ Normal distribution with mean μ and variance σ^2, page 44.

$n[t]$ Number of wells with $\overline{\text{OD}} > 0.1$ at time t, see equation (3.2), page 52.

$\mathbf{P}$ PC loading matrix (matrix of eigenvectors), see equation (A.5), page 133.

$\hat{\mathbf{P}}$ First A columns of $\mathbf{P}$, see equation (A.9), page 134.

$\mathbf{S}$ Sample covariance matrix, see equation (A.6), page 133.

s^2 Estimate of σ^2, see equation (3.3), page 54.

s_1^2 Estimate of σ_1^2, see equation (3.3), page 54.

s_2^2 Estimate of σ_2^2, see equation (3.3), page 54.

s_T^2 Estimate of σ_T^2, see equation (3.3), page 54.

s_0^2 Model Pooled Residual Variance, see equation (A.17), page 136.

s_i^2 Observation SPE, see equation (A.16), page 136.

$\mathbf{T}$ PC score matrix, see equation (A.7), page 133.

$\hat{\mathbf{T}}$ First A columns of $\mathbf{T}$, see equation (A.9), page 134.

$\mathbf{T^2}$ Vector of Hotelling's T^2 statistics, see equation (A.10), page 135.

v Correction factor in calculating SPE to account for the influence of data on the model, see equation (A.20), page 136.

$\mathbf{X}$ Data matrix for PCA, see equation (A.3), page 133.

$\bar{\mathbf{X}}$ Vector of observation means, see equation (A.6), page 133.

$\hat{\mathbf{X}}$ Estimate of $\mathbf{X}$ based on the first A PCs, see equation (A.9), page 134.

$\mathbf{x_i}$ Vector of observations, see equation (A.3), page 133.

List of Acronyms

AWCD	Average Well Colour Development
ARDRA	Amplified rDNA Restriction Analysis
ANOVA	Analysis of Variance
ARISA	Automated rRNA Intergenic Spacer Analysis
bp	base pair
BTEX	Benzene, Toluene, Ethylbenzene and Xylene
BTX	Benzene, Toluene and Xylene
CA	Cluster Analysis
CCA	Canonical Correspondence Analysis
CFU	Colony Forming Unit
CLPP	Community-Level Physiological Profiling
CSUP	Carbon Source Utilisation Profile
DCA	Detrended Correspondence Analysis
DICM	Differential Interference Contrast Microscopy
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
cDNA	Complementary DNA (DNA synthesised from mRNA)
rDNA	DNA encoding for rRNA
DRE	Destruction and Removal Efficiency

EC Elimination Capacity

EEA European Environment Agency

EPA Environmental Protection Agency (in the United States)

EPER European Pollutant Emission Register

EPS Extracellular Polymeric Substances (or Extracellular Polysaccharides)

ESEM Environmental SEM

EU European Union

FAME Fatty Acid Methyl Ester

FID Flame Ionisation Detector

FISH Fluorescent *in-Situ* Hybridisation

FRET Fluorescence Resonance Energy Transfer

GAC Granular Activated Carbon

GC Gas Chromatograph

GN Gram Negative

GP Gram Positive

HAP Hazardous Air Pollutant

HMCM Hoffman Modulation Contrast Microscopy

ID Internal Diameter

MEK Methyl Ethyl Ketone

MIBK Methyl Isobutyl Ketone

MM Mineral Medium

MPN Most Probable Number

NMVOC Non-Methane Volatile Organic Compound

dNTP deoxynucleoside Triphosphate

OD Optical Density

PB	Phosphate Buffer
PC	Principal Component
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PLFA	Phospholipid Fatty Acid
ppb	parts per billion
ppm	parts per million
PTC	Programmable Thermal Controller
QSAR	Quantitative Structure-Activity Relationship
t-RF	terminal-Restriction Fragment
t-RFLP	terminal-Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
mRNA	Messenger RNA
rRNA	Ribosomal RNA
RT-PCR	Real-Time PCR
SEM	Scanning Electron Microscopy
SIMCA	Soft Independent Modelling of Class Analogy
SPE	Squared Prediction Error
SSCP	Single-Strand Conformation Polymorphism
SUP	Substrate Utilisation Profile
TEM	Transmission Electron Microscopy
TES	Trace Element Solution
TGGE	Thermal Gradient Gel Electrophoresis
VIC	Volatile Inorganic Compound
VOC	Volatile Organic Compound

Chapter 1

Introduction

1.1 Volatile Organic Compound (VOC) Air Pollution

1.1.1 Sources

VOCs find their way into the atmosphere from a variety of anthropogenic and natural sources. There is considerable uncertainty in the scale of biogenic emissions (principally isoprene and α-pinene with other mono-terpenes and VOCs) but it appears that these emissions, mainly from vegetation, are greater than anthropogenic emissions on a global scale (Fehsenfeld *et al.*, 1992; Lamb *et al.*, 1993). The Environmental Protection Agency (in the United States) (EPA) estimated that biogenic sources accounted for more than four-fifths of total VOC emissions in the US during 1999 (Lin *et al.*, 2005) and global hydrocarbon emissions from vegetation are estimated at $1.2 * 10^6$ kt C per year (Fuentes *et al.*, 2002). However, on a regional scale anthropogenic emissions may exceed natural ones (Lamb *et al.*, 1987) and it is these that are of interest with regard to emissions control. The European Pollutant Emission Register (EPER), maintained by the European Environment Agency (EEA), estimates European Union (EU)-wide industrial emissions of Non-Methane Volatile Organic Compound (NMVOC) in excess of 545 kt for 2001 (European Environment Agency Website, Accessed August 4th, 2005).

Anthropogenic emissions include synthetic and natural compounds and are associated with various industries, including: synthetic VOCs from the manufacture of various items, including paints, dyes, flavourings, pesticides, pharmaceutical products, pulp and paper, printing, refined products and organic chemicals; and natural VOCs from the food processing industry (both for animal and human consumption), slaughterhouses, wastewater treatment works, breweries, distilleries, landfill sites and waste incinerators (Leson and Winer, 1991; Riem, 1992).

1.1.2 Health and Environmental Implications

VOCs are associated with various atmospheric reactions including the production of low-level ozone (in the presence of NO_x) and carbon monoxide (Amann, 2001; Fehsenfeld *et al.*, 1992). They contribute to acidification of local ecosystems (Amann, 2001). Many VOCs and Volatile Inorganic Compounds (VICs) are malodorous at low concentrations (in the parts per million (ppm) or sometimes parts per billion (ppb) range) and so even trace amounts may generate complaints from local residents. Some VOCs are carcinogens. They, or secondary aerosols formed from them, may be respiratory irritants or smog precursors (Amann, 2001). An increasing awareness of these implications has driven government regulatory agencies to implement stringent rules requiring the removal of these compounds from effluent air streams (Deshusses, 1997); for example, VOC emissions in the US declined 20 % between 1970 and 1998 and the 2010 target calls for a reduction of 40 to 45 % below the 1990 emission level (Amann, 2001). This has generated interest in biological control techniques as cost-effective treatment technologies (Deshusses, 1997).

1.2 Biofiltration

A biofiltration unit consists of a biologically active packed bed through which contaminated air flows. The contaminated air is conditioned before entering the bed, typically by filtering to remove particulates that may cause clogging, heat exchange to bring the temperature within a range that supports biological activity, and humidification to limit the amount of moisture picked up by the gas as it passes through the bed, which would otherwise lead to drying, the cessation of any biologically activity and the consequent loss of contaminant removal (Brauer, 1986; Leson and Winer, 1991). So treated, the gas is transported by forced convection through the bed; interphase mass transfer occurs between the bulk gas and a thin, aqueous biofilm supported on the surface of the packing material. The VOC and oxygen are partitioned into the biolayer, where they are consumed by aerobic microbial activity creating a concentration gradient to maintain a flow of the components from the gas phase into the biofilm (Ottengraf, 1986).

Biofiltration has been applied to a wide variety of compounds, including ammonia, hydrogen sulphide, mono-aromatics, alcohols, halogenated hydrocarbons and gasoline vapours; biofiltration units are installed at a wide variety of locations, for example ethanol plants, paint spraying operations, meat rendering plants, leaking fuel storage tanks and bakeries (Swanson and Loehr, 1997).

In Europe over 600 installations were reported by 1991 at a variety of industrial sites (Fouhy, 1992). In the U.S., biofiltration has mainly found application in treating off-gas from publicly owned wastewater treatment facilities (POTWs) and it is estimated that seven major vendors have over 300 installations at such facilities (Iranpour *et al.*, 2005).

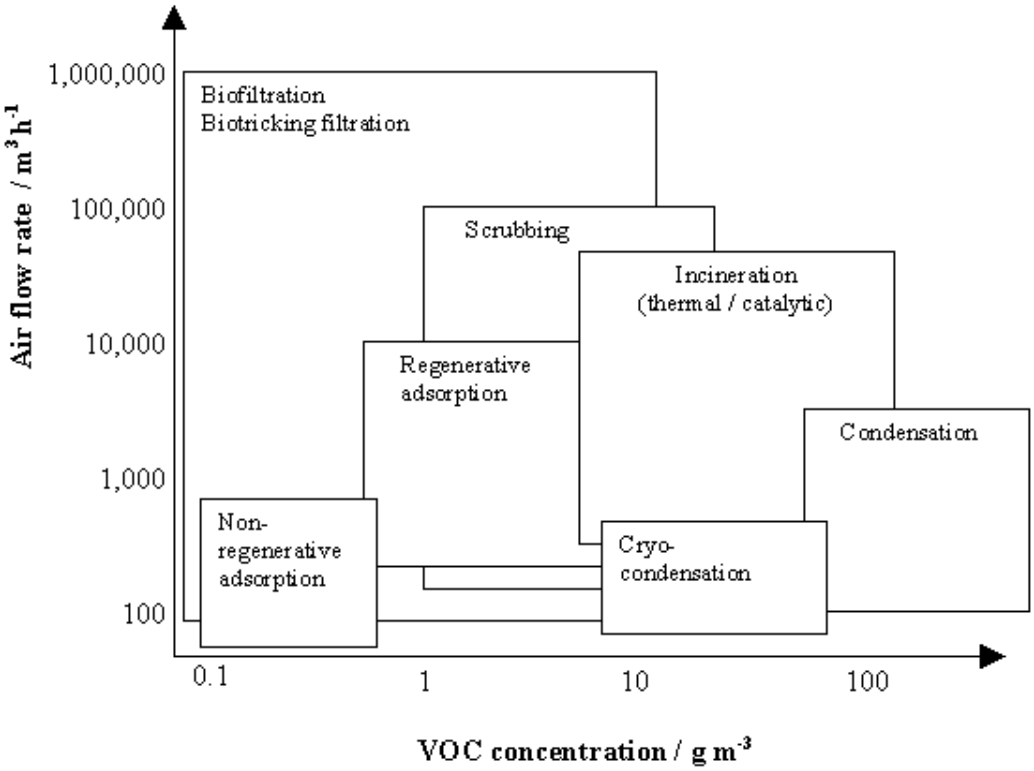


Figure 1.1: Techniques for the control of VOCs at various concentrations and flow rates. Reproduced from Deshusses and Cox (2002).

1.3 Methods for Control of VOC Air Pollution

Various methods may be employed to control VOC emissions; the economic feasibility of each depends on a combination of the VOC concentration and size of the waste air stream. Figure 1.1 illustrates this and shows that biological techniques are most suited to treating low concentration effluent gas at various total flow rates.

1.3.1 Biological Methods

There are three closely related biological technologies: biofiltration, biotrickling filtration and bioscrubbing. In a biofilter, air passes through a biologically active packed bed; the pollutant partitions into a thin, aqueous biofilm phase where it is removed by biological degradation. The biotrickling filter works on a similar principle, but liquid nutrient medium is continuously trickled through the bed, allowing improved control over

the reaction conditions such as pH and nutrient concentrations. Bioscrubbers consist of two units: the contaminated air is contacted with an aqueous phase into which the VOC is transferred; the aqueous phase then flows into a separate bioreactor where microbial degradation of the compound occurs. Bioscrubbers allow even closer control over reaction conditions than biotrickling filters, at the expense of reduced surface area for mass transfer and more complex operation. Both bioscrubbers and biotrickling filters are more complex to operate than biofilters, requiring increased capital and operating expenditure for the supplementary equipment and disposal of the increased amount of biologically active waste generated (van Groenestijn and Hesselink, 1993).

1.3.2 Non-Biological Methods

Alternative technologies for VOC removal include incineration, catalytic incineration, chemical scrubbing, adsorption, absorption, condensation and membrane separation (Jorio and Heitz, 1999). Incineration requires significant amounts of fuel and generates secondary pollutants such as NO_x; this is reduced in catalytic incineration or by heat recovery at the expense of higher capital costs (Bohn, 1992). Chemical oxidation by scrubbing involves hazardous compounds which must be purchased and later disposed of (Bohn, 1992). Adsorption using activated carbon transfers the pollutant from the air to a solid phase, which then requires further treatment (Devinny *et al.*, 1998). Condensation is not suitable for compounds with a low boiling point (below about 38 °C) (Jorio and Heitz, 1999). Membrane techniques require a high pressure differential across the membrane and remain to an extent experimental (Devinny *et al.*, 1998).

1.3.3 Selection of a Control Technology

Many factors will influence the selection of a suitable air pollution control technology. Biological techniques are well-suited to air flows contaminated with relatively low concentrations of biodegradeable compounds. They may represent the only cost-effective option for large flows of air (see Figure 1.1). Biofiltration compares very favourably with other technologies in terms of cost (see Table 1.1) the principal drawback being the long residence time, and hence large reactor volume, required (although this may be overcome using, for example, multi-layer beds where space is at a premium) (Bohn, 1992).

1.4 History of Biofiltration

Biofiltration has been used for a more than half a century as a technology for odour control, a soil biofiltration system being employed for odour control in Long Beach, California in 1953 (Wani *et al.*, 1997), and may have been in use as early as the 1920s.

Table 1.1: Costs of VOC removal technologies, taken from Bohn (1992)

	Total cost per 10^6 ft^3 of air, 1991 US dollars
Incineration	$130
Chemical scrubbing with chlorine or ozone	$60
Adsorption/regeneration of activated carbon	$20
Biofiltration	$8

More recently, the technology has been applied to other easily biodegraded volatile compounds and more complex mixtures. Since the 1980s, significant research effort has been expended in an attempt to extend the application to more recalcitrant compounds, such as chlorinated and sulphurous species, and to mixtures of compounds and to improve the packing material in terms of its nutrient composition, pore structure and mechanical integrity – important in prolonging the life of the system. There is a great variation in packing material used industrially: it may be compost, wood chips, or other organic and inorganic materials. Biofilters also vary greatly in size, the treated quantity of gas varying from 300 $m^3 h^{-1}$ at a landfill site, to over 200 000 $m^3 h^{-1}$ at an animal rendering facility (Fouhy, 1992).

Increasing regulatory stringency with respect to air emissions, such as the Clean Air Act Amendments (1990) in the U.S., the Air Quality Framework Directive, its daughter and relevant national legislation in Europe, and the Canadian Environmental Protection Act (1999), is driving further research into all air pollution control technologies; biofiltration is cheap, does not result in secondary pollution problems (such as by-products associated with incineration) and does not require extensive processing facilities (Wani *et al.*, 1997). The suitability and cost-effectiveness of biofiltration for treating VOCs has led to increased acceptance and use by industry; biofilters have been evaluated for treatment of many compounds, using a variety of packing media and bed configurations (van Groenestijn and Hesselink, 1993). Latterly, researchers have developed numerical models of the process and begun to consider microbial dynamics and characterisation (Deshusses, 1997). Recent contributions have included attempts at modelling long-term performance (Song and Kinney, 2002) and various operational strategies, such as nutrient supplementation, the use of thermophilic bacteria and cometabolism.

1.5 Advantage and Disadvantages

Biofiltration has some advantages over competing technologies:

- the pollutant is mineralised, forming principally carbon dioxide and water plus a little additional biomass;

- there are no secondary pollutants formed as a result of the treatment process – such as NO_x from incineration – and the pollutant is not simply transferred to a different phase for further treatment – as in scrubbing or adsorption;
- capital and operating costs are both modest compared with competing technologies, including biological technologies such as biotrickling filters or bioscrubbers, which are more complex and incur additional utility and maintenance expenses;
- large volumes of gas can be treated economically; and,
- degradation of sparingly soluble pollutants or those in very low concentrations is possible.

There are limitations however:

- only gas streams at moderate temperatures can be treated;
- application is limited to fairly low concentrations of contaminant – higher concentrations inhibit metabolism and may injure the micro-organisms;
- long-term control is difficult;
- recovery times after periods of non-use or on initial start-up can be long; and,
- long residence times and consequently large units can be required for treatment of recalcitrant compounds.

1.6 Research Objectives

Recent research efforts have resulted in significant improvements in the technology, in particular with regard to reliability, reducing start-up time, media life and the range of compounds and concentrations able to be treated. Despite this, the fundamental structure of the biofilm remains uncertain and the transport and kinetic phenomena controlling biofiltration on a microscopic scale remain unclear (Devinny *et al.*, 1998). The microbial population is at the heart of the technology yet relatively little is known about its structure or dynamics. The dynamic behaviour of the community in terms of fluctuations over time, response to perturbations (such as temperature, nutrient concentrations, pH, moisture content, shutdown period, VOC concentration, change of VOC, *etc.*) and the timescale of changes, is poorly understood. It is unclear to what extent efforts at intervention, such as inoculation, are effective and what characteristics may identify a "healthy" community or precede biofilter failure.

The research presented in this thesis characterises the microbial community in a biofilter using Community-Level Physiological Profiling (CLPP) based on Carbon Source

Utilisation Profiles (CSUPs) with Biolog EcoPlates™. The goal of this research is to provide insight into the variation of the community with bed depth and over time and to assess the behaviour of beds operated in parallel under the same initial and operating conditions. A number of experiments were performed seeking to address the following hypotheses:

- that changes in the bacterial population may be assessed by CLPP based on the pattern of Carbon Source Utilisation Profiles (CSUPs) measured with Biolog EcoPlates™ (a measure of potential functional diversity);
- that the community in a local area (small relative to the size of the biofilter) at any one time is similar;
- that the community remains stable over time;
- that the community is similar over the depth of the biofilter; and,
- that the community in parallel biofilters initially at the same state remains similar over time.

Chapter 2

Literature review

2.1 Fundamentals

2.1.1 Introduction

Biofiltration may be used for the treatment of air contaminated with one or more biodegradable compounds. The air is passed through a packed bed; microorganisms are immobilised on the surface or in the pore space of this packing in a thin biolayer (Ottengraf and Diks, 1992); the Volatile Organic Compound (VOC) and oxygen partition into the biolayer, where diffusion and biological oxidation occur. VOCs and Volatile Inorganic Compounds (VICs) may be treated, either singly or in mixtures (Bohn, 1992); some examples of treatable compounds are listed in Table 2.1. By-products of the process include water, carbon dioxide, new biomass and sometimes organic acids (Leson and Winer, 1991). The process is illustrated in Figure 2.1.

2.1.2 Terminology

The following terms, commonly employed in biofilter literature, are used in this thesis:

- Mass loading (or volumetric loading): the mass flowrate of VOC entering the biofilter per unit volume of packed bed (typical units: g VOC m^{-3} h^{-1}).
- Elimination Capacity (EC): the rate of VOC removal per unit volume of packed bed (analogous to loading) (typical units: g VOC m^{-3} h^{-1}).
- Destruction and Removal Efficiency (DRE): the fractional removal of the VOC (typical units: %).

Note that EC is the product of loading and DRE.

Table 2.1: Examples of compounds treated and packing media used

Compound(s)	Media	Reference
Acetone, toluene and trichloroethylene	Organic material	Gidas *et al.* (2001)
Acetone	Compost and clay	Hwang *et al.* (1997)
Alkylbenzene	Pearlite	Veiga *et al.* (1999)
Ammonia and VOCs	Heather	Pearson *et al.* (1992)
Benzene, toluene and chlorinated alkanes/alkenes	Compost and pearlite	Ergas *et al.* (1995)
Benzene and toluene	Activated carbon	Li *et al.* (2002)
BTEX	Sugar cane bagasse and compost	Kapse *et al.* (2004)
Ethanol	Compost and polystyrene	Arulneyam and Swaminathan (2000)
Hexane	Peat/Pearlite	Kibazohi *et al.* (2004)
Isopentane	Peat moss or ceramic	Togna and Singh (1994b)
Methane and trace VOCs	Porous clay	Gebert *et al.* (2004)
Methanol	Various	Shareefdeen *et al.* (1993)
MEK/MIBK/Mixture	Compost and polystyrene	Deshusses *et al.* (1995a,b)
Octane	Compost and pearlite	Zhang (2000)
α-Pinene	Wood chips, compost and pearlite	de Castro *et al.* (1997)
Styrene	"Biomass pellets"	Jorio *et al.* (2000)
Toluene	Wood bark	Andreoni *et al.* (1997)

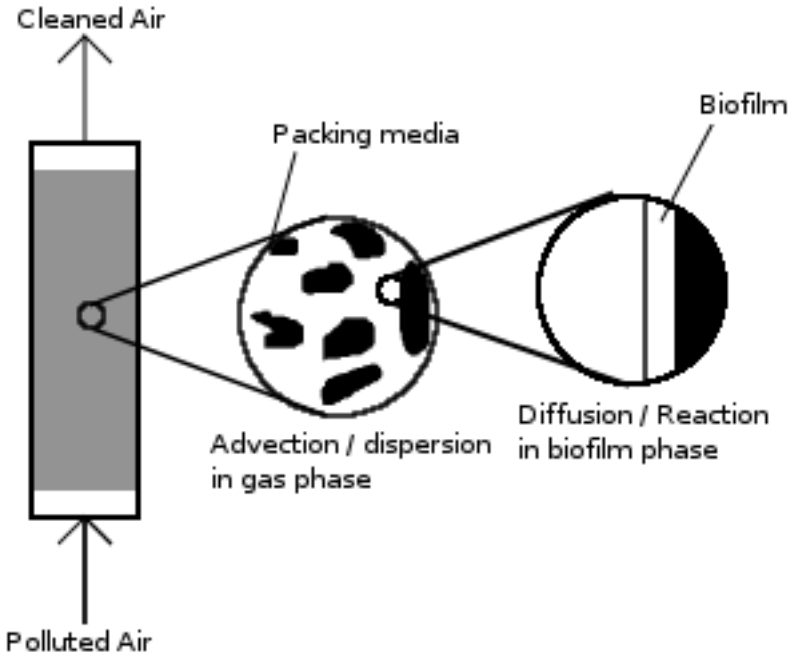


Figure 2.1: Internal mass transfer in a biofilter

2.1.3 Applications

Biofiltration has been applied for waste-gas treatment in many industries; some of these are listed in Table 2.2; other notable uses include: waste gas from soil remediation (Dawson, 1993; Oosting *et al.*, 1992), board mills and printing (Pond, 1999), investment casting (Leson, 1993; Leson *et al.*, 1993, 1995) and livestock husbandry (Pearson *et al.*, 1992). More unusual applications include cleaning air in the space station (van Groenstijn and Kraakman, 2004) and the removal of mercury vapours (Philip and Deshusses, 2004). Biofiltration is suitable for low-to-moderate concentrations of biodegradable compounds, either alone or as a mixture, and may be used for a wide range of air flow rates; indeed, for large air flows at low concentrations it may represent the only cost-effective solution (Devinny *et al.*, 1998); Techniques for VOC removal and the concentration/flow rates they are suitable for are illustrated in Figure 1.1 (Page 3).

Biofiltration of ethanol. Various laboratory-scale biofilter studies have used ethanol as a model VOC; the type of packing media used and maximum ECs obtained are summarised in Table 2.3. Bioscrubbers have also been used to treat ethanol-contaminated air (Granström *et al.*, 2002; le Cloirec *et al.*, 2001). Table 2.3 shows some high values of EC (up to 250 g VOC m^{-3} h^{-1}); however, overloading of ethanol biofilters may lead to the production of acidic intermediary metabolites and result in the failure of the biofilter (Devinny and Hodge, 1995).

The removal of ethanol from air is of industrial interest and it may be present alone or as part of a mixture in the emissions from various processes including: aerobic composting (Smet *et al.*, 1999); the food and drink industry (Arulneyam and Swaminathan, 2000; Passant *et al.*, 1993); printing (Granström *et al.*, 2002) and foundries (Passant *et al.*, 1992). It has widespread use as a solvent in the manufacture of cosmetics, surface coatings, inks, household cleaners, pharmaceuticals, insecticides and disinfectants and is used as a feedstock in the production of various chemicals (Canadian Centre for Occupation Health and Safety Website, Accessed August 16th, 2005). Biofiltration has been successfully used to treat ethanol emissions from investment foundries at full-scale (Leson *et al.*, 1993, 1995).

Although exposure to ethanol vapour is not thought to have serious or long-lasting consequences, prolonged exposure at levels above 1000 ppm may lead to short-term effects such as headaches and loss of concentration (Sax, 1989). Exposure at higher levels (above 1800 ppm) may cause respiratory irritation. Ethanol is odorous (detection threshold estimated in the range 49-716 ppm) and the industrial exposure limit in the US has been set at 1000 ppm (Canadian Centre for Occupation Health and Safety Website, Accessed August 16th, 2005).

Table 2.2: Industries using biofiltration in Europe, reproduced from Leson and Winer (1991)

Adhesive production	Chemical manufacturing	Chemical storage
Coating operations	Coca roasting	Coffee roasting
Composting facilities	Film coating	Fish frying
Fish rendering	Flavours and fragrance	Investment foundries
Industrial wastewater treatment	Residential wastewater treatment	Pet food manufacturing
Print shops	Landfill gas extraction	Slaughter houses
Tobacco processing	Waste oil recycling	

2.1.4 Typical Industrial Design

The waste air to be treated is first conditioned to: 1. bring it to appropriate temperature – between 10 and 40 °C is typical (van Lith *et al.*, 1990); 2. remove particulate matter that may otherwise clog the bed; 3. equalise VOC loading by passing it through a carbon bed (if highly variable loads are expected) (Devinny *et al.*, 1998); and, 4. bring the relative humidity close to 100 % to prevent drying of the bed. It then flows via a distribution system to the packed bed; the bed may be open or closed, may include multiple layers of packing or be separated into different units (Leson and Winer, 1991). The packed height is typically between 1 and 1.5 m (van Groenestijn and Hesselink, 1993). The main challenge in operating the bed is to maintain the moisture content – the bed dries due both to incomplete humidification of the incoming gas and heat generated through biodegradation – and regular spraying, either manual or automated, is required for moisture balance; problems with moisture may be the main reason for biofilter failure (van Lith *et al.*, 1997). Optimal values for media water content seem to be in the range 40 to 60 % and particularly any drop below the lower limit leads to a dramatic loss of DRE (Auria *et al.*, 1998; Pinnette *et al.*, 1993; Quinlan *et al.*, 1999). The packing near the inlet dries out more quickly and so downflow is generally preferred since it is easier to replace moisture at the top of the bed; upflow must sometimes be used (if the bed is open to atmosphere for example) and may also be preferred if acidic by-products are formed (these by-products are formed near the inlet – the region with highest loading – and in downflow may trickle down through the bed) (Schroeder, 2002).

2.1.5 Mass Transport

The gas phase is generally considered to be in plug flow (Schroeder, 2002). Kibazohi (2000) tested this assumption monitoring the concentration of a tracer gas injected into a laboratory-scale biofilter and found it reasonable. There is evidence of axial dispersion in some systems (Shareefdeen *et al.*, 1998). Substrates (the VOC(s)) partition into the

Table 2.3: Summary of biofilter and biotrickling filter studies using ethanol as a model VOC. ECs indicated are the maximum referred to in the paper and have sometimes been estimated from other information.

Media	Maximum EC (g VOC m^{-3} h^{-1})	Reference
Compost and polystyrene	195	Arulneyam and Swaminathan (2000)
Peat	30	Auria *et al.* (1998)
Peat/Pearlite	40	Baltzis and Androutsopoulou (1994)
Sugar Cane Bagasse	94	Christen *et al.* (2002)
Pall rings[a,b]	138	Cioci *et al.* (1997)
Pall rings[a]	220	Cox *et al.* (2001)
GAC	156	Devinny and Hodge (1995)
Wood bark	73	Eszényiová *et al.* (2001)
Peat moss	70	Grove *et al.* (2004a,b)
GAC	220	Hodge *et al.* (1992)
Compost or GAC	220	Hodge and Devinny (1994, 1995, 1997)
Wood bark	230	le Cloirec *et al.* (2001)
Bark and compost[c]	150	Leson *et al.* (1993, 1995)
Compost and GAC	100	Lim and Park (2004, 2005)
Lava rock	N/A	Nukunya *et al.* (2005)
Sugar cane bagasse	250	Pérez *et al.* (2002)
Inert silicon[a,d]	N/A	Pirnie *et al.* (2004)
Wood bark	110	Ramirez-Lopez *et al.* (2000)
Fixed-film spiral bioreactor	185	Shim *et al.* (1995)
Sand and lava rock	N/A	Steele *et al.* (2004)
Not stated	175	Togna and Singh (1994a)

[a] Biotrickling filter
[b] Pilot-scale study
[c] Full-scale biofilter
[d] Fungal biofilter; mixture of VOCs

biofilm phase. There may be a mass transfer resistance at the interface but this is usually regarded as negligible, mass transfer usually being limited by diffusion in the biofilm (Ramesh and Devinny, 2004). In the biofilm, diffusion and biological oxidation occur. Oxygen is similarly transferred and other nutrients (for example, nitrogen and phosphorous) may diffuse from the support material into the biofilm (Swanson and Loehr, 1997). End-products include additional biomass, water, carbon dioxide (which diffuses back out of the biofilm) and sometimes inorganic acids (particularly for sulphurous or chlorinated compounds) (Leson and Winer, 1991).

2.1.6 Packing Media

The packing media serves as a support and source of nutrients for the biofilm. The material must be suitable for the development of a biofilm, have sufficient mechanical strength to prevent compacting, may act as a source of nutrients for the biofilm and may also provide some buffering capacity to limit changes in pH ($CaCO_3$ (lime) is often added as a buffer) (Schroeder, 2002; Swanson and Loehr, 1997). The most common media in use is compost plus an inert bulking agent (Schroeder, 2002); various other media are suitable, including: peat, compost, speciality commercial media, inorganic materials such as pearlite and plastics or mixtures of organic and inorganic material (Wani *et al.*, 1997).

Over time, media decomposition and biomass accumulation may lead to clogging which causes increased pressure drop across the bed and channelling; the media must eventually be replaced, although there are examples of biofilters operating for ten years or more (Easter *et al.*, 2004).

2.1.7 Numerical Modelling

Overview

Various efforts to model biofiltration have been made over the past two decades. Whilst many of these show good agreement with laboratory-based studies, no model has yet been developed that has widespread application in the design of industrial units; nor has a consensus been reached in the literature with regards to the scientific principles underpinning the technology (Devinny *et al.*, 1998). This may be due to a number of particular difficulties: the simple form of reaction kinetics (shown by the concentration profiles) being fitted by a number of mechanistic models; experimental difficulties due to variation in and between biofilters; the inaccessibility of the biofilm – a thin, heterogeneous and evolving matrix of microorganisms; and, the presence of phenomena operating at different timescales – empty-bed residence times being in the order of minutes, sorption hours to days and biomass growth weeks to months – leading to computationally-intensive simulations (Devinny *et al.*, 1998).

The first major effort modelling effort was made by Ottengraf and van den Oever (1983) (a more detailed description of the same work may be found in Ottengraf (1986)); this has formed the basis for much of the subsequent work. Two phases are considered: a gaseous phase and a biologically-active aqueous phase. The gas phase moves through the biofilter in plug flow and the pollutant and oxygen partitions into the biofilm where steady-state diffusion and degradation, by first- or zeroth-order kinetics, are considered. Many researchers have adopted and adapted this model, retaining the concept of the two-phase (or sometimes three-phase including the packing) partitioned system whilst relaxing other assumptions by including, for example: substrate-inhibition kinetics and oxygen limitation (Shareefdeen *et al.*, 1993); transient effects and incomplete biofilm coverage (Hodge and Devinny, 1995; Shareefdeen and Baltzis, 1994); sorption and inhibition with multiple VOCs (Baltzis *et al.*, 1997; Deshusses *et al.*, 1995a,b; Shareefdeen *et al.*, 1997); growth and decay of biomass (Morgenroth *et al.*, 1995); nutrient limitation and biomass growth (Nukunya *et al.*, 2005; Song and Kinney, 2002); and the effect of drying (Morales *et al.*, 2003; Mysliwiec *et al.*, 2001).

Here, an overview of steady-state mechanistic modelling as developed by Ottengraf and van den Oever (1983) is presented; more information may be found in the references, the book by Devinny *et al.* (1998) or review articles (Shareefdeen and Shaikh, 1997; Ramesh and Devinny, 2004).

An alternative modelling strategy, based on Quantitative Structure-Activity Relationships (QSARs), was proposed by Choi *et al.* (1996); this model attempts to predict performance in eliminating compounds with unknown behaviour from that of a few well-characterised compounds.

Ottengraf's Model

Ottengraf and van den Oever (1983) developed the first model and much of the subsequent work is based on this. Assuming the flow and concentrations are axisymmetric (no variation in the radial direction) and at pseudo-steady-state[a] then,

$$u\frac{dc_i}{dh} = A_s D_{eff,i}\frac{ds_i}{dx}\bigg|_{x=0} \tag{2.1}$$

where u is gas superficial velocity, c is gas phase concentration for component i, h is axial distance from inlet, A_s is packing material specific surface area (area per unit volume), $D_{eff,i}$ is effective diffusivity in the biofilm, s is biofilm phase concentration, and x is distance into the biofilm. The inlet concentrations provide boundary condition, $c_i = c_{i,0}$ at $h = 0$. This model makes a number of assumptions:

1. Mass transfer in the gas phase is by convection only;

[a]This is reasonable if the inlet conditions can be considered constant, because the timescale relating to changes in the biomass is long relative to that associated with the diffusion-reaction process.

2. Local equilibrium exists between the gas and biofilm phase (i.e. interfacial resistance is negligible);

3. Pollutant transport occurs by diffusion in the biofilm, described using Fick's second law employing an effective diffusivity; and

4. The thickness of the biofilm is small compared with the packing material particle diameter (so that planar geometry may be considered).

The value of the differential at the surface is obtained by considering diffusion and biological reaction within the biofilm,

$$D_{eff,i}\frac{d^2 s_i}{dx^2} = -\frac{X\mu(s_{ox}, s_{voc})}{Y_{X|i}} \tag{2.2}$$

where X is biofilm density, $\mu(s_{ox}, s_{voc})$ is a biodegradation rate kinetic function and $Y_{X|i}$ is the yield coefficient for biomass on substrate i. The boundary conditions are:

1. $s_i = c_i/m_i$ at $x = 0$, where m is the gas-biofilm distribution coefficient (Henry's coefficient); this corresponds to local gas-biofilm equilibrium at the surface; and,

2. $\frac{ds_i}{dx} = 0$ at $x = \delta^*$, where δ^* is the effective biofilm thickness – the active thickness before one substrate is depleted, as suggested by Williamson and McCarty (1976).

The solution to the above coupled equations depends upon form of the rate function, $\mu(s_{ox}, s_{voc})$. Ottengraf and van den Oever (1983) assumed Monod kinetics applied with respect to the VOC only,

$$\mu(s_{ox}, s_{voc}) = \mu_{max}\frac{s_{voc}}{s_{voc} + K_{s,voc}} \tag{2.3}$$

Analytical solutions are possible if the reaction is assumed to be first- or zeroth-order:

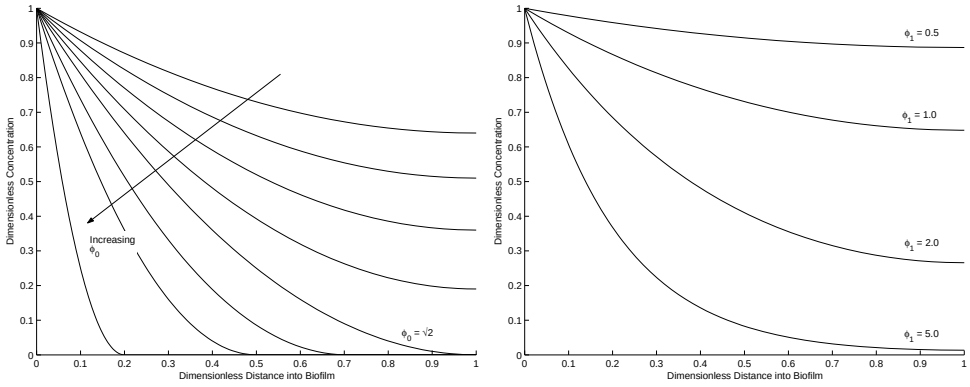
- First order, $K_{s,voc} \gg s_{voc}$, giving

$$\mu(s_{ox}, s_{voc}) \approx \mu_{max}\frac{s_{voc}}{K_{s,voc}} \tag{2.4}$$

  in which case $\delta^* = \delta$, where δ is the total biofilm thickness, for the second boundary condition above; or,

- Zeroth-order, $K_{s,voc} \ll s_{voc}$, giving

$$\mu(s_{ox}, s_{voc}) \approx \mu_{max} \tag{2.5}$$



(a) Zeroth-order kinetics (b) First-order kinetics

Figure 2.2: Illustration of concentration profiles in the biofilm for (a) Zeroth-order kinetics and (b) First-order kinetics. ϕ_0 and ϕ_1 are the Thiele moduli for zeroth- and first-order kinetics respectively[b].

which may be either diffusion- or reaction-limited, corresponding to insufficient mass-transfer into the biofilm resulting in the depletion of the substrate within the biofilm thickness ($\delta^* < \delta$) and sufficient mass-transfer resulting in the reaction kinetics being limiting ($\delta^* = \delta$).

Concentration profiles in the biofilm (solutions to Equation 2.2) are illustrated in Figure 2.2. The concentration profiles found along the axis of the biofilter (solution to Equation 2.1) obtained are illustrated in Figure 2.3. Note that the first-order solution does not theoretically permit complete VOC removal. Experimental results have been found to be in good agreement with model predictions in many studies, for example: Kibazohi (2000); Ottengraf and van den Oever (1983); van Lith *et al.* (1990); and, Zilli *et al.* (1996).

Parameter Estimation. A major challenge in modelling biofiltration is obtaining good estimates of the various kinetic and physical parameters required. Empirical lumped-parameter models exist that overcome this difficulty (for example, Smith *et al.* (2002)), but at the expense of physical interpretability of the measured parameters. The models rely on estimations of one or more of the partition coefficient, diffusivity, adsorption isotherms and microbial yield and kinetic information. Values are typically taken from similar systems (for example, air/water systems for partition coefficient; growth in suspended culture of pure organisms for yield and microbial kinetics) or estimated (for example, effective diffusivity based on a correlation with biomass density developed

[b]The Thiele modulus is a dimensionless quantity representing the relative importance of reaction rate and diffusivity (Bird *et al.*, 2002; Thiele, 1939); it is defined as $\phi_0 = \delta\sqrt{\frac{km_i}{D_{eff}C_0}}$ and $\phi_1 = \delta\sqrt{\frac{k}{D_{eff}}}$ for zeroth- and first-order kinetics, respectively (Ottengraf, 1986).

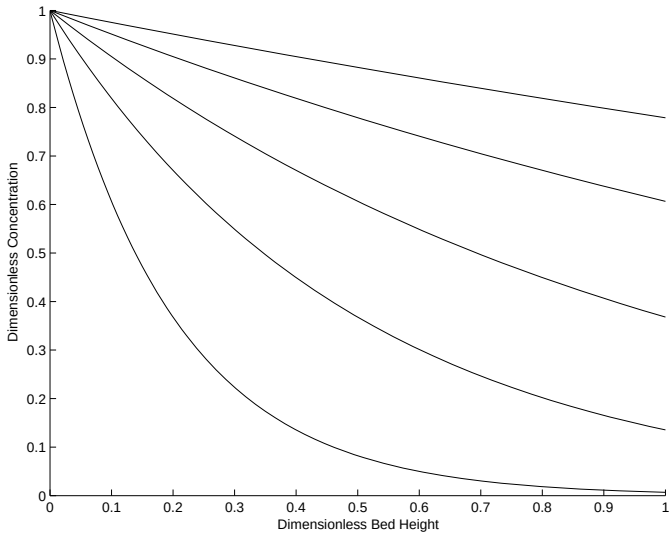


Figure 2.3: Typical concentration profiles for the gas phase assuming first-order kinetics. This results in exponential decay; zeroth-order kinetics with reaction- and diffusion-limitation would give quadratic and linear decays respectively.

by Fan *et al.* (1990)). It may also be necessary to fit the value of one parameter to experimental results and the limited information that may conveniently be collected from experimentation (concentration profiles) make it difficult to verify the accuracy of the estimates.

Quantitative Structure-Activity Relationship Models

Choi *et al.* (1996) proposed the use of QSARs to predict the performance of biofilters in treating specific compounds. This approach assumes that it is possible to determine quantitative measures of relevance to the biological activity of a compound from its chemical structure (Martin, 1981). Choi *et al.* (1996) found the first-order connectivity index $({}^1\chi)^{\text{c}}$ to be the best descriptor of DRE, with Henry's constant and the octanol-water partition coefficient also helpful; physically, these parameters correspond to a characterisation of the compound's structure, the partitioning equilibrium of the compound between the gas and aqueous phases and a measure of compound polarity which may be indicative of bioavailability. Aizpuru *et al.* (2002) determined that the same parameters were of interest in a study of eleven VOCs and note that a single-parameter description is not adequate

[c]Connectivity indices may be calculated from a hydrogen-suppressed graph of the molecule. The graph is valence-weighted: each C-atom is assigned a number, δ_i, which is the sum of adjacent C-atoms. Considering a connectivity of order m, the value $\prod_i (\delta_i)^{-0.5}$ may be calculated for all subgraphs containing m edges; the overall mth-order connectivity index, ${}^m\chi$, is given by the sum of these values over the molecule (Kier and Hall, 1976).

for a statistically significant model; they conclude that, whilst these models are satisfactory in predicting performance on the compounds studied, there is a need to gather many experimental data in order to find descriptors able to predict biofilter performance for mixtures of compounds due to substrate inhibition and co-metabolism.

2.2 Biofilter Ecology

2.2.1 Introduction

Although the presence of a healthy microbial community is essential to the operation of a biofilter, relatively little research effort has been expended in characterising this community; indeed, it is not immediately clear what is meant by a "healthy" microbial community in this context. Studying the ecosystem in a biofilter is complex: a diverse mix of organisms is present and interacting; this mixture is subject to environmental stresses (including changes in pH, temperature, moisture content and VOC concentration); the system is likely to be heterogeneous and may take a long time to reach steady-state, if it does so at all; new organisms may establish; and, established species may die off. An ecological approach can be taken to attack this missing information (Devinny *et al.*, 1998). Since a biofilter is essentially a soil bed, techniques from the field of soil ecology would seem to be directly applicable to biofiltration research.

Approaches and Difficulties in Microbial Ecology

Mixed microbial communities are complex entities and the choice of approach to adopt in studying them is not clear-cut. One may examine the *structure* of the community – the taxonomic constitution of the species present – or, one may study the *function* of the community – its response to some stimulus. The former corresponds to a reductionist view – that the community may be described by its constituent members – whilst the latter is a holist view regarding the community as a functional team rather than a collection of independent entities. The holist view is taken to an extreme in the book by Sonea and Panisset (1983), who argue that the bacteria may be regarded as a "superorganism" with global reach and that species in the traditional sense have no meaning in the prokaryotic world due to the phenomena of horizontal gene transfer (via plasmids and so on); this is refuted by Young (1994) who argue that, although this transfer is possible, there are practical barriers to the uninhibited exchange of chromosomal DNA which allow genomes to diverge and so distinct species to develop, as evidenced by molecular techniques. Regardless of the outcome of this philosophical debate, it is important to remember that the bacteria maintain a complex interaction both with each other and the environment and that the relationship between structure and function remains unclear.

Both approaches have pitfalls: in studying the structure, it is unclear how much weight should be attached to species richness (total number of species) versus rank abun-

dance (number of each species present) and rank abundance data may be difficult to obtain; it is also unclear how the results should be interpreted – what is the implication of one species disappearing from the community, or the replacement of one species with another? Furthermore, symbiotic effects may be difficult to assess from purely structural information. Attempts to study the function necessarily (at present) involve significant changes in the environment; what is then being studied is not functional diversity but *potential* functional diversity and it is not clear how this relates to the *in-situ* functional diversity of the community. Furthermore, those significant environmental changes constitute a bias in the technique and any functional assay will tend to favour certain types of organism, often culturable ones, and so the response may reflect only the changes in that sub-population.

Both types of studies are further complicated by the presence of such a wide variety of organisms (different assays must be used to investigate changes in, say, the fungal and bacterial populations) and wide number of environmental factors that may influence the population (moisture level, temperature, pH, higher organisms, *etc.*). A further challenge exists in identifying and applying appropriate statistical methods for analysing the very rich data sets that are generated; most authors opt for some sort of multivariate ordination technique but the choice is often subjective.

Objectives for Biofilter Research

As engineers, a pragmatic approach should be adopted and questions of practical interest addressed. The following broad areas are of interest, regardless of whether structural, functional or a combination of approaches is utilised:

- Which methods from soil ecology can be applied to biofilter research?
- Can the microorganisms in a biofilter be characterised as a community on a local scale? Over what scale do changes in those communities occur?
- Does the community tend in some sense, either functional or structural, towards stability, or is it in a constant state of change?
- Does inoculation of the biofilter have any influence on the community? Can improved inoculation strategies be developed that might, for example, promote more rapid acclimation or greater resistance to environmental stresses?
- Can any relationship between the community and biofilter operation be identified? For example, can the ability of the community to withstand environmental stresses without "failure" be assessed? *I.e.*, can some method for assessing the overall "health" of the community be developed?

As shown in the following section, a wide variety of ecological tools have been successfully applied to biofilter research and so so the first question is answered: most

techniques from soil ecology appear to be applicable to biofilter research. Developing these techniques into tools able to answer questions of practical interest in the laboratory and industry remains a significant challenge; indeed, given the number of techniques available even selection of the most appropriate is not easy. The next step, and the focus of most related research to date, is to characterise communities in various biofilters to gather data and gain experience in interpreting the information available; this may lead to a better idea of how microbial communities may be monitored and manipulated, and what constitutes a "healthy" community.

Health of Biofilter Microbial Populations

Clearly it is desirable that the community in a biofilter be healthy; but what exactly is meant by this? The purpose of a biofilter is to remove certain pollutants from the air flowing through it; this suggests that *a healthy community is one that can maintain biofilter operation (i.e., DRE) when exposed to reasonable environmental stresses*. Here "reasonable," refers to environmental changes that might be expected during normal operation (seasonal variations in temperature and rainfall, changes in pH as a result of organic acid formation, fluctuations in pollutant concentration or moisture and nutrient levels, *etc.*) rather than sudden or severe stresses (such as extremes of temperature or excessive drying).

Microbial Ecology Methods Applied to Biofiltration

A number of techniques from microbial ecology have been used to examine biofilter ecosystems (Table 2.4). The techniques are briefly described in Section 2.2.2, biofiltration ecology studies reviewed in Section 2.2.3 and studies of some other, similar systems in Section 2.2.4. A more detailed description of Community-Level Physiological Profiling (CLPP), which is used in this thesis, is presented in Section 2.2.5.

Table 2.4: Techniques in microbial ecology used for biofiltration research

Type	Name	Contributions to biofilter research	References
Direct Observation	Transmission Electron Microscopy (TEM)	Biofilm may be active (alive) only near the surface	Cox *et al.* (1996)
	Scanning Electron Microscopy (SEM)	Biofilm structure irregular and thickness variable	Acuña *et al.* (1999)
Culture-Based	Plate culture	Microbial density varies with depth	*e.g.* Ergas *et al.* (1995); Smith *et al.* (1996); Li *et al.* (2002)
	Community-Level Physiological Profiling (CLPP)	Potential functional diversity varies over time	Grove *et al.* (2004a)
Molecular Methods	DNA/RNA-Based	Community is stratified; diversity varies with time; biofilter may be a *K*-environment; operating units may have reduced diversity; degree of functional redundancy may depend on compound being treated and temperature (thermophilic systems being more similar than mesophilic)	*e.g.* Juteau *et al.* (1999); Kong *et al.* (2001); Li and Moe (2004); Sakano and Kerkhof (1998); Steele *et al.* (2004)
	Phospholipid Fatty Acid (PLFA) / Fatty Acid Methyl Ester (FAME) Analysis	Community is diverse and varies with depth; formation of a stable community may take 10's to 100's of days; viable plate counts may be inaccurate as pH declines; acclimation times depend on inoculum source	Gebert *et al.* (2004); Webster *et al.* (1996); de Castro *et al.* (1997)

2.2.2 Methods in Microbial Ecology

Direct Observation

Direct observation of the biofilm is possible using a microscope. Very briefly, microscopy may be divided into two categories, and each type has various advantages and disadvantages:

Light Microscopy. A traditional brightfield microscope may be used to classify organisms by their morphology (coccus, rod, *etc.*) or Gram-nature and to perform direct cell-counts on detached microbes (note that it is not possible to distinguish between living and dead organisms) (Madigan *et al.*, 2002); however, examination of an *in-situ* biofilm is difficult for practical reasons. Other light-based microscopes may be employed for biofilter research, for example: Hoffman Modulation Contrast Microscopy (HMCM) allowing *in-situ* three-dimensional imaging without artefacts; and Differential Interference Contrast Microscopy (DICM) to give topographical information and (with fluorescent staining) to provide information about the total and active number of cells (Surman *et al.*, 1996).

Electron Microscopy. This provides high-magnification imaging of prepared samples; however, the high level of preparation required may introduce artefacts into the images. There are three types: TEM can provide cross-sectional information about the spatial distribution of organisms; SEM can provide topological information at very high magnification; and Environmental SEM (ESEM), a variant of SEM, which allows direct imaging of hydrated organisms (Surman *et al.*, 1996).

Culture-Based Methods

Culture-based methods include all those involving the cultivation of microorganisms outside the environment under consideration.

Traditional Plating Techniques. In traditional plating techniques, aliquots of suspended samples are incubated on agar plates. The resulting colonies may be removed and transferred to other plates so that pure isolates may be obtained, the so-called streak-plate method (Madigan *et al.*, 2002). Species may then be identified or characterised by a variety of techniques (for example, Gram-staining, observing morphology, *etc.*). A measure of the community density may be obtained using a dilution series to estimate the number of Colony Forming Units (CFUs). This technique suffers from the serious limitations that it is laborious and biased in that many, and perhaps most, species are not culturable under these conditions (Amman *et al.*, 1995).

Community-Level Physiological Profiling (CLPP). CLPP using Carbon Source Utilisation Profile (CSUP) is an adaptation of a technique developed for the identification of pure bacterial cultures (Konopka *et al.*, 1998). Communities are assayed by assessing their ability to degrade various sole carbon sources as a measure of potential functional diversity; further details are found in Section 2.2.5 (Page 35).

Molecular Methods

Many methods have been developed recently to monitor the microbial population using molecular tools. These have the advantage of removing the culture bias inherent in the methods described previously although this may be at higher expense in equipment and expertise and biases also exist in molecular techniques, such as incomplete extraction from environmental samples and preferential amplification of DNA from certain species (Muyzer and Ramsing, 1995).

Polymerase Chain Reaction (PCR) Based Techniques. Environmental DNA or RNA may be extracted from the packing material and a portion of the genome amplified using PCR. Briefly, two oligonucleotide primers (oligos) are added in excess to the extracted DNA; these primers, typically around 20 base pairs (bps) match highly-conserved regions on the DNA (for example, and often, the gene encoding bacterial 16s Ribosomal RNA (rRNA)). A heat-resistant DNA-polymerase enzyme (isolated from the thermophilic bacteria *Thermus aquaticus* and known as Taq polymerase) and bases (dNTPs) are also added. The mixture is heated, melting the DNA into two strands, and then allowed to cool causing the strands to anneal; the excess of primers ensures that the majority of strands will anneal to a primer and not each other. The DNA-polymerase extends the primers, using the DNA strand as a template. The denaturation-annealing-extension cycle takes a few minutes and can be automated using a Programmable Thermal Controller (PTC); this allows many copies of the target region to be made (Madigan *et al.*, 2002).

A number of PCR-based applications exist to assess the microbial community:

- **Denaturing Gradient Gel Electrophoresis (DGGE)**. Different species are separated by electrophoresis of the PCR products using a gel with a denaturing gradient; the different sequences in the species cause the denaturation to be complete at different points on the gel. Each "band" formed on the gel is therefore indicative of a different species; further taxonomic information can be obtained by analysis of these bands using targeted oligonucleotide probes (Muyzer and Ramsing, 1995);

- **terminal-Restriction Fragment Length Polymorphism (t-RFLP)**. Either or both of the primers used in the PCR is labelled with a fluorescent marker on one end. The primers are typically targeted at a gene (in bacteria, often the 16s DNA encoding for rRNA (rDNA) which has a highly-conserved length in the range 500 to 2000 bp). A digestion enzyme is added to the PCR product, which cleaves the DNA in response to a specific sequence of a few bases; different species, or groups of species, have this sequence at different points in the amplified portion DNA and so the terminal-Restriction Fragments (t-RFs), which contain the fluorescent labels, will be of differing lengths. The lengths of the t-RFs can be determining using capillary electrophoresis on an automated DNA sequencer. The resulting fingerprint provides a measure of the phylogenetic diversity in the sample and can be used to estimate species richness (Liu *et al.*, 1997);

- **Automated rRNA Intergenic Spacer Analysis (ARISA)**. PCR is performed to amplify the spacer region between two genes (typically 16s and 23s for bacteria). One of the primers is fluorescently labelled and, because the intergenic spacer region varies in size between species, the resulting fragments are of different sizes. This is similar to t-RFLP but appears to provide a better resolution, apparently distinguishing at the species level (Fisher and Triplett, 1999);

- **Gene Sequencing**. Copies of a target gene (in bacteria, often the 16s small-subunit rRNA) are amplified using PCR and the nucleotide sequence determined using an automated sequencer. The sequences obtained are compared to a database, allowing identification of the species present (Snyder and Champness, 2003).

- **Fluorescent *in-Situ* Hybridisation (FISH)**. A library of single-stranded, fluorescently-labelled DNA probes is constructed. The environmental sample is treated *in-situ* to melt the DNA and incubated with the probe library to allow hybridisation of matching sequences. Unhybridised DNA is removed by washing and the location of the hybridised probes can then be determined with a fluorescent microscope; because it is carried out *in-situ*, this technique allows the localisation of target species or genes (Karp, 1999).

- **Single-Strand Conformation Polymorphism (SSCP)** A variable region of a gene (again, typically 16s small-subunit rDNA for bacteria) is amplified using PCR. The conformation (shape) of single-stranded DNA is influenced by the sequence; this influences its mobility in non-denaturing electrophoresis gels. Electrophoresis of the PCR product is performed and the differences in mobility due to conformation allow DNA fragments with different sequences to be separated, even if they are of the same size (Orita *et al.*, 1989; Lee *et al.*, 1996).

- **Real-Time PCR (RT-PCR)**. Reverse transcriptase is used to produce a Complementary DNA (DNA synthesised from mRNA) (cDNA) strand from Messenger RNA (mRNA). PCR then proceeds based on a region of the cDNA strand in the range 100 to 400 bp. Fluorescence Resonance Energy Transfer (FRET) is used to observe the initial, exponential, phase of the PCR process allowing quantification of the initial mRNA present[d]. RT-PCR provides information on both the diversity and rank abundance of species; furthermore, the presence of mRNA indicates that the species observed are active and not dormant. The technique requires more costly equipment that traditional PCR and requires careful handling of samples due to the widespread occurrence of RNAase enzymes.

[d] PCR proceeds in three phases: exponential growth, linear growth and a plateau. There is high variability in the linear phase. Traditional PCR applications record data after the plateau and so cannot be used for quantification; RT-PCR records data during the exponential phase and provides quantitative information.

Other Molecular Methods.

Phospholipid Fatty Acid (PLFA) Analysis. PLFAs are found in the membranes of microorganisms. Extraction and analysis provides a fingerprint of the total microbial community present, and a change in that fingerprint indicates a change in the community although individual species may not be resolved. Certain communities express signature lipids, which may be used as indicators of their presences, and environmental stresses may induce changes in the ratios of lipid types (for example, cis- to trans-monoenoic unsaturated fatty acids or saturated to unsaturated lipids) (Zelles, 1999).

2.2.3 Studies of Biofilter Ecology

Overview

Species. The microorganisms in a biofilter may include bacteria, fungi, actinomycetes and yeasts (Leson and Winer, 1991); bacteria and fungi predominate and, under favourable conditions, the bacteria are able to uptake nutrients and grow more rapidly and so will represent the majority of the biomass (Devinny *et al.*, 1998). Fungi are able to degrade a wider variety of compounds and tolerate more extreme conditions – for example a pH as low as 2.5 (van Groenestijn and Hesselink, 1993; Veiga *et al.*, 1999) or temperatures between 60 and 71 °C (de Castro *et al.*, 1997). Predators are also common, including protozoa (de Castro *et al.*, 1997; Cox *et al.*, 1999; Cox and Deshusses, 1999) and viruses (Steele *et al.*, 2004). The system is highly heterogeneous and may take a long time to reach steady-state – Webster *et al.* (1996) found that it took 500 days for microbial densities, measured by plate counts, to reach stable values; this was perhaps due to low VOC loading and slowly declining pH.

Biofilms. The various species coexist within a biofilm on the surface of the packing material. There are few studies dealing with biofilm architecture specifically in biofilters, due to the difficulty in examining a very thin biolayer on an ill-defined and heterogeneous support (Deshusses, 1997). Even where biofilm monitoring is possible, it may be difficult to interpret the results due to the lack of robust mathematical models able to accept the information (Lewandowski and Beyanal, 2003). Biofilms in general may be characterised as a microbial aggregate formed at a phase boundary; the microorganisms exist in a matrix of Extracellular Polymeric Substances (or Extracellular Polysaccharides) (EPS) that serve various functions including adhesion to the surface, protection from environmental fluctuations and acting a matrix through which interaction may occur (Wimpenny, 2000). Picioreanu *et al.* (2000) note four types of heterogeneity that may be of interest: geometrical (*e.g.* thickness and porosity); chemical (*e.g.* substrate concentrations and pH); biological (*e.g.* species diversity and activity – growth, death, *etc.*); and, physical (*e.g.* permeability, EPS properties). Certainly the biofilm provides significant levels of protection from environmental stresses (Allison *et al.*, 2000b) but our understanding of its structure, interactions and dynamics is still developing (Costerton, 2000).

Inoculation. The packing material may be inoculated with a mixed microbial community to help during start-up (Leson and Smith, 1997); often, activated sludge from wastewater treatment plants, which contains a wide variety of organisms, is used (Malhautier *et al.*, 2005). For example, Chan and Grennberg (2001) prepared cultures from three mixed-culture environments to try and prepare an inoculum suitable for a biofilter degrading air-phase rape-seed (canola) oil through acclimation in shake-flasks over 77 days; they found all three could successfully degrade the oil as a sole-carbon source and characterised the predominant bacteria as mesophilic, aerobic, Gram-negative rods. Massol-Deyá *et al.* (1997) note that inoculating a microbial habitat with a strain or strains to encourage a particular process has typically been found to be difficult; in a study of a fixed-film bioreactor degrading BTX-contaminated groundwater, they found the laboratory-prepared inocula to be poor competitors compared with the native microflora.

Higher Organisms. The presence of higher organisms in biofilters may be deliberate or accidental. Protozoa have been observed (de Castro *et al.*, 1997) and have been deliberately introduced to control biofilm growth (Cox *et al.*, 1999; Cox and Deshusses, 1999). Insect larvae may be present and sometimes hatch into flying insects within closed units; Elsie (1999) reported pomace flies (*Diptera drosophilidae*) in closed biofilters and Devinny *et al.* (1998) report one instance of a spider colony establishing to feed on the insects. The presence of these organisms does not appear to adversely affect performance and their removal of some biomass may in fact be beneficial in terms of reduced clogging; this has been exploited by introducing mites into fungal biofilters treating toluene (Woertz *et al.*, 2002a,b).

Emission and capture of microorganisms. Ottengraf *et al.* (1991) found concentrations of microorganisms, principally bacteria with some moulds, in the exhaust gas from a full-scale biofilter installation to be in the range 10^3 to 10^4 CFUs m^{-3}; this is similar to the values typically found in indoor air. The authors used a bench-scale system and found that the concentration varies with gas flow rate and also that if the inlet gas is highly loaded then the biofilter also functions as a capture system. Sanchez-Monedero *et al.* (2003) investigated the use of a biofilter, originally installed for odour control, as such a capture system for fungal spores and bacteria; they found over 90 % of spores and 39 % of bacteria were captured in each of seven biofilters surveyed at different composting facilities. Martens *et al.* (2001) investigated the effects of different filter media and found reductions in viable cell count of between 70 and 95 %, total bacteria count of 25 to 90 % and fungal count of 60 % in all five media-types tested; they note that the biofilters most effective at reducing odour (VOCs from a pig farm) emitted slightly more bacteria.

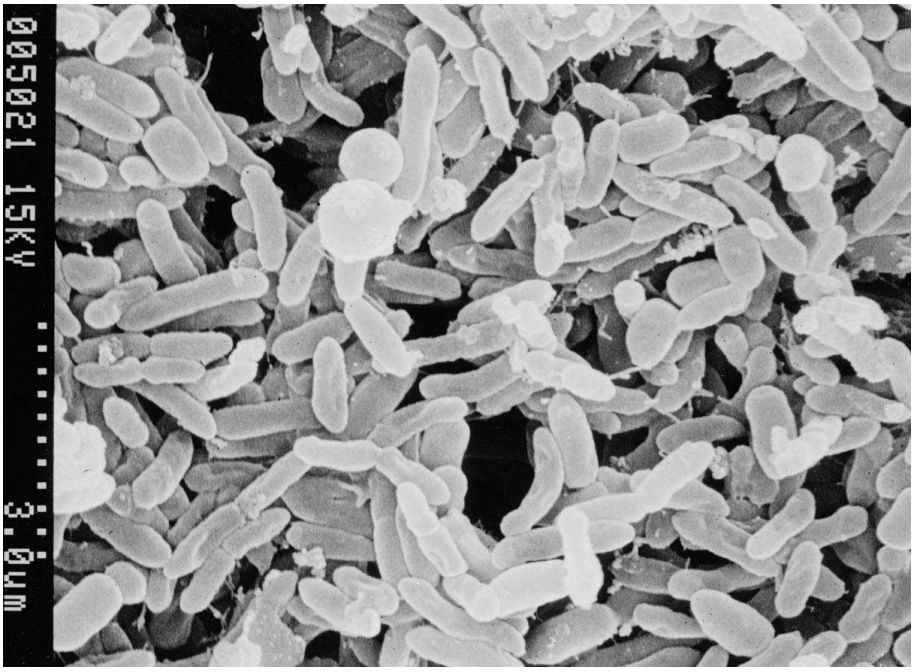
Direct Observation

Drawing conclusions from direct observation of biofilters is difficult because the heterogeneities in packing, microbial distribution, VOC concentration, *etc.* are on a very much

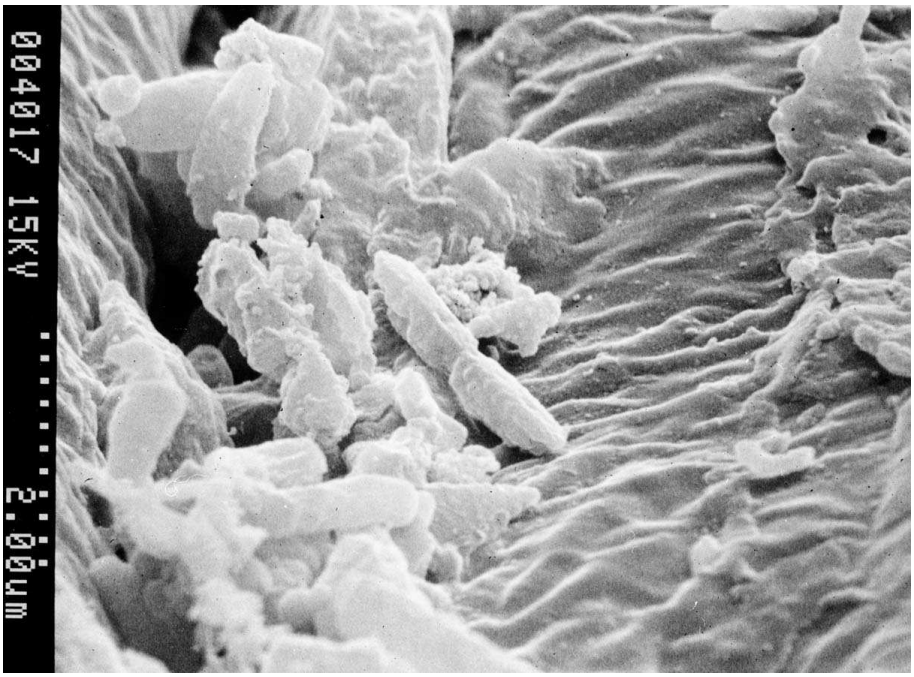
larger scale than the observations themselves. Nevertheless, observations are possible and provide some general information (Devinny *et al.*, 1998).

Light microscopy. de Castro *et al.* (1997) found active bacteria and protozoa in three biofilters treating α-pinene; a comparison of the units did not indicate differences. Protozoa sizes ranged from 5 to 50 µm but after repacking of the units only small (< 10 µm) protozoa were observed.

Electron microscopy. The electron microscope provides much greater magnification and may be suitable, for example, for investigating the nature of the biofilm (Devinny *et al.*, 1998) although the problems of scale are even more apparent. Cox *et al.* (1996) used TEM to show that the active biomass may be concentrated near the surface of the biolayer, with deeper areas having dead cells. Acuña *et al.* (1999) used SEM to observe a biofilm irregular in structure and varied in thickness and later (Acuña *et al.*, 2002) to see higher cell densities, salt crystals and extracellular polymer formation in a peat biofilter with high nutrient supplementation, and scarce colonisation and changes in cell morphology for one with low nutrient supplementation. Examples of scanning electron micrographs taken of a compost biofilter are shown in Figure 2.4.



(a)



(b)

Figure 2.4: Scanning electron micrographs from a compost biofilter degrading ethanol and illustrating the heterogeneous nature of the biofilm. Note the different scales. Images are previously unpublished and were prepared by H. Kautola and S. Javadpour.

Culture Based Methods

Traditional Plating Techniques. Various authors have applied plate-counting techniques to biofilters; some results are summarised in Table 2.5. As shown, a considerable range of values is obtained. Some authors (Li *et al.*, 2002; Smith *et al.*, 1996) reported results reducing with distance from the inlet (and so changing VOC concentration). Krailas *et al.* (2000) found a relationship between bacterial count and methanol loading: an initial increase is followed by a plateau and a decrease. Medina *et al.* (1995) monitored counts over time in a Granular Activated Carbon (GAC) column loaded with gasoline vapours; fungal numbers increased rapidly to a plateau near the inlet but more slowly and only after a delay (around 50 days) near the outlet whilst bacterial counts were initially stable for the same period before increasing rapidly and slowly near the inlet and outlet, respectively. Sun *et al.* (2002) investigated the impact of different moisture content (between 30 and 70 %) in the media and found increasing moisture content favoured bacterial growth, with more moulds and actinomycetes found at lower values; 50 % moisture content was most favourable for yeast growth.

Kibazohi (2000) isolated organisms from a Hexane-degrading biofilter and identified them using the Biolog system (with Gram Negative (GN) or Gram Positive (GP) plates as appropriate) for bacteria and physiological characteristics for fungi. Of the species isolated (see Table 2.6), the *Pseudomonad* species were the most abundant and only *P. corrugata* was able to utilise hexane as a sole-carbon-source when grown in suspended culture. Conde *et al.* (2001) found variations between two units degrading paint thinner exposed to simulated summer and winter temperatures; they report differences in the plate counts between the units and over the 30 day duration of the experiment. They isolated four *Bacillus*, one *Micrococcus* and one *Pseudomonas* species. Alba *et al.* (2003) identified *Bacillus*, *Micrococcus* and *Pseudomonas* species and the fungus *Scopulariopsis brevicaulis* in similar units operating with sterile feed conditions.

Bendinger *et al.* (1992) isolated and characterised coryneform bacteria from a biofilter treating animal-rendering plant emissions, identifying strains with novel characteristics. Veiga *et al.* (1999) isolated five dominant microbial genera in biofilters degrading alkylbenzene vapours at pHs between 3.5 and 7 – two dominant bacteria (*Bacillus* and *Pseudomonas*), a *Trichosporon* and two unidentified.

Community-Level Physiological Profiling (CLPP). The applicability of CLPP with CSUP to a biofilter has been demonstrated in a previous study showing a shift in community structure following a change in the VOC (Grove *et al.*, 2004a).

Molecular Methods

Polymerase Chain Reaction (PCR) Based Techniques.

- **DGGE.** Sercu *et al.* (2003) used DGGE with 16s rRNA to show differences in the community structure between the inlet and outlet of a biofilter treating hydrogen

Table 2.5: Viable plate count results from published studies

Organism	Support Type	CFUs g^{-1} support	Reference
Bacteria	Compost	10^6 to 10^8	Ergas *et al.* (1995)
Bacteria	Compost	10^8 to 10^{10}	Elsie (1999)
Bacteria	Peat	10^9 to 10^{13}	Kiared *et al.* (1996, 1997)
Bacteria	Compost and pall rings	10^7 to 10^8	Krailas *et al.* (2000)
Bacteria Fungi	Peat, pearlite or mixture	10^{12} to 10^{13} 10^9 to 10^{10}	Kibazohi (2000)
Bacteria Yeasts Moulds Actinomycetes	Compost and pearlite	10^8 10^3 to 10^5 10^5 10^5 to 10^6	Sun *et al.* (2002)
Bacteria Toluene Degraders	Inert packing	10^8 to 10^9 10^7 to 10^8	Smith *et al.* (1996)
Bacteria Fungi	GAC	10^7 to 10^{10} 10^3 to 10^6	Medina *et al.* (1995)
Bacilli Spore Bacilli Fungi	Cylindrical activated carbon	10^4 to 10^5 10^5 to 10^6 10^5 to 10^6	Li *et al.* (2002)
Bacteria Moulds Yeast	Rice husks	10^8 to 10^{10} 10^9 to 10^{10} 10^8 to 10^{11}	Conde *et al.* (2001)

Table 2.6: Genera of microorganisms isolated from a biofilter degrading hexane (Kibazohi, 2000)

Bacteria	Yeasts & Fungi
Acinetobacter	*Aspergillus*
Bacillus	*Candida*
Clostridium	*Fusarium*
Corynebacterium	*Mucor*
Micrococcus	*Penicillium*
Pseudomonas	*Phichia*
Rhodococcus	*Polypæcium*
Staphylococcus	*Tricoderma*
	Rhodotorula

sulphide; they identify a sub-group (ammonia-oxidising bacteria) and suggest that this group, although not directly metabolising the pollutant, can act as an indicator for optimal environmental parameters in the biofilter. Sercu *et al.* (2004) investigated community dynamics using the same technique in a two-stage biotrickling filter removing dimethyl sulphide and hydrogen sulphide; they found differences in the planktonic community when compared with the biofilm, the first biofilter had a stable community from the start whilst the second took a period of 60 days to reach stability.

Li and Moe (2004) found stratification of the microbial population in two biofilters degrading Methyl Ethyl Ketone (MEK) under different conditions; differences were also observed between the units. Bruns *et al.* (2001) found strains from the β-*Proteobacteria* to be dominant using DGGE, Thermal Gradient Gel Electrophoresis (TGGE) and an intergenic spacer region analysis; they found the profile developed with the latter method to yield the most information.

Kong *et al.* (2001) examined mesophilic and thermophilic units operating in parallel treating either methanol or α-pinene; they found greater similarity between the high-temperature communities than the low-temperature ones and a greater functional redundancy in the mesophilic methanol-degrading community than the mesophilic α-pinene community.

- **t-RFLP**. Grove *et al.* (2004b) investigated the variation with height in a biofilter treating ethanol; experimental difficulties with PCR, possibly due to enzyme inhibition by humic content of the packing, led to an incomplete data set but results indicated the establishment of at least one species due to inoculation with a mixed community.

- **ARISA**. Steele *et al.* (2004) tracked the bacterial community in two biofilters degrading ethanol over a 68 day inoculum acclimation period and 29 days of biofilter operation. They found decreasing diversity over the acclimation period. In an analysis of operational and failed biofilters, they found greater diversity in the failed units; this suggests that biofilter failure in this case was not due to microbial death but to restructuring of the community.

- **Gene sequencing**. Sakano and Kerkhof (1998) sequenced 16s rRNA and the ammonia monooxygenase gene (*amoA*) in an ammonia biofilter; they found a 38 % decrease in heterotroph diversity over 102 days with the predominant members initially being from both the β- and γ- subdivisions of the *Proteobacteria* and at the end from only the γ- subdivision.

  Ahrens *et al.* (1997) tested isolates from several biofilters treating emissions from animal-rendering plants using partial 16s rRNA sequencing and fatty acid analyses; they classified 70 Gram-negative, non-fermenting isolates as belonging to the α- and β- subdivisions of the *Proteobacteria*.

  Juteau *et al.* (1999) identified eleven genera from a toluene-degrading biofilter; two groups, *Pseudonocardia* and *Rhodococcus*, were 34 times more numerous and the authors suggest that the biofilter is a *K*-environment – with crowding and restricted nutrients – and so these *K*-strategists out-compete faster growing *r*-strategists such as *Pseudomonas* and *Acinetobacter* which are adapted to uncrowded, nutrient-rich *r*-environments[e].

- **FISH**. Friedrich *et al.* (2002, 2003) demonstrated that the community diversity in a biofilter is high and investigated the vertical profile in a full-scale biofilter; they report the bottom 50 cm to be particularly active in treating aldehydes. The authors could find only weak and non-significant correlations between waste-gas and microbial variables; this is consistent with the high level of diversity demonstrated and suggests it may be difficult to correlate biofilter performance (DRE) with the microbial structure.

- **SSCP**. Khammar *et al.* (2005) used SSCP analysis with plate-counting techniques to show differences in diversity and community density in two biofilters treated with different inocula degrading a mixture of VOCs. The authors report significant variations in both diversity and density with depth, rapid changes during the first fortnight of operation and a generally high diversity.

[e] A crowded environment may favour so-called *K*-strategists while an uncrowded one favour *r*-strategists; these species may have different characteristics such as high affinity for nutrients *vs.* high rate of acquisition, specialisation *vs.* generalisation and so on. (Andrews and Harris, 1986)

Other Molecular Methods.

PLFA Analysis. Gebert *et al.* (2004) used PLFA to compare two biofilters oxidising methane and other trace VOCs, showing that community structure varied with depth and that both biofilters were dominated by type II methanotrophs – a highly specific population attributed to the continuous trace methane exposure; a previous study (Gebert *et al.*, 2003) found methane degradation rates to be well correlated with methanotrophic cell count. Lipski and Altendorf (1997) used fatty acid analyses to identify 62 bacterial strains, mostly from the *Proteobacteria*, in a compost biofilter degrading a complex mixture of VOCs; these were mostly similar to 31 isolates previously characterised (Lipski *et al.*, 1992) with two groups missing – the authors speculate that this is due to a more restricted mixture of VOCs.

Webster *et al.* (1996) showed that environmental stresses (*e.g.* from starvation or toxic compounds) were generally low but increased over time in five biofilters degrading hydrogen sulphide and a mixture of VOCs; they attribute this to declining pH levels. The authors found it took hundreds of days to find a stable community, which was dominated by Gram-negative species. They also found viable plate counts were initially comparable with PLFA data but became unreliable as the pH declined; they report values consistent with those in Table 2.5: between 10^6 and 10^8 CFUs g^{-1} based on viable plate count and 10^7 and 10^{10} CFUs g^{-1} based on PLFA.

2.2.4 Studies of Other Systems

There are a number of other applications for removal of contaminants by biodegradation with a mixed community. Victorio *et al.* (1996) used CLPP with Biolog plates to demonstrate differences in the heterotrophic community in wastewater treatment systems and suggest the system might find application in assessing the ability of a community to degrade specific substrates by testing those substrates on the microplate. Kaewpipat and Grady Jr. (2002) used DGGE to investigate the behaviour of replicate laboratory activated sludge systems; they found divergence in one set of parallel runs but not another and suggest the divergence may be due to the degree of perturbation experienced and note that the diverged communities displayed similar overall performances. Zumstein *et al.* (2000) used SSCP to monitor the bacteria and archaea in an anaerobic digester over two years; they found the bacterial community was subject to fast and significant shifts throughout the study, while the archaeal community was more stable (again, overall system performance was maintained throughout the shifts).

In investigations of methanogenic bioreactors with Amplified rDNA Restriction Analysis (ARDRA), Fernández *et al.* (1999) found greater variations in the bacterial than archaeal communities and observed two phases distinguished by different dominant methanotrophs over 605 days of operation; throughout these changes the performance was stable. Subsequently, Fernández *et al.* (2000) identified changes in reactors after perturbation with a glucose spike with an increase in diversity following the perturbation and

illustrating the importance of undetectable members of the community; two initial classes of community were identified (based on the dominant morphotypes) and the degree of perturbation varied between the classes, although both types of community returned to the state before perturbation; in a companion paper, Hashsham *et al.* (2000) showed communities with parallel substrate processing to be more stable in response to a perturbation than those with serial processing.

Kooi *et al.* (1997) modelled a tri-trophic food chain, consisting of substrate, bacteria (prey), ciliates (predator) and carnivore (top predator), in chemostatic operation based on energy-budget considerations for individuals; they suggest that the behaviour of the system is chaotic with the top-predator/predator population following a slowly oscillating path and the predator/prey population a fast one.

2.2.5 Community-Level Physiological Profiling (CLPP)

History and Applications

Biolog plates were initially developed to allow the identification of pure cultures, initially Gram-negative species of clinical interest (GN plates) and subsequently GP plates were developed to help distinguish Gram-positive species (Konopka *et al.*, 1998). The 96-well plates are inoculated with a suspension of cells. Each well contains a tetrazolium dye, nutrients and a carbon source; the colourless tetrazolium dye reduces to a coloured formazan in response to respiration. Identification is possible based on the pattern of response or no-response. The 95 substrates (one is a water blank) on the GN plates were selected from a survey of 500 substrates using 6000 bacterial strains (Bochner, 1989).

CLPP of a microbial consortium based on CSUPs was first demonstrated by Garland and Mills (1991) and there have been a number of subsequent studies to characterise different environmental communities (Konopka *et al.*, 1998). In the technique, a microtitre plate with multiple sole-carbon-sources is inoculated with a mixed microbial sample. Biolog EcoPlates™, containing three replicates of 31 carbon sources (plus control), were produced for ecological studies as a means of providing convenient replication of data and with the carbon sources selected to allow greater discrimination between communities (Choi and Dobbs, 1999; Insam, 1997; Preston-Mafham *et al.*, 2002). A reduced number of substrates appears sufficient for distinguishing between environmental samples (Haack *et al.*, 1995) and is advantageous in reducing the number of replications required for statistically meaningful analysis (Insam, 1997). The reduced set available commercially on Biolog EcoPlates™ (listed in Appendix B) was selected by Insam (1997) based on studies using the 95 substrates on the GN plate by Hitzl *et al.* (1997) and another 125 substrates not featured on the GN plate by Campbell *et al.* (1997).

Microbial respiration is assessed by the colour response of a tetrazolium salt present in each well. As the carbon substrate is oxidised, it provides electrons to form NADH within the cell; these flow through the organism's electron transport mechanism with the colourless/faint yellow soluble salt acting as an artificial final electron acceptor. The re-

duced product is an insoluble, purple formazan and the reaction is therefore essentially irreversible; thus, the amount of colour development in each well, assessed spectrophotometrically, may be used as an indicator of the utilisation of that carbon source (Bochner and Savageau, 1977; Bochner, 1989; Seidler, 1991). These CLPPs may be used to characterise the functional diversity of microbial communities using multivariate statistical analysis (Preston-Mafham *et al.*, 2002).

Fungal functional diversity is not assessed using this system, since some fungi cannot reduce the tetrazolium indicator dye (Dobranic and Zak, 1999); Biolog FF, for use with fungi, do not contain the indicator, are assessed turbidimetrically and would provide a means to assess variation in the fungal community (Garland, 1996a) and Dobranic and Zak (1999) found a different tetrazolium dye to be a suitable alternative. The use of MT plates, in which the carbon substrate is selected and added by the experimenter, would allow the use of single or mixed sources tailored to a particular system and might give greater resolving power if some basis for selecting those carbon sources can be found (Preston-Mafham *et al.*, 2002). Although this may simplify the analysis, this approach should be treated with caution because an assumption is made that differences in the community in future experiments will be similar to that in previous ones; the retention of apparently redundant C-sources would allow this assumption to be checked.

The technique has been applied in a variety of situations; Konopka *et al.* (1998) note that many of the studies are distinguishing between very different ecological environments but that some studies have found differences between very similar environments, (for example, communities in different drinking water filters (Moll and Summers, 1999) and rhizosphere communities from different species of plants (Garland, 1996b)); some examples of studies using the technique are given in Table 2.7.

Table 2.7: Examples of ecological studies using CLPP with Biolog plates

Environment	Plate Type	Statistical Method	Findings	Reference
Agricultural soil	GN	Principal Component Analysis (PCA)	Showed differences between different soils; no difference with reading time of plates is not provided it is not very early (before 10 h) or late (after 72 h)	Glimm *et al.* (1997)
Agricultural soil	GN	Canonical Correspondence Analysis (CCA)	Communities similar on different dates, differences seen with moisture content (winter flooding) and environmental carbon (straw incorporation)	Bossio and Scow (1995)
Cultured mixed communities	GN	Cluster Analysis (CA), PCA	Used area under the curve to differentiate between three cultured communities; colour development curve is sigmoidal	Hackett and Griffiths (1997)
Desert plant communities	GN GP	CA, PCA	Detected differences between six plant communities and with moisture gradient	Zak *et al.* (1994)
Drinking water biofilter	GN	Counting C-sources used	Able to distinguish communities based on ozonation, contact time, pH and temperature	Moll and Summers (1999)
Plant rhizosphere communities	GN	Detrended Correspondence Analysis (DCA)	Distinguished between four different plant communities over two years.	Garland (1996b)

Continued on Next Page...

Table 2.7: Examples of ecological studies using CLPP with Biolog plates, cont'd

Environment	Plate Type	Stat. Method	Findings	Reference
Semi-arid soils	GN2 GP2 ECO SFN2 SFP2	Non-metric multidimensional scaling	Differences between soils are found; assay is not sensitive to differences in incubation temperature; all bacterial plates (GN2, GP2, ECO) distinguish between soils; also showed differences between fungal communities (SFN2 and SFP2 plates)	Classen *et al.* (2003)
Wastewater	GN GP MT	PCA	Used area under curve in place of a single reading time; found GN plates more suitable that GP; MT plates (no carbon source) suggest carry-over of carbon is not a problem but evaporation from peripheral wells may be of concern	Guckert *et al.* (1996)

Use and Reproducibility

The results from CLPP with Biolog plates has been found to be highly reproducible (Garland and Mills, 1991, 1994; Gomez *et al.*, 2004; Haack *et al.*, 1995; Bossio and Scow, 1995; Classen *et al.*, 2003). Studies have found that the results are not significantly affected by the time chosen for analysis (Glimm *et al.*, 1997) or the incubation temperature of the plates (Classen *et al.*, 2003). Balser *et al.* (2002) concluded that most of the methodological variability is found at the level of soil sample replication; replicate plates accounting for relatively little of the variation and being of less importance in studies. The authors also found that studies that allow the soil to settle before plating may underrepresent the Substrate Utilisation Profile (SUP), since only species easily detached from the solid particles are represented. The "lag time" between inoculation and response seems to be mainly a function of cell density in the inoculum and efforts must be made to standardise this in any study (Garland and Mills, 1991; Haack *et al.*, 1995).

Advantages and Disadvantages

The technique has advantages over traditional cell culture techniques, which are time-consuming and biased due to the small number of species that may be cultured; and over molecular-level techniques such as RNA amplification, which may be time-consuming and require significant expertise (Garland, 1997). The Biolog plate technique is relatively easy, rapid and inexpensive; however, it is still unclear to what extent it is dissimilar to traditional culture techniques (Degens and Harris, 1997). Konopka *et al.* (1998) notes that the technique is culture-based since the inoculation cell density is low and enrichment biases may make the results unrepresentative; furthermore, because the assay substrates are not found in the environment, it is not clear how fundamental information about ecological differences can be ascertained from the results. Widmer *et al.* (2001) compared CLPP with DNA and PLFA analyses and found all three techniques produced reproducible results and were able to distinguish between three different soil types; the authors warn that characterisation by one single method should be approached with caution in particular if the relative degree of similarity between different communities is to be ascertained. O'Connell and Garland (2002) found differences in response between GN plates and the same carbon sources in a differently formulated GN2 plate; they note that CLPP is not limited to use with Biolog plates and suggest that different microplates might be developed for ecological work.

Statistical Methods

A variety of techniques for analysing the data have been employed by different researchers. Some authors describe the results from each plate using one or two measures; for example, Tam *et al.* (2001) use the Shannon-Weaver measures of diversity and evenness of response and Zak *et al.* (1994) use similar indices (both studies subsequently

Table 2.8: Multivariate statistical techniques applied to CLPP data. More detailed descriptions of the analysis may be found in ter Braak (1995).

Method	Description	Example study
Detrended Correspondence Analysis (DCA)	Theoretical axes that best separate (create the greatest distance between) groups are constructed.	Garland (1996b)
Canonical Correspondence Analysis (CCA)	Axes that best separate the groups (as in DCA) are constructed but these are correlated with measured environmental variables.	Bossio and Scow (1995)
Principal Component Analysis (PCA)	A few Principal Components (PCs) in the direction of maximum variance are extracted from the data.	Glimm *et al.* (1997)
Most Probable Number (MPN)	The MPN – the initial cell density required for growth in each well – is estimated and PCA performed on the resulting data.	Gamo and Shoji (1999)

apply multivariate techniques). These techniques have the advantage that simple univariate techniques may be applied to them, but the major disadvantages that much of the information in the plate is lost and different SUPs may generate the same values. For this reason, most authors adopt some form of multivariate ordination technique; techniques that have been used are summarised in Table 2.8.

Multivariate Statistics

Univariate statistics allows rigourous hypothesis testing at a given confidence level on differences in, for example, the means and variances of variables. In considering ecological data, univariate statistics are generally unsuitable because of the large number of variables involved; two considerations influence this unsuitability: firstly, the variables tend to be highly correlated and, secondly, there are difficulties in performing multiple t- or F-tests at an appropriate significance (α-) level because the overall, or family, α-level (*i.e.* the probability of making at least one Type I error over *all* hypothesis tests performed) is significantly higher than the nominal α-level of each test.

To overcome this difficulty, most ecological researchers turn to ordination methods; that is, methods that arrange data along ordinates such that data that are close together are somehow ecologically similar and data that are far apart are somehow different; ter Braak (1995) provides an overview of multivariate methods as applied in ecology. In this

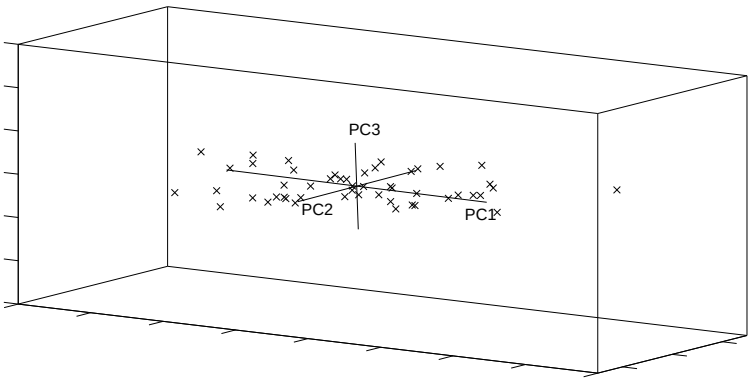


Figure 2.5: Example illustrating PCA. Constructed data set with PC axes superimposed; successive axes account for less and less of the total variance in $\mathbf{X}$.

work, in common with a number of other published works using CLPP based on CSUPs with Biolog plates (Glimm *et al.*, 1997), PCA is adopted as a method of ordination. Exact statistics (in terms of the α- level) can be developed for hypothesis testing based on the extracted PCs even when the number of observations is small compared with the dimensionality of the data (Läuter *et al.*, 1996; Läuter, 1996). Mathematical details of the statistical methods used are given in Appendix A; the following sections describe their use and interpretation.

PCA – Use and Interpretation

PCA is a technique to reduce multi-dimensional data into a limited number of PCs – linear combinations of the original variables created such that the first PC accounts for the maximum possible amount of variance, the next PC the next most, and so on. This is illustrated in Figure 2.5. Thus, the first few PCs are usually able to describe most of the variation in the data. Analysis is usually performed on the scale-invariant correlation matrix (all the variables are first normalised to have the same variance) but where all the scales are the same, as is the case with CLPP data, the scale may contain information so the covariance matrix is employed. PCA has the advantage that it does not require *a priori* assumptions about the grouping of observations – the technique is entirely data based.

Selection of the Numbers of PCs to retain

A method must be found to decide how many PCs should be retained. It is convenient to retain two or three for graphing; however, the number to retain should be made on some defined criteria and not for convenience. Various techniques are available and include selecting (Joliffe, 2002):

- The minimum number of PCs needed to account for a pre-selected proportion of

the variance;

- Only those PCs with a variance greater than some threshold, usually close to unity (Kaiser's Rule) [f];
- The most significant PCs from a scree plot, illustrated in Figure 2.6, or log-eigenvalue plot;
- PCs with unequal eigenvalues; and,
- As many PCs, calculated based on a prototype set, as are able to improve the estimation of observations in a prediction set (cross-validation).

The first three methods are highly subjective. The fourth, testing for unequal eigenvalues appears to have the advantage of using formal hypothesis tests however the significance level of those tests is extremely hard to estimate and, in practice, the method normally results in the retention of more PCs than is necessary. The final method is powerful when sufficient observations are available to form of prototype and prediction sets; in practice, there is also a degree of subjectivity in the method due to the need to establish a cut-off value for the improvement in prediction generated by a PC for it to be retained.

PCA Interpretation. The main way to interpret PC data is via *loading* and *score* plots. Each PC is a linear combination of the original x-variables and the contribution of each variable to a PC is referred to as the loading. The score values are obtained by projecting an observation into the PC-space; *i.e.* a score (t) for each observation is calculated by taking the the x-values and the applying the weights for the appropriate PC (a vector of t-scores, $\mathbf{t}$, corresponding to each successive PC can be made for each observation). If the data can be separated by a factor model, this will be reflected in one or more of the PC scores; these can be plotted either individually or against one another in scatter plots and grouping of observations assessed visually. The loading indicates the weight of each x-variable on that factor; analogous plots of loadings may be used to identify the most important variables. It is sometimes possible to assign an interpretation to a PC based on some other available information about the observations; such interpretations must be treated with caution, particularly if the presence of hidden independent variables is suspected.

PCA Diagnostics. Two diagnostics may be used to identify outliers. The first, Hotelling's T^2, is a multivariate generalisation of "Student's" t-test and identifies outliers that have the same underlying factor structure as the data (*i.e.*, outliers that lie on the PC plane or hyperplane but are far from the origin) (Hotelling, 1931). Due to the data-based nature of the model, a strong outlier may cause one or more of the PCs to turn towards it; such data are referred to as having high *leverage* on the model and a single erroneous value

[f] This rule should only be applied when PCA has been performed using the correlation matrix

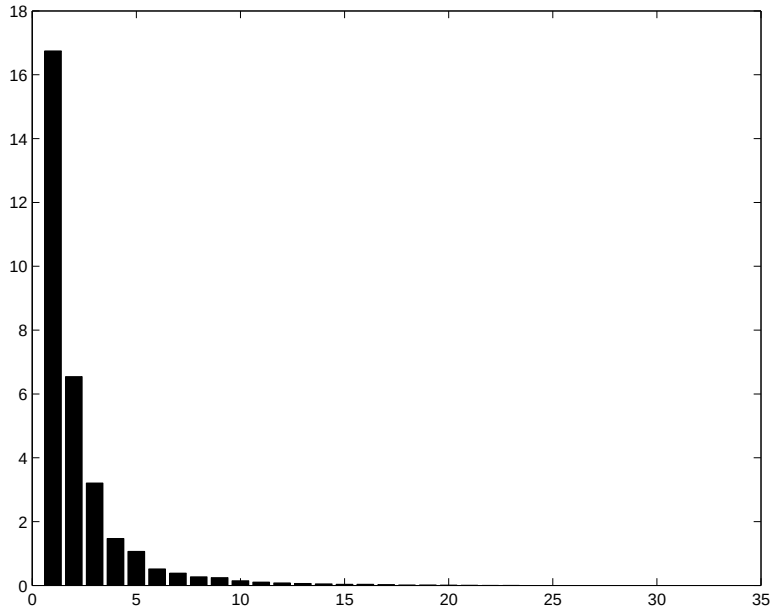


Figure 2.6: Example scree plot to illustrate use; this plot is from data previously published (Grove *et al.*, 2004a). The eigenvalues (the amount of variation explained by each PC) are plotted against the PC number. The curve typically follows a downward exponential shape followed by a straight, often near horizontal, line – a "cliff" followed by the "scree" (Jobson, 1992); The "scree" are discarded. Here, 3 or 4 PCs would be a suitable number to retain (there is some discussion on whether to retain or reject the first component in the "scree" and either scheme may be adopted; Joliffe, 2002).

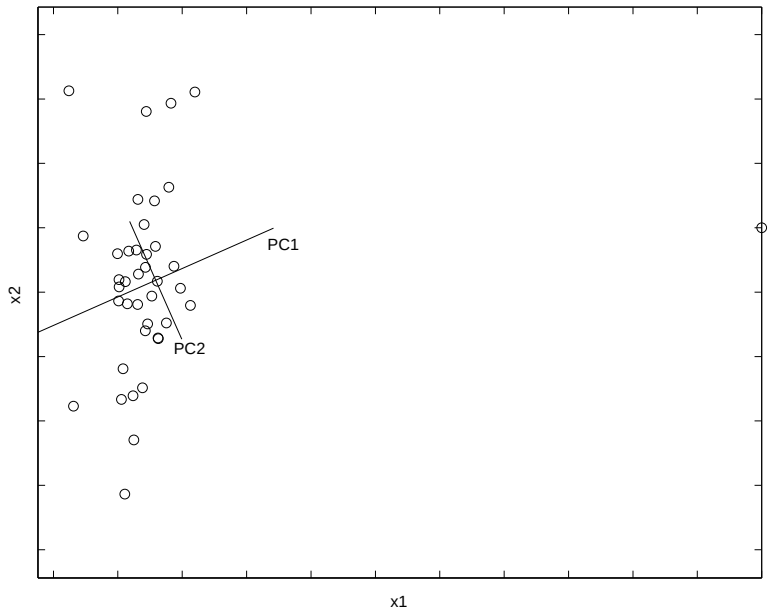


Figure 2.7: PCA performed on a constructed data set ($x_1 \sim N(\mu, \sigma^2)$ and $x_2 \sim N(\mu, 3\sigma^2)$) to illustrate high leverage. A strong outlier has been introduced on the far right and the effect on the first PC, which would otherwise align in the direction of maximum variance (parallel to the x_2 axis), is apparent.

may "occupy" an entire PC – this is illustrated in Figure 2.7. The T^2-statistic is beta-distributed and this allows formal testing at a given confidence interval for outliers (Tracy *et al.*, 1992); if the number of data is small it may be necessary to identify and remove very strong outliers and perform the analysis again due to the strong effect on the PCs. The locus of the confidence boundary is an ellipse on a scatter plot of two PCs (generally only the upper boundary is of interest); note that if three or more PCs are selected for retention, an observation outside the confidence interval may not appear so on all scatter plots.

The second type of outlier has a differing underlying factor structure. These data have a high residual when projected into the PC (hyper)plane; *i.e.*, the data fall close to the centre of the score plot but there is a large perpendicular distance from this plane to the observation. Outliers are identified by calculating the Squared Prediction Error (SPE) for each observation and comparing this with the pooled residual variance for all observations using an F-test.

Both diagnostics may be examined graphically using bar graphs with the confidence limit superimposed (each observation appears in both graphs). Such plots are convenient because they provide information about outliers from any number (A) of retained PCs (elliptical confidence intervals on two-component scatter plots indicate only outliers having a high T^2 as a result of high leverage *on one of the plotted components*).

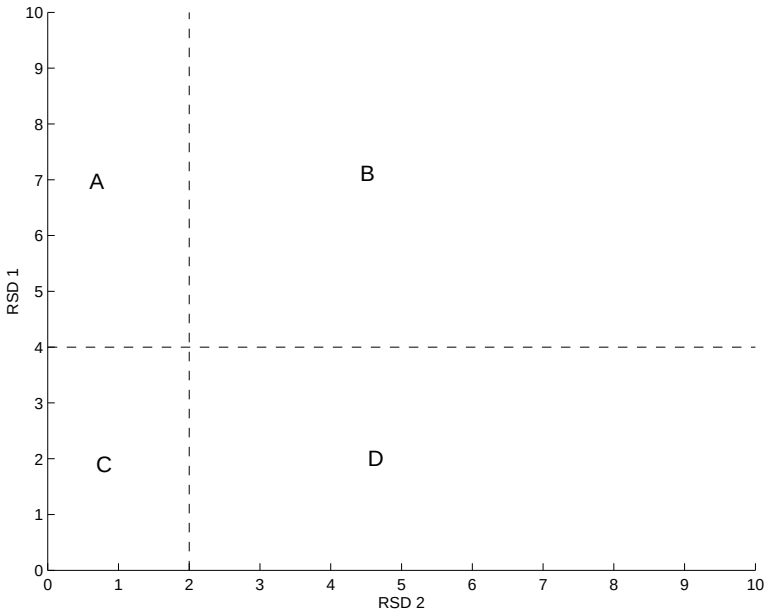


Figure 2.8: Cooman's plot: observations SPEs for two models are plotted against each other with the critical distance indicated. Each observation may fit both class models (area C), one model (areas A and D) or neither model (area B).

Multivariate Data Modelling. PCA may be used to compare data according to explicit class models; this technique, known as Soft Independent Modelling of Class Analogy (SIMCA), was developed by Wold (1978) and Wold *et al.* (1986) and is described by Eriksson *et al.* (2001) and Martens and Martens (2001). Briefly, a PC model is developed from prototype data in one class and the probability of membership of other observations is calculated according to the outlier detection procedures above[g,h].

The probability of class membership may be used to determine whether the two classes are similar. For comparison of two classes, a Cooman's scatter plot of observation SPEs for the two classes shows observations falling into either, both or neither class; this is illustrated in Figure 2.8.

2.3 Concluding Remarks

The presence of a healthy microbial community is the engine for successful biofilter operation. Previous studies have shown that the ecosystem is mixed – supporting archaea,

[g]The distance to the model (SPE) for data *in* the model prediction set is slightly inflated to account for their influence on the model PCs; this maintains the significance level of subsequent hypothesis tests.

[h]For observations *not* in the model, note that Hotelling's T^2 follows an F-distribution while for observations *in* the model it follows a B-distribution as before; this results in different confidence limits (the values converge as $N \rightarrow \infty$)

bacteria, moulds and yeasts – and may vary with location and over time. Zak *et al.* (1994) note that, "evaluating the biodiversity of microbial communities remains an elusive task because of taxonomic and methodological difficulties." These difficulties apply also to the microbial community in a biofilter and further complications are added by the need to consider dynamic variations in the community.

The literature reviewed in the preceding pages indicates that community interactions in a biofilter are complex and dynamic. Differences may be seen with location in the bed and over time while overall system function, in terms of DRE, may be maintained despite these changes; it may therefore be difficult or impossible to correlate the state of the microbial community with system performance. However, it may be that there are identifiable characteristics associated with "healthy" communities. Perturbations in environmental parameters, such as temperature, may trigger changes in the community structure. The degree of diversity may be affected by the compounds being treated, with certain compounds leading to specialist organisms being dominant (for example, methanotrophs in response to methane exposure); biofilters treating more easily degradable compounds may exhibit a higher diversity but the crowded, nutrient-limited ("*K*-type") environment is likely to favour certain types of organism.

Studies in mixed-community bioreactors have found similarly complex with fluctuations in the community diversity over time while overall system function is maintained. Perturbation response sometimes, but not always, leads to divergence and removal of the perturbation may or may not cause a return to the state before it was applied. The presence of some functional redundancy in the community, in terms of parallel or shared processing paths may be indicative of a more stable community. The fluctuations observed in such community structures – changes over time, different behaviour in response to perturbations and after the perturbation is removed – is symptomatic of a chaotic system and mathematical modelling based on a predator-prey description supports this hypothesis.

The purpose of this work is to investigate changes in the microbial community over time at different depths in the biofilter bed and in units operating in parallel. Changes in the potential functional diversity of the community are assessed based on CLPP using Biolog EcoPlates™.

Chapter 3

Methods and Materials

3.1 Introduction

Changes and differences in the bacterial community were investigated using a functional approach based on Community-Level Physiological Profiling (CLPP) using Carbon Source Utilisation Profiles (CSUPs). The applicability of this approach to compost biofilters has been previously demonstrated (Grove *et al.*, 2004a). Four experiments were performed, the purposes being:

1. To investigate the reproducibility and variability with sample size of the CLPP assay;
2. To investigate changes with time and position in a single biofilter;
3. To investigate differences between two biofilters operating in parallel after a short acclimation period; and,
4. To investigate differences in two biofilters operating in parallel over time.

3.2 Selection of a Volatile Organic Compound (VOC)

Ethanol was selected as a model VOC. Ethanol is convenient to work with and has been used successfully in our laboratory (Grove *et al.*, 2004a,b), in other laboratory-scale studies and at industrial sites (see Table 2.3 on Page 13). Efforts were made to keep ethanol loading below the maximum values indicated to avoid formation of acidic intermediates that has been observed at high loading rates in other studies (le Cloirec *et al.*, 2001; Devinny and Hodge, 1995).

3.3 Biofilter Set-Up and Operation

An existing laboratory-scale biofiltration apparatus was used and has been previously described (Kibazohi, 2000). Air flows through a humidification column and VOC bubbler in parallel and is recombined before flowing to the biofiltration columns. The apparatus is illustrated in Figure 3.1 and included:

- A humidification column constructed of glass tubing (8 cm Internal Diameter (ID); 1.3 m height) and packed with 16 mm plastic pall rings (tap water is added periodically during operation to maintain the water level close to the top of the column);
- A VOC bubbler consisting of a glass vessel connected to a VOC reservoir designed to maintain an approximately constant level of liquid;
- A 1 L plastic mixing chamber;
- "Rotameter"-type flow meters (Cole-Parmer Inc., Illinois) of assorted capacities; and,
- Four biofilters: two larger, glass units (11.5 cm ID; packed height approximately 65 cm) and two smaller, plastic units (5 cm ID; packed height approximately 30 cm).

All biofilters were operated in a down-flow mode and defined Mineral Medium (MM) (see Table 3.1) was added to the top periodically using a peristaltic pump. Air sampling ports were available in the flow line between the mixing unit and the biofilters and at the exhaust of each unit. Screw-topped sampling ports (approximately 2 cm diameter) for the extraction of media samples were available on the sides of the larger units (position is given relative to packed bed height in the text, where applicable); no such ports exist on the smaller units. Packing media was supported on wire meshes held above the bottom of the columns. Drains allowed the removal of leachate for recirculation to the top of the bed, or for disposal.

3.3.1 Inoculation

Experiments 1, 3 and 4 were performed without microbial inoculation of the packing media. Inoculation was used in Experiment 2 and the method used is described in the section for that experiment.

3.3.2 Packing Media

The packing material was obtained locally and was either peat (White Rose Garden Centre or Home Hardware, Waterloo) or wood-based compost (Plant Operations, University of Waterloo). Natural lime (White Rose Garden Centre, Waterloo) was added to the peat to control pH in the ratio 8 kg lime : 1 m^3 packed volume (Kibazohi, 2000). All media was soaked in MM (1 L MM:4 L dry packing) overnight prior to packing.

Table 3.1: Composition of Mineral Medium (MM).

Component	Amount
K_2HPO_4 (Merck)	1.00 g
KH_2PO_4 (Fisher)	0.10 g
$(NH_4)_2SO_4$ (BDH)	1.00 g
$MgCl_2.6H_20$ (BDH)	0.50 g
$FeSO_4.7H_20$ (Merck)	0.02 g
Dissolved in, Deionised water	1 L
TES (see Table (b) below)	2 mL

(a) Mineral Medium (MM). This solution was not sterilised.

Component	Amount
$CaCl_2$ (BDH)	200 mg
$MnSO_4.5H_20$ (BDH)	200 mg
$CuSO_4.5H_20$ (BDH)	200 mg
$ZnSO_4.7H_20$ (Baker)	200 mg
$(NH_4)_6Mo_7O_{24}.45H_20$ (Baker)	200 mg
Dissolved in, Deionised water	1 L

(b) Composition of Trace Element Solution (TES). This solution was filter sterilised

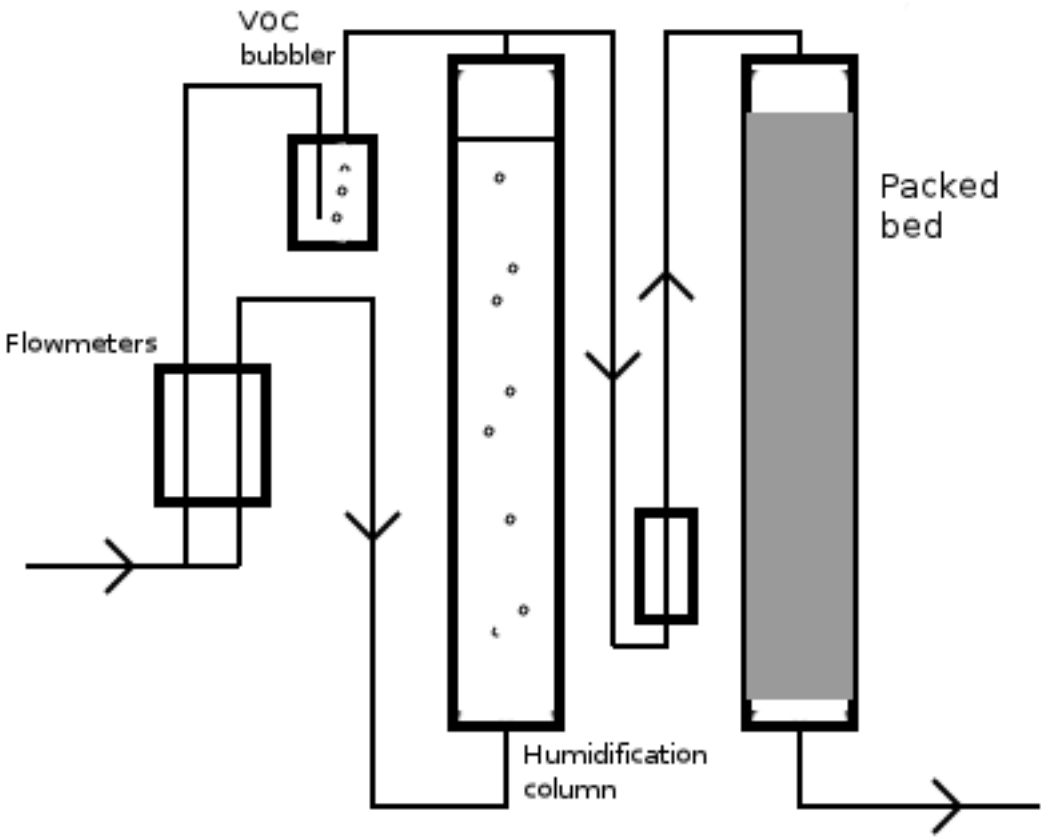


Figure 3.1: Illustration of biofilter apparatus. Air flows via two flow meters to a VOC bubbler and humidification column in parallel. The flow is recombined, passes through a further flow meter and then to the packed column where biofiltration occurs. The set-up allows for several biofilters to be operated in parallel.

3.4 Analytical Methods

3.4.1 Gas Analysis

VOC concentration in the gas phase was determined using a Hewlett-Packard (Avondale, Pennsylvania) 5890 Series II Gas Chromatograph (GC) equipped with a Flame Ionisation Detector (FID) and RTX-502.2 capillary column (Chromatographic Specialties, Inc., Brockville, ON). The column was operated in split-flow mode and data collected on a personal computer using the Peak Simple Chromatography Data System (Laballiance, Pennsylvania). Samples were collected using 500 cm^3 Tedlar® bags (Environmental Express, South Carolina) fitted with a flexible tube and needle inserted through a rubber septum into the inlet or outlet air lines from the biofilter. Sub-samples from these bags were injected into the GC using a Hamilton (Nevada) gas-tight syringe. Calibration was performed by injecting measured volumes of liquid VOC and air into Tedlar® bags reserved for the purpose and allowing the VOC to evaporate to give a known concentration. The Tedlar® bags were reused but only for samples taken in the same place (*i.e.* bags used to sample from the inlet line were not reused for the exhaust line) to avoid errors arising from absorption of the VOC to the walls of bag.

3.4.2 pH

Approximately 0.5 to 1 g of packing was removed from a side sampling port, mixed thoroughly with deionised water and a pH probe used to measure the pH.

3.5 Community-Level Physiological Profiling (CLPP)

3.5.1 Sampling

Biolog EcoPlates™ (Biolog Inc., Hayward, CA) were used for CLPP. Packing samples of 0.5 to 1 g (wet) were removed from the columns. For the large columns: the sampling ports and a spatula were first disinfected with 70 % ethanol and samples removed from the ports into sterile tubes. For the small columns, the entire column was taken to a laminar flow hood, the rubber plug in the top of the column removed and the top 5 cm of packing removed and discarded; samples were then taken directly from the top of the exposed packing using a spatula.

The samples were suspended in 10 cm^3 of Phosphate Buffer (PB) (see Table 3.2) and shaken for 3 h at room temperature. Aliquots (2 cm^3) of the resulting suspension were diluted in buffer to control the Optical Density (OD) close to 0.2 at 420 nm. Each well of the EcoPlates™ was inoculated with 150 µL using a multichannel autopipettor (Eppendorf, NY). The plates were incubated at room temperature and the OD at 590 nm

Table 3.2: Composition of Phosphate Buffer (PB). This solution was sterilised by autoclaving.

Component	Amount
Na_2HPO_4 (BDH)	1.24 g
NaH_2PO_4 (Sigma)	0.18 g
NaCl (Caledon)	8.50 g
Dissolved in,	
Deionised water	1 L

of each well read on a multi-spectrophotometer (Labsystem, Multiskan Ascent) at 12, 16, 20 and 36 h (Experiments 1 to 3) or 12, 24, 36, 48 and 60 h (Experiment 4).

3.5.2 Statistical Methods

Selection of Analysis Time and Data Pre-Treatment

The OD readings were first truncated to lie in the range $\{0, 2\}$, since values below 0 are clearly erroneous and values above 2 have been shown to be dominated by measurement error (Glimm *et al.*, 1997). The reading for each well was then corrected by subtracting the value of the water blank for that replicate, and standardised by dividing by the Average Well Colour Development (AWCD) for the replicate, in line with the recommendations of Garland (1996a). If OD_{ijt} represents the corrected OD for well i of replicate j at time t, then the AWCD for replicate j at time t is given as,

$$\mathrm{AWCD}_{jt} = \frac{1}{31} \sum_{n=1}^{31} \mathrm{OD}_{ijt} \tag{3.1}$$

The standardised OD values are then calculated by dividing the OD by the AWCD,

$$\overline{\mathrm{OD}}_{ijt} = \frac{\mathrm{OD}_{ijt}}{\mathrm{AWCD}_{jt}} \tag{3.2}$$

The time point for analysis is selected by counting the number of wells for which standardised OD exceeds 0.1 (over all plates) and selecting the time with the greatest change, *i.e.* choosing the time t such that $(n[t] - n[t-1])$ is maximised, where $n[t]$ represents the number of wells (counting over all plates) with $\overline{\mathrm{OD}} > 0.1$ at time t.

Principal Component Analysis (PCA)

Overview of Data. Statistical analysis of the normalised data ($\overline{\text{OD}}$ values) was performed using PCA; an explanation of PCA is provided in the literature review (Section 2.2.5, Page 41) and mathematical details in Appendix A (Page 132) and this section concentrates on a description of the analysis performed. PCA was performed, using Matlab on a personal computer, at the selected time point using the covariance matrix to maintain scale. Analyses were performed separately for each experiment.

The number of components to analyse was determined using a scree plot (see Section 2.2.5 and Figure 2.6, Pages 41-43 for an explanation). Observations were examined visually using scatter plots of Principal Component (PC) scores. Outliers were identified by developing confidence intervals using Hotelling's T^2 statistic (for outliers having the same underlying factor structure) and the Squared Prediction Error (SPE) (for outliers having a different underlying factor structure); bar graphs of these statistics, with the confidence interval indicated, are presented. The confidence interval for Hotelling's T^2 statistic is also presented as an ellipse on the PC score plots.

Soft Independent Modelling of Class Analogy (SIMCA). SIMCA was performed to compare observations forming different classes within each experiment. This is described in Appendix A; briefly: a PC-model is created by performing PCA on a subset of the experimental data. The other observations are projected into the PC-space generated and may be classified as being a member or not of the prototype class using T^2 and SPE statistics[a]. This can be accomplished visually by using score plots and bar graphs of the statistics. Comparison of different classes can be achieved via a "Cooman's" plot – a scatter plot of SPEs generated from two different class models.

3.6 Experiment 1 – Determination of Sample Variability

One of the small biofilters was operated without microbial inoculation. Ethanol was the sole VOC. The packing material was peat moss (White Rose Garden Centre, Waterloo) and the packed height 30 cm. VOC concentrations were monitored until significant Destruction and Removal Efficiency (DRE) was obtained. At this point, the top 5 cm of packing was removed and discarded (moisture control in the smaller biofilters is problematic and the top of the reactor is most susceptible to changes in moisture content) and five samples of 0.5-1 g and two samples of approximately 6 g were taken from the exposed surface. CLPP was performed as described in Section 3.5 (for the 6 g samples, PB dilution was maintained at the same ratio). One EcoPlate™ was prepared from each of the 0.5-1 g samples and two from each of the 6 g samples.

[a]For observations that are not members of the prototype class, T^2 follows an exact F-distribution; SPE for the members of the prototype class is increased to account for their influence on the model.

3.6.1 Univariate Statistical Methods

A nested Analysis of Variance (ANOVA) (see Section A.2 Appendix A) was performed following the method of Balser *et al.* (2002) to determine the contribution to the variance of the first PC of: sample size, media sub-sample and replication (provided by the three sets of carbon sources on each plate). To perform this, PCA was initially performed using the three replicate OD values on the plates without averaging. The contribution to the variance of each component is modelled as:

$$\sigma_T^2 = \sigma_1^2 + \sigma_2^2 + \sigma^2 \tag{3.3}$$

where σ_T^2 is the total variance, σ_1^2 is the variance due to the size of the media sample, σ_2^2 is the variance due to the sub-sample and σ^2 is the variance due to replication of the C-sources on the plate (this represents methodological variation).

An estimate s^2 of each variance was calculated and univariate F-tests used to test the hypotheses,

$H_0 : \sigma_1^2 = 0$ with $H_1 : \sigma_1^2 \neq 0$
and,

$H_0 : \sigma_2^2 = 0$ with $H_1 : \sigma_2^2 \neq 0$
this gives an indication of the relative contribution of each source of variation to the total.

3.7 Experiment 2 – Spatial and Temporal Variation in One Biofilter

One large biofilter packed with compost (Plant Service, University of Waterloo) was operated using ethanol as the VOC. The packing media was inoculated with a mixed microbial community. This was prepared by incubating activated sludge (obtained from the City of Waterloo Municipal Waste Water Treatment Plant) for 24 h at room temperature in shaker flasks (20 mL sludge with 150 mL MM plus 5 mL VOC in a 500 mL flask at 150 rpm) and then for 27 h at room temperature with bubbled aeration (100 mL of growth culture with 1 L MM and 20 mL VOC; no shaking).

The initial packed bed height was 63 cm. Five samples of 0.5-1 g were taken for CLPP from each of three sampling ports on days 10, 21, 31, 42 and 53. The sampling ports were located 7, 34 and 59 cm (referred to as bottom, middle and top respectively) from the bottom (exhaust) of the bed. PCA and SIMCA analyses were performed using mean data from the EcoPlates™; classes were based on location in the biofilter and sample day.

3.8 Experiment 3 – Acclimation of Parallel Biofilters

Two small biofilters packed with peat (White Rose Garden Centre, Waterloo) were operated with ethanol as the sole VOC and without microbial inoculation. After significant VOC DRE was attained, the top 5 cm of packing were removed and five samples of 0.5-1 g removed from the exposed surface for CLPP. PCA and SIMCA were performed using mean data from the EcoPlates™; classes were based on the two different biofilters.

3.9 Experiment 4 – Spatial and Temporal Variations in Parallel Biofilters

Two large columns were operated in parallel using ethanol as the sole VOC and without microbial inoculation. The packing material was peat (Home Hardware, Waterloo; this is a different peat to that used for Experiments 1 and 3) and initial packed height 67 cm. Humidified air only (no VOC) flowed through the column for the first 14 days. Five samples of 0.5-1 g at two locations – 20 (bottom) and 60 cm (top) from the bottom of the packing – were taken from each column on days 14, 26, 40 and 54 for CLPP analysis. PCA and SIMCA were performed using mean data from the EcoPlates™; classes were based on the two different biofilters, location (top or bottom) and sample day.

3.10 Overall Comparison of Biofilters

PCA and SIMCA was performed on the combined data set formed from average data from all experiments to examine the variability between assays.

3.11 Summary of Experiments

A summary of the experiments performed is presented in Table 3.3 and reproduced for reference in Appendix D on the final page of this thesis.

Table 3.3: Description of Experiments Performed

Experiment Number	Name	Purpose
1	Determination of sample variability	To determine whether the microbial community in a biofilter can be characterised using CLPP, whether similar results are found from samples taken over a range of a few centimetres and to investigate the effect of sample size.
2	Spatial and temporal variation in one biofilter	To examine the spatial and temporal variation of the potential functional diversity in a biofilter over time.
3	Acclimation of parallel biofilters	To examine the parallel development of two biofilters over their acclimation period.
4	Spatial and Temporal Variations in Parallel Biofilters	To examine the acclimation and development of the community in two parallel biofilters over time at different vertical locations in the bed.

Chapter 4

Results and Discussion

4.1 Experiment 1 – Determination of Sample Variability

4.1.1 Rationale

Community-Level Physiological Profiling (CLPP) has mainly been applied in the comparison of bulk soil samples where relatively large samples may be used; because of the relatively small size of a laboratory-scale biofilter taking large samples would involve the removal of an excessive amount of material from the columns. The purpose of this experiment was:

1. To determine whether the microbial community in a biofilter can be characterised using CLPP and whether community results are similar in samples taken over a range of a few centimetres; and,

2. To compare results obtained from media sub-samples of different sizes.

(1) is important because a question arises as to the heterogeneity of the bacterial community; work on the community proceeds on the premise that the population may be characterised through assays applied locally. Since it is not readily apparent that the community in a biofilter meets this requirement (*i.e.*, it has not been demonstrated that the community in a biofilter may be considered similar over the scale of a few centimetres), it is worthwhile verifying this assumption.

(2) is important in establishing a suitable sample size and, by establishing the relative contribution to the variation of sub-sampling and replication of C-substrate values, focusing replication efforts in the most useful direction.

4.1.2 Biofilter Operation

One small biofilter was operated with an inlet ethanol concentration of 1270 ppm ($\pm$ 80) giving a loading of 240 g VOC m^{-3} h^{-1} ($\pm$ 20). After 14 days of operation, the Destruction

and Removal Efficiency (DRE) was 85 % (± 7) corresponding to an Elimination Capacity (EC) of 210 g VOC m^{-3} h^{-1} (± 20); at this point the experiment was stopped and samples for CLPP taken near the inlet of the bed. The pH of the media (Sample taken from the same vicinity as samples for CLPP) declined from 7 to 5 during the experiment; this may be due to the production of acidic by-products (ethanoic acid) as a result of overloading the biofilter.

4.1.3 Principal Component Analysis (PCA)

PCA was performed on the whole data set treating the three subsamples on each plate as independent samples. The time selected for analysis, determined by the method described in Section 3.5.2 (Page 52), was 16 h. The first two Principal Components (PCs) were selected for analysis from a scree plot (Figure 4.1) and were able to account for 63 % of the variance in the data. A score plot is provided in Figure 4.2 and bar graphs of T^2 (Figure 4.3a) and Squared Prediction Error (SPE) (Figure 4.3b). A visual examination of the score plot shows that the results from different sample sizes cannot be separated based on the first two PCs and the majority of observations fall within the 95 % confidence intervals for both T^2 and SPE tests; note that in this instance there are 27 samples and so about 2 samples would be expected to fall outside the confidence intervals.

The results of the nested Analysis of Variance (ANOVA) based on the first PC are shown in Table 4.1a. This indicates that the majority of the variance in the data is due to the media sample with comparatively small contributions made by the size of the media sample and the C-course replication on the plate.

Based on this small contribution to the variance of the different sample sizes, the analysis is repeated without considering this source of variation; results, given in Table 4.1b), show the sample selected account for the majority of the variation and there is little variation between on-plate replicates of the C-sources.

The results of the ANOVA suggest that the mean of C-source replicates should be used as input to the PCA. This was performed and a scree plot (Figure 4.4), score plot (Figure 4.5) and bar graphs of T^2 (Figure 4.6a) and SPE (Figure 4.6b) are provided. It is still not possible to separate the sample sizes based on the first 2 PCs.

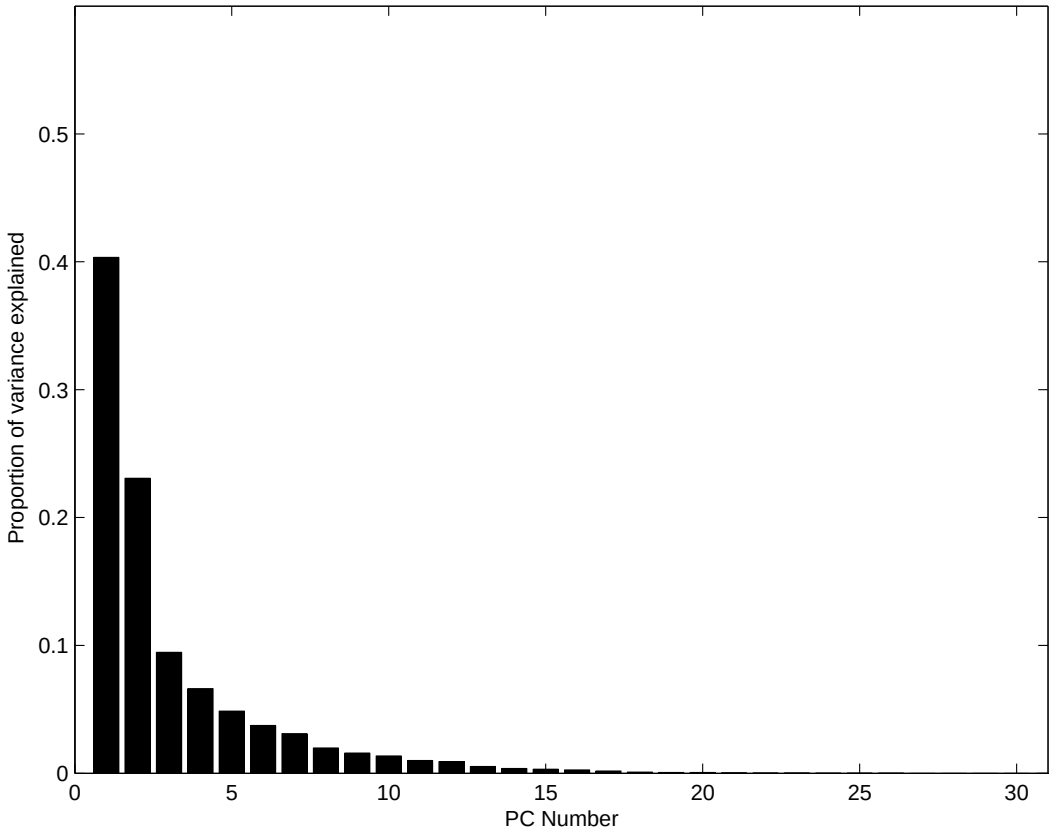


Figure 4.1: Scree plot for Experiment 1 (determination of sample variability) based on data without averaging. The first 2 PCs account for 63 % of the variance in the data set.

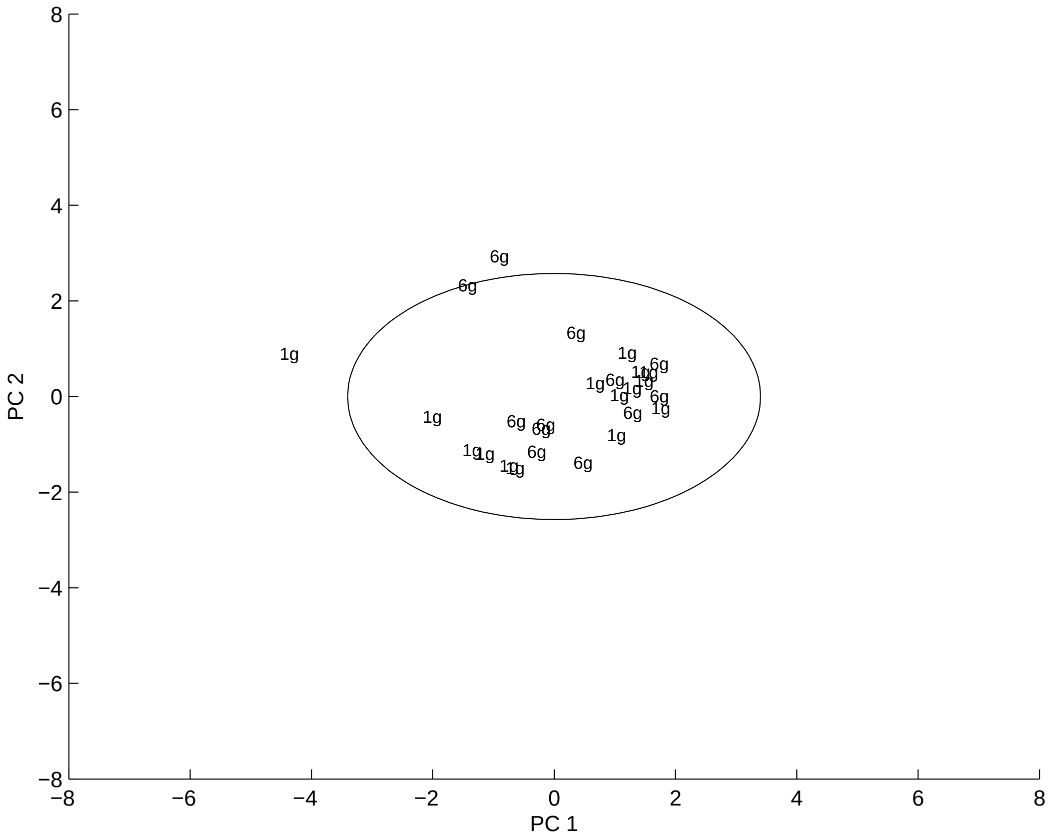
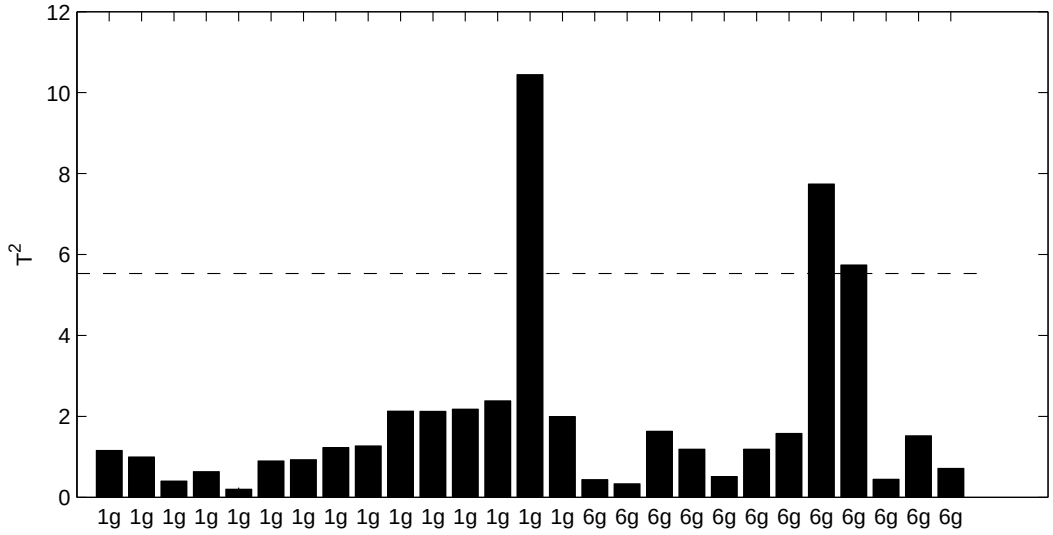
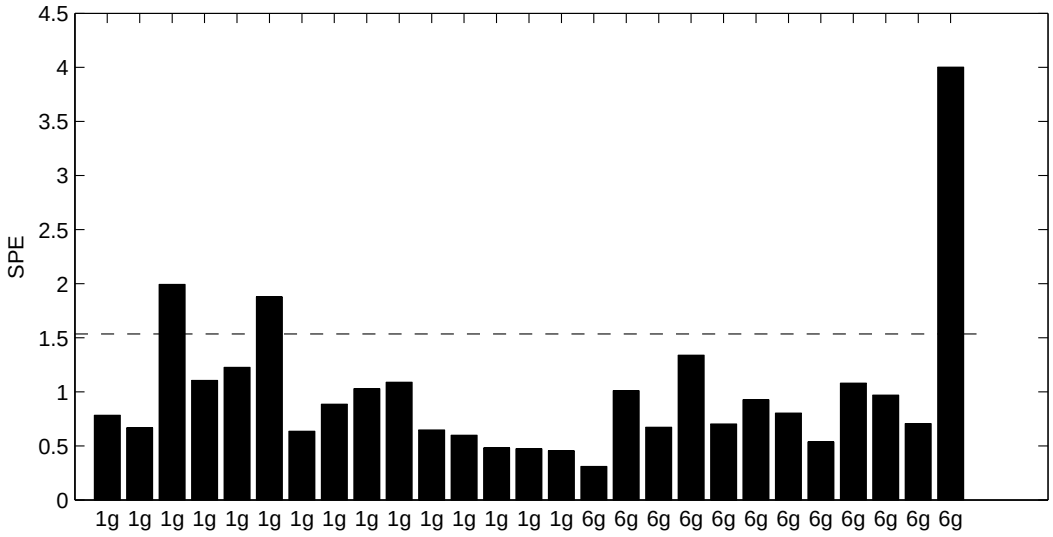


Figure 4.2: Score plot for Experiment 1 (determination of sample variability). The ellipse represents the 95 % confidence interval based on Hotelling's T^2 statistic for the model generated by 2 PCs. Observations are of either 1 g or 6 g samples as indicated.



(a) Bar graph of Hotelling's T^2



(b) Bar graph of SPE

Figure 4.3: PCA diagnostic plots for Experiment 1 (determination of sample variability). The dashed lines represent 95 % confidence intervals for the model generated by 2 PCs. Observations are of either 1 g or 6 g samples as indicated.

Table 4.1: ANOVA results from Experiment 1 (determination of sample variability), showing the relative contribution to the variance in the 1st PC due to various sources: sample size (1 g or 6 g), media sample or on-plate replication of C-source.

Source	df	SS	F	% of Variance
Size	1	2.5	0.11	<0.1
Sample	5	115.2	8.46	98.0
Plate	20	54.5		2.0
Total	26			

(a) ANOVA treating each replicate as a separate sample

Source	df	SS	F	% of Variance
Sample	8	14.4	4.75	95.8
Plate	18	54.5		4.2
Total	26			

(b) ANOVA repeated without considering sample size as a separate source of variation.

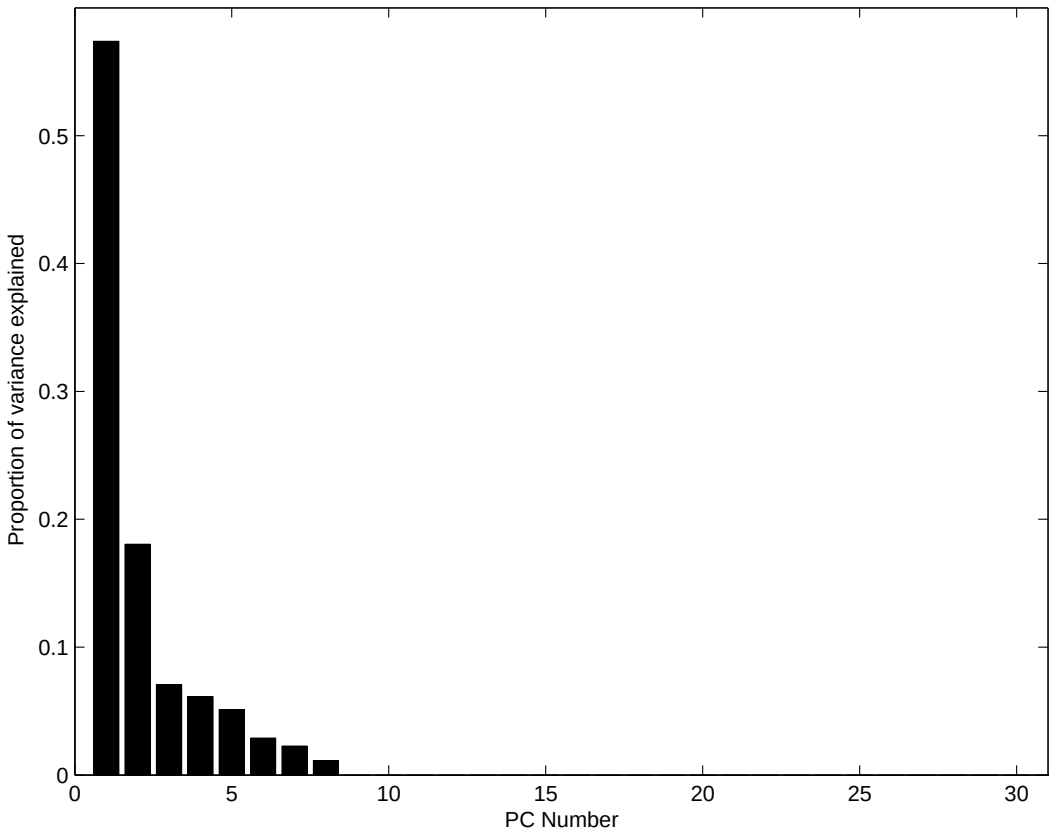


Figure 4.4: Scree plot for Experiment 1 (determination of sample variability) based on mean values of C-source replicates. The first 2 PCs account for 75 % of the variance in the data set.

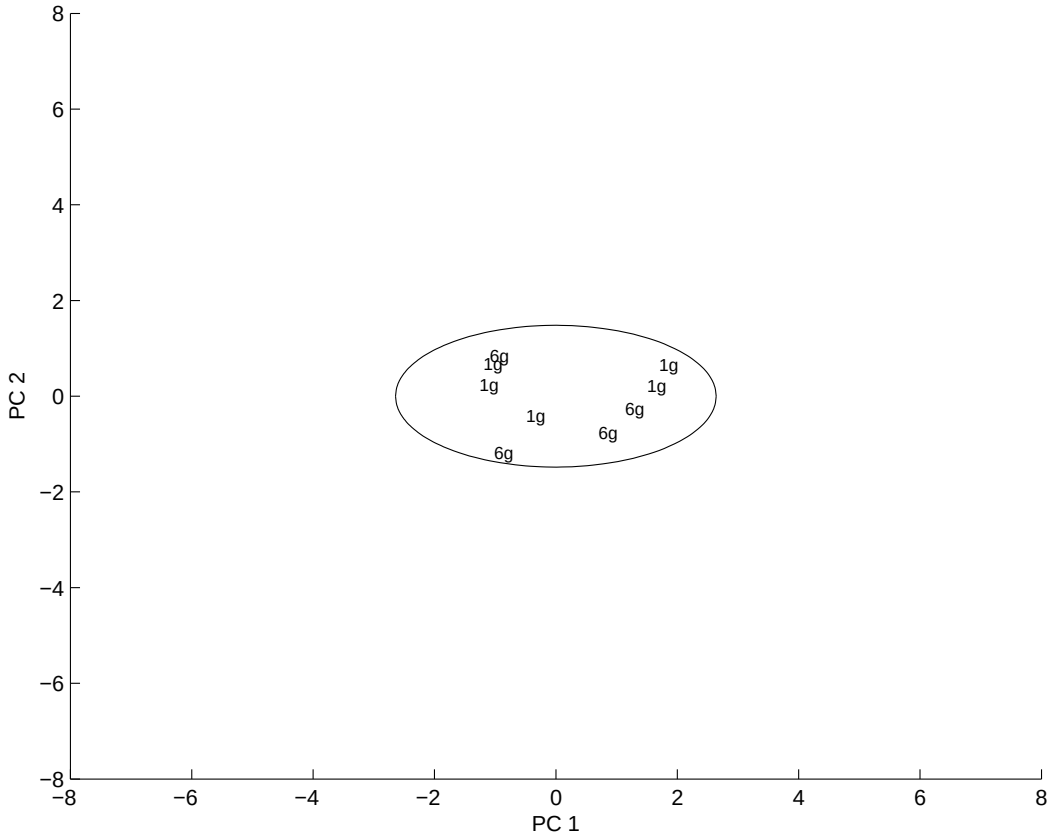
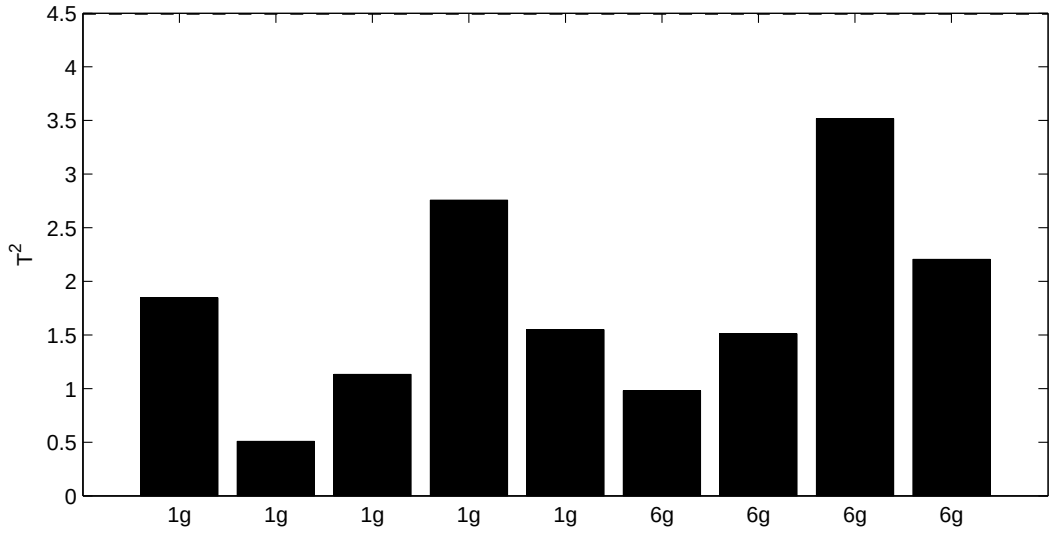
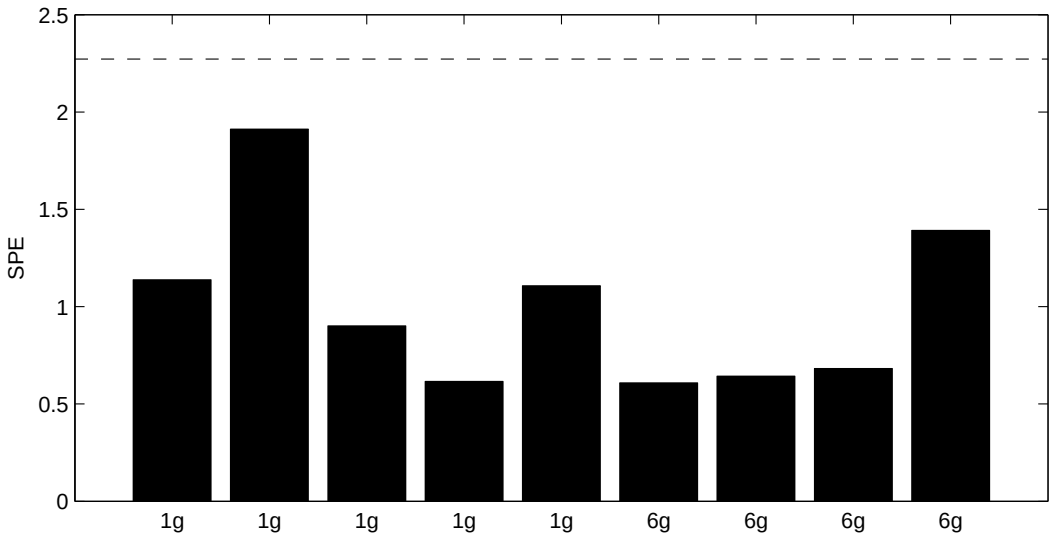


Figure 4.5: Score plot for Experiment 1 (determination of sample variability) based on mean values of C-source replicates. The ellipse represents the 95 % confidence interval for the model generated by 2 PCs. Observations are of either 1 g or 6 g samples as indicated.



(a) Bar graph of Hotelling's T^2



(b) Bar graph of SPE

Figure 4.6: PCA diagnostic plots for Experiment 1 (determination of sample variability). The dashed lines represent 95 % confidence intervals for the model generated by 2 PCs. Observations are of either 1 g or 6 g samples as indicated.

4.1.4 Discussion

This unit was overloaded with ethanol (loading of 206 g VOC m^{-3} h^{-1}). The decline in pH is attributed to the formation of acidic metabolites and insufficient buffer capacity of the media. Despite the overloading, function of the biofilter was maintained, as would be expected over the short-term.

The results suggest that the use of 0.5-1 g samples instead of larger samples is acceptable. This is consistent with the sample sizes used in other studies of biofilter microbial communities (for example, 0.5 g samples: Conde *et al.* (2001); 1 g: Krailas *et al.* (2000), Kiared *et al.* (1996); 2 g: Khammar *et al.* (2005)) but not for many CLPP studies with Biolog plates where subsamples are typically taken from larger, homogenised samples (for example, Balser *et al.*, 2002, used triplicate 5 g subsamples from a homogenised 500 g sample). The results further suggest that replication efforts are best focused on additional media samples and not inoculating multiple plates from the same sample; this is consistent with the findings of the study by Balser *et al.* cited above, who found the main source of variation to be in the triplicate 5 g subsamples rather than a dilution series or Biolog plate replication.

The PC score plot (Figure 4.5) shows the samples clustered closely together. Since all samples were taken from a similar location in the same biofilter, this indicates that the variation in community structure, as measured by the potential functional diversity, was small over the length scale of the sampling area (a few centimetres). This supports the hypothesis that the community in a biofilter may be regarded as similar over such a scale. This is important, because although this scale is small compared with the overall size of a biofilter, it is large relative to the scale of the biofilm and it justifies the assumption in the remainder of this work that the bacterial population in any localised area of the biofilter may be regarded as a community and that samples taken from that location are representative of it.

4.2 Experiment 2 – Spatial and Temporal Variation in One Biofilter

4.2.1 Rationale

Previous studies have suggested that the structure of the microbial community in a biofilter varies both spatially and temporally. This experiment examines these variations using potential functional diversity based on CLPP. The relationship between community structure in a taxonomic sense and CLPP remains unclear; it is therefore not apparent that CLPP will be able to distinguish between communities established from the same starting position as they change due to environmental conditions even if there are underlying taxonomic changes.

4.2.2 Biofilter Operation

Ethanol concentrations at the inlet and outlet of the biofilter are shown in Figure 4.7; a DRE greater than 97 % was achieved within 10 days of start-up. Ethanol removal at locations through the bed on day 53 is shown in Figure 4.8. EC varied between 20 and 140 g VOC m^{-3} h^{-1} (mean of 70 g VOC m^{-3} h^{-1}). Samples for CLPP were taken from sample ports located near the top (inlet), middle and bottom (exhaust) of the biofilter 10, 21, 31, 42 and 53 days after start-up. The pH varied between 7.1 and 7.5 during the experiment.

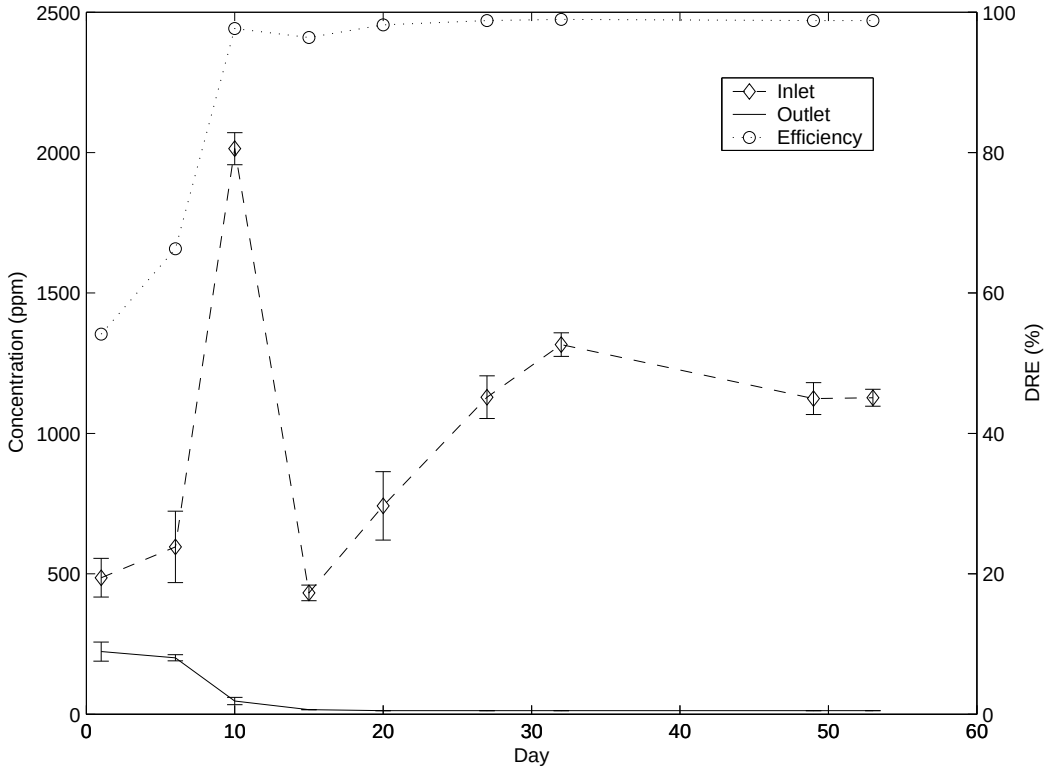


Figure 4.7: Ethanol concentrations at inlet and outlet and DRE during Experiment 2 (spatial and temporal variation in one biofilter). Error bars indicate 95 % confidence intervals based on 4 replicate samples.

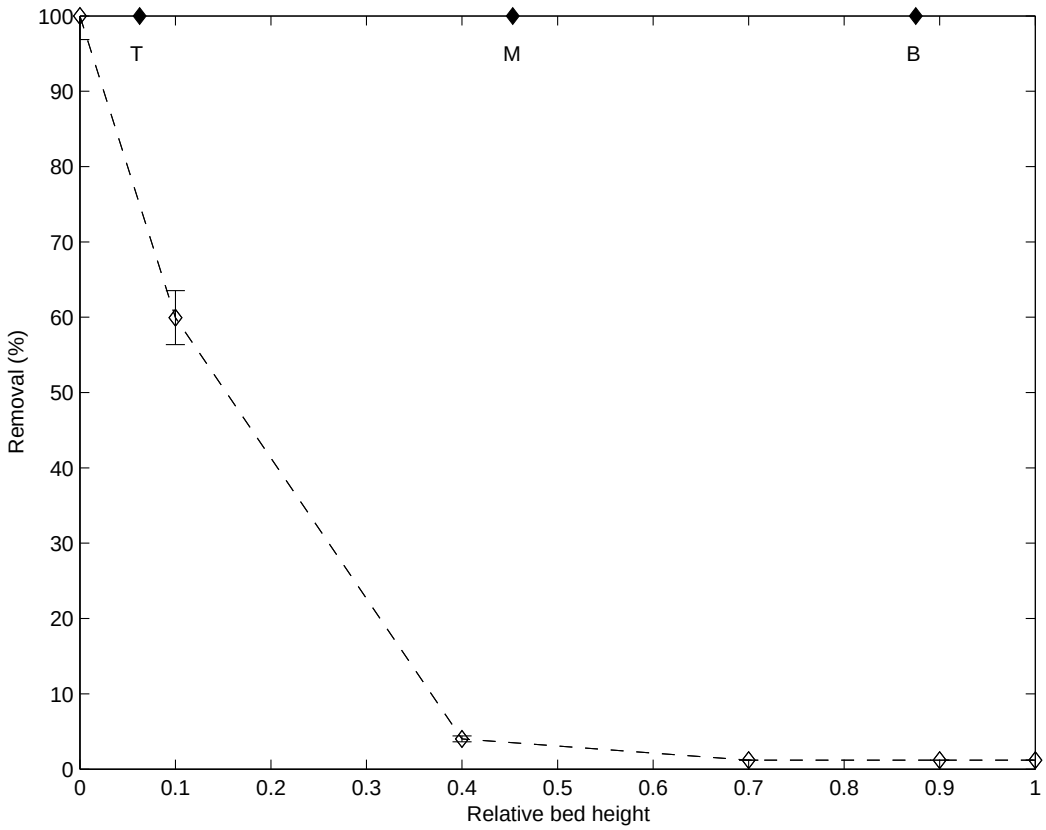


Figure 4.8: Ethanol removal with relative bed height during Experiment 2 (spatial and temporal variation in one biofilter) on day 53. Error bars indicate 95 % confidence intervals based on 3 or 4 replicate samples. The location of media sampling ports is indicated by ♦.

4.2.3 PCA

PCA was performed on the data using the mean values of the three on-plate C-source replicates. The time selected for analysis, determined by the method described in Section 3.5.2 (Page 52), was 16 h. The first 2 PCs were chosen for analysis from a scree plot (Figure 4.9) and were able to account for 70 % of the total variance in the data. PC scores for the first two principal components are plotted in Figure 4.10 and bar graphs of T^2 (Figure 4.11a) and SPE (Figure 4.11b).

Examination of the score plot (Figure 4.10) indicates that the first PC accounts mainly for differences between samples M5 and B5 – taken from the middle and bottom of the biofilter on day 53 – and all other samples. This suggests a shift in the biofilter community in that location between 42 and 53 days. The second PC differentiates samples T2 to T5 – taken from the top of the biofilter – from all other samples. This suggests a functional shift in the community near the inlet of the biofilter and therefore exposed to the highest ethanol concentration. Samples from the packing media before inoculation (S) fall between the samples taken from the top (T2 to T5) and all others; this suggests differences between the inoculated media before and after inoculation.

Values of Hotelling's T^2 (Figure 4.11a) indicate significant differences in some observations from the M5 and B5 groups, as suggested by inspection of the first PC above. The SPE (Figure 4.11b) indicates a few observations outside the 95 % confidence interval, as would be expected for this number of observations; however, on day 42 many observations fall outside this confidence limit for samples taken near the top (4 out of 5), middle (2) and bottom (5) respectively. This indicates that the variance structure of those data differs from the remainder of the observations.

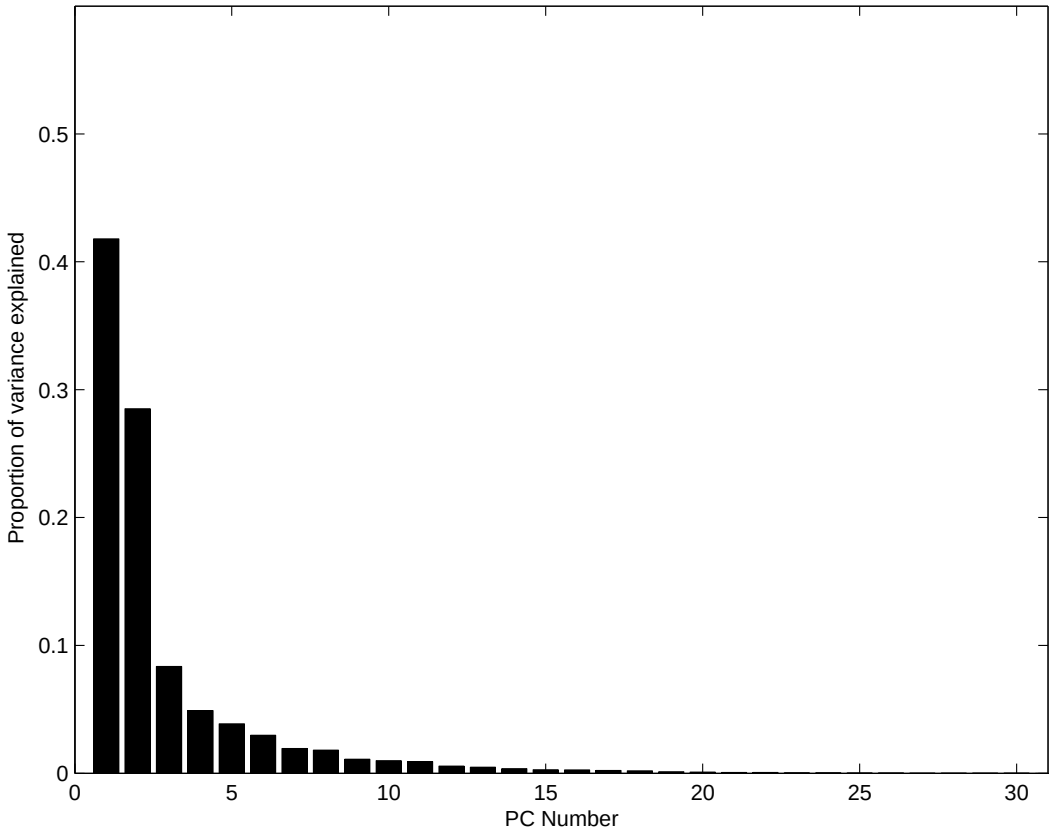


Figure 4.9: Scree plot for Experiment 2 (spatial and temporal variation in one biofilter). The first 2 PCs account for 70 % of the variance in the data set.

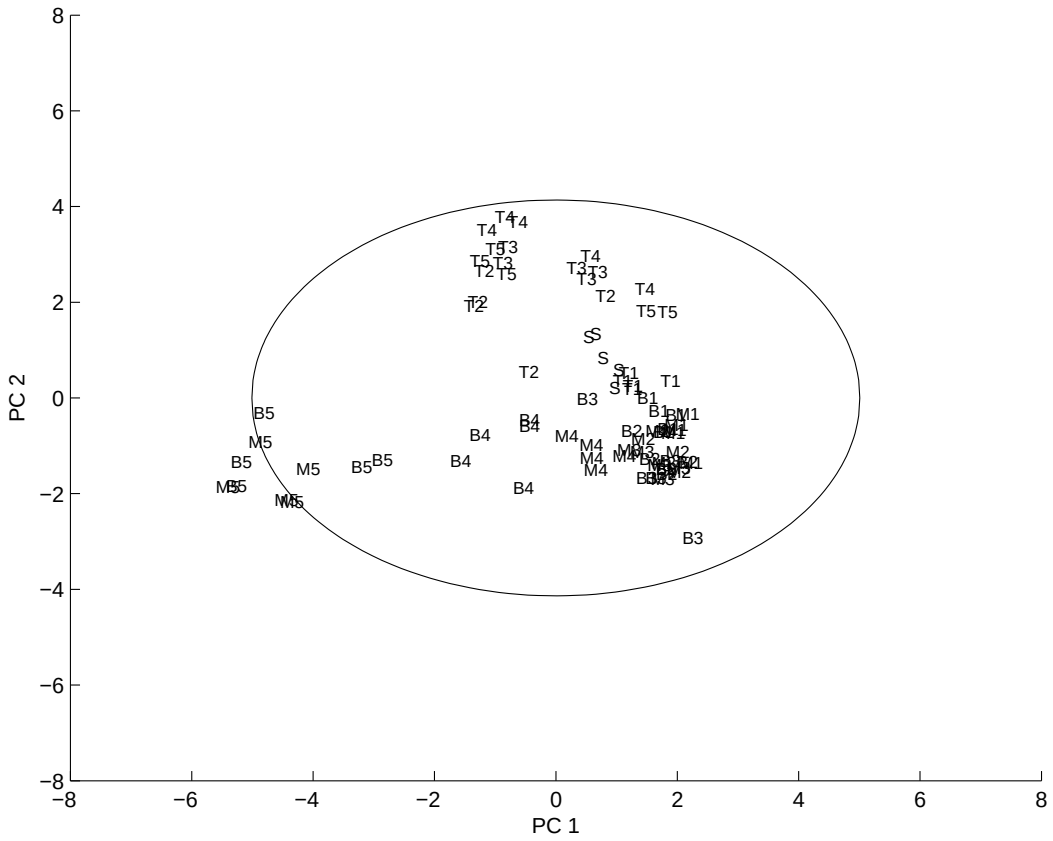
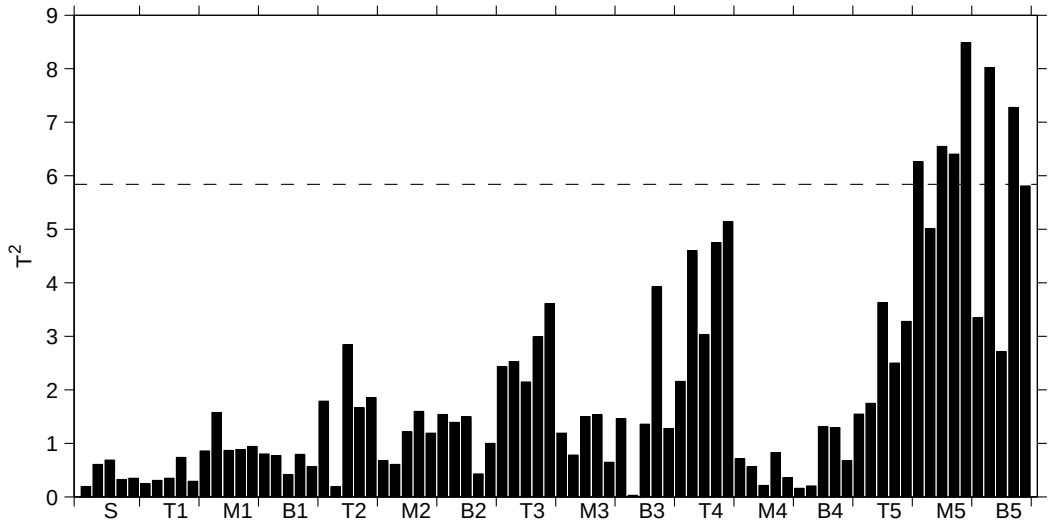
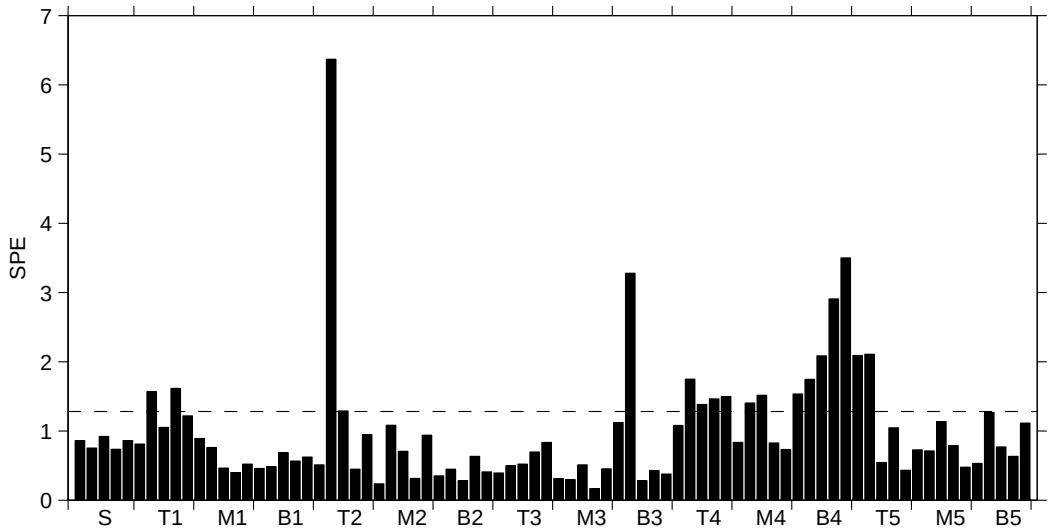


Figure 4.10: Score plot for Experiment 2 (spatial and temporal variation in one biofilter). The ellipse represents the 95 % confidence interval based on Hotelling's T^2 statistic for the model generated by 2 PCs. The observations are from near the top (T), middle (M) or bottom (B) of the unit with samples 1, 2, 3, 4 and 5 being taken on days 10, 21, 31, 42 and 53, respectively. Samples were also taken from the media before inoculation (S).



(a) Bar graph of Hotelling's T^2



(b) Bar graph of SPE

Figure 4.11: PCA diagnostic plots for Experiment 2 (spatial and temporal variation in one biofilter). The dashed lines represent 95 % confidence intervals for the model generated by 2 PCs. The observations are from near the top (T), middle (M) or bottom (B) of the unit with samples 1, 2, 3, 4 and 5 being taken on days 10, 21, 31, 42 and 53, respectively. Samples were also taken from the media before inoculation (S).

4.2.4 Soft Independent Modelling of Class Analogy (SIMCA)

Visual inspection of the score plot (Figure 4.10) suggests comparisons according to grouping of the data:

- Location based on the top (T) *vs.* middle (M) and bottom (B); and,
- Date based on observations from the middle (M) and bottom (B) on days 10, 21 and 31 *vs.* days 42 and 52.
- Date based on observations from the top (T) on day 10 *vs.* days 21, 31, 42 and 52.

Class models for each case were developed by performing PCA on the relevant data subsets using the method described in Section 3.5.2 (Page 53) and Appendix A (Page 136). Cooman's plots are presented for each comparison.

Classes Based on the Sample Location

Models were developed based on whether the samples came from the top (T) or the middle (M) / bottom (B) of the biofilter; 2 PCs were retained in each case, accounting for 68 % and 78 % of the total variance in the models respectively. A Cooman's plot of the SPEs (Figure 4.18) illustrates a very clear division based on class; the number of observations classified into each group is given in Table 4.2. Samples of media before inoculation (S) are not included in the table but do not appear to conform to either class based on the plot.

Table 4.2: SIMCA classifications for Experiment 2 (spatial and temporal variation in one biofilter) based on sample location. The number of observations classified as belonging to the model developed using 2 PCs from samples taken from the top (T; 25 observations) or middle/bottom (MB; 50 observations) of the biofilter.

	Observation Class			
Model	T		MB	
T	19	(76 %)	0	(0 %)
MB	0	(0 %)	43	(86 %)

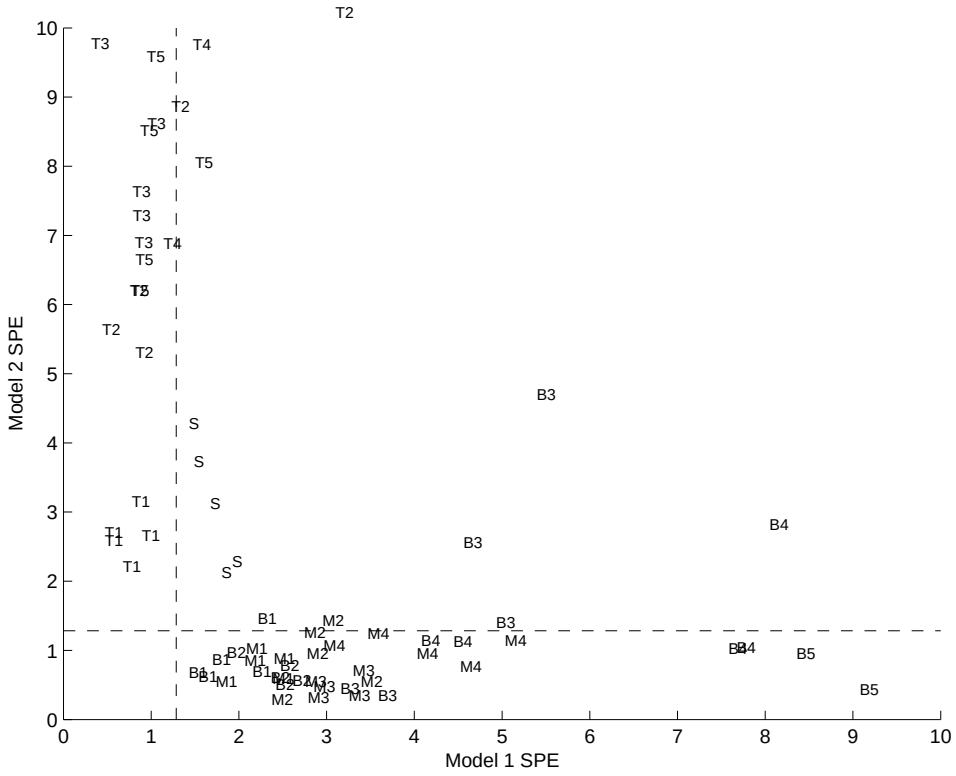


Figure 4.12: Cooman's plot for Experiment 2 (spatial and temporal variation in one biofilter) with classes based on sample location. The SPEs for 2-PC models generated based on samples from the top (x-axis) and middle/bottom (y-axis) of the biofilter are plotted. Dashed lines represent 95 % confidence intervals.

Classes Based on the Sample Date

From the overall score plot (Figure 4.10) the population at the top appears to shift between samples 1 and 2, then remaining similar, with the population in the middle and bottom of the biofilter undergoing a function shift around sample 4 or 5. These classes were used for SIMCA analysis.

Top section. SIMCA was performed based on the top observations only with one class defined as sample date 1 and the other sample dates 2, 3, 4 and 5. One PC was retained in the former and two PCs in the latter, able to account for 64 % and 69 % of the total variance respectively. A Cooman's plot (Figure 4.13 shows the SPEs of the observations. Visual inspection reveals a clear difference between the two classes.

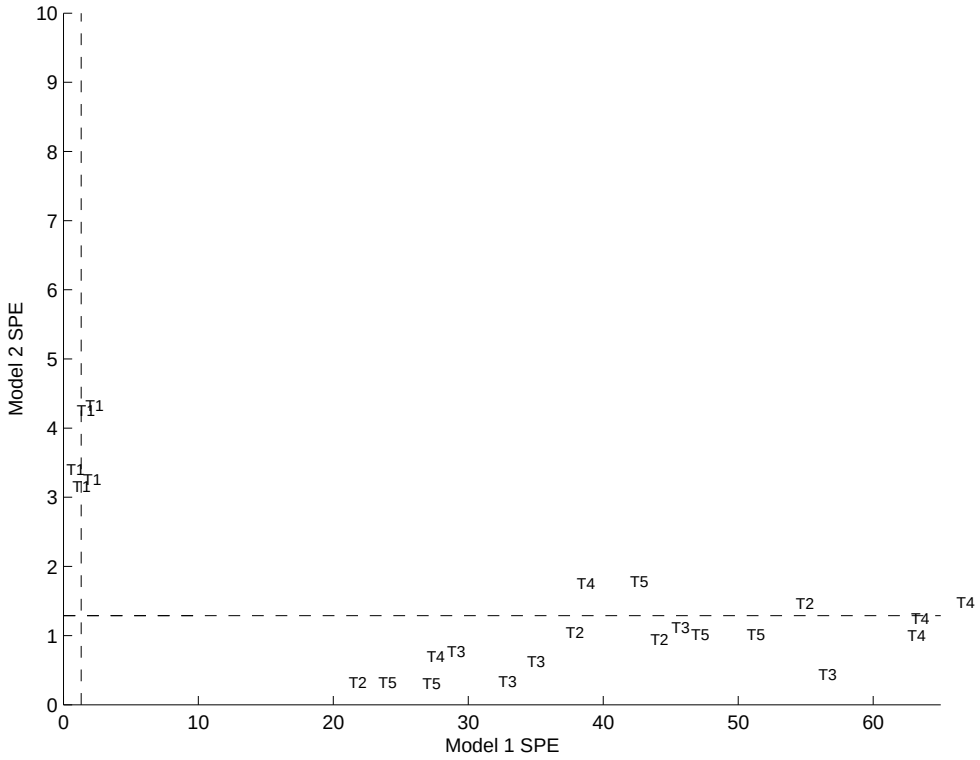


Figure 4.13: Cooman's plot for Experiment 2 (spatial and temporal variation in one biofilter) with classes based on sample date. The SPEs for the 1-PC model generated based on samples 1 (x-axis) and 2-PC model samples 2, 3, 4 and 5 (y-axis) are plotted; the models were able to account for 64 % and 69 % of the variance, respectively. Dashed lines are 95 % confidence intervals.

Middle and bottom sections. SIMCA was performed based on the middle and bottom observations only with one class defined as sample dates 1, 2 and 3 and the other sample dates 4 and 5. Two PCs were retained in each model able to account for 61 % and 82 % of the total variance respectively. A Cooman's plot (Figure 4.14 shows the SPEs of all observations (including those in neither class)). As expected from the results of the previous section, observations from the top (T) at all times fall outside both class boundaries; this is also true for observations of the media before inoculation (S). The observations of the middle and bottom split into the defined classes; the classifications are summarised in Table 4.3.

Table 4.3: SIMCA classifications for Experiment 2 (spatial and temporal variation in one biofilter) based on sample date. The number of observations classified as belonging to the model developed using 2 PCs from samples taken from the middle and bottom of the biofilter on dates 1, 2 and 3 (MB123; 30 observations) or dates 4 and 5 (MB45; 20 observations).

	Observation Class			
Model	MB123		MB45	
MB123	22	(73 %)	0	(0 %)
MB45	0	(0 %)	16	(80 %)

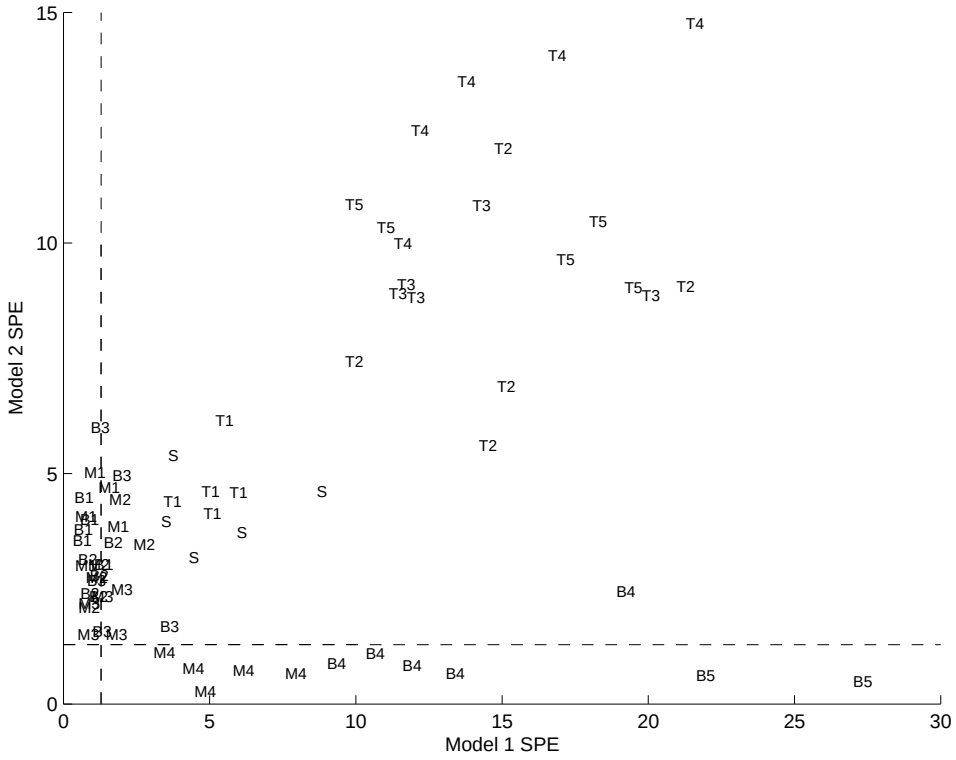


Figure 4.14: Cooman's plot for Experiment 2 (spatial and temporal variation in one biofilter) with classes based on sample date. The SPEs for 2-PC models generated based on samples from the middle and bottom of the biofilter on dates 1, 2 and 3 (MB123; x-axis) or dates 4 and 5 (MB45; y-axis). Dashed lines represent 95 % confidence intervals.

4.2.5 Discussion

The results suggest stratification of the biofilter, with the community at the top of the unit diverging from that at the middle and bottom. Stratification has been observed in previous studies: Khammar *et al.* (2005) found shifts in community structure correlated with biodegradation activity in a biofilter treating a mixture of Volatile Organic Compounds (VOCs); Li *et al.* (2002) and Medina *et al.* (1995) found increased plate counts near the inlet of the bed; and Li and Moe (2004) found stratification over the long-term (hundreds of days) noting more differences in species relative abundance (size of population) rather than richness (number of species). Friedrich *et al.* (2003) found no vertical stratification in the community structure despite the portion nearest the inlet being more active in the degradation of some compounds. In this study, the biofilter is significantly under-loaded (mean EC below 70 g VOC m^{-3} h^{-1}) and the majority of ethanol removal is achieved near the inlet to the bed (see Figure 4.8). The divergence of the community is presumably attributable to acclimation to ethanol occurring only in the upper part of the bed. This is consistent with the findings of the structural study by Khammar *et al.* (2005) since biodegradation activity occurs only in the first half of the bed.

There is a shift in the community in the portion of the biofilter near the inlet (and so exposed to significant ethanol loading) after between 10 and 21 days of operation; this is discussed further in Section 4.6.1 (Page 104).

Following about 40 days of operation, the community in the middle and bottom of the unit undergoes a significant shift. This may be due to starvation or various hidden environmental factors (changes in moisture and so on). This raises questions about the affect of sustained periods of shut-down. Previous studies (for example, Zhang, 2000) have found biofilters able to restart quickly after a period of non-use (up to 30 days in the cited study). Here, a sudden change in the community is seen following approximately 40 days without VOC loading (note that the community here has never experienced VOC loading). An opportunity exists for studies into changes in the community during extended periods of shut-down. Specifically, it is possible that such shifts may have an impact on the length of adaptation period (if any) required on restart and on the long-term viability of biofilter operation.

4.3 Experiment 3 – Acclimation of Parallel Biofilters

4.3.1 Rationale

Most previous studies have examined the community in a biofilter at one location. Stratification of the community has been observed but little is known about the variation of the community at the same depth but distant horizontal points within the unit. Noting the large scale of operating units, two biofilters were operated in parallel under similar conditions to simulate locations far enough apart to be considered isolated in a full-scale installation.

4.3.2 Biofilter Operation

Two small biofilters were operated with an inlet VOC concentration of 440 ppm ($\pm$ 100), giving a loading of 80 g VOC m^{-3} h^{-1} ($\pm$ 20). After 10 days of operation, the DREs for units A and B were 87 % ($\pm$ 28) and 82 % ($\pm$ 27) giving ECs of approximately 70 g VOC m^{-3} h^{-1}; at this point the experiment was stopped and samples for CLPP taken near the inlet of the bed.

4.3.3 PCA

PCA was performed on the data using the mean values of the three on-plate C-source replicates. The time selected for analysis, determined by the method described in Section 3.5.2 (Page 52), was 16 h. The first 2 PCs, accounting for 85 % of the total variance, were chosen for analysis from a scree plot (Figure 4.15). PC scores for the first two principal components are plotted in Figure 4.16 and bar graphs of T^2 (Figure 4.17a) and SPE (Figure 4.17b). Examination of the score plot shows the data grouped within each class with the first PC distinguishing between the two biofilters. One sample from biofilter B falls outside the 95 % confidence interval based on Hotelling's T^2 (also see Figure 4.17a). A second observation from unit B falls outside the 95 % confidence interval defined by the SPE (Figure 4.17b). These two observations may indicate a higher variability in the samples taken in that unit.

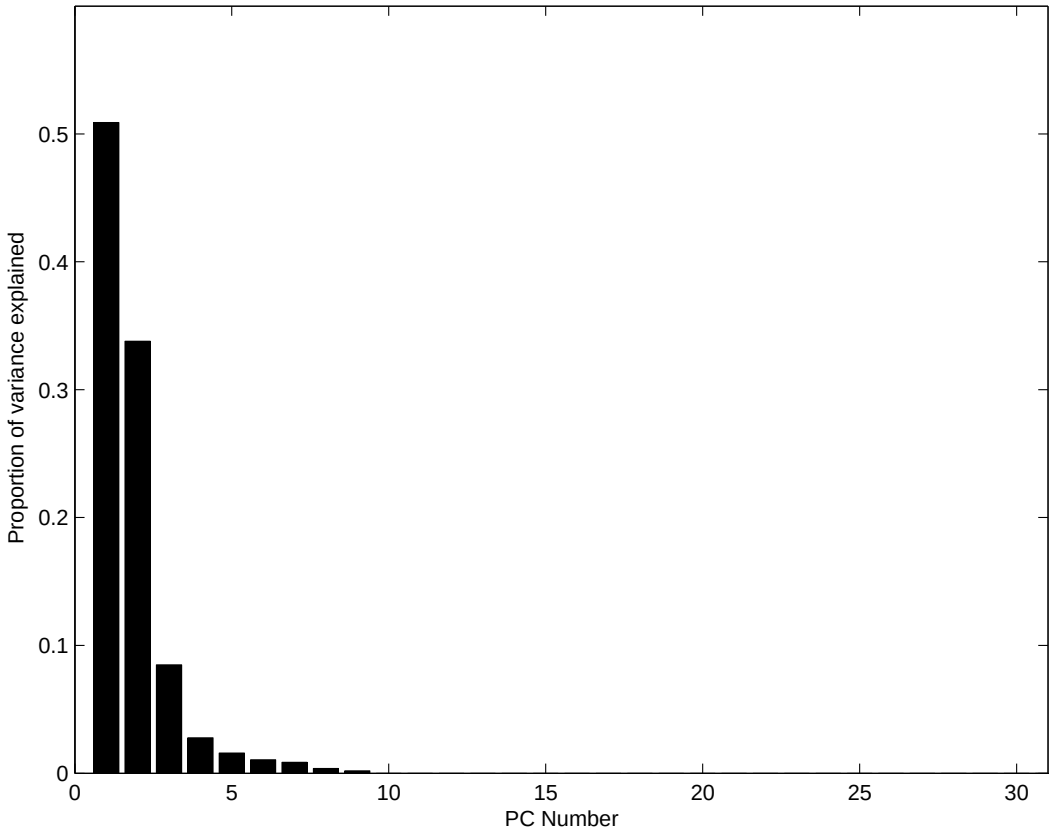


Figure 4.15: Scree plot for Experiment 3 (acclimation of parallel biofilters). The first 2 PCs account for 85 % of the variance in the data set.

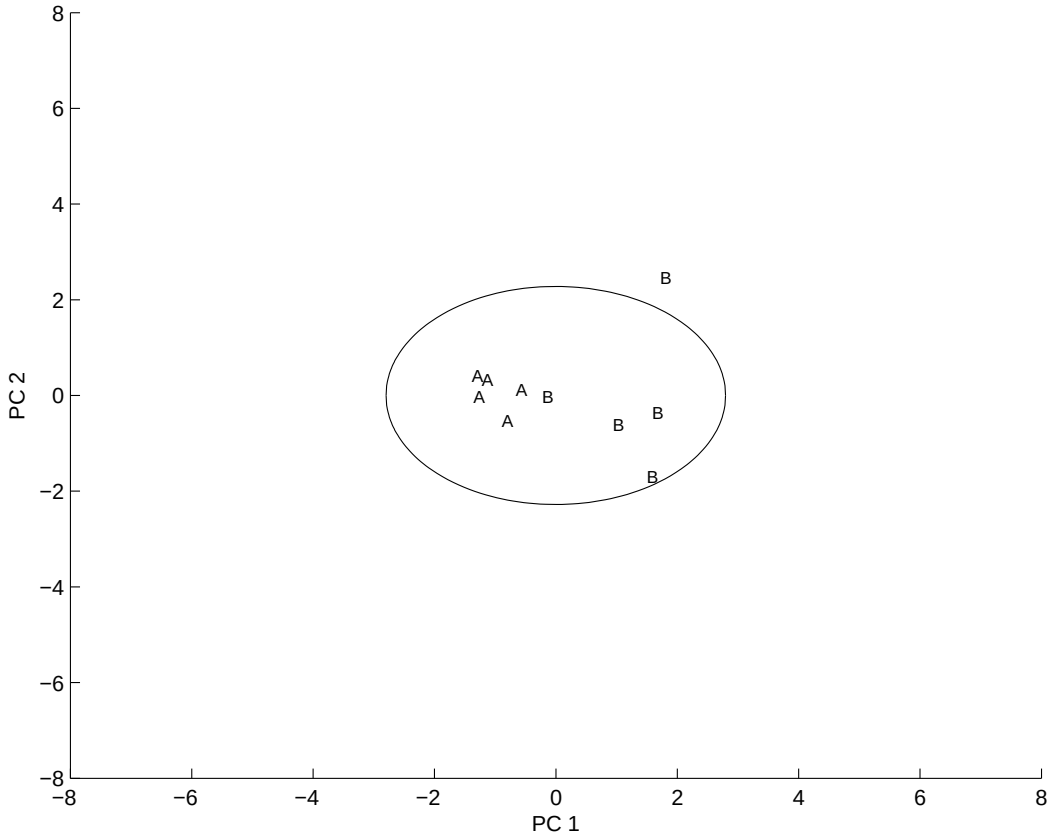
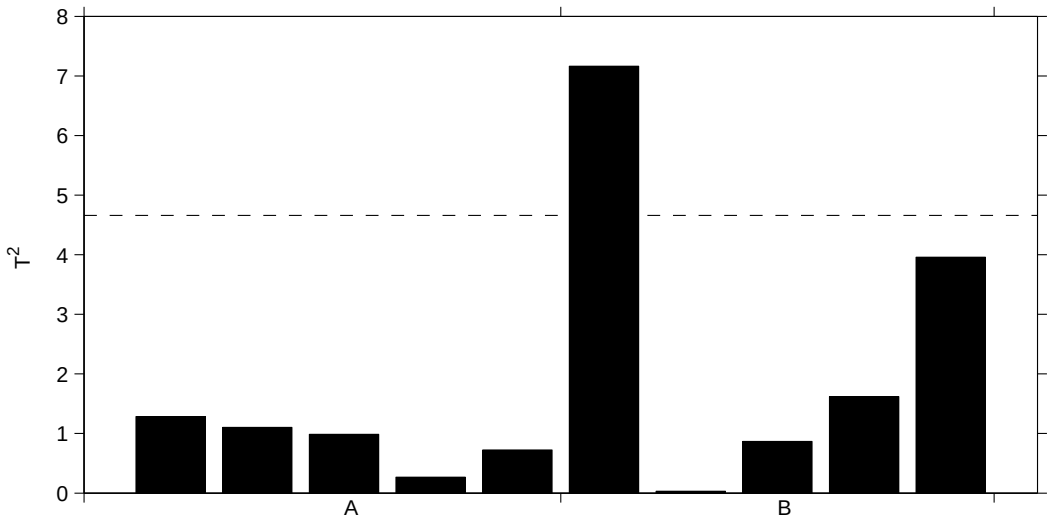
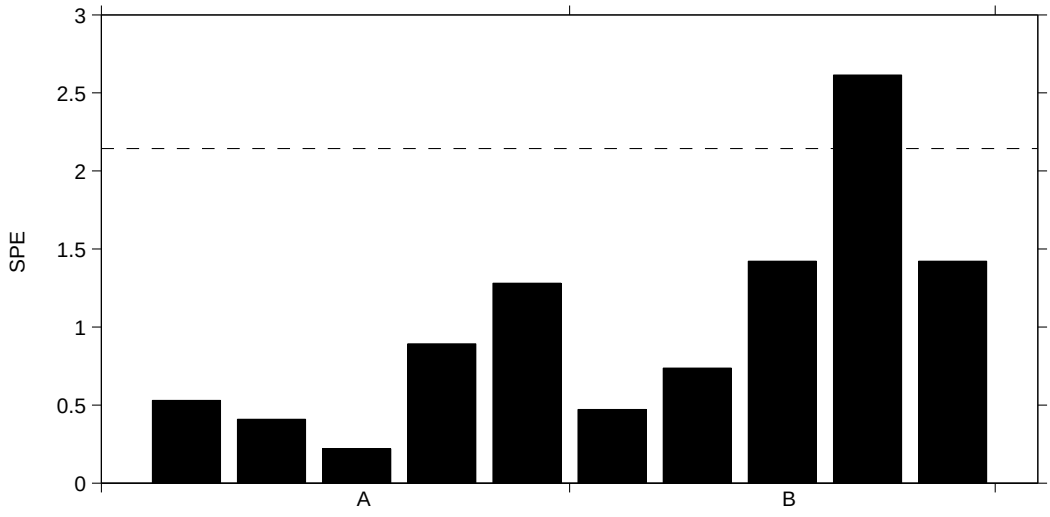


Figure 4.16: Score plot for Experiment 3 (acclimation of parallel biofilters). The ellipse represents the 95 % confidence interval based on Hotelling's T^2 statistic for the model generated by 2 PCs. Observations are from biofilter A or B as indicated.



(a) Bar graph of Hotelling's T^2



(b) Bar graph of SPE

Figure 4.17: PCA diagnostic plots for Experiment 3 (acclimation of parallel biofilters). The dashed lines represent 95 % confidence intervals for the model generated by 2 PCs. Observations are from biofilter A or B as indicated.

4.3.4 SIMCA

Class models were developed for each biofilter based on 1 PC (due to the restricted number of observations) using the method described in Section 3.5.2 (Page 53) and Appendix A (Page 136).

Classes Based on the Biofilter Sampled

One PC models were able to account for 55 and 53 % of the total variance for biofilters A and B respectively. The observations from unit A form a tight group, with all observations falling with the 95 % confidence intervals based on Hotelling's T^2 and SPE. The observations from unit B do not form such a tight group (this can be seen in the score plots of the original data) and one observation with high leverage was removed. Class memberships are shown in Table 4.4.

Table 4.4: SIMCA classifications for Experiment 3 (acclimation of parallel biofilters) based on biofilter sampled. The number of observations classified as belonging to the model developed using 1 PC from biofilters A and B. Class A has 5 observations; class B has 4 observations and class X is an outlier removed from class B.

Model	Observation Class A		Observation Class B		Observation Class X	
A	4	(80 %)	0	(0 %)	0	(0 %)
B	5	(100 %)	4	(100 %)	0	(0 %)

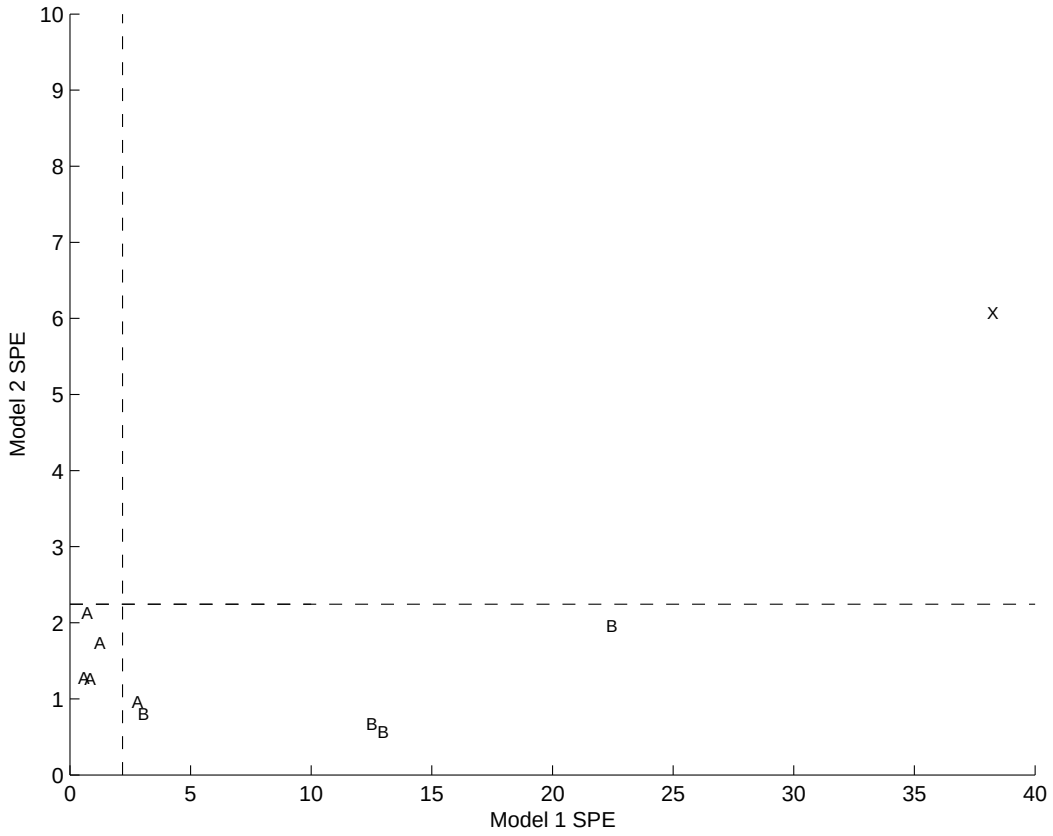


Figure 4.18: Cooman's plot for Experiment 3 (acclimation of parallel biofilters) with classes based on biofilter sampled. The SPEs for 1-PC models generated based on samples from biofilters A (x-axis) and B (y-axis) of the biofilter are plotted. The observation marked X is from biofilter B but was excluded from the class model due to high leverage (it is an outlier and does not fit the model). Dashed lines are 95 % confidence intervals.

4.3.5 Discussion

Based on the overall PCA of all data or developing PC-models from each of the biofilters, it is possible to distinguish between the two units. This suggests that the potential functional diversities of the communities have diverged.

SIMCA classifications for the model generated by biofilter A support this interpretation but those based on the model for biofilter B do not. There is an increased variability in the observations of biofilter B (illustrated by the overall score plot); this variability contributes to the difficulties in the classification analysis. The reason for this increased variability between samples cannot be ascertained: both biofilters were packed with similar media and subject to similar conditions; it may be due to the community in that biofilter undergoing a change, experimental error (such as contamination of some samples) or some other reason. SIMCA analysis proceeds on the basis that the class can be distinguished by some underlying variance structure; the data for biofilter B do not appear to support this requirement and so the class model must be rejected.

Differences in the community are found based on visual interpretation of the score plot (Figure 4.16) and the class model developed from biofilter A; this is discussed further in Section 4.6.2 on Page 105.

4.4 Experiment 4 – Spatial and Temporal Variations in Parallel Biofilters

4.4.1 Rationale

This experiment is a continuation of Experiment 3, investigating the development of the microbial community in two biofilters running in parallel over time.

4.4.2 Biofilter Operation

Two of the large biofilters were used in this study; ethanol concentrations at the inlet and outlet are shown in Figure 4.19; the biofilters were operated for the first 14 days without ethanol loading (humidified air only). EC varied between 50 and 120 g VOC m^{-3} h^{-1} (mean of 100 g VOC m^{-3} h^{-1}). Both biofilters were subject to the same inlet concentration and air flowrates. DREs close to 100 % were achieved in both units from start-up. The pH of the media, measured near the centre of the column, declined from 7.2 to 6.9 over the course of the experiment.

4.4.3 PCA

PCA was performed on the data using the mean values of the three C-source replicates. The time selected for analysis, determined by the method described in Section 3.5.2 (Page 52), was 36 h; this is significantly different to the time chosen for the other experiments (16 h). It was observed that the media used in this experiment (peat moss) disintegrated more readily on shaking than media used in the other experiments (compost or peat moss from a different supplier), resulting in the need for increased dilution (by approximately 50 %) to control the Optical Density (OD) close to 0.2 prior to inoculation of the EcoPlates™; this may have resulted in the reduction of the initial cell density in the inoculum, which has been shown to increase lag time before colour development (Garland and Mills, 1991; Haack *et al.*, 1995).

The first 3 PCs, accounting for 63 % of the total variance, were chosen for analysis from a scree plot (Figure 4.20). PC scores for the first two principal components are plotted in Figure 4.21 and bar graphs of T^2 (Figure 4.22a) and SPE (Figure 4.22b). Examining the score and Hotelling's T^2 plots, it is not possible to differentiate the data based on location (top or bottom) or unit (A or B). The first PC appears to separate the data based on time, differentiating samples 1 and 2 (days 14 and 26) from samples 3 and 4 (days 40 and 54).

Five observations fall outside the 95 % confidence interval but this is expected in a data set of this size ($N = 80$, so about 4 observations are expected to fall outside the confidence limits). A larger number of observations fall outside the 95 % confidence limit for SPE, in particular from the third sample point (day 40); this is coincident with the

time of the change in the community structure indicated by the score plot (Figure4.21) and may represent a higher degree of variation in the community while it is undergoing some sort of reorganisation.

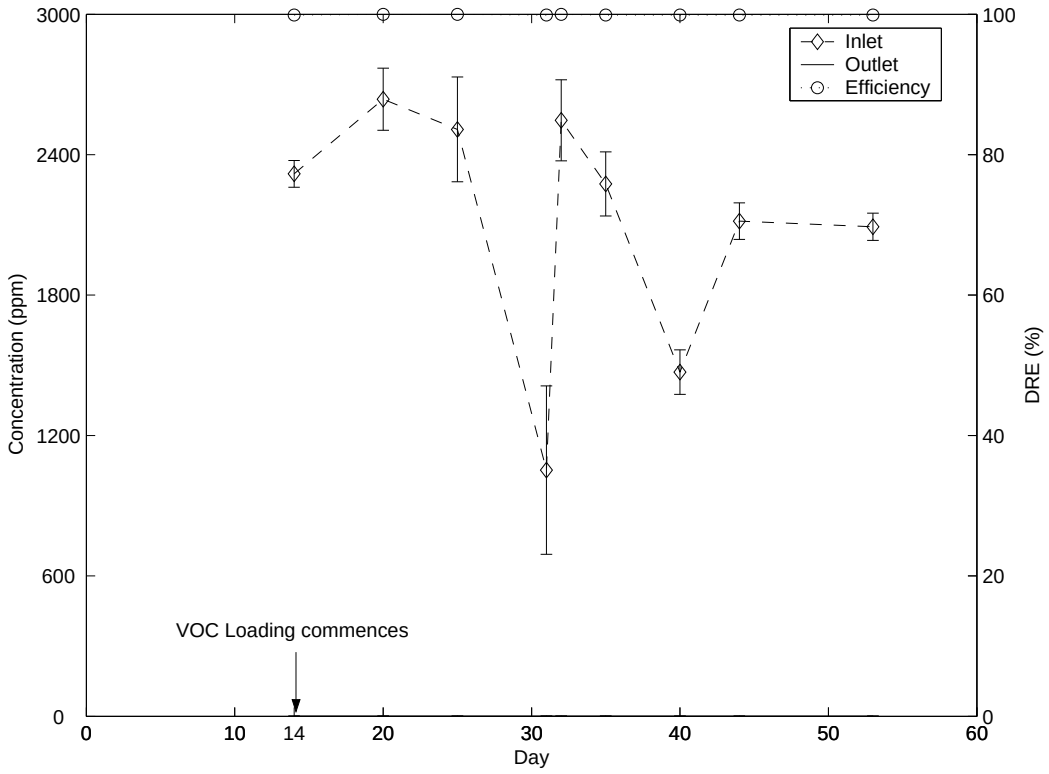


Figure 4.19: VOC concentrations at inlet and outlet and DRE during Experiment 4 (spatial and temporal variations in parallel biofilters). Outlet VOC concentrations were below the detection threshold. Error bars indicate 95 % confidence intervals based on triplicate samples. For the first 14 days, humidified air without ethanol was flowed through the biofilter.

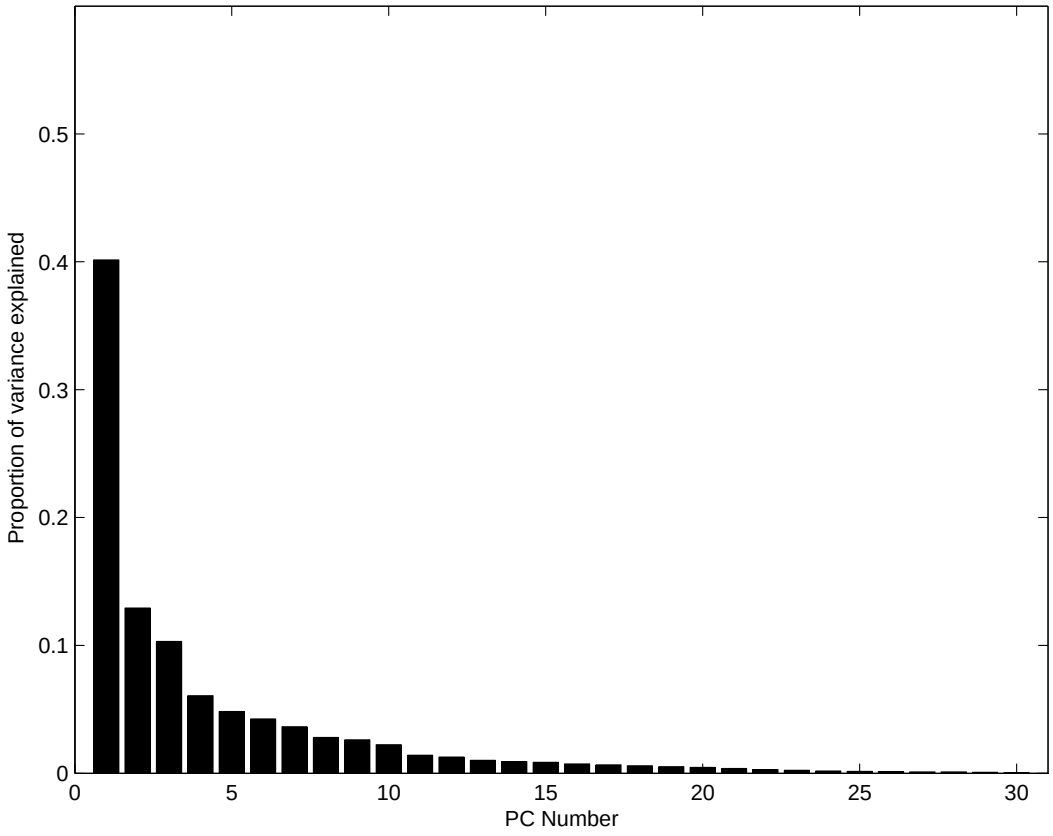


Figure 4.20: Scree plot for Experiment 4 (spatial and temporal variations in parallel biofilters). The first 3 PCs account for 63 % of the variance in the data set.

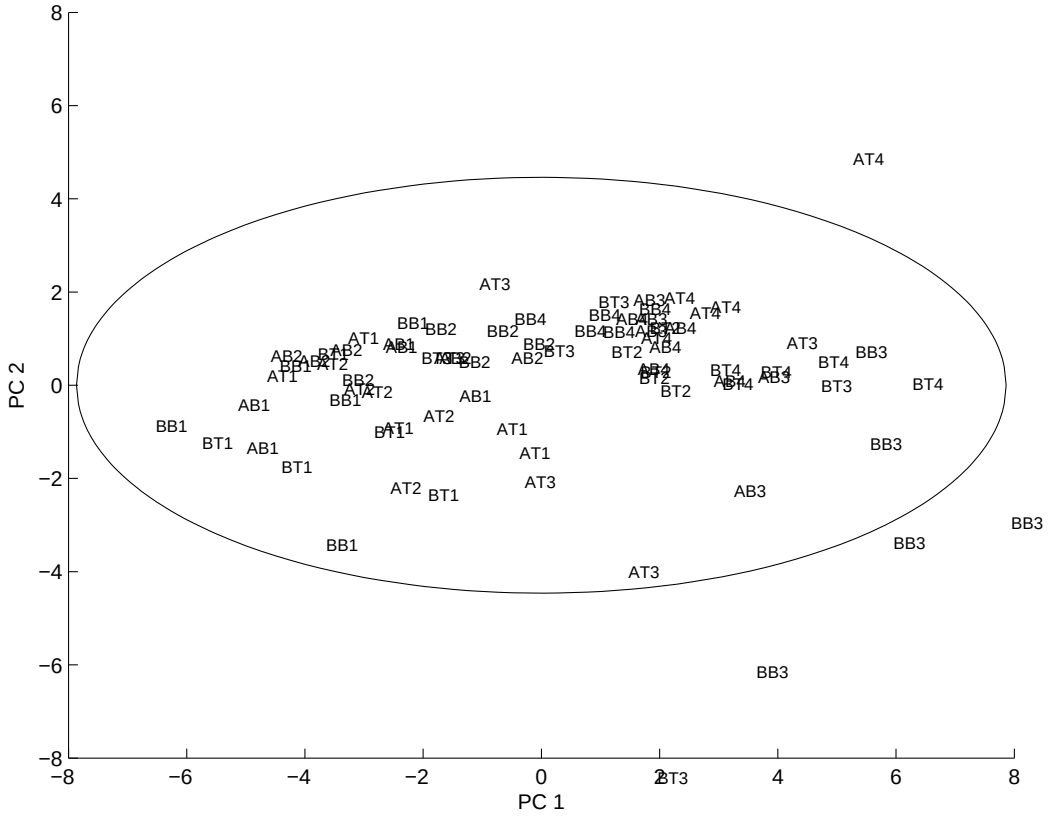
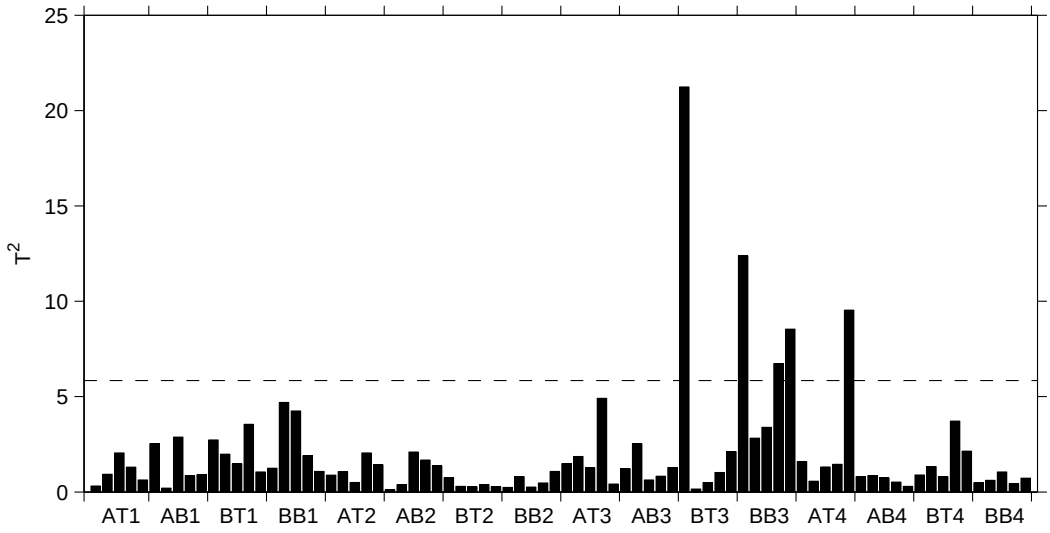
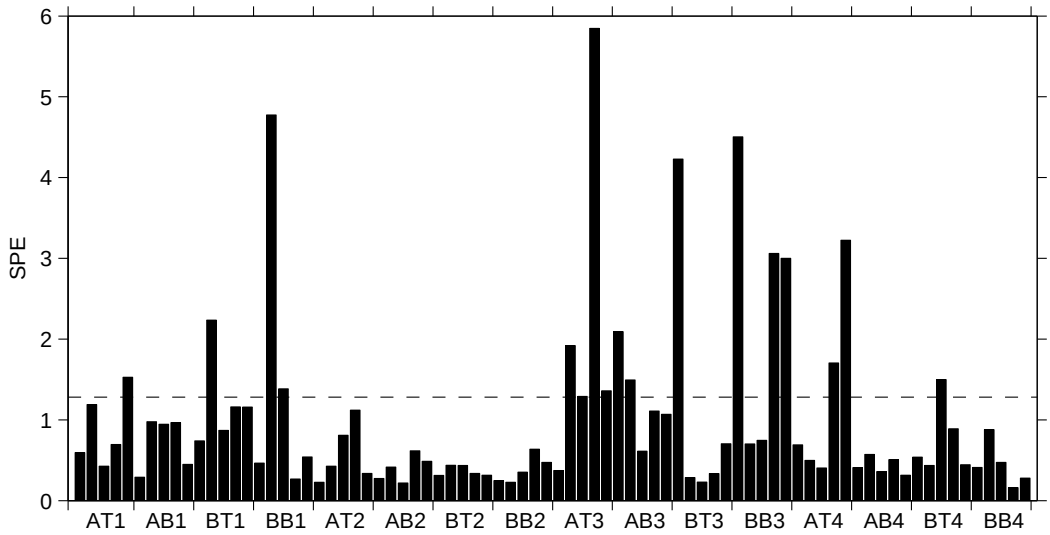


Figure 4.21: Score plot for Experiment 4 (spatial and temporal variations in parallel biofilters). The ellipse represents the 95 % confidence interval based on Hotelling's T^2 statistic for the model generated by 3 PCs (projected into the plane shown). Observation labels indicate the biofilter (A or B), location (top – T or bottom – B) and the day sampled (1, 2, 3 and 4 for days 14, 26, 40 and 54 respectively). Ethanol loading commenced after sample 1 was taken (day 14).



(a) Bar graph of Hotelling's T^2



(b) Bar graph of SPE

Figure 4.22: PCA diagnostic plots for Experiment 4 (spatial and temporal variations in parallel biofilters). The dashed lines represent 95 % confidence intervals for the model generated by 3 PCs. Observations labels indicate the biofilter (A or B), location (top – T or bottom – B) and the day sampled (1, 2, 3 and 4 for days 14, 26, 40 and 54 respectively). Ethanol loading commenced after sample 1 was taken (day 14).

4.4.4 SIMCA

Class models were developed for the following groups using the method described in Section 3.5.2 (Page 53) and Appendix A (Page 136):

- Biofilter: A *vs.* B;
- Location: top T *vs.* bottom B; and,
- Date: samples 1 and 2 (days 14 and 26) *vs.* samples 3 and 4 (days 40 and 54).

Classes Based on the Biofilter Sampled

Three PCs, accounting for 63 % (A) and 72 % (B) of the variance, were retained in the models. Score plots and Hotelling's T^2 statistic do not show the data separating into classes (data not shown). A Cooman's plot of the model SPEs is shown (Figure 4.23). The majority of observations fall within the bottom left quadrant of the Cooman's plot, indicating membership of both classes. A number of observations poorly described by both classes (upper right quadrant); these observations do not belong predominantly to any one group of observations and their presence is attributed to random variation in the observations. Class memberships are shown in Table 4.5.

Table 4.5: SIMCA classifications for Experiment 4 (spatial and temporal variations in parallel biofilters) based on biofilter sampled. The number of observations (out of 40) classified as belonging to the model developed using 3 PCs from biofilters A and B.

	Observation Class			
Model	A		B	
A	28	(70 %)	23	(58 %)
B	24	(60 %)	33	(83 %)

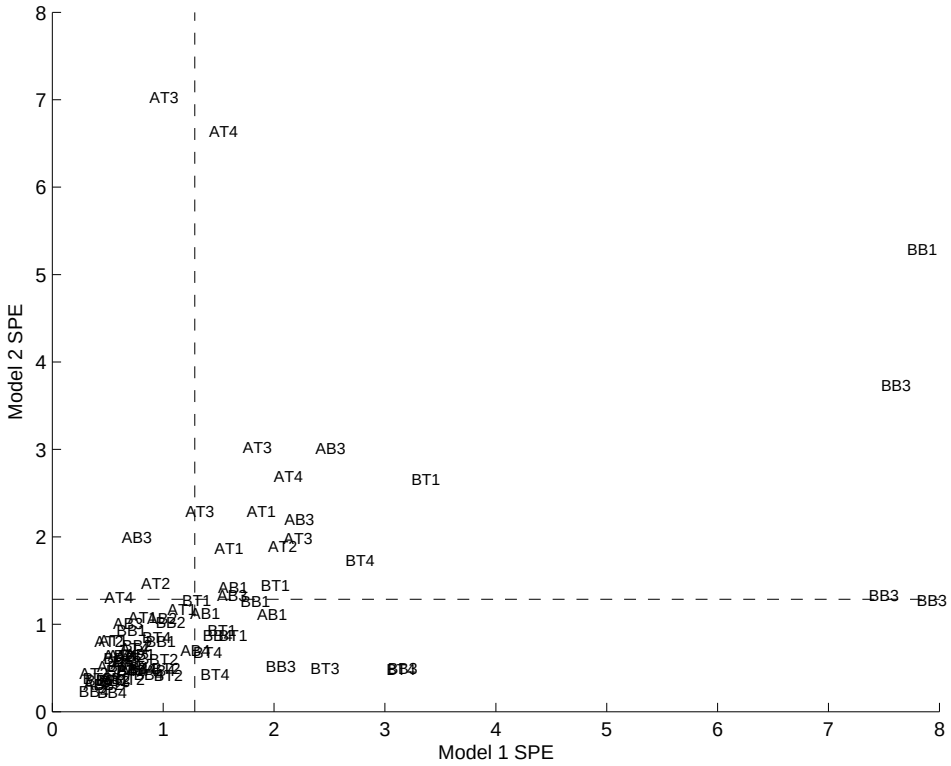


Figure 4.23: Cooman's plot for Experiment 4 (spatial and temporal variations in parallel biofilters) with classes based on biofilter sampled. The SPEs for 3-PC models generated based on biofilters A (x-axis) and B (y-axis) are plotted. Dashed lines are 95 % confidence intervals.

Classes Based on the Sample Location

Three PCs, accounting for 67 % (T) and 74 % (B) of the variance, were retained in the models. Score plots and Hotelling's T^2 statistic do not show the data separating into classes (data not shown). A Cooman's plot of the model SPEs is shown (Figure 4.24). The majority of observations fall within the bottom left quadrant of the Cooman's plot, indicating membership of both classes. Class memberships are shown in Table 4.6.

Table 4.6: SIMCA classifications for Experiment 4 (spatial and temporal variations in parallel biofilters) based on sample location. The number of observations (out of 40) classified as belonging to the model developed using 3 PCs from the top (T) and bottom (B).

	Observation Class			
Model	T		B	
T	30	(75 %)	32	(80 %)
B	26	(65 %)	34	(85 %)

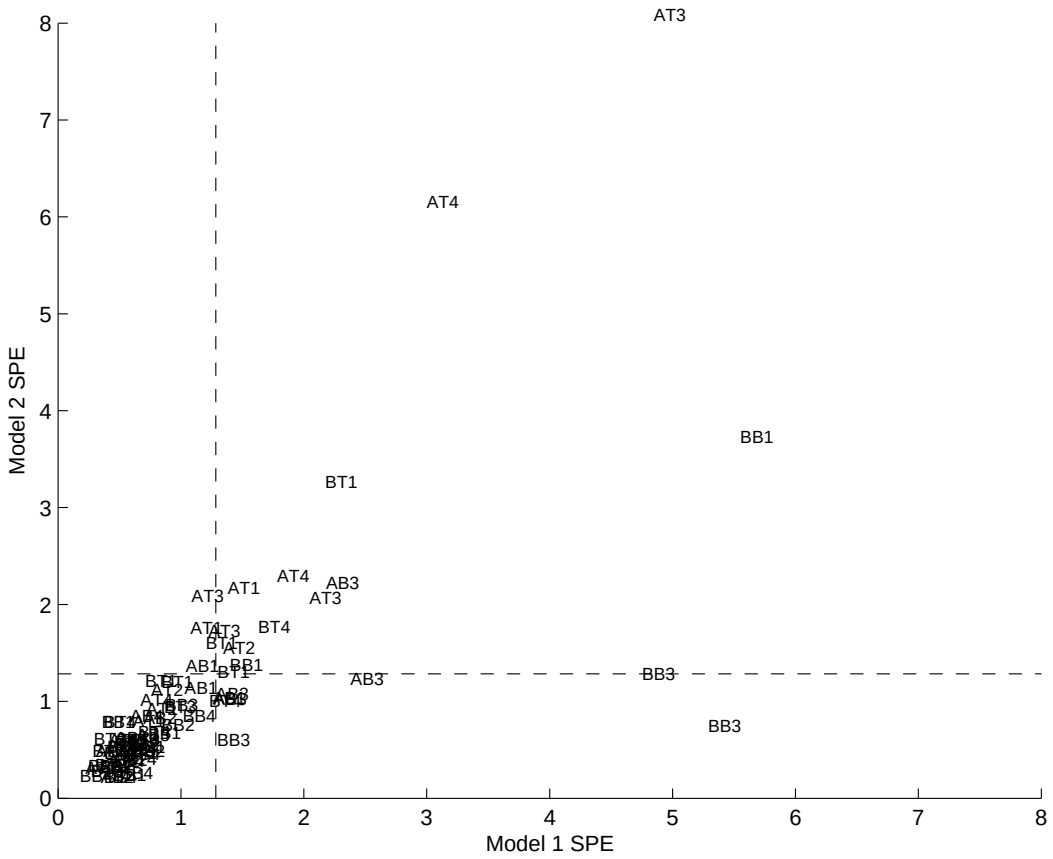


Figure 4.24: Cooman's plot for Experiment 4 (spatial and temporal variations in parallel biofilters) with classes based on sample location. The SPEs for 3-PC models generated based on sample location T(op) (x-axis) and B(ottom) (y-axis) are plotted. Dashed lines are 95 % confidence intervals.

Classes Based on the Sample Date

Four PCs, accounting for 72 % (samples 1 and 2) and 71 % (samples 3 and 4) of the variance, were retained in the models. A Cooman's plot of the model SPEs is shown (Figure 4.25). Observations in either model are mostly classified correctly, while observations not in the model are mostly rejected, *i.e.* the data appears to fall into the two classes as described (these classifications are summarised in Table 4.7).

Table 4.7: SIMCA classifications for Experiment 4 (spatial and temporal variations in parallel biofilters) based on sample date. The number of observations classified as belonging to the model developed using 4 PCs from samples on days 1 & 2 or 3 & 4 based on the observation groups (part (a)) or classes (part (b)).

Model	AT1	AB1	BT1	BB1	AT2	AB2	BT2	BB2	AT3	AB3	BT3	BB3	AT4	AB4	BT4	BB4
1 & 2	1	4	2	4	4	4	5	5	0	0	2	0	0	1	1	2
3 & 4	1	1	0	1	2	2	5	4	1	3	5	4	3	5	4	5

(a) Classification by group of observations (5 per group)

	Observation Class			
Model	1 & 2		3 & 4	
1 & 2	29	(73 %)	6	(15 %)
3 & 4	16[a]	(40 %)	30	(75 %)

[a] 9 of these observations come from groups BT2 and BB2

(b) Classification (%) by class of observation

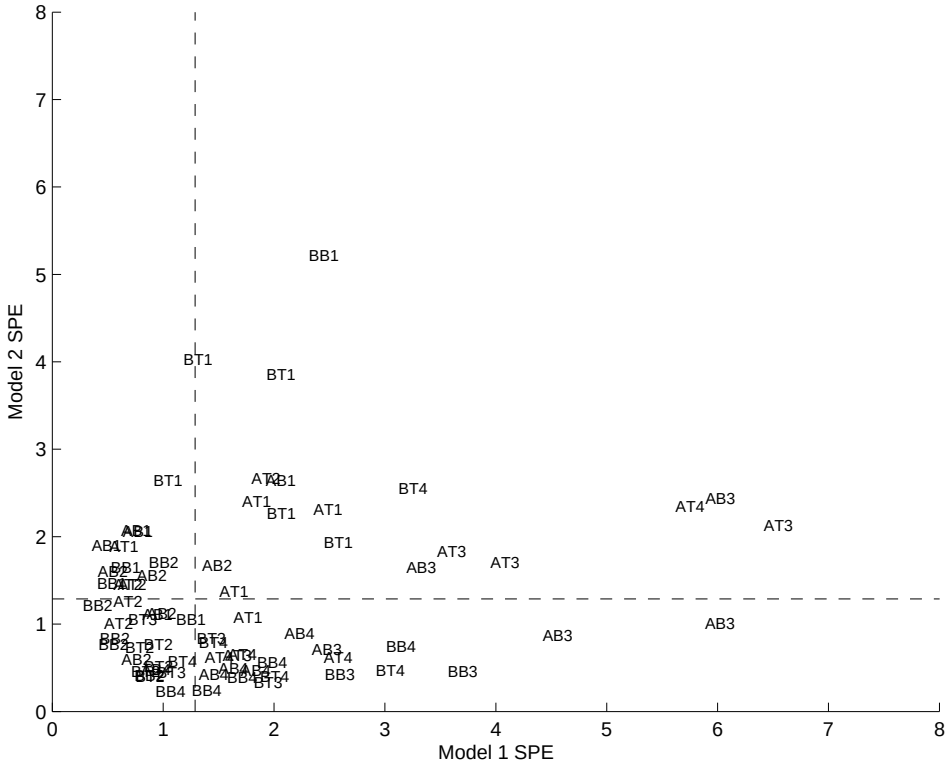


Figure 4.25: Cooman's plot for Experiment 4 (spatial and temporal variations in parallel biofilters) with classes based on sample date. The SPEs for 4-PC models generated based on samples 1 and 2 (x-axis) and samples 3 and 4 (y-axis) are plotted. Dashed lines are 95 % confidence intervals.

4.4.5 Discussion

No acclimation period is observed with respect to DRE. This may be due to biofilm formation during the period that only humidified air was flowing through the unit. This is discussed further in Section 4.6.3.

The data do not indicate differences based on bed height (stratification); this is in contrast to the results found in Experiments 2 (spatial and temporal variation in one biofilter) where stratification was observed. Other studies have found contrasting results: Friedrich *et al.* (2003) found no stratification while Khammar *et al.* (2005) observed stratification correlated with degradation of different components in a VOC mixture. In this experiment, ethanol loading was higher than in Experiment 2 (spatial and temporal variation in one biofilter) (102 *vs.* 70 g VOC m^{-3} h^{-1}); this would cause more of the column to experience VOC loading and it is suggested that no divergence took place due to both sampling locations experiencing ethanol exposure. Li and Moe (2004) found differences in a biofilter treating Methyl Ethyl Ketone (MEK) due to concentration changes through the bed; however, the experiment lasted several hundreds of days providing a longer period for selective pressure to cause the communities to diverge.

Differences in the community between the units were not observed. The data indicate that a change has occurred in the potential functional diversity of the community sometime between 26 and 40 days. It is interesting that the two units appear to have changed in the same manner and over the same timescale. This is in contrast to the results in Experiment 3 (acclimation of parallel biofilters) and is discussed further in Section 4.6.2 on Page 105.

A change in the community is seen with time, summarised in Table 4.7. This change appears to occur sometime after 26 days of operation (including the first 14 days without ethanol loading). Observations from BT2 and BB2 belong to both classes – this perhaps suggests a transition phase – while observations from biofilter A do not fall into the second class until the final sample, perhaps suggesting a similar transition but at a later date. This is discussed further in Section 4.6.1 (Page 104).

4.5 Overall Comparison of Experiments

4.5.1 Rationale

Although each experiment was designed to stand alone, a question arises as to whether this technique can be used to compare different biofilters (for example, operating in different locations, treating different VOCs, *etc.*).

4.5.2 PCA

PCA was performed on the data set based on observations for all experiments at the time chosen for analysis in those experiments (16 h for 1, 2 and 3 and 36 h for 4). It should be possible to compare results from different times because of normalisation of the ODs via Average Well Colour Development (AWCD). The method of normalising initial cell densities (based on the OD of the inoculum) is of concern – due to the different media used, it is likely that this is consistent within each experiment but it may not be so between experiments. Again, normalisation of the ODs allows comparisons to be made however the results should be interpreted cautiously.

Scree, score and PCA diagnostic plots are shown in Figures 4.26, 4.27 and 4.28. The first 2 PCs account for 53 % of the total variance. PC 1 appears to account for variance within Experiments 2 and 4. PC 2 separates Experiments 2 and 4. Experiments 1 and 3 are separate from one another but overlap with Experiment 4. The plot of SPE shows that the PCs are not adequate in describing Experiment 1 or many observations from Experiment 4; these therefore have a different underlying variance structure.

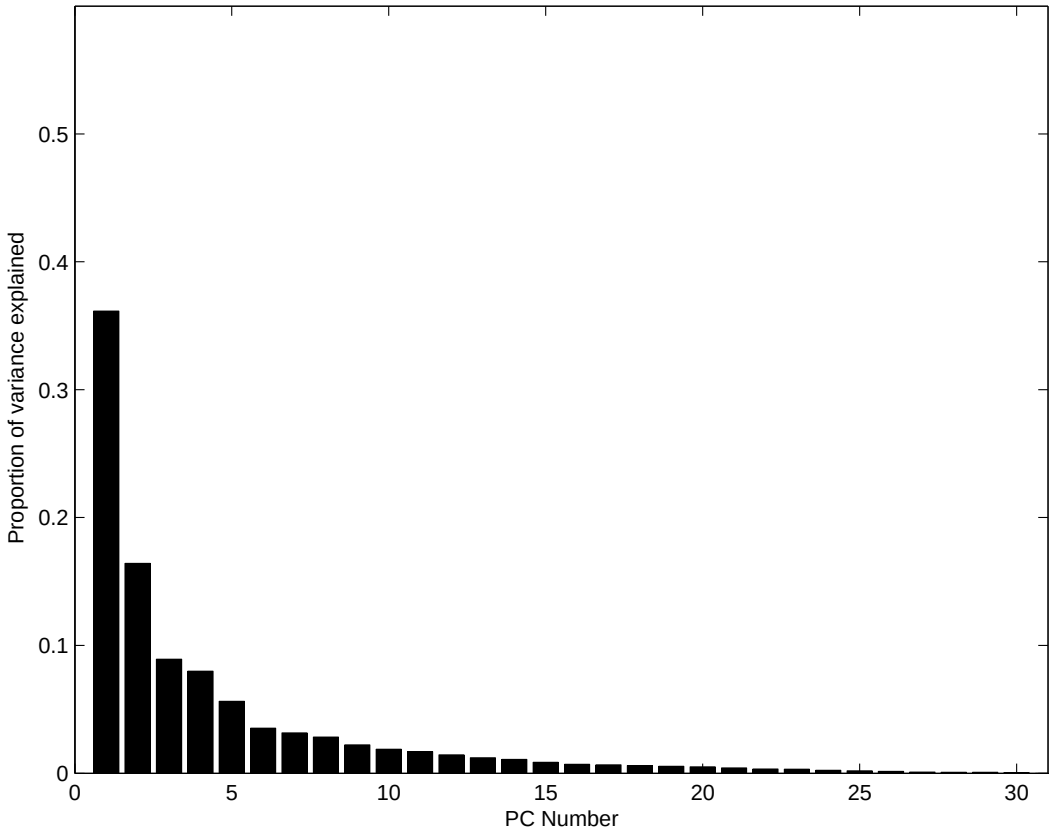


Figure 4.26: Scree plot for all experiments. The first 2 PCs account for 53 % of the variance in the data set.

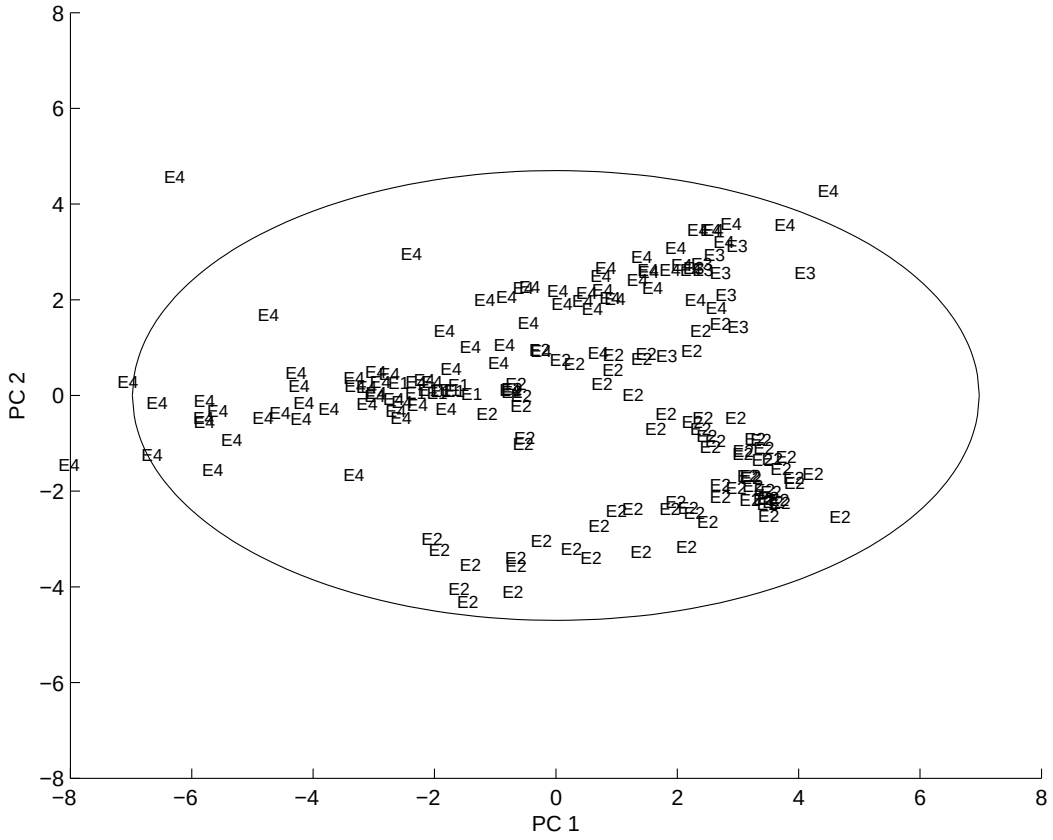
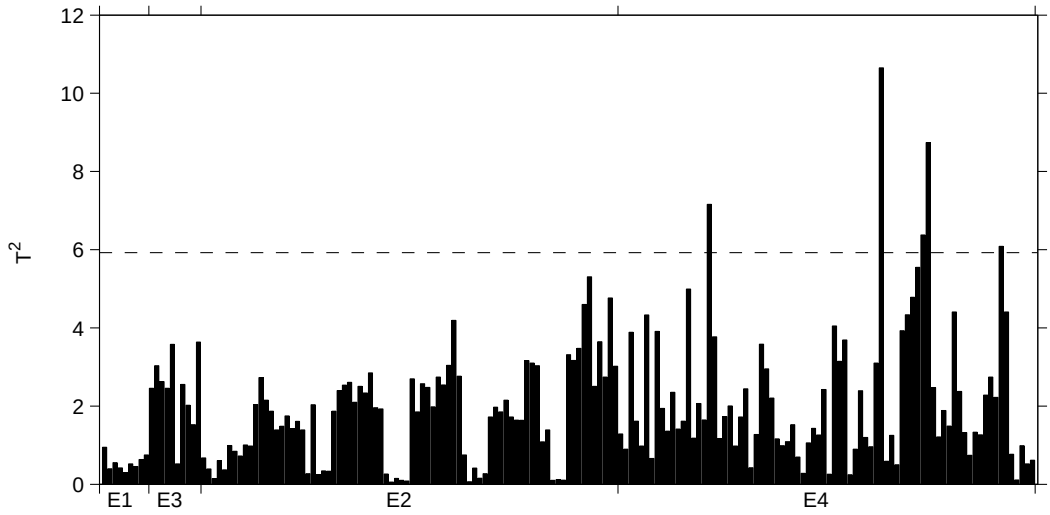
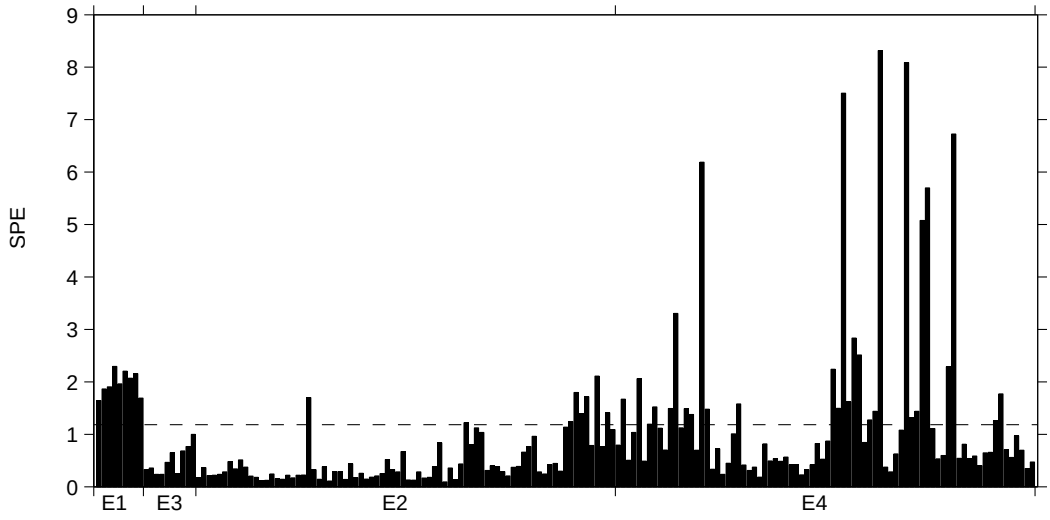


Figure 4.27: Score plot for all experiments. The ellipse represents the 95 % confidence interval based on Hotelling's T^2 statistic for the model generated by 2 PCs. Observations labels indicate the experiment number.



(a) Bar graph of Hotelling's T^2



(b) Bar graph of SPE

Figure 4.28: PCA diagnostic plots for all experiments. The dashed lines represent 95 % confidence intervals for the model generated by 2 PCs. Observations labels indicate the experiment number.

4.5.3 Discussion

Noting that the different types of packing media used in the studies may have caused differences in inoculation densities, it is difficult to draw firm conclusions from these results. Experiment 2 is clearly separated from the others. It is tempting to conclude that this is due to different packing media (wood compost *vs.* peat) or the lower VOC loading, however it may be an artefact due to differences in sample preparation as a result of the different media. Experiments 1 and 3 used the same peat media but are separated. Experiment 4 used a different peat media and overlaps with both Experiments 1 and 3.

Comparison between experiments was not an intention of this study and consequently is difficult; this does illustrate that if comparisons of different biofilters is desired care must be taken to address the influence of packing media type and sample preparation on the observations. The overlap of some groups from different experiments suggests that such studies may be of interest if sample preparation issues are properly addressed.

4.6 Collective Discussion

4.6.1 Variation of Community Structure over Time

In Experiment 2 (spatial and temporal variation in one biofilter) and considering only samples from the top (portion of the biofilter with significant ethanol loading), visual examination of the score plot (Figure 4.10 on Page 72) indicates a shift between samples 1 (day 10) and the remainder (days 21 to 53). Performing SIMCA based on these classifications results in the Cooman's plot shown in Figure 4.13, which supports the visual interpretation. In Experiment 4 (spatial and temporal variations in parallel biofilters) a separation between samples 1 and 2 (days 14 and 26) and samples 3 and 4 (days 4 and 54) is seen from the score plot (Figure 4.21 on Page 91) and supported by the Cooman's plot (Figure 4.25 on Page 98); since ethanol loading commenced after sample 1 was taken (day 14), this suggests a shift in the community between 12 and 26 days after exposure. In both experiments a change in the community is seen in the range between 10 to 26 days after ethanol loading commences.

A number of studies from the literature investigate changes in the community over time: de Castro *et al.* (1997) found variations in the similarity index in a biofilter treating α-pinene over the first 28 days of operation followed by no significant changes over the remaining 110 days of operation; Li and Moe (2004) found long-term changes (hundreds of days) for units degrading MEK; Sakano and Kerkhof (1998) found a reduction in diversity in an ammonia biofilter between samples taken on days 15 and 102; Sercu *et al.* (2004) found gradual changes in the biofilm community of a biofilter degrading reduced sulphur compounds over the first 60 days of operation before stability was reached; Steele *et al.* (2004) found the community in biofilters treating ethanol to have stable evenness and diversity indices to one another and the inoculum over 2 weeks of operation,

followed by a strong divergence between overloaded (which subsequently failed) and non-overloaded units between 2 and 4 weeks; and, Webster *et al.* (1996) observed stability of the microbial community in biofilters degrading VOCs and hydrogen sulphide took hundreds of days but note this was influenced by slowly declining pH. All studies agree that there are variations in the community structure with time. Three biofilters in this study show differences between approximately 10 and 20 days of operation followed by a period of stability of some weeks and changes over a similar period are reported in several of the above studies; presumably, this reflects the timescale over which the biofilm reaches maturity: there is an initial period of biofilm growth followed by a rearrangement of the community in which certain organisms find specific ecological functions and flourish.

4.6.2 Comparison of Units Operating in Parallel

Studies of biofilters operating in parallel under similar conditions are limited. Krailas *et al.* (2000) used identical units and reported differences in total bacteria count at the top of the bed; however, one unit was operating in upflow and the other in downflow so differences may be attributable to VOC availability. Khammar *et al.* (2005) found differences in diversity based on Single-Strand Conformation Polymorphism (SSCP) between two biofilters, but the units were subject to different inocula (different activated sludges). Studies of similar units are of interest because the scale of industrial installations means that distantly separated points within the biofilter may be in effective isolation; therefore, a need exists to establish whether the community at isolated points is similar. The comparison of different biofilters also requires some understanding of differences that may occur in parallel installations. Experiments 3 and 4 were intended to address these issue.

Experiment 3 suggests a divergence in the community between two units over a period of 10 days. Experiment 4 suggests similarities in two units operating over a 14 day period without VOC loading and a further 39 days with VOC loading; during the period of loading a shift in both units was seen at similar times. These results are somewhat contradictory: the results of Experiment 4 demonstrate that it is possible for isolated communities exposed to very similar environmental conditions to maintain similar community profiles over a period of weeks; while, results from Experiment 3 demonstrates that the profiles may diverge over a period of days. It is only possible to speculate about the reason for divergence but possibilities include heterogeneity in the packing, poor environmental control (for example, moisture content or pH) or the accidental introduction of different microorganisms; such perturbations would not be surprising in a full-scale installation. The interaction between microorganisms in a biofilter is complex and includes many species competing for a limited food resource and predator-prey interactions; the dynamic response of such a system to a small perturbation may be chaotic in nature (Kooi *et al.*, 1997). If the system is chaotic, it might be characterised by a set of pseudo-steady states with small perturbations sometimes causing a transition from one

to another; Kaewpipat and Grady Jr. (2002) found similar results with one set of parallel communities diverging and another remaining similar in a study of activated sludge reactors.

This has demonstrated that while isolated communities may develop in a similar manner, this is not necessarily the case. Opportunities for future work exist in investigating the behaviour of similar units exposed to different environmental conditions (such as pH, moisture content and loading) to simulate variations found within operating units. The effect of extreme differences in the biofilter environment were investigated by Steele *et al.* (2004) using parallel studies of biofilters treating ethanol with one overloaded unit to induce acidic by-product accumulation and hence failure (failure appeared to be associated with an increase in diversity). Similar studies where the variations are less extreme (*i.e.* at levels that might be found within an operating unit) may provide insight into changes in the community across industrial biofilters and influence whether biofilters should be envisioned as a single community distributed across the whole area or many, parallel communities behaving independently.

4.6.3 Inoculation and Acclimation

Inoculation of the biofilter with a mixed microbial population was performed in Experiment 2. There was an acclimation period of about 10 days before a DRE close to 100 % was observed. No inoculation was performed in the other experiments and DREs over 80 % were found after 14 days (Experiment 1) or 10 days (Experiment 3). In Experiment 4, the biofilter was operated for 14 days with humidified air only (no ethanol); a DRE close to 100 % was observed immediately when ethanol loading commenced.

One purpose of microbial inoculation is to reduce acclimation time. Studies of biofilters treating ethanol have found variation in the length of the start-up period: Arulneyam and Swaminathan (2000) observed a startup period of 15 days while a biofilm developed in a biofilter packed with compost and polystyrene particles inoculated with a previously-acclimated consortium; Christen *et al.* (2002) and Pérez *et al.* (2002) observed very brief acclimation periods for biofilters inoculated with *Candida utilis*; Cox *et al.* (2001) observed nearly complete removal after a few days in a thermophilic biotrickling filter inoculated with a mixed community obtained from compost; Devinny and Hodge (1995) found inoculation eliminated the initial period of poor DRE for biofilters packed with Granular Activated Carbon (GAC); and, Kiared *et al.* (1996) observed a reduced DRE of around 80 % over the first 6 days using peat as the packing material.

In this work, inoculation of the biofilter with a mixed culture does not appear to have been beneficial in terms of reducing the acclimation time; this is in agreement with the findings of Arulneyam and Swaminathan (2000) and Kiared *et al.* (1996) but contrasts with the experiences of other researchers cited above. In Experiment 4, after operation with humidified air no period of acclimation to ethanol was observed. This suggests that the biomass growth is of most importance during the acclimation period

and the biofilm is able to adapt to ethanol rapidly after exposure. If sufficient biomass is provided through inoculation presumably this period of biomass growth can be reduced or eliminated; this may explain the absence of acclimation periods in the studies cited above. Cioci *et al.* (1997) monitored microorganism concentration during the operation of a pilot-scale study and found a rapid increase over the first 20 days of the experiment to a stable value around 10^8 CFUs g^{-1}. Similarly, Kiared *et al.* (1996) found a rapid increase over the first 6 days of experimentation followed by a decrease to stable values around 10^8 CFUs g^{-1} after 18 days. The 10 to 14 day acclimation periods observed are consistent with these studies.

Note that ethanol is a relatively easy compound to degrade and for more recalcitrant compounds the provision of species able to degrade the compound inoculation may be of more importance; for example, de Castro *et al.* (1997) found reduced acclimation times for biofilters treating α-pinene depending on the inoculum source and Smet *et al.* (1996) found an increase in EC for methyl sulphides following inoculation with an enriched culture.

4.6.4 Applications in Research and Industry

An opportunity exists to develop monitoring methods based on CLPP or other techniques to investigate whether any general characteristics of failing biofilters may be observed; this would provide a chance to intervene in failing units (for example, re-inoculation) prior to failure; a significant difficulty to be overcome is in identifying which shifts are symptomatic of a community in "poor health" and which may be transitions between "healthy" states. This difficulty is exacerbated if, as may be the case, the dynamic response of the community to a perturbation is chaotic. However, it may still be possible to define underlying characteristics indicative of the state of the community; to draw a comparison with another chaotic system – the weather – it is complex, changing, uncontrollable and difficult to predict future values; however, characteristic values of variable that indicate whether or not it is raining *at the moment* can be identified. In the same way, there may be indicators of "good" and "ill" health of the community; if such indicators can be found, they would be invaluable in examining differences in operational performance between biofilters in different locations and in determining the mechanism of biofilter failures. For example, there is evidence (Steele *et al.*, 2004) that a reduction in the community diversity is associated with biofilter failure. Further investigation of operating and failed biofilters is necessary to properly identify such indicators.

CLPP provides a method to monitor the stability of the biofilter community; the bar graphs of Hotelling's T^2 and SPE statistics (for example, Figure 4.22) in fact constitute multivariate control charts able to monitor for changes in potential functional diversity. A shift in the biofilter community may reflect poor control of the operating environment and provide an opportunity for problems to be identified and controlled but the comments about chaotic behaviour above should be noted: it is not clear at present how

to distinguish functional shifts that indicate problems from those that are simply transitions between operating states. Such monitoring may find application in industry for monitoring and control or in the laboratory as a mechanism for studying the timescale and response to environmental perturbations – for example, pH, VOC concentration or temperature variations, drying, change of compounds, period of shutdown – and in identifying the efficacy of inoculation with a mixed community. Such research may lead to changes in industrial practice; for example, can improved methods of culturing the inoculum (or delivering it to the biofilter media) be developed and are there operational practices that help maintain the "health" of the community under different conditions? Questions in this area might include how best to manage a period of shut down (should it be stopped and started slowly or should air still be supplied?), how additional or different compounds should be introduced to the bed and identifying when reinoculation is necessary.

Chapter 5

Conclusions

- This work has demonstrated that Community-Level Physiological Profiling (CLPP) using Carbon Source Utilisation Profile (CSUP) with EcoPlates™ is a sensitive assay for measuring variation and changes in the bacterial community in a biofilter. It was found that the community in a local area (scale of a few centimetres) is similar based on this assay.
- The acclimation period for a biofilter treating ethanol to reach maximum Destruction and Removal Efficiency (DRE) is in the range 7 to 14 days and may be due primarily to biofilm formation rather than adaptation to ethanol. In two units operated without Volatile Organic Compound (VOC) loading for 14 days, near complete removal of ethanol was observed when loading commenced.
- A further acclimation period of a similar length was observed before some stability in the bacterial community was obtained. This may reflect a re-organisation of the community as certain species find specialist roles. Stability of the resulting community was observed over a period of weeks.
- A sudden shift in potential functional diversity after several weeks was observed in a biofilter section with minimal ethanol loading. This may be due to starvation and has implications for the impact of periods of shut-down on the microbial community.
- Stratification of the community may occur and was observed between areas of the bed exposed or not exposed to ethanol; different exposure concentrations did not appear to result in stratification over a period of weeks.
- The community in two separate biofilters operating in parallel was observed to diverge in one experiment. In a second experiment, similarity between the two units was maintained despite changes in both communities over the course of the experiment. This has implications for the reproducibility of laboratory studies and variations with horizontal location in industrial biofilters.

Chapter 6

Recommendations

1. Industrial biofilters may cover large areas. There is little work that has addressed variations in the community horizontally (a number of studies have found vertical stratification). This work has shown two similar units to maintain similar community function over a period of about two months. Industrial units operate for much longer periods and may be subject to significant variations in the conditions of the packing media and environmental variables across the bed. Further work is necessary to determine the similarities and differences in horizontally-separated communities using both field investigations and parallel laboratory studies.

2. The relationship between microorganism community structure and function remains unclear. Studies combining functional and structural approaches may provide a more complete picture of the microbial community.

3. Similarly, the community is not restricted to bacteria but contains a variety of other microorganisms such as fungi and yeasts. Further work is needed to address the role of these different organisms in biofiltration.

4. An interesting question from a practical standpoint is the reasons for biofilter failure. Comparisons of failed and operating biofilters are of interest where opportunities exists. Monitoring of the microorganism community during simulated variations in environmental conditions (loading, pH, moisture content, *etc.*) in the laboratory may contribute to a fundamental understanding of failure in the biofilter community. Such knowledge may direct future work on methods for monitoring the microbial community and suggest strategies for intervention (such as re-inoculation) if symptoms preceding failure can be identified.

5. Inoculation of biofilters with microorganisms from either natural or specialised populations is common and may lead to a reduction in the acclimation period or an improvement in the Elimination Capacity (EC) observed. It is unclear to what

extent introduced species are able to establish themselves as part of the community over the long-term. Investigations with molecular techniques may be able to resolve some of these queries by characterising the media before and after inoculation and the inoculum source. Studies using contaminants that are metabolised only by certain specialised microorganisms are particularly of interest with regard to the ability of introduced species to be established within the native microflora. A comparison of methods used for the preparation of the inoculum (for example, cultivation with various concentrations of the compound to be treated) might be made to try to optimise establishment of species from the inoculum into the biofilter.

6. The microbial community forms a biofilm. The nature of this is fundamental to biofilter function but little information is available about the biofilm and investigations remain difficult. The biofilm is significant because it provides protection from environmental stresses and a matrix through which microbial interactions may occur. Studies of the biofilm are needed to properly understand the mechanism by which a biofilter operates.

7. Biofilters are sometimes subject to periods of non-operation. Studies have shown that units are able to rapidly recover operation (DRE) without long acclimation periods but little information exists about the changes in the microbial community that occur during this time. Such studies may provide an indication of the maximum shut-down period that may be sustained without the need for re-acclimation of the community and suggest strategies for maintaining community function (for example, periodically air flow or nutrient feeding).

8. There are difficulties in comparing biofilters operating under different conditions due to sample preparation issues. Future studies should take care to record relevant information to allow comparisons to be made more easily (for example, heterotrophic plate counts of the EcoPlate™inocula or DNA yields for PCR-based techniques).

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Appendices

Appendix A

Statistical Appendix

A.1 Introduction

This appendix contains a brief overview of the statistical methods used in this thesis; there are many textbooks providing an in-depth treatment of multivariate statistical methods, including the books by Eriksson *et al.* (2001), Jackson (1991) and Martens and Martens (2001).

A.2 Nested Analysis of Variance (ANOVA)

Consider a random variable x with mean μ. The measurement of x is subject to error; in a nested ANOVA, it is assumed that there are several random effects contributing to these errors so that

$$x = \mu + a_a + b_{b(a)} + c_{c(ba)} + \dots \tag{A.1}$$

where each effect $(a, b, c \dots)$ is "nested" within the previous effects. For example, if multiple replicates of an experiment are performed and multiple samples are taken each time then there is an error due to differences between replicates; nested within this is a error due to the sample taken from that replicate and further nested within this are measurement and analytical errors.

Assuming each effect is normally distributed

$$i_i \sim N(0, \sigma_i^2);\ i = \{a, b, c \dots\} \tag{A.2}$$

then the contribution to the variability in x of each level of nesting can be estimated by considering the sum of differences squared between nesting levels in an ANOVA table.

This is instructive because it allows the performance of an F-test at each level that indicates the significance of that level's contribution to the overall variability in the data. This can be used to identify the major source of variability in the data and to focus

efforts to reduce error (for example, if it is determined that most of the variability is due to experimental replicates, there will be relatively little reward for increasing the number of samples from each replicate or number of measurements taken on each sample).

A.3 Principal Component Analysis (PCA) – Mathematical Details

Considering a data matrix $\mathbf{X}$ in which each row $\mathbf{x_i}$ corresponds to an observation i of length K (*i.e.* K variables are being observed),

$$\mathbf{X} = \begin{bmatrix} \mathbf{x_1} \\ \mathbf{x_2} \\ \vdots \\ \mathbf{x_N} \end{bmatrix} \tag{A.3}$$

where K is the number of variables, N is the number of observations and $\mathbf{x_i}$ is given by,

$$\mathbf{x_i} = \begin{bmatrix} x_{i1} & x_{i2} & \dots & x_{iK} \end{bmatrix} \tag{A.4}$$

where x_{ik} is the value of x_k for observation i. Principal Components (PCs) are extracted by solving the eigenvalue-eigenvector problem,

$$\mathbf{\Lambda} = \mathbf{P}'\mathbf{S}\mathbf{P} \tag{A.5}$$

Where $\mathbf{P}$ is a matrix of PCs loadings (the eigenvectors) and the lead diagonal of $\mathbf{\Lambda}$ contains the corresponding eigenvalues (off-diagonal elements are 0) and $\mathbf{S}$ is the covariance matrix [a]:

$$\mathbf{S} = \frac{1}{n-1}\sum_{i=1}^{n}(\mathbf{X_i} - \bar{\mathbf{X}})(\mathbf{X_i} - \bar{\mathbf{X}})' \tag{A.6}$$

where $\bar{\mathbf{X}}$ is the vector of means. PC scores are then given by the solution to

$$\mathbf{T} = \mathbf{P}'(\mathbf{X} - \bar{\mathbf{X}}) \tag{A.7}$$

[a] More commonly, PCs are extracted from the correlation matrix. In general, the scale of the x_i varies and so the scale-invariant correlation matrix is employed to prevent the variables with the largest absolute values from dominating the first PCs; in this work the scale of all x variables is the same and therefore can be regarded as information (this is also the reason that the absolute value of the PCs can exceed unity).

where $\mathbf{T}$ is a matrix of PC *scores* approximating the data ($\mathbf{P}$ is a matrix of *loadings* describing the influence of the original X-variables on the T-scores).

Geometrically, this corresponds to a rotation of the axes in the original X-space so that the variance is concentrated in the higher PCs. The relative contribution to explaining the variance of each PC $\mathbf{P_j}$ is given by $\lambda_j / Tr(\mathbf{\Lambda})$, where λ_j is the eigenvalue corresponding to PC j and Tr is the trace of the matrix (sum of the lead diagonal); these may be plotted in a scree plot.

If p variables are observed, $(p-1)$ PCs may be formed to fully describe the data; an estimate of $\mathbf{X}$ may be formed using a subset of the first A PCs,

$$\mathbf{X} = \mathbf{1} * \bar{\mathbf{X}} + \hat{\mathbf{T}}\hat{\mathbf{P}}' + \mathbf{E} \tag{A.8}$$

$$= \quad \hat{\mathbf{X}} \quad + \mathbf{E} \tag{A.9}$$

where $\hat{\mathbf{T}}$ and $\hat{\mathbf{P}}$ are the first A columns of the corresponding matrices and $\hat{\mathbf{X}}$ is an estimate of $\mathbf{X}$.

Geometrical Interpretation. The loading matrix $\mathbf{P}$ may be interpreted as the maximum variance line ($A = 1$), plane ($A = 2$) or hyperplane ($A > 2$) through the X-space. Euclidean distances may calculated between points in P-space; these correspond to Maholinibis distances in the original X-space.

Diagnostics

Two types of outlier may be identified. This is suggested by the geometrical interpretation above: outliers may be either well or poorly described by the PC model. The former case corresponds to an outlier having the same underlying variance structure; such outliers will be represented within P-space (on the maximum-variance plane) but far from the origin. In the latter case, the underlying variance structure is different and so the observation in X-space is poorly described by the approximation in P-space (*i.e.*, the orthogonal distance from the observation to the maximum-variance plane is large)[b]; these are identified by large residual vectors $\mathbf{e_i}$.

Hotelling's T^2 Statistic. Hotelling's T^2-statistic (the multivariate equivalent to "Student's" t-statistic) gives a measure of the leverage each observation has on the PCs, *i.e.* it is a method of determining which observations have had the greatest influence on the direction of the PCs and may be used to identify outliers of the first type (same underlying factor structure) (Hotelling, 1931). It is calculated as,

$$\mathbf{T^2} = (\mathbf{X} - \bar{\mathbf{X}})\mathbf{S^{-1}}(\mathbf{X} - \bar{\mathbf{X}}) \tag{A.10}$$

[b]These outliers are more common where PC analysis has been performed on a training set of data and a test set of data is being projected into the prototype P-space; if strong outliers exist in the original X-space, these outliers will exert high *leverage* on the PCs (by virtue of their high contribution to the total variance), tending to rotate the PCs axes so causing the outlier to fall into the first category.

Where $\mathbf{T^2}$ is a vector of T^2 statistics corresponding to the observations in in $\mathbf{X}$. Those observations with a high value of T^2 should be examined to check for bad data and, if the observation is significantly affecting the PCs it may be best to note this and remove it from the analysis. Most authors approximate T^2 as either a χ^2 or an F-distribution[c], but Tracy *et al.* (1992) note that it is in fact a beta-distribution multiplied by a constant,

$$T^2 \sim \frac{(N-1)^2}{N} B(\alpha; A/2, (N-A-1)/2) \tag{A.11}$$

Values of the beta distribution may be calculated from values of the F-distribution,

$$B(\alpha; A/2, (N-A-1)/2) = \frac{(A/(N-A-1)) * F(\alpha; A, N-A-1)}{1 + (A/(N-A-1)) * F(\alpha; A, N-A-1)} \tag{A.12}$$

Using the distribution defined above, an ellipsoidal confidence interval for the PC model may be calculated. If $A = 2$, this is an ellipse and may be added to a PC score plot.

Testing of Model Residual. The normal distance of an observation to the model provides a measure of how well the extracted PCs describe that observation; it is therefore a means to identify outliers having a different underlying factor structure. First, the model residuals are calculated,

$$\mathbf{E} = \hat{\mathbf{X}} - \mathbf{X} \tag{A.13}$$

$$= \begin{bmatrix} \mathbf{e_1} \\ \mathbf{e_2} \\ \vdots \\ \mathbf{e_N} \end{bmatrix} \tag{A.14}$$

The normalised Squared Prediction Error (SPE) for each observation is then given by the sum of the squares of the residuals (equivalent to finding the lengths of the PCs not included in the model), normalised by $K - A$.

$$s_i^2 = \frac{\sum_{k=A+1}^{K} e_{ik}^2}{K-A} \tag{A.15}$$

$$= \frac{\sum_{k=A+1}^{K} \mathbf{P'_k}(\mathbf{x_i} - \bar{\mathbf{X}})^\mathbf{2}}{K-A} \tag{A.16}$$

[c]The approximation as a χ^2 distribution is exact where the estimates of the mean vector, $\bar{\mathbf{X}}$, and covariance matrix $\mathbf{S}$ are the true population values μ and $\mathbf{\Sigma}$ respectively; the approximation as an F-distribution is exact where the observation is independent of $\bar{\mathbf{X}}$ and $\mathbf{S}$. Either approximation is appropriate if $N >> K$ but, due to the small sample size available, would here result in a highly conservation confidence interval.

A pooled residual variance, s_0^2, for the model can be calculated from the observation SPEs,

$$s_0^2 = \sum_{i=1}^{N} s_i^2 \tag{A.17}$$

This suggests the F-statistic,

$$F = \frac{s_i^2}{s_0^2} \tag{A.18}$$

which is distributed as $F(\alpha; K - A - 1, (K - A - 1) * N)$ and is test of the probability of the observation belonging to the model.

Diagnostic Plots. Values of Hotelling's T^2 and SPE for each observation, together with the confidence limits, may be conveniently visualised using scatter plots or bar charts, for example Figure 4.3 on Page 61 (observations on the two charts are paired).

Soft Independent Modelling of Class Analogy (SIMCA)

This method may be used as a way to classify observations using the SIMCA scheme, explained in Eriksson *et al.* (2001) and Martens and Martens (2001). PC analysis as described above is performed on a training set of data to formulate a model. This may be used to classify other observations by projecting them on to the model PCs.

Analysis proceeds as described above except that only the observations in the training set are used in forming the PCs. For observations in the training set, the T^2 statistic maintains a beta-distribution as above (see Equation A.11); observations not in the training set are independent of the estimates of the mean vector and covariance matrix used in the model and follow an exact F-distribution:

$$T^2 \sim \frac{A(\tilde{N}^2 - 1)}{\tilde{N}(\tilde{N} - A)} F(\alpha; A, (\tilde{N} - A)) \tag{A.19}$$

where $\tilde{N}$ is the number of observations in the training set. This provides a higher (more conservative) confidence limit (as N increases the two limits will converge).

For the observation SPEs, a correction factor is used on the training set observations to account for the influence of the data point on the model; Equation A.15 becomes,

$$s_i^2 = \frac{\sum_{k=A+1}^{K} e_{ik}^2}{K - A} * v \tag{A.20}$$

For observations not in the training set, v is unity as before; for data in the training

set, v is always greater than unity and is given by,

$$v = \frac{\tilde{N}}{\tilde{N} - A - 1} \tag{A.21}$$

Observations may be classified as members of the training class or not based on the above statistics. Often the SPE values are of most interest; two classes may be conveniently compared by plotting the SPE statistics against one another, together with lines indicating confidence limits, in a Cooman's plot which indicates which observations belong to neither, one or both classes.

Appendix B

Carbon Sources on the Biolog EcoPlate™

Table B.1: Carbon(C)-sources on the Biolog EcoPlate™. Each well contains the C-source listed and a tetrazolium violet indicator dye. The set of 32 wells is repeated 3 times. The C-sources fall into the following categories: amines ($*$), amino acids (†), carbohydrates (‡), carboxylic acids ($**$), polymers (††) and phenolic compounds (‡‡) (Insam, 1997).

Water	β-Methyl-D-Glucoside‡	D-Galactonic Acid γ-Lactone‡	L-Arginine†
Pyruvic Acid Methyl Ester**	D-Xylose‡	D-Galacturonic Acid**	L-Asparagine†
Tween 40††	i-Erythritol‡	2-Hydroxy Benzoic Acid‡‡	L-Phenylalanine†
Tween 80††	D-Mannitol‡	4-Hydroxy Benzoic Acid‡‡	L-Serine†
α-Cyclodextrin††	N-Acetyl-D-Glucosamine‡	γ-Hydroxybutyric Acid**	L-Threonine†
Glycogen††	D-Glucosaminic Acid**	Itaconic Acid**	Glycyl-L-Glutamic Acid†
D-Cellobiose‡	Glucose-1-Phosphate‡	α-Ketobutyric Phosphate**	Phenyl ethylamine*
α-D-Lactose‡	D,L-α-Glycerol Phosphate‡	D-Malic Acid**	Putrescine*

Appendix C

Sample Calculations

C.1 Data Pre-Treatment

Data pretreatment is illustrated by an example plate (replicate plate 1 taken from the top sampling port on day 10 Experiment 2; reading time: 16 h). The Optical Density (OD) readings are shown below; the locations in the matrix correspond with the well locations:

$$\begin{bmatrix} 0.107 & 1.392 & 0.234 & 0.312 & 0.107 & 1.252 & 0.323 & 0.241 & 0.109 & 1.350 & 0.280 & 0.285 \\ 0.633 & 0.361 & 0.443 & 0.926 & 0.738 & 0.323 & 0.534 & 0.896 & 0.738 & 0.329 & 0.568 & 0.851 \\ 0.424 & 0.118 & 0.077 & 0.135 & 0.391 & 0.144 & 0.077 & 0.117 & 0.359 & 0.126 & 0.076 & 0.145 \\ 0.491 & 1.033 & 0.524 & 0.637 & 0.391 & 1.202 & 0.497 & 0.593 & 0.404 & 0.960 & 0.481 & 0.461 \\ 0.560 & 1.510 & 0.840 & 0.108 & 0.568 & 1.522 & 0.686 & 0.108 & 0.590 & 1.699 & 0.931 & 0.104 \\ 1.230 & 0.110 & 0.161 & 0.131 & 1.039 & 0.111 & 0.116 & 0.126 & 1.149 & 0.110 & 0.136 & 0.118 \\ 0.853 & 0.109 & 0.091 & 0.286 & 0.926 & 0.111 & 0.100 & 0.330 & 0.992 & 0.114 & 0.095 & 0.256 \\ 0.615 & 0.459 & 0.288 & 0.689 & 0.756 & 0.441 & 0.297 & 0.951 & 0.712 & 0.437 & 0.282 & 0.544 \end{bmatrix}$$

The mean of the three replicates is taken,

$$\begin{bmatrix} 0.1077 & 0.7030 & 0.3913 & 0.4287 \\ 0.5727 & 1.1393 & 0.9237 & 0.6943 \\ 1.3313 & 0.3377 & 0.1293 & 1.0650 \\ 1.5770 & 0.1103 & 0.1113 & 0.4457 \\ 0.2790 & 0.5150 & 0.0767 & 0.5007 \\ 0.8190 & 0.1377 & 0.0953 & 0.2890 \\ 0.2793 & 0.8910 & 0.1323 & 0.5637 \\ 0.1067 & 0.1250 & 0.2907 & 0.7280 \end{bmatrix}$$

And the value of the water blank subtracted and values below 0 truncated to 0,

$$\begin{bmatrix} & 0.5953 & 0.2837 & 0.3210 \\ 0.4650 & 1.0317 & 0.8160 & 0.5867 \\ 1.2237 & 0.2300 & 0.0217 & 0.9573 \\ 1.4693 & 0.0027 & 0.0037 & 0.3380 \\ 0.1713 & 0.4073 & 0 & 0.3930 \\ 0.7113 & 0.0300 & 0 & 0.1813 \\ 0.1717 & 0.7833 & 0.0247 & 0.4560 \\ 0 & 0.0173 & 0.1830 & 0.6203 \end{bmatrix}$$

The number of wells, $n[t]$ for which corrected OD exceeds 0.1 can then be calculated (see Page 52); here it is 22. The Average Well Colour Development (AWCD) is then calculated (Equation 3.1 on Page 52),

$$AWCD = 0.391$$

And the OD values divided by this to give the corrected OD (according to Equation 3.2 on Page 52),

$$\begin{bmatrix} & 1.4769 & 0.7037 & 0.7963 \\ 1.1535 & 2.5593 & 2.0243 & 1.4554 \\ 3.0356 & 0.5706 & 0.0537 & 2.3749 \\ 3.6450 & 0.0066 & 0.0091 & 0.8385 \\ 0.4250 & 1.0105 & 0 & 0.9749 \\ 1.7646 & 0.0744 & 0 & 0.4498 \\ 0.4259 & 1.9432 & 0.0612 & 1.1312 \\ 0 & 0.0430 & 0.4540 & 1.5389 \end{bmatrix}$$

The 31 values represent one observation and are used for PCA (each observation is one row of the data matrix $\mathbf{X}$).

C.2 Principal Component Analysis (PCA)

For simplicity, PCA will be demonstrated using only 3 of the 31 variables. First, a matrix ($\mathbf{X}$) of observations is formed; each column corresponds to a variable and each row an observation. For this example, the 5 observations are the 5 replicate plates taken from the top sampling port on day 10 of Experiment 2 (reading time: 16 h) and the variables are the 3 with the greatest contribution to the first PC for the analysis of all data from that experiment (wells A2, D1 and E2).

$$\mathbf{X} = \begin{bmatrix} 1.4769 & 3.6450 & 1.0105 \\ 1.2842 & 3.3539 & 1.0195 \\ 1.3521 & 3.5788 & 1.1420 \\ 0.9106 & 4.1699 & 1.0899 \\ 1.4699 & 3.4125 & 1.0358 \end{bmatrix}$$

First, the data are mean-centred,

$$\mathbf{X} - \bar{\mathbf{X}} = \begin{bmatrix} 0.1781 & 0.0130 & -0.0490 \\ -0.0145 & -0.2781 & -0.0400 \\ 0.0534 & -0.0532 & 0.0825 \\ -0.3881 & 0.5379 & 0.0304 \\ 0.1712 & -0.2195 & -0.0237 \end{bmatrix}$$

And the covariance matrix ($\mathbf{S}$) is calculated[a] (Equation A.6 on Page 133),

$$\mathbf{S} = \begin{bmatrix} 0.0537 & -0.0607 & -0.0049 \\ -0.0607 & 0.1045 & 0.0069 \\ -0.0049 & 0.0069 & 0.0031 \end{bmatrix}$$

PCs are then calculated by an eigenvalue-eigenvector decomposition (Equation A.5 on Page 133),

$$\mathbf{\Lambda} = \begin{bmatrix} 0.1454 & 0.0133 & 0.0026 \end{bmatrix}; \mathbf{P} = \begin{bmatrix} -0.5532 & -0.8314 & -0.0522 \\ 0.8309 & -0.5552 & 0.0366 \\ 0.0594 & 0.0231 & -0.9980 \end{bmatrix}$$

Where $\mathbf{\Lambda}$ is a vector of eigenvalues representing the amount of variance explained by the corresponding PC given as a set of loadings in $\mathbf{P}$. The set of PC scores is then given by projecting the mean-centred data ($\mathbf{X} - \bar{\mathbf{X}}$) into the PC space (*i.e.* the PCs scores (in $\mathbf{T}$) are a projection of the data in P-space, as suggested by Equation A.7 on Page 133),

$$\mathbf{T} = \mathbf{P}'(\mathbf{X} - \bar{\mathbf{X}}) = \begin{bmatrix} -0.0401 & -0.1564 & 0.0906 \\ -0.0305 & 0.1656 & 0.2255 \\ 0.0870 & -0.0129 & 0.0689 \\ -0.0096 & 0.0248 & -0.6635 \\ -0.0067 & -0.0210 & 0.2785 \end{bmatrix}$$

The results are illustrated in Figure C.1. Note that the data are described entirely by the 3 PCs – the analysis has simply rotated the axes of the mean-centred data where the loading matrix ($\mathbf{P}$) describes the rotation.

Hotelling's T^2 statistic can then be calculated (see Equation A.10 on Page 134) to give a measure of the leverage of each observation – this is a measure of the distance from the observation to the origin in the PC-space. If SIMCA is performed, the data are projected into a subset of the PC-space. The 2-PC projection is illustrated in Figure C.2; Hotelling's T^2 for each observation is a measure of the lengths of the lines shown. The 95 % confidence interval would be an ellipse on this plot but, due to the restricted number of observations in the example, is much larger than the values on the plot. The projection is an approximation of the data and results in a residual error; this is illustrated in Figure C.3. The 95 % confidence interval is again not shown due to scale but would be two planes parallel to and equidistant from the model plane shown.

[a]The calculation of $\mathbf{S}$ uses the mean-centred $\mathbf{X} - \bar{\mathbf{X}}$ in any case.

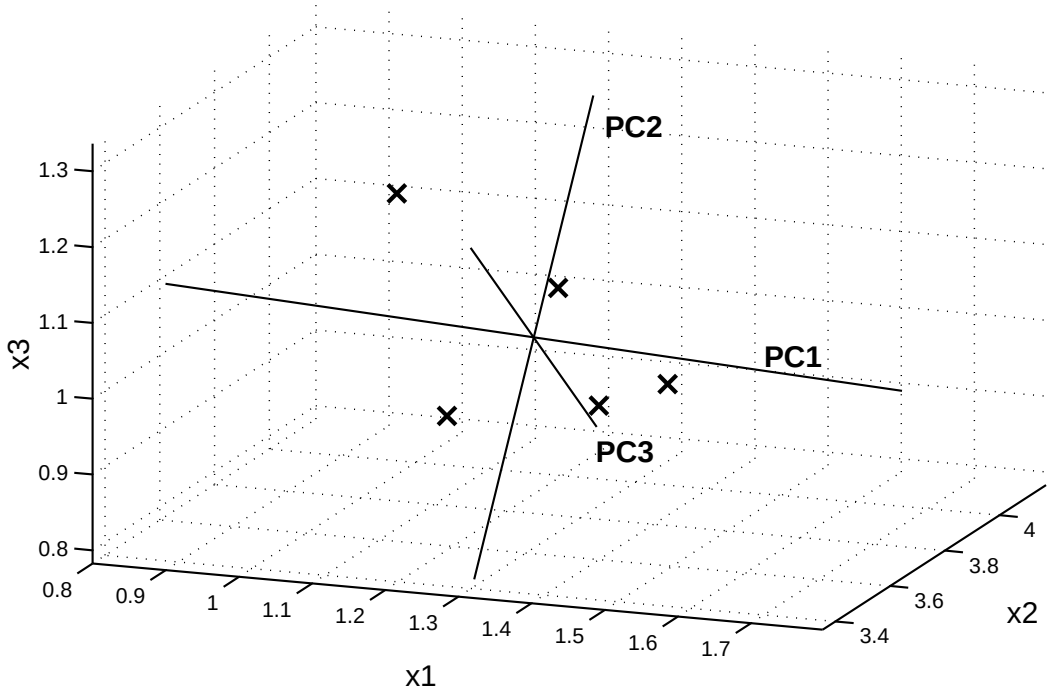

Figure C.1: Plot to illustrate sample data. The main axes represent the original x-variables. PC axes are superimposed. Note the first PC axis is in the direction of highest variance, all PC axes are orthogonal and the origin of the PC-space is at the mean.

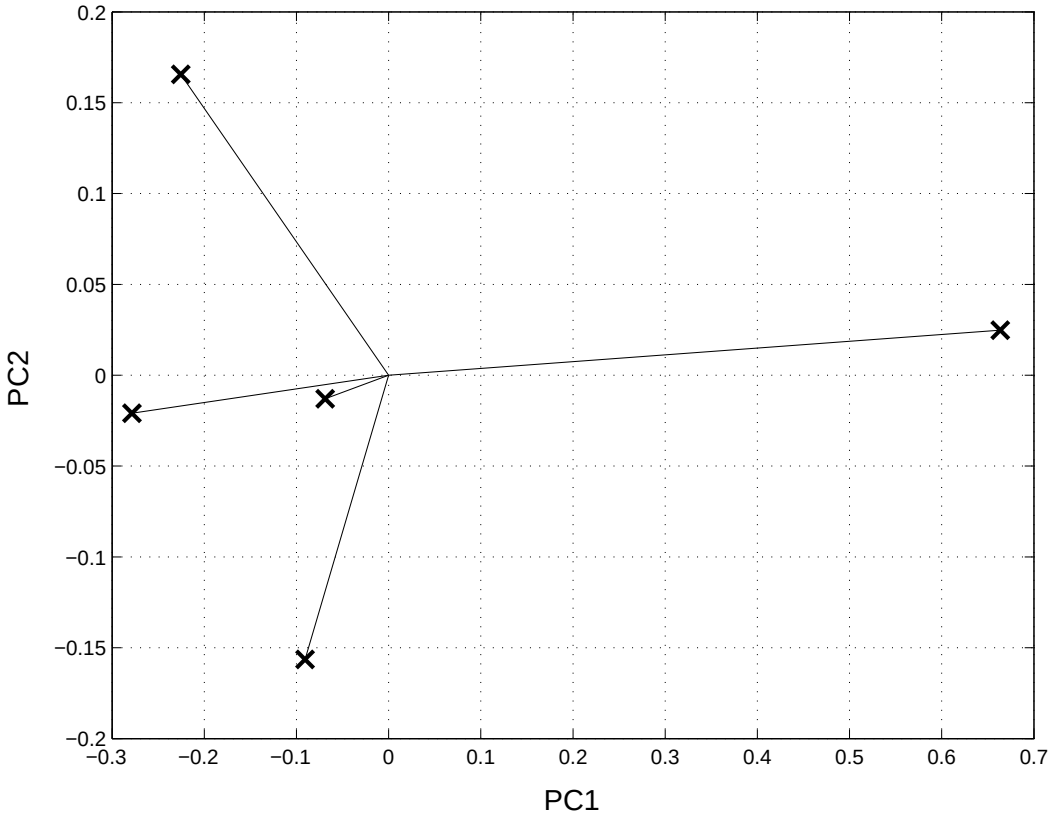

Figure C.2: Plot to illustrate leverage of data measured by Hotelling's T^2 statistic. The axes represent the first two PCs. Hotelling's T^2 is a measure of the distance of each observation from the origin.

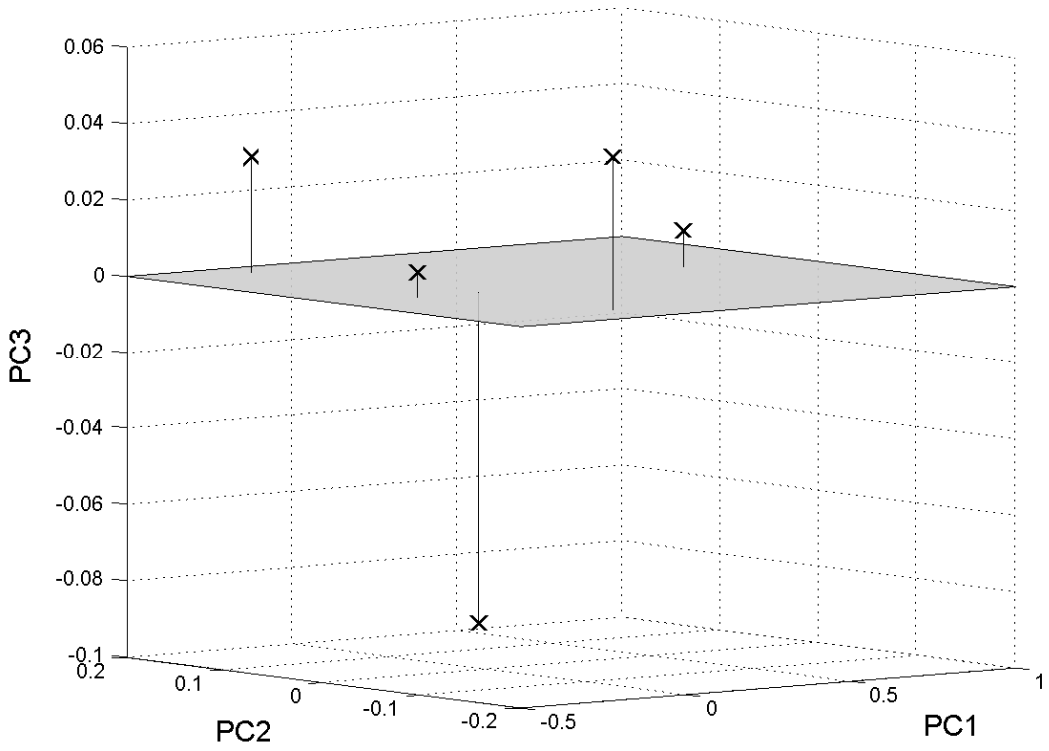

Figure C.3: Plot to illustrate residual error measured by SPE. The axes represent the PCs. In the two-component model, the data (x) are modelled by their projection into the PC1-PC2 plane (end of the lines corresponding with each observation) and the prediction error is the length of the line.

Appendix D

Description of Experiments

Table D.1: Description of Experiments Performed

Experiment Number	Name	Purpose
1	Determination of sample variability	To determine whether the microbial community in a biofilter can be characterised using Community-Level Physiological Profiling (CLPP), whether similar results are found from samples taken over a range of a few centimetres and to investigate the effect of sample size.
2	Spatial and temporal variation in one biofilter	To examine the spatial and temporal variation of the potential functional diversity in a biofilter over time.
3	Acclimation of parallel biofilters	To examine the parallel development of two biofilters over their acclimation period.
4	Spatial and Temporal Variations in Parallel Biofilters	To examine the acclimation and development of the community in two parallel biofilters over time at different vertical locations in the bed.