Assessing the impact of biodiversity on ecosystem function in clinically derived bacterial communities

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by

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Abstract

Background: Cystic fibrosis is the most common inheritable genetic disease affecting 1 in 2500 new born Caucasian infants. The primary cause of morbidity and mortality in these individuals is respiratory failure resulting from chronic airway infections. Previous studies have shown that many bacterial species colonise the airways at any one time. These results have suggested the need for understanding the ecological mechanisms occurring within these communities. This thesis uses ecological experiments to assess the impact of biodiversity on the activity of bacterial assemblages.

Methods: Bacterial species and *P. aeruginosa* ecotypes, isolated from expectorated sputum samples and identified using 16S rRNA gene sequence variation, were assembled into combinations of increasing diversity. These assemblages were inoculated into different environments to assess their effect on respiration. Total respiration was monitored using the MicroResp™ or the BIOLOG EcoPlate™ systems. A general linear model approach was used to analyse the data and investigate the ability of three ecological processes (species richness, composition and interactions) to account for the observed variance.

Results: Richness was generally shown to be statistically significant in all environments tested with mixed species present ($p < 0.005$). Conversely, when *P. aeruginosa* ecotypes were present no effect of richness was observed ($p > 0.272$). Composition was found to account for significant variation in the data in all environments and species combinations ($p < 0.001$). Altering the environment was shown to affect the significance of productivity and interactions among the species.

Conclusions: The experiments reported within show a novel application of established ecological models. These identify a complex interplay of mechanisms within the bacterial assemblages that are dependent on the environment and relatedness of the bacteria present. This thesis identifies key areas of future work to use ecology to provide insight into respiratory infections.

Words: 290

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Herman Melville, *Moby Dick*

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"*The best things in life are beyond money; their price is agony and sweat and devotion…"*

Lt. Col. Jean V. Dubois (Ret.)
Robert A. Heinlein, *Starship Troopers*

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Abbreviations

A572nm	Absorbance at a wavelength of 572 nm
AIC	Akaike information criterion
ANOVA	Analysis of variance
BA	Blood agar
bp	Base pair
C	Composition (species)
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
cfu	Colony forming units
CO_2	Carbon dioxide
CU	Coefficient units
d_{Jac}	Distance based on Jaccard's coefficients
DHB	Defibrinated horse blood
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminotriacetic acid
GLM	General linear model
HCl	Hydrochloric acid
HPA	Health Protection Agency
IFA	Inoculating fluid A
KCl	Potassium chloride
LB	Luria-Bertani
LR	Linear richness
M9	M9 minimal salt media
MH	Mueller-Hinton
$MgCl_2$	Magnesium chloride
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NLR	Non-linear richness
OD_z	Optical density (measured at a wavelength of z nm)
P	Partitions
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
RDP	Ribosomal Database Project
REP	Replicates
RPD	Random partition design
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SD	Standard deviation
se	Standard error
SS	Sums of squares
T-RF	Terminal restriction fragment
T-RFLP	Terminal restriction fragment length polymorphism
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TukeyHSD	Tukey's honest significant difference
UCO_2	Units of respired CO_2 over 24 hours
v/ v	Volume per volume in ml/ ml
w/ v	Weight per volume in g/ l

Glossary

The following glossary presents terms and symbols used within this thesis. Certain of these terms and symbols are defined in specific relation to the work presented here.

Assemblage – An artificially created group of bacterial species of manipulated diversity

Biodiversity – The extent of genetic or phenotypic variation present within the bacterial isolates within the system.

Community – A group of bacterial species that inhabit the same space at the same time and are shown/ hypothesised to interact between themselves.

Constrained – A type of environment of reduced mixing.

Diversity – See "*Biodiversity*".

Ecosystem – The biotic and abiotic structure of a spatial construct.

Ecosystem function – Any process of an ecosystem associated with the biota.

Ecotype – A population within a species possessing phenotypic differences.

Fully factorial design – A type of experimental design in which all of the entities being investigated are present, in every possible combination, with all of the other entities.

Indirect manipulation – Methods that control bacterial biodiversity in an experimental microcosm by contracting the existing diversity without culturing.

Isolate – A colony of bacteria derived from a pure culture first isolated on solid medium prior to identification.

Microcosm – An experimental unit consisting of a nutrient source inoculated with one or more bacterial species.

Microcosm mix – See "*Assemblage*".

Mixed – A type of environment that allows the bacterial cells present to freely intersperse with other entities within the media.

Overyielding – A microcosm exhibits *overyielding* when its productivity is greater than the average productivity of its constituent species in monoculture.

Partitioned species pool – The combination of the species, across all microcosms, at each richness level. At each level, each species is present only once.

Random partition design – The experimental design used here states that the number of species richness levels is dictated by the numerical factors of the total species pool. As such, each species is present once at each richness level.

Realised species richness – The species richness of the microcosms at the end of the experiments.

Species – A population of bacteria in which the 16S rRNA gene nucleotide sequence shows over 97% identity with the corresponding 16S rRNA gene sequence from a reference strain.

Species richness level – The number of species present within the assemblages.

System function – see "*Ecosystem function*"

Total species pool – The total number of species.

UCO_2 – The units of respired CO_2 measured at the end of the experiment in which values are presented that are equivalent to $\%CO_2$ v/v in the headspace of the microcosm chamber

Analytical terms

Replicate – Each experimental measurement was replicated twice with this entered as a factorial variable "*REP*" in the analysis.

Partitions – A single Partition represents a single set of 28 microcosms which has each species present in each richness levels once. This term is coded in the analysis as a factorial variable "*P"*. Each Partition was independently replicated twice given a total number of 56 microcosms in this study.

Linear richness – This term investigates the amount of variance accounted for by an increase in the number of species present using the richness levels as a continuous variable.

Non-linear richness – This term investigates the amount of variance accounted for by an increase in species number using the richness levels as a factorial variable with six levels.

Species composition – This term uses a presence absence matrix for each of the species in each of the microcosms. This allows the mean respiration to be assigned to each of the species in the analysis.

Q – Each *Q* term within a Partition represents the unique ordering (partitioned species pool) of the species at a given species richness level. As a factorial variable, each level of *Q* represents an arrangement of species at a single species richness level in a single Partition. The values of *Q* are numbered reciprocally to the species richness level i.e. the highest species richness level was assigned the lowest *Q* level.

M – As a factorial variable, each level of *M* represents a single arrangement of species within a Partition. Each value of *M* is replicated twice. As with *Q,* the values of *M* are numbered reciprocally to the species richness level i.e. the highest species richness level was assigned the lowest *M* level.

Symbols

Symbol	Description
y	The independent variable of the model. Throughout this thesis this will represent respiration
β_0	The intercept of the general linear model. This term is the point at which the regression line crosses the y-axis. This is the calculated value of respiration in microcosms with no species.
β_{LR}	The linear model coefficient for the linear richness variable. As linear richness is entered as a continuous variable this equates to the gradient of the regression line.
x_{LR}	The values of x for the linear richness term as entered into the model as a continuous variable.
β_{REP}	The linear model coefficient for the replicate term. The coefficients represent the mean respiration for each of the levels entered.
x_{REP}	The values for x of the replicate term. This term is entered as a factorial variable with two levels; 1 and 2.
β_P	The linear model coefficient for the Partition term. The coefficients represent the mean residual for each of the levels entered.
x_P	The values for x of the Partition term. This term is entered as a factorial variable with three levels; 1, 2 and 3.
β_C	The linear model coefficient for each individual species. This value represents the mean residual for each of the species across all the assemblages in which it was present.
x_C	The presence of each individual species was coded into the general linear model by either a binary code or by weighted values, between 0-1. These were multiplied by the residuals to give values for only the assemblages in which a given species was present
β_{NLR}	The linear model coefficient for the non-linear richness term. The coefficients represent the mean value for each of the levels entered.
x_{NLR}	The values for x of the non-linear richness term. This term is entered as a factorial variable with six levels; 1, 2, 3, 4, 6 and 12.

Symbol	Description
β_Q	The linear model coefficients for the Q term. This represents the different combinations of species at each richness level across the Partitions. The coefficients are the mean residual values for each level.
x_Q	The values for x of the replicate term. This term is entered as a factorial variable with 18 levels; 1 to 18.
β_M	The linear model coefficients for each of the unique species assemblages in the experiment. These represent the mean residual values for each level entered into the model
x_M	The values for x of the replicate term. This term is entered as a factorial variable with 168 levels; 1 to 168.
e	The error term

Equations

Miscellaneous equations used in the analysis

$$d_{Jac} = 1 - \frac{a}{a+b+c} \quad [1]$$

Equations used in the calibration of the respirometer (Chapter 3b)

$$CO_{2(gas)} + H_2O + HCO_3^- \leftrightarrow 2CO_3^{2-} + 3H^+ \quad [2]$$

$$HCl + NaHCO_3 \rightarrow H_2O + NaCl + CO_2 \quad [3]$$

$$\log(absorbance_{572nm}) = -0.266 * \log(\%CO_2) - 0.804 \quad [4]$$

$$\%CO_2 = e\text{^}\frac{(\log(absorbance_{572nm})+0.804)}{-0.266} \quad [5]$$

Equations used in the description of the general linear model (Chapter 3c)

$$y = \beta_o + \beta_{LR}x_R + (\sum_1^N \beta_C x_C) + \beta_{NLR}x_R + \beta_Q x_Q + \beta_M x_M + e \quad [6]$$

$$y = \beta_o + \beta_{LR}x_R + e \quad [7]$$

$$y = \beta_o + \beta_{LR}x + (\sum_1^{12} \beta_C x_C) + e \quad [8]$$

$$y = \beta_o + \beta_{LR}x_R + (\sum_1^{12} \beta_C x_C) + \beta_{NLR}x_R + e \quad [9]$$

Equations of general linear models used in the statistical analysis of the data:

$$y = \beta_o + \beta_{REP}x_{REP} + \beta_P x_P + \beta_{LR}x_R + (\sum_1^N \beta_C x_C) + \beta_{NLR}x_R + \beta_Q x_Q + \beta_M x_M + e \quad [10]$$

$$y = \beta_o + \beta_{LR}x_R + \begin{bmatrix} \beta_{Sp1} \\ \vdots \\ \beta_{Sp12} \end{bmatrix} x_C + \begin{bmatrix} \beta_{NLR1} \\ \vdots \\ \beta_{NLR12} \end{bmatrix} x_R \quad [11]$$

$$y = \beta_0 + \beta_{REP}x_{REP} + \beta_{LR}x_R + \sum_1^6(\beta_c x_c) + \beta_{NLR}x_R + e \quad [12]$$

Chapter 1

1. General Introduction

1.1 Introduction

The impact of bacterial biodiversity on the activity of assemblages is an area of research which is the subject of increasing importance for microbial ecologists. To date, most of the work on this subject has examined bacterial species from natural environmental contexts. This thesis however, will apply ecological techniques to an environment that as yet, has not been investigated in this manner, the airways of cystic fibrosis (CF) patients.

The primary disease related cause of mortality and morbidity in patients with CF is pulmonary failure, therefore maintaining the health of the CF airways is of great importance (Hauser *et al.,* 2011). Morbidity occurs through progressive lung damage arising from chronic airway infections by a typically complex mix of bacterial species. Although many studies have surveyed the airways of individuals with CF to establish the composition of the bacterial community, few have directly investigated ecological mechanisms in this context. The CF airways provide a constrained and selective habitat for the study of ecological mechanisms within chronic polybacterial infections. The increased understanding of chronic infections that emerges from this may be of future importance in terms of the treatment of these infections.

The aim of this study was to better understand the ecological mechanisms within CF-associated airway infections by the application of microbial ecology methods. By using the relatively constrained diversity of the CF airways, it will be possible to study the biodiversity - ecosystem function relationships of a clearly bounded habitat containing a community with relatively limited diversity.

1.2 Biodiversity and Ecosystem Function

The relationship between biodiversity and ecosystem function has been an important area of study for over a century. Darwin, in "*On the Origin of Species*", has been credited as making the first published observation that an increase in biodiversity corresponded to an increase in plant biomass yield (Tilman, 1999; Tan *et al.,* 2012). The mechanisms that drive the impact of biodiversity on ecosystem function are, however, highly complex. Studies of biodiversity involve a similarly complex set of

evolutionary, biogeographical and physical processes (Huston, 1997). As such, understanding the impact of biodiversity is often concerned with how the different mechanisms contribute to the plurality of processes (Lawton, 1996).

The class of experiments created to study this relationship are termed biodiversity-ecosystem function (BEF) experiments. The six most cited papers relating to BEF experiments in 2009 were reported by Solan *et al.* (2009). In every case, these were based on the manipulation of terrestrial plant assemblages. The experiments reported in four of these studies were of particular importance (Naeem *et al.* 1994; Hooper & Vitousek, 1997; Tilman *et al.,* 1997; Hector *et al.,* 1999). Of these, Tilman *et al.* (1997) and Hector *et al.* (1999) reported two of the largest-scale BEF experiments published so far. These studies manipulated plant communities in the Cedar Creek and the pan-European "BIODEPTH" experiments respectively. Both of these studies and subsequent studies radiating from these data (e.g. Loreau & Hector, 2001; Tilman *et al.,* 2012) support Darwin's observation that an increase in biodiversity equates to an increase in ecosystem function.

Experimental manipulation therefore allows insight into this relationship, or as stated by Kareiva (1994):

> "*…the influence of biodiversity can be elegantly dissected through experimental manipulations."*

A review by Tilman *et al.* (2002) stated that most studies before 1990 postulated that productivity of an ecosystem was driven by a single dominant species. As further work was conducted in assessing ecosystem productivity, a paradigm shift occurred and the biodiversity of a system began to be considered to be as important as a dominant species (Tilman *et al.,* 1996). As more species combinations and ecosystems were tested, it also became apparent that a positive relationship was the most common function of biodiversity on productivity (i.e. where biodiversity increases tended to correlate with increase productivity; as reviewed in Duffy, 2002). Current models, supported by empirical data, suggest that there is no single relationship that underpins the impact of biodiversity on a measure of ecosystem function (Bell *et al.,* 2009a). Vitousek & Hooper (1993) hypothesised three possible effects of biodiversity on ecosystem function (Figure 1a). These suggested that the relationship could be positive linear, positive asymptotic or neutral. Based on more recent findings, the positive linear model was replaced by a negative asymptotic relationship (Figure 1b; Jiang *et al.,* 2008).

To analyse which of these relationships are present, BEF experiments have been, and continue to be, employed. These BEF experiments are conceptually very simple; a

measure of ecosystem function (e.g. primary productivity; biomass turnover, generation of CO_2) is plotted as the response variable, against an increasing measure of diversity. The trajectory of these data shows the relationship of the ecosystem function to the specific change in biodiversity (Naeem *et al.,* 2002). Around this trend, a notable degree of variation within the data is usually observed at each level of biodiversity. As will be discussed below, the methods for explaining, and reasons for, this variation is still a matter of debate (Hector *et al.,* 2009).

Three primary mechanisms have been proposed to affect the biodiversity impact upon productivity. These are termed the "sampling", "selection" and "complementarity" effects (Tilman *et al.,* 1997; Loreau & Hector, 2001; Hector *et al*., 2009; Becker *et al*., 2012). The sampling effect dictates that as higher diversity assemblages are created, the more likely it will be to include a dominant species. In contrast, the selection effect reflects the performance and abundance of a species in a mixture. Finally, the complementarity effect calculates the extent to which the species are coordinated with one another. This complementarity has been frequently related to niche differentiation (Tilman *et al.,* 1997). As it is unclear how to separate ecological processes between species, any observed complementarity effects have however been regarded as being synonymous with interactions between the species (Hector *et al.,* 2009).

Plant BEF experiments have concluded typically that there are mainly positive relationships between biodiversity and productivity (Duffy, 2002; Jiang *et al.,* 2008). In these studies, the predominant mechanism thought to cause these positive effects was the selection effect (Loreau & Hector, 2001), although, "transgressive overyielding", a form of complementarity effect where the assemblage productivity is greater than the best-performing monoculture, was also generally observed (Kirwan *et al.,* 2009).

The BEF experiments discussed so far have been based on plant communities. In this thesis however, the focus has been on the manipulation of bacterial biodiversity in relation to productivity. The focus of this Introduction will therefore, now turn to bacterial BEF experiments.

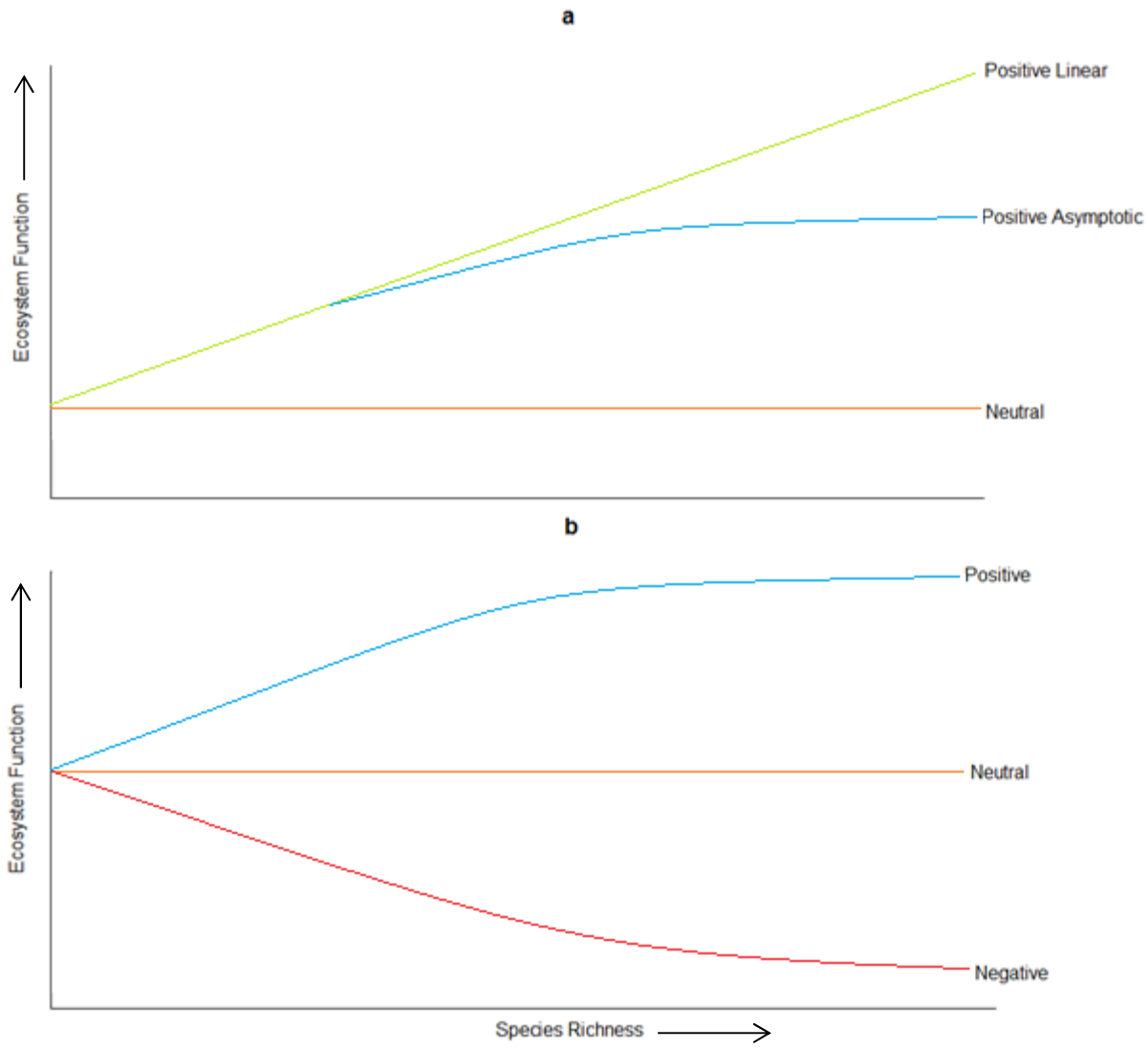

Figure 1.1: Hypothesised BEF relationships as adapted from (a) Vitousek & Hooper (1993) and (b) Jiang *et al.* (2008)

Bacterial Biodiversity and Ecosystem Function

The use of bacterial species in BEF experiments was traditionally rooted in the testing of theory or mathematical models (Bell *et al.,* 2009a). The field of environmental bacterial BEF research however, has recently begun to provide important information as to how bacterial richness and community structure effects ecosystem function in its own right (Salles *et al.,* 2009; Frossard *et al.,* 2012). Previous bacterial BEF experiments, whilst based on bacteria derived from different habitats, have yielded examples of all hypothesised possible outcomes (Figure 1.1b; Jiang *et al.,* 2008); namely positive (Bell *et al.,* 2005), neutral (Zhang *et al.,* 2009) and negative (Becker *et al.,* 2012). Prior to this study, such experiments had not been performed on clinically-derived bacterial species.

There are marked differences between BEF experimentation in macro- and micro-ecology. One difference that needs the most thought, as it poises real difficulties for the bacterial ecologist, is how biodiversity is calculated (Cohan, 2002). Established practice in macro-ecology is that either the functional or genetic diversity can be used. The identification of bacterial species now increasingly exploits conserved region(s) of the genome, with many studies based on the phylogenetically-informative 16S rRNA gene (Frossard *et al.,* 2012; Lawrence *et al.,* 2012). These forms of bacterial species identification avoid problems associated with phenotypic assessments that can vary, depending on the character being studied, between members of a single species.

Another important consideration for the inclusion of species is the habitat from which they were obtained. Previously, in plant BEF experiments, species were included in the experiment with the determining factor for selection being the ability to grow in the environment tested (Naeem *et al.,* 2009). To infer meaningful conclusions from data using bacterial species, the habitat from which they were isolated needs to be relevant to the environment under investigation (Schimel *et al.,* 2007; Frossard *et al.,* 2012).

Whilst recent studies have widened the scope of bacterial BEF experiments (Becker *et al.,* 2012; Frossard *et al.,* 2012), they have not fully assessed whether an increase in biodiversity impacts on the ecosystem function. The choice of ecosystem function to measure is also important. Broadly, there are two approaches to measuring bacterial ecosystem function. The first is to target a specific mechanism and observe how biodiversity impacts on it (e.g. Frossard *et al.,* 2012). The second takes a more holistic function, such as respiration. Although less informative about a specific process, measures such as respiration allow fundamental questions to be asked of the

data. In addition this can also assess the significance of the ecological mechanisms affecting the measured outcomes from a community (Bell *et al.,* 2005; Salles *et al.,* 2009; Lawrence *et al.,* 2012).

There are other points relevant to the choice of habitat. No previous BEF experiment has selected to study a clinically-important setting. To the best of my knowledge, the only references to infection in BEF experiments published to date have been found in studies from Ostfeld *et al.* (e.g. Ostfeld & Keesing, 2000; Ostfeld, 2009) who discuss how reducing biodiversity results in an increased risk of zoonotic infections. More recently, Bell *et al.* (2009a) proposed that the use of BEF experiments to understand ecosystems could have implications for microbial disease treatment. Outside of BEF experiments, other recent studies are linking bacterial biodiversity to human allergy development (Hanski *et al.,* 2012).

Previous bacterial BEF experiments illustrated the effect of biodiversity on a measure of ecosystem function within a specific environment (Table 1.1). Many of these studies however, manipulate the biodiversity of the systems by contracting the existing diversity by using methods such as dilution (Peter *et al.,* 2011) or fumigation (Griffiths *et al.,* 2000). These methods, known here as "indirect manipulation" methods, allow a subset of the species in the original community to be established. In contrast another method by which biodiversity can be manipulated is through the artificial assembly of communities (Bell *et al.,* 2005). This strategy, known here as "direct manipulation" methods, requires the mixing of pure cultures in order to create a community of the desired diversity and composition.

Questions have arisen about the relevance of these direct manipulation methods of biodiversity establishment to natural environments (Bell *et al.,* 2009a). Indirect manipulation methods are thought to allow more of the community to be present in the experiments and therefore, have been considered by some as more applicable to nature (Frossard *et al.,* 2012). This is because the direct manipulation of diversity requires the isolation of species from the habitat being studied. Only in very few habitats can these methods provide a complete representation of the species within a given environment (Bell *et al.,* 2005). Direct manipulation allows the investigation of individual species in desired assemblages as dictated by the researcher. Further advantages of the direct manipulation methods are that the process of cultivation of the bacterial species allows a better insight into the ecology of the system investigated (Nichols, 2007).

Reference	Environment	Diversity	Productivity measure and other remarks
Wohl *et al.,* 2004	Water (River)	10	Monitored cell density in four richness levels at six time points over 25 days. Reported a positive relationship between richness and biomass.
Bell *et al.,* 2005	Water (Tree-hole)	72	Bacterial species identified using fatty acid methyl ester profiling. The first study to use the Random Partitions Design. Reported a positive relationship between richness and evolved CO_2.
Salles *et al.,* 2009	Soil	16	Primary investigation into niche differentiation for the continuing of communities. Reported a positive relationship between richness and both denitrification and CO_2 production .
Zhang *et al.,* 2009	Soil	6	Used a single species and isolated six morphologically different "species analogues" from the same ancestor. Reported a neutral relationship between richness and growth rate.
Langenheder *et al.,* 2010	Soil	6	Focused on "transgressive overyielding" in six richness levels utilising three single carbon sources. Reported a time-dependent relationship between richness and metabolic activity.
Becker *et al.,* 2012	Soil	8	Used a single species with these isolates chosen to cover all genetic subgroups. Reported a negative relationship between richness and both root colonisation and host plant protection.
Lawrence *et al.,* 2012	Water (Tree-hole)	5	Ecosystem function was assessed after 15 days of perturbation & evolution. Reported a positive relationship between richness and evolved CO_2.
Tan *et al.,* 2012	Water (Fresh)	8	Primary aim was to investigate whether phylogenetic relatedness was a good surrogate for ecological similarity. Reported a positive relationship between richness and biomass.

Table 1.1: List of bacterial BEF studies based on direct manipulation of biodiversity. All eight bacterial BEF studies used direct manipulation to alter the biodiversity of an assemblage. A measure of ecosystem function was measured and related to the manipulated biodiversity. The majority of these studies showed that an increase in species diversity was matched by an increase in ecosystem function. This finding is both supported (Peter *et al.,* 2011) and contradicted by (Langenheder *et al.,* 2005) studies using indirect manipulation methods.

BEF models and their analysis

Hector *et al.* (2009) stated that theoretical, semi-mechanistic and mechanistic models were the three main types of models for BEF experiments. The models have acquired these monikers based upon the method used to separate biodiversity effects. Theoretical models assign unmeasureable processes to be the cause of biodiversity effects. Semi-mechanistic models fit parameters that can be loosely related to biological processes. The final model type was termed mechanistic models and these have been championed in the literature (Loreau, 1998; Loreau & Hector, 2001) because they attribute parameters in the model to quantifiable biological processes (Hector *et al.,* 2009). Information about the actual biological processes present in the microcosms assembled can be obtained using mechanistic models. These models were used exclusively throughout this study.

With a hypothesis-driven, mechanistic experimental design, a null hypothesis is also needed. The starting point of many models is that all species are equivalent in their effect on ecosystem function (Loreau *et al.,* 2002; Bell *et al.,* 2009b). This results in there being no general effect on ecosystem function with changing biodiversity; the majority of statistical models take this as the null hypothesis. A key challenge was to conduct an experiment that could distinguish and assess the mechanisms by which species richness influences ecosystem function. The effects that result in variation from this hypothesis are the reason for the application of complex statistical analyses.

There is no "gold-standard" of analysis for data produced from BEF experiments with many different techniques having been previously implemented (Schmid *et al.,* 2002). Tilman *et al.* (1996) used simple analyses of variance (ANOVA), whereas later studies have utilised general linear models to analyse the data generated (e.g. Loreau & Hector, 2001; Bell *et al.,* 2005; Kirwan *et al.,* 2009) and so to separate out aspects of biodiversity impacting on ecosystem function.

The choice of statistical analysis and design of experiments to distinguish the three mechanisms by which diversity influences ecosystem function is complicated. The classical solution has been to design fully factorial experiments in which species are tested in all different combinations. These however, require large numbers of experimental microcosms for even modest numbers of species. For example, a study with 12 species would require, without replication, over 4000 different microcosm mixes to be assembled. Bell *et al.* (2005) overcame this limitation by developing a design based on random partitioning.

In the Random Partitions Design, species were randomly sorted so that they were all selected exactly once at each level of species richness. This was repeated to provide new random assemblages, where again, each species was selected once per species richness level, but now in new combinations. By selecting a number of species richness levels, this avoided the need for an arduous fully factorial design. Moreover, by using this method, the knowledge of individual species productivity, in monoculture, is no longer essential. This model allows the user to assess the contribution of both species richness and species identity to ecosystem function and to infer the presence of interspecies interactions (Bell *et al.*, 2009b).

1.3 Cystic Fibrosis

The experimental models used throughout this thesis are based upon the chronic airway infections that are common in individuals with CF. Having introduced the underlying ecology in this thesis, the following sections will next introduce CF as a disease.

CF is one of the most common life-shortening autosomal recessive diseases in Caucasian populations, (Davies *et al.,* 2007, LiPuma, 2010) with an incidence of approximately one in 2500 infants (Davies *et al.,* 2007) and a carrier rate of one in 25 of the UK Caucasian population (Bilton, 2008). The disease is systematic, causing a range of endocrine and gastrointestinal abnormalities (Lyczak *et al.,* 2002; Davis *et al.,* 2007); however the most severe clinical manifestation of CF is in the lung (Lyczak *et al.,* 2002; Goss & Burn 2007; Davis *et al.,* 2007). In the airways of individuals with CF, altered physiological states typically lead to repeated chronic bacterial lung infections. These bacterial infections, and the subsequent immune response, cause damage that leads to a reduction in lung function (Lyczak *et al.,* 2002; Goss & Burn, 2007). Ultimately, lung function can reduce to the point where respiratory failure occurs. Despite advances, this remains by far the largest cause of mortality in this patient group (Lyczak *et al.,* 2002; Davies *et al.,* 2007; LiPuma, 2010; Callaghan & McClean, 2012).

Genetic basis of CF and physiological implications in the respiratory tract

CF is caused by a mutation in the CF transmembrane conductance regulator (*CFTR*) gene. The *CFTR* gene encodes for an adenosine triphosphate binding cassette transporter protein and cyclic adenosine monophosphate activated chloride channel (Callaghan & McClean, 2012). The most common mutation (over 70% of mutated genes) in this gene is a deletion of phenylalanine at position 508 (Davies *et al.,* 2007).

Non-mutated *CFTR* genes encode for a protein which is expressed on the surface of epithelial cells which is responsible for sodium ion absorption and chloride ion secretion (Boucher, 2004). Individuals homozygous for this mutation, ~ 55% of Caucasian individuals with CF, produce a non-functional *CFTR* protein which is degraded in the cytoplasm of the cells (Lyczak *et al.,* 2002).

In the CF lung, the dysfunctional *CFTR* protein leads to altered fluid balance across epithelial cells and an impaired mucociliary clearance system (Bals *et al.,* 1999). The altered ability to control the movement of ions across the epithelial cells causes an accumulation and thickening of mucus and the formation of mucus plaques (Boucher, 2004). The inability to remove the mucus and cellular debris causes a viscous matrix to be formed in the CF airway. This allows the establishment of chronic infections by colonising microbes (Callaghan & McClean, 2012).

CF lung infections

The airways, in both health and disease, are exposed to a large number of foreign bodies, including bacteria, present in inhaled air. With an impaired mechanism for the removal of mucus and other debris, the host immune response to these microbes results in inflammation of the nearby tissue and ultimately, permanent lung damage (Goss & Burns, 2007). A number of different types of microbes may be implicated in disease progression. Viruses have been considered detrimental in terms of contributing to lung damage (Wat *et al.,* 2008). In this study, the focus will be on the bacteria present in the CF airways. As bacterial infections are the most important factor in the reduction of CF airway function (LiPuma, 2010). It is now important to understand the manner in which the colonisation of the CF airways develops. Understanding these processes may have implications for directing therapy, and both modelling and modulating the acquisition of bacterial species.

Colonisation of the airways is thought to begin at an early age; approximately 65% of CF patients have been considered by traditional culture-based diagnostic microbiology, to be colonised by at least one pathogen by one year of age (Rosenfeld *et al.,* 2001). To assess what species are present however, it is important to first focus on the means of characterisation. The traditional means of doing so has relied on "classical" microbiological diagnostic strategies. These strategies have focused on the cultivation of a small number of bacterial species which are considered to be "key" pathogens in the CF airways. These species include the *Burkholderia cepacia* complex, *Haemophilus influenzae, Pseudomonas aeruginosa, Staphylococcus aureus* and

Stenotrophomonas maltophilia (Lyczak *et al.,* 2002). Other bacterial species including *Achromobacter xylosoxidans* and members of the *Streptococcus* genus have been more recently associated with the progression of CF lung disease (Sibley *et al.,* 2009; LiPuma, 2010; Rudkjøbing *et al.,* 2012).

Current treatment for chronic CF lung infections

A range of different symptom management strategies are used to maintain the health of patients with CF, with many important treatments focusing on reducing damage to the CF lung. These treatments can broadly be categorised into those that are drug- and non-drug based. Of the non-drug based therapies, exercise, nutritional regimes and regular physiotherapy have all been shown to be important for the health of CF patients (Quon & Goss, 2011). A range of drug based approaches are also important. These include the use of mucolytic agents, anti-inflammatory agents and both inhaled and intravenous antibiotics (Tang *et al.,* 2005; Davis *et al.,* 2007).

Whilst all treatments are of benefit, the use of antibiotics has been argued to be the most important contributing factor to the increase in life expectancy of CF patients (Lyczak *et al.,* 2002). Despite the benefit seen by the use of these agents to CF patient health overall, there are many important issues that need to be considered. These issues include which antibiotic(s) to use in relation to the pathogenic agent perceived to be important (Aaron *et al.,* 2004), when to start the course of antibiotics, the role of the antibiotic – as either a prophylactic (Flume *et al.,* 2007) or to suppress an exacerbation (Taccetti *et al.,* 2008). These issues need also to be considered in relation to the stage of lung disease which often correlates with the age of the CF patient and their previous health care.

Bacterial species in CF airways

The current treatments for CF airway infections highlight the role of antibiotics in relation to the increase in life expectancy for CF patients. By extension, this also stresses the importance of bacteria in CF airway disease progression. Therefore, it is equally important to describe what is known about the bacteria in the CF airways.

A range of bacterial species have been found in lung infections (Huang *et al.,* 2010), particularly in CF (Rogers *et al.,* 2003; Guss *et al.,* 2011). The presence of multiple bacterial species raises interesting questions for researchers. These include: what species are present, why are there so many different species present and how do these species affect the disease? The majority of the published literature focused on the

"What is there" element whilst hypothesising the "Why are they there" (Filkins *et al.,* 2012). Few studies have experimented on the "How do they affect the disease" (although Sibley *et al.,* 2008 showed that supposed non-pathogenic bacterial species could increase the pathogenicity of *P. aeruginosa* in a *Drosophila* model). The use of BEF experiments using clinically isolated bacteria could allow researchers to gain insights into the ecology of the community and shape potential future treatments of the disease (Bell *et al.,* 2009a).

Assessing biodiversity in CF

Currently in CF microbiology research, the issue of biodiversity has been correlated to the severity of the disease indicating that as the species diversity increases the disease severity decreases (Delhaes *et al.,* 2012). This drop in diversity has however, also been linked with age, or length of infection (Cox *et al.,* 2010). Indeed, in a study by Goddard *et al.* (2012) it was shown that the lungs at transplantation, typically the end-stage of the airway disease, were infected by a maximum of three detectable species. Therefore, the severity of the disease may not be affected by the diversity, but simply by the prolonged infection of the airways by colonising species. As such, the presence of mixed species communities in the CF airways and ultimately their role in disease and stability is currently unknown (Filkins *et al.,* 2012).

Identification of bacterial species present in mixed samples

Traditionally, the presence of bacterial species in the CF airways has been assessed by culture-based methods. These relied on the bacterial species being able to grow in the conditions chosen. More recently, however, culture-independent methodologies have been implemented to assess the diversity of bacterial species in the CF airway.

Culture-independent methods have become the standard to obtain measures of species richness for research for many years, especially as they allow detection of "uncultured" species (Rogers *et al.,* 2003; Goss *et al.,* 2010). The most regularly used gene for the identification of species is the 16S ribosomal RNA (rRNA) gene sequence (Cohan, 2002). The 16S rRNA gene is of particular interest to those studying the phylogenetic relatedness of prokaryotes (Woese & Fox, 1977; Clarridge, 2004). This gene is present at least once in every prokaryotic organism and is part of the 30S subunit of the prokaryotic ribosome (Stryer *et al.,* 2002; Acinas *et al.,* 2004). Regions of this gene are either conserved or variable. Conserved regions keep the functionality of the gene intact and also allow the binding of primers for polymerase chain reaction

(PCR) across a wide range of bacterial species for specific amplification (Yong & Qian, 2009). The variable areas of the gene are regions that have altered in sequence slowly over time, allowing phylogenetic relatedness between organisms to be calculated (Woese & Fox, 1977; Weisburg *et al.,* 1991). The variable regions of the gene sequence have been widely used to characterise which bacterial species are present within a sample (Liu *et al.,* 1997).

To characterise the bacterial species present, a process consisting of the extraction of nucleic acids from cells in a clinical sample is required. These nucleic acids serve as templates for the amplification of the rRNA gene. Depending on the sample, a mix of species is present. As a mix of different rRNA gene amplicons will result, some means of resolving which amplicons, or species, are present is therefore required. The identities of the bacterial species present can be either inferred or derived by a number of different DNA based methods. Of these, Terminal-Restriction Fragment Length Polymorphism (T-RFLP) profiling has been used commonly in assessing the diversity of species in CF samples (Rogers *et al.,* 2003; Sibley *et al.,* 2011; Stressmann *et al.,* 2011a). This method resolves amplicons from different species as bands visualised in a single electrophoretic lane (Dickie & FitzJohn, 2007). Here, each band has been generated by restriction endonuclease digestion of the amplicons. Different restriction endonuclease sites are formed in the hypervariable (i.e. species specific) regions of the 16S rRNA gene. By detecting a tag incorporated into one primer prior to PCR, DNA sequencing machinery can only detect tagged DNA fragments. These are therefore constrained to one end of the PCR product. The length of the fragment depends on the first site of the restriction endonuclease in the PCR product (Bruce & Hughes, 2000). A mix of these bands (known as Terminal-Restriction Fragments; T-RFs) is produced from PCR products amplified from DNA extracted from a community. A virtual image of the gel is created by the DNA sequencer with the size and intensity of the T-RFs calculated. T-RF sizes can then be matched with *in silico* predicted T-RF libraries (Rogers *et al.,* 2003) for an inferred identification of species. Whilst it is possible that two species will have the same sized fragment, this is unlikely in CF sputum sample analysis (Rogers *et al*., 2003).

To achieve a more conclusive identification of the bacterial species present, defining the 16S rRNA gene sequences that are present is required (Dickie & FitzJohn, 2007). This allows direct comparison with reference sequences of known species (Souza *et al.,* 2006). These reference sequences are from known bacterial species and

uploaded into extensive databases such as the Ribosomal Database Project (Maidak *et al.,* 1994) and GenBank (Altschul *et al.,* 1990).

There are a number of ways to generate this 16S rRNA sequence information, including clone library or next-generation sequencing. Both of these methods are based upon the amplification of a region of the 16S rRNA gene as described above. Differences lie in the method used to separate the individual gene sequences.

These techniques have successfully investigated the presence of bacterial species in samples taken from the CF airways. They cannot however, inform directly as to the type or degree of ecological mechanisms present. As such, one approach to this is by direct manipulation of the bacterial species through culture based methods.

1.4 Experimental approach

Obtaining a relevant measure of biodiversity

To construct BEF experiments using direct manipulation of bacterial species, an isolation step is first required. Isolation of bacteria from CF sputum samples is routinely performed by hospital pathology laboratories using a set of standard operating procedures (UK Standards for Microbiology Investigations, *www.hpa.org.uk*). A range of solid media under specified growth conditions is used to isolate species that have been considered important in lung disease progression. Information from culture is relayed to the clinicians for assessment and treatment of the patient.

In the context of ecological experimentation, the ecology of any system is intricately related to factors such as the evolution of each component species. This becomes a circular phenomenon with the evolutionary path of an organism being driven by the organism's immediate ecology (Figure 1.2). Therefore, to infer meaningful parallels with communities in CF airway infection, deriving bacteria with "experience" of the CF airways and bacterial communities may be important when establishing BEF experiments (Frossard *et al.,* 2012).

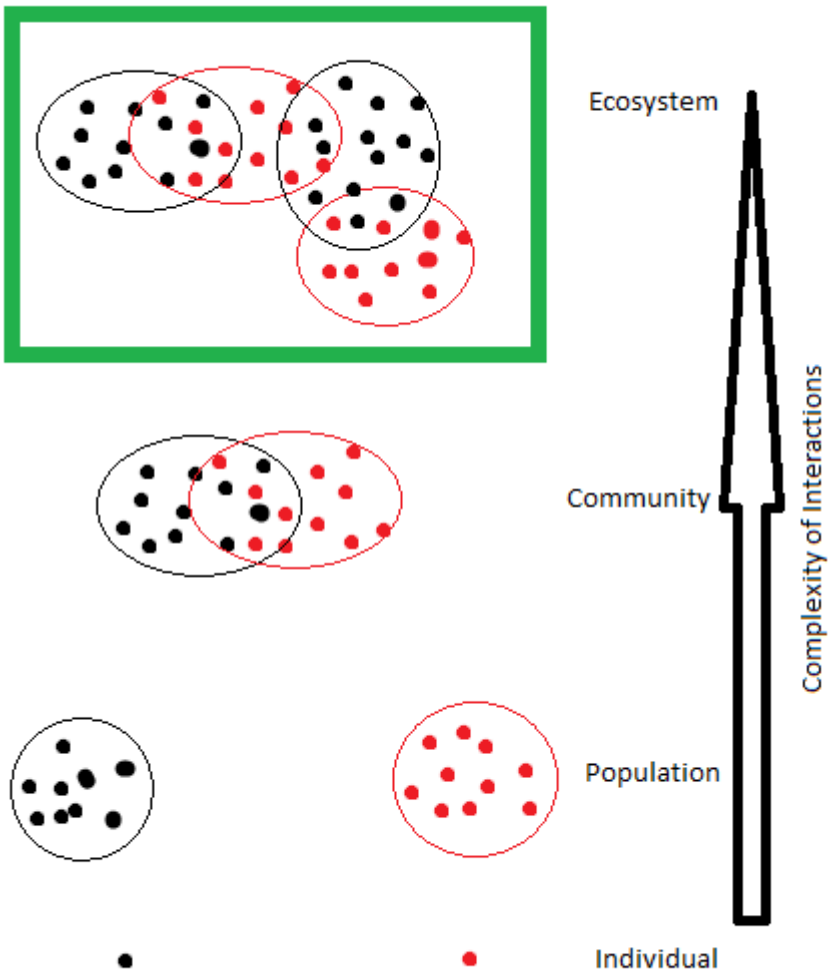

Figure 1.2: Levels of ecological organisation as adapted from Atlas & Bartha (1998). This Figure illustrates basic ecological organisational levels. The individual is the simplest; a population is made up of many individuals of the same species. The community, is a product of two, or more, populations interacting. Finally the ecosystem is a result of the community interactions whilst taking into account the interactions with the environment.

Culture methodologies for clinical microbiology and direct manipulation

The species regarded traditionally as pathogens, described above as *B. cepacia* complex, *H. influenzae, P. aeruginosa, S. aureus* and *S. maltophilia* (Lyczak *et al.,* 2002), are frequently cultured from CF sputa. Culture methods were the only way of detecting and, in turn, identifying bacterial species prior to the wide-spread use of culture independent techniques. Despite the range of species detected, *P. aeruginosa* has been considered to be the single most important pathogenic species by many researchers (Aaron *et al.,* 2004; Goddard *et al.,* 2012) in the airways. Culture-based techniques have however, been developed further at least as research tools. Through application, an increased diversity of bacterial species have been isolated from samples taken from the CF airway (Coenye *et al.,* 2002; Sibley *et al.,* 2011). Although the number of bacterial species isolated can be increased with the use of anaerobic culturing conditions, the majority of dominant bacterial species can be cultured using aerobic conditions alone (Guss *et al.,* 2011).

Identification of isolates

The identification of bacterial isolates is primarily performed in a research context using 16S rRNA gene sequence analysis (Dickie & FitzJohn, 2007) as above and matching the unknown sequence to a reference sequence in a database (Souza *et al.,* 2006).

Assessing the community function is different to the biodiversity

Assessing the diversity within a system and actually obtaining information about the ecological process are different. Although information about specific processes can be found by genotypic means (e.g. Siciliano *et al.,* 2003), the most common way is to investigate the phenotype of the community. This has been commonly accomplished using respiration, detected by either the BIOLOG (Yang C. *et al.,* 2011) or MicroResp™ systems (Lawrence *et al.,* 2012).

Although both of these systems monitor the respiration of the community, the mechanism by which they do this is different. The MicroResp™ system uses a colorimetric change to detect the percentage of carbon dioxide (CO_2) present within the closed system. This method has been considered to give a rapid measurement of the productivity of the bacterial assemblage present in the experimental environment (Campbell *et al.,* 2003). An advantage of this method is the ability for the researcher to manipulate diversity of the starting inoculum. In addition to this manipulation, the experimental substrate and other factors can also be controlled.

The substrate in which bacterial assemblages grow has been shown to affect how the species present interact with each other (Kuemmerli *et al.,* 2009) and changes in the environment have been shown to promote stress responses by mechanisms such as gene regulation (Schimel *et al.,* 2007). In a clinical setting, bacterial species and assemblages have been shown to adapt to the host environment over the course of chronic infections such as those found in CF airways (Yang L. *et al.,* 2011). It has also been shown that in spatially limited environments, such as those created in biofilms, that synergistic interactions allow a bacterial community to better utilise nutrients (Elias & Banin, 2012).

The BIOLOG system uses fixed single carbon sources thus allowing large amounts of phenotypic data to be generated. The measure of respiration in this system also uses a colorimetric detection system. In contrast to the MicroResp™ system, the BIOLOG system measures a redox reaction linked with respiration through the utilisation of a redox reaction indicator which is retained as a purple solid inside bacterial cells once reduced (Garland & Mills, 1991). Therefore, this method also takes into account the cell density of the bacterial species.

1.5 **Aims**

The current literature on the microbiology of the CF airways has detailed a spatially constrained environment with a relatively high diversity of bacterial species. To date, few studies have identified how the bacterial species interact between within an assemblage. This study will focus on the interplay between bacterial species in *in vitro* microcosms as shaped by a set of defined variables for testing.

The studies in the following chapters distinguished the effects of biodiversity in terms of three components; first, the increased function observed as a result of more species. This was called linear richness with new species potentially bringing new functions and extending the ability of the assemblage to utilise resources. Second, the change in function as related to specific species or combinations thereof. This was termed composition. Third, with increased numbers there is increased scope for interactions. A key challenge here was to conduct an experiment that could distinguish and assess these three mechanisms by which species richness influences ecosystem function.

The overall aim was to assess the impact of bacterial biodiversity on the total respiration. The hypothesis tested over the course of this study was that increasing the number of species will have a positive effect on the respiration measured.

The aim of Chapter 3 was to create a pool of bacterial species isolated from CF sputum samples, and to optimise a reproducible method to measure accurately the respired CO_2 generated. The final aim of Chapter 3 was to pilot and adapt a statistical model for the accurate analysis of data generated from BEF experiments. Chapter 4 aimed to assess the impact of biodiversity on total assemblage respiration and to investigate the effect of environmental changes, nutrient source and viscosity, on the impact of biodiversity. The aim of Chapter 5 was to assess the impact of phenotypic diversity, using assemblages of *P. aeruginosa,* on total microcosm respiration. Finally, the aim of Chapter 6 was to assess the type of interactions present within communities of increasing species richness.

Chapter 2

2. Materials and Methods

2.1 Clinical samples

Sputum samples were provided under full ethical approval (NHS REC number: 08/H0502/126) by adult CF patients attending the Adult Cystic Fibrosis Centre at Southampton General Hospital, UK. Three sputum samples were obtained from two stable CF patients (both female, ages 29 and 38 years). In order to disrupt the matrix of the sputum, samples were processed immediately by mixing with an equal volume (~2 ml) of Sputasol (Oxoid Ltd., Basingstoke, UK) and held at room temperature for five minutes. A ten-fold dilution series of this sputum - Sputasol mixture was created by serial dilution in sterile Phosphate Buffered Saline (PBS, pH 8.0; Oxoid). A 2 ml portion of the sputum-Sputasol mixture was stored at -80°C in 40% v/v glycerol (Sigma-Aldrich, Gillingham, UK) and 0.25M NaCl (Sigma-Aldrich).

Mueller-Hinton broth (MH; Oxoid Ltd.) and Blood Agar (BA; Oxoid Ltd), were chosen as suitable non-selective media (Coenye *et al.,* 2002). Both BA and MH agar plates were inoculated with 20 µl of the serially diluted suspension and were incubated at 33˚C for 36 hours using standard operating procedures (UK Standards for Microbiology Investigations, *www.hpa.org.uk*) from the Health Protection Agency (HPA) Southampton. After incubation, colonies (~ 15 per dilution) were randomly selected. Random selection was performed by using an approach with randomly placed line advancing across the plate. To ensure that pure bacterial cultures were collected, repeated streak plating of single colonies was performed. Pure colonies were stored at -80°C in a bead-based cryo-protection system (Protect™ tubes, Technical Service Consultants Ltd., Lancashire, UK).

2.2 Growth media

Bacterial isolation

Two solid media were used to isolate bacterial species from sputum samples (described above). These were BA and MH agar. These media were chosen in order to use "non-selective" media. These media have also been used previously in studies

(Coenye *et al.,* 2002) and at the HPA Southampton to facilitate the isolation of a broad range of bacterial species from CF sputum samples.

MH broth (Oxoid Ltd.) was prepared according to the manufacturer's instructions (21 g/l) and sterilised by autoclaving at 121°C for 15 minutes. For BEF experiments and for DNA extraction, bacterial isolates were grown in 20 ml sterile MH broth overnight at 37°C shaking at 110 rpm. MH agar plates were prepared with MH broth supplemented with 1.2% (w/v) technical agar (both Oxoid Ltd.) and sterilised by autoclaving at 121°C for 15 minutes. Blood Agar (BA; Oxoid Ltd.), plates were purchased pre-prepared from Oxoid.

Experimental substrates

Two liquid growth substrates were chosen as the media for BEF experiments:

1. **Tryptone water** (Oxoid Ltd.) 30 mg/ml in (x1) M9 buffer (Sigma-Aldrich), sterilised at 121°C for 15 minutes
2. **Defibrinated Horse Blood** (DHB; Oxoid Ltd.) 2.4% (v/v) in (x1) M9 buffer (Sigma-Aldrich). The DHB was purchased sterile from Oxoid and added to sterilised M9 buffer when cooled to 50°C

The sterility of all components was assessed by plating 50 µl of the medium prepared onto a MH agar plate. Sterility was inferred if no growth was observed on the plates after overnight incubation at 37°C. This was further supported by no growth in the control wells of each experiment.

Choice of Experimental Substrates

For the experiments presented in Chapters 4 and 5, the aim was to conduct them on two contrasting growth substrates which had a relevance to the lung environment. Given the nature of the CF lung, various mucin based media were evaluated but were found to be both difficult to manipulate and standardise. This was due to variability in the supply, incomplete dissolving in water and issues arising from lack of sterility. A specialised medium that aimed to imitate the nutrient content in the CF sputum (Palmer *et al.,* 2007) was also evaluated. This medium was not taken forward due to the high number of components that were easily utilisable by many species.

Tryptone Water and DHB were chosen as two contrasting growth substrates for the BEF experiments. M9 buffer was used to dilute and buffer both nutrient sources. It was decided that the first nutrient that would be investigated was a simple carbon source yet one that still was capable of supporting growth of a broad range of species isolated

from the human body. Tryptone Water provided amino acids, in various peptide lengths, vitamins and salt at neutral pH (~7.2). Vitamins are included to support the growth of bacterial species and NaCl is included for osmotic balance. The assorted amino acids are created from tryptic digests of casein (in dried milk), a complex of proteins found in mammalian milks.

The second nutrient source tested was required to contrast the first. Therefore, a medium that was predicted to have a more recalcitrant carbon source that required high-energy expenditure for utilisation was chosen. DHB is widely used in blood agar for clinical samples, although generally it is used as a nutrient source to investigate iron acquisition. Moreover, it has been suggested that during infections, some bacterial species actively target erythrocytes and haemoglobin for nutrient iron (Pishchany & Skaar, 2012). Horse blood is taken from specially-bred animals maintained on a high protein diet. The blood is extracted aseptically to avoid sterilization that would lyse the erythrocytes and cause haemolysis. Fibrin is mechanically removed, to prevent clotting, without lysis of other blood components. This was chosen as a contrast to the Tryptone Water as the DHB possessed a more recalcitrant carbon source. DHB is used in the isolation of many human pathogens therefore it is known to support bacterial growth as a solid medium.

Altering the viscosity of the experimental substrates

Enhanced viscosity versions of the experimental substrates were made by addition of 0.1% (w/v) of purified agar (Oxoid Ltd.) to liquid media. In the case of Tryptone Water, all the constituents were autoclaved together (Tryptone water, M9 and purified agar) at 121°C for 15 minutes. For the DHB, the M9 buffer with purified agar was autoclaved at 121°C for 15 minutes prior to the addition of the horse blood.

Preliminary data (not shown) indicated that a small increase in the viscosity of the environment was able to change the significance of at least one ecological mechanism on respiration. The increased viscosity environment was included to reduce potential bacterial movement and diffusion of nutrients. This environment was called the "constrained" environment. In the liquid environments, the bacteria had a greater opportunity to mix with other species. This environment was referred to as the "mixed" environment hereafter.

2.3 DNA extraction

DNA was extracted using a protocol adapted from Rogers *et al.* (2003) outlined briefly below. All chemicals used in this procedure were sterilised prior to use either by autoclaving or sterile filtration (0.2 µm pore, Sartorius AG, Gottingen, Germany).

Bacterial cells from liquid cultures were pelleted by centrifugation at 5,600 x *g* for five minutes at room temperature. The pellet was resuspended in 900 µl PBS (Oxoid). A single sterile 3 mm tungsten bead (QIAgen, Crawely, UK) and 0.2 g of 0.2 mm acid washed glass beads (Sigma-Aldrich, Gillingham, UK) were added together with 200 µl of 500 mM guanidium thiocyanate (Sigma-Aldrich), 100 mM EDTA pH 8.0 (Applied Biosystems, Carlsbad, CA, USA), 0.5% (w/v) N-lauryl-sarcosine (Sigma-Aldrich) to the bacterial suspension. The suspension was homogenised for 45 s at 6.0 m/s in a FastPrep™ machine (MP Biomedicals, LLC, Solon, OH, USA). Samples underwent two cycles of heating and cooling to 90˚C and -20˚C respectively for five minutes at each temperature. Cell debris was pelleted by centrifugation at 13,100 x *g* for 10 minutes at room temperature (all subsequent centrifugation steps were performed at room temperature unless otherwise stated). The supernatant was removed and was added to polyethylene glycol (final concentration 10%; Sigma-Aldrich) and sodium chloride (final concentration 0.5 M; Sigma-Aldrich) and stored at 4˚C for one hour. The precipitate was pelleted by centrifugation at 13,100 x *g* and the supernatant was discarded. The pellet was resuspended in 500 µl distilled water and 300 µl phenol: chloroform: isoamyl alcohol (proportions 25:24:1; Fisher Scientific Ltd., Loughborough, UK) was added to the resuspended pellet. The mixture was vortexed until "creamy" and centrifuged at 13,100 x *g* for 5 minutes. The resulting aqueous phase was drawn off, mixed with one volume isopropanol (Sigma-Aldrich) and 0.1 volumes ammonium acetate (final concentration 0.25 M, Fisher Scientific Ltd.) and stored at -20˚C for one hour. A pellet was obtained by centrifugation at 13,100 x *g* for 10 minutes. The supernatant was discarded and the pellet washed in 70% ethanol and air-dried before being resuspended in 50 µl nuclease-free water (Invitrogen, Paisley, UK). For every round of extractions, a negative control sample, containing fresh nuclease-free water, was processed.

2.4 Amplification of a region of the 16S rRNA gene

Amplification of the bacterial 16S rRNA gene was carried out using universal primer set; 8f (5'-AGA GTT TGA TCC TGG CTC AG-3'; Liu *et al.,* 1997) and pH' (5'-AAG GAG GTG ATC CAG CCG CA-3'; Bruce *et al.,* 1992). For T-RFLP work,

primer 8f was labelled at the 5'end with IRD700 dye (VHBio, Gateshead, UK); primer pH' (Invitrogen) was unlabelled. For amplicons that were to be to be sequenced, the forward primer (8f) was also unlabelled (Invitrogen). These primers bound to regions close to either end of the gene. This set was therefore able to amplify almost the entire 16S rRNA gene.

PCR mixtures comprised of 12.5 µl of Red*Taq* Readymix (Sigma-Aldrich; 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatine, 0.2 mM of each deoxynucleotide triphosphate and 1 unit of *Taq* DNA polymerase) and 0.2 µM of both primers. Template DNA (typically *c*. 50-100 ng) and sterile nuclease-free water was used to make the final reaction volume a total of 25 µl. PCR conditions were those described in Liu *et al.* (1997); initial denaturation at 94˚C for three minutes, followed by 35 cycles of 94˚C for 30s, 56˚C for 45s and 72˚C for two minutes and ended by a seven minutes final extension at 72˚C. All PCRs were carried out using an AB 2720 Thermal Cycler (Applied Biosystems)

2.5 Agarose gel electrophoresis

All amplified DNA products were separated by using Tris-acetate-EDTA (TAE)-1% (w/v) agarose gel (Bioline, London, UK) electrophoresis at 160 volts for 35 minutes in TAE buffer. DNA was stained with 10 µg/ml GelRed™ (Biotinium, Hayward, CA, USA) and visualised on a UV (λ = 362nm) transilluminator (Alpha Innotech, Santa Clara, CA, USA). Restriction digest fragments (see below) were visualised after TAE-2% (w/v) agarose gel electrophoresis at 120 volts for 65 minutes in TAE buffer. The DNA size marker (100 - 10,000 bp) used for all TAE-agarose gel electrophoresis procedures was the MassRuler™ Express DNA Ladder, Mix, Forward (Fermentas, York, UK).

2.6 Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis

Restriction endonuclease digestion

PCR products (*c*. 150 ng) were digested to completion using 1 unit of the restriction endonuclease *Hha*I (New England Biolabs, Ipswich, MA, USA) for 5 hours at 37˚C, in accordance with manufacturer's instructions. Restriction reactions were carried out in an AB 2720 Thermal Cycler (Applied Biosystems) followed by heating to 65°C for 10 minutes.

T-RFLP profiling

T-RFLP analysis was performed as previously described in Rogers *et al.* (2003). Digestion of the amplified products was performed as above. Approximately 0.7 µg T-RFLP PCR products were separated by length using a 25x25x0.2cm polyacrylamide gel (8% v/v SequaGelXR denaturing acrylamide gel (National Diagnostics, Hessle, UK), 8.3 M urea, 10% formamide and 0.5x Tris-Borate-EDTA (National Diagnostics) buffer. Polymerisation was initiated using 125 µl 10% ammonium persulphate (National Diagnostics) and 12.5 µl TEMED (BioRad, Hemel Hempstead, UK)). Data were generated using a LI-COR IR^2 automated DNA sequencer (LI-COR Biosciences, Nebraska, USA) set at 55°C and 1,200 V. Bands were included in the analysis if they were >0.1% of the total DNA signal in a given lane (Rogers *et al.,* 2004). The position of each band was calculated in relation to the microSTEP 15a (700 nm) size marker (Microzone, Haywards Heath, UK) using the image analysis software Phoretix™ 1D (Nonlinear Dynamics Ltd, Newcastle, UK).

2.7 16S rRNA gene sequence analysis

Following amplification of the 16S rRNA gene region as described above, sequencing was performed commercially (Macrogen Ltd., Seoul, South Korea). The sequences were trimmed to a uniform length of 550 bp. Species nomenclature was assigned to the closest match on GenBank by using BLASTN (*http://ncbi.nlm.nih.gov/blast/*) and confirmed using the Ribosomal Database Project (RDP) using SeqMatch (*http://rdp.cme.msu.edu/).* Taxonomic identities were assigned by >97% sequence identity to an existing sequence present in the GenBank database.

2.8 Growth and construction of bacterial assemblages

For BEF experiments, bacterial broth cultures were grown and prepared for inoculation into assemblages where the initial densities and species diversity were controlled. Prior to the start of the experiment, the species selected (details in Chapter 3a) were each inoculated into 20 ml MH broth. These cultures were incubated at 37°C and shaken overnight at 110 rpm in a Unitron shaking incubator (Infors-HT, Reigate, Surrey, UK). The overnight cultures were inoculated, 1:20, into 2 x 20ml fresh portions of sterile MH broth. The two cultures were used as independent replicates. These were incubated for 1.5 hours at 37°C, shaken at 110 rpm, until broth optical densities measured at 600nm (OD_{600}) reached between 0.8-1.0 using a Jenway 6300 spectrophotometer (Bibby Scientific Ltd, Staffordshire, UK). Bacterial suspensions

were pelleted by centrifugation for 5 minutes at 5,000 x *g* and the supernatant discarded. Cell pellets were washed twice in 1x M9 minimal salt solution (M9; Sigma-Aldrich). The OD_{600} was adjusted to 0.1 (approximately $1x10^5$ colony forming units (cfu) per ml) by resuspending the pellet in an appropriate volume of M9. The diversity of the experimental inocula was manipulated by mixing these cultures. Of these mixtures, 100 µl portions were inoculated into 400 µl of the experimental medium resulting in a 1:4 dilution of the culture. The number of bacteria entered into each experiment was kept constant at approximately $1x10^4$ cfu per 500 µl experimental microcosm. This was kept constant regardless of the number or composition of species. Therefore as the number of species increased, the size of each of the individual species inocula decreased. Microcosms were inoculated with one, two, three, four, six or 12 species (see Chapter 3c for full details) into a 1.2 ml-deep 96-well plate containing the experimental medium in the volumes stated above. Each set of microcosm mixes comprised of 28 microcosms (Partitions), with each independently replicated twice.

2.9 Measurement of CO_2 production

Full details of the CO_2 measurements and calibration of the method can be found in Chapter 3b, briefly; CO_2 production was measured by the MicroResp™ Soil Respiration System assay (Macaulay Scientific Consulting Ltd., Aberdeen, UK). This assay used a pH indicator, Cresol Red, within a buffered gel to measure the concentration of CO_2 dissolved in the gel. The deep-96-well, containing the inoculated medium, and 96-well plates, containing the indicator gel, were sealed together using specifically designed rubber seal and clamps (Macaulay Scientific Consulting Ltd.). All indicator plate absorbance readings were taken (λ = 572 nm) using a Spectramax 190 plate spectrophotometer (Molecular Devices, LLC, Sunnyvale, CA, USA).

2.10 EcoPlate™ community level phenotypic profiling

Bacteria were grown overnight from frozen stocks in sterile Luria-Bertani (LB; Oxoid Ltd.) broth at 37°C shaking at 110 rpm. Overnight cultures were centrifuged at 5,000 x *g* for 5 minutes and the supernatant was discarded. The pellet was re-suspended in sterile 1x M9 buffer (Sigma-Aldrich) and the centrifugation step repeated. OD_{600} readings were taken and the appropriate volume diluted in Inoculating Fluid-A (IFA; *www.biolog.com*, BIOLOG, Hayward, CA, USA) to reach an equivalent OD_{600} of 0.01. Of the IFA-culture, 100 µl was pipetted into each well of the EcoPlate™ (BIOLOG) in accordance with manufacturer's instructions.

The EcoPlates™ were 96-well plates comprised of 31 sole carbon sources and one negative, no-substrate, control, in triplicate. According to manufacturer's instructions, the EcoPlates™ and sterile IFA were stored in the dark at 4°C until use. Each set of assays was independently replicated twice. The EcoPlates™ were incubated at 37°C with OD_{590} readings (Yang C. *et al.,* 2011), measured using a SpectraMax 190 spectrophotometer (Molecular Devices). The lengths of incubation varied between studies but were either 24 or 48 hours.

2.11 **Statistical analysis**

All statistical analyses were performed using the R statistical programming package (v2.12.2 - 2.13.0; *www.R-project.org*). Statistical analyses using general linear models are described in detail in Chapter 3c. The general linear models were tested for the homogeneity of variances and the normality of the residuals. These were assessed visually and confirmed using the Fligner-Killeen test for homogeneity of variances and the Shapiro-Wilk test for normality of errors (further details in Chapter 4).

All hierarchical cluster diagrams, drawn using the complete linkage method, in this thesis were calculated using the Jaccard's similarity coefficients converted to give the Jaccard's distances (eq.1):

$$d_{Jac} = 1 - \frac{a}{a+b+c} \qquad [1]$$

where: a = the number of positive values shared between two microcosms, b = number of positive values unique to the first microcosm, and c = number of positive values unique to the second microcosm.

Chapter 3

Experimental methodologies

Chapter 3 reports the choice and optimisation of methodological procedures essential for the biodiversity and system function experiments reported in this thesis. The chapter is divided into three sections; a) the isolation and choice of bacterial strains, b) the optimisation and calibration of the respiration assay and c) the explanation of the experimental design and general linear model analysis.

Chapter 3a

3. **a. Isolation of bacterial species**

3.1 **Introduction**

This study sought to manipulate bacteria from CF airways into assemblages varying in diversity. Here, the isolation of CF sputum associated bacteria is reported. Bacteria have been isolated from the CF airways since the recognition of the disease by Andersen in 1938 (in Mearns *et al.,* 1972). Although these initial studies predominantly identified *Staphylococcus aureus* and *Pseudomonas aeruginosa* as common, the role of bacteria in the progression of lung damage was widely discussed (Mearns *et al.,* 1972). More recently, some other species have been considered pathogenic due to correlations with their routine culture from CF patients and worsening symptoms (Burns *et al.,* 1998). CF lung disease has been considered both a polybacterial & polymicrobial infection (Harrison, 2007).

Culture-independent techniques have provided additional information on the species present. These methods have also shown that there is a higher diversity of bacterial species in the CF lung than previously identified by culture (Rogers *et al.,* 2003). Culture-independent methodologies have identified as yet "uncultured" bacterial species, which in turn resulted in culture-independent techniques being increasingly used over culture methods for the study of bacterial communities (Rogers *et al.,* 2009). Despite this, studies have shown that the culture of bacterial isolates from CF samples can yield a large degree of diversity (Coenye *et al.,* 2002; Guss *et al.,* 2011; Sibley *et al.,* 2011).

3.2 Materials and Methods

Clinical samples and bacterial isolation

Three clinical CF sputum samples were taken in this study to isolate bacteria (Chapter 2.1). An isolate of *Stenotrophomonas maltophilia* from a third patient (female, age = 56) attending the same CF Centre (Chapter 2.1) was also included in this study.

DNA extraction and amplification

DNA extraction and amplification was performed as previously described (Chapter 2.3 and 2.4 respectively).

16S rRNA gene sequence analysis

The 16S rRNA gene was analysed using Terminal Restriction Fragment Length Polymorphism (T-RFLP) and sequence analysis as previously described (Chapter 2.6 and 2.7 respectively).

3.3 Results

Bacterial cultures

Following the inoculation of BA and MH agar with dilutions of sputum samples diverse bacterial colonies were detected after 36 hours incubation. From the colony counts and dilutions it was determined that there was between 1 x 10^6 and 1 x 10^8 cfu per ml present in the sputum samples. Using data from a pilot study, where 60 isolates yielded eight isolates with distinct 16S rRNA gene sequences, a total of 155 isolates were randomly selected for further study. Isolates were purified by streak-plating and single colonies selected for cryo-storage and DNA extraction. DNA was extracted from these 155 purified isolates and a region of the 16S rRNA gene was amplified in each case.

Bacterial identification

Later experiments relied on T-RFLP to distinguish rapidly between different species of bacteria within microcosm assemblages. As such, it was important from the outset to be able to differentiate the species by using T-RFLP profiling. Initially, T-RFLP profiling was performed on the amplified 16S rRNA gene from each isolate. Each amplification and endonuclease restriction was independently replicated twice on the DNA extracted from each isolate. The Terminal-Restriction Fragment (T-RFs) lengths were found for each isolate and T-RFLP profiling provided tentative species identification utilising a database developed by Rogers *et al.* (2003). A total of 155 random isolates were selected between the two patients with 126 and 29 isolates picked from the cultured samples from the first and second patient respectively. Of the 155 isolates, 69 were identified as Gram-negative bacterial species (Table 3.1). These were divided into five distinct species from five genera. The remaining 86 isolates were identified as 11 distinct Gram-positive species from four genera. These isolates were representative of 5.6% the total number of isolates cultured from the sputum samples.

For a more detailed phylogenetic classification, the 16S rRNA gene for 94 isolates was sequenced. These 94 isolates spanned across all of the different T-RF groups, with at least one of each group sequenced. The 16S rRNA genes sequences were matched to reference sequences, as previously described, to assign a taxonomic identity to each isolate group (Table 3.1). From the 155 isolates, the most frequently isolated bacterial species was identified as *Pseudomonas aeruginosa* with 40 isolates. Of these,

phenotypic analysis showed that it was possible to obtain 12 phenotypically distinct isolates (ecotypes) within the same species. These *P. aeruginosa* ecotypes were further investigated (Chapter 5). The next most common isolate was found to be *Streptococcus salivarius* with 28 isolates. *Burkholderia cepacia*[1], *Staphylococcus aureus* and *Streptococcus pseudopneumoniae* were found to be the next most abundant species with 15, 14 and 12 isolates respectively.

From the sequence analysis, 17 species were identified. Of these species, five were Gram negative; *Achromobacter xylosoxidans, Acinetobacter baumannii, B. cepacia, Serratia marcescens* and *P. aeruginosa.* Twelve species were Gram positive; *Enterococcus faecium, Micrococcus luteus, S. aureus, Staphylococcus capitis* & *Staphylococcus haemolyticus* and *Streptococcus gordonii, Streptococcus mitis, Streptococcus oralis, Streptococcus pneumoniae, S. pseudopneumoniae, S. salivarius* and *Streptococcus sanguinis*.

An isolate of *Stenotrophomonas maltophilia* was added to this collection from a third female CF patient (see section 3.2) isolated by the HPA Pathology Laboratory in Southampton General Hospital. The isolate was confirmed to be *S. maltophilia* by 16S rRNA sequencing as described previously.

Selection of bacterial species for experimentation

From the species pool above, a group of the bacterial species were chosen to be included in the BEF experiments. Whilst the data are not included in this thesis, initial experiments were conducted using assemblages of up to eight species. The results of this pilot study indicated that at the maximum richness level of eight, the respiration continued to rise with no clear indication of reaching an asymptote.

To enable the experimental system to gather the maximum amount of ecologically relevant data, 12 species were chosen. As will be explained further in Chapter 3c, the highest number of richness levels that could be created with a collection of 18 isolated species is six. The same number of richness levels can also be achieved by using 12 species. The decision to increase from eight to 12 species, rather than from eight to 18, was based on the most efficient yield of ecological data. Twelve species was also more practical when starting the experiment at this required fewer assays to be measured. As such, 12 were selected at random for use in the later experiments. These 12 species are indicated in Table 3.1 by the addition of a "Sp" followed by a number.

[1] Members of the B*urkholderia cepacia* complex have been shown to not be differentiated fully by the 16S rRNA gene sequence (Mahenthiralingam *et al.,* 2000). This species was termed *B. cepacia* here for simplicity.

In some cases, multiple isolates had been identified as isolates of the same species (Table 3.1). From each set of isolates, one was selected at random. By selecting a single isolate from each taxonomic group, the effect of diversity between species could be investigated (Chapters 4 and 6). Further study (Chapter 5) was designed to investigate the diversity within a species. Confirmation of the taxonomic identity of the selected isolates was performed by 16S rRNA gene sequencing and matching to reference sequences as previously described.

Group	Abundance	T-RF (bases)	Taxonomic identifier	Acc. No.	Seq.ID	Sp. ID.
1	40	156	*Pseudomonas aeruginosa*	HQ454496	99.0	Sp1
2	n/a[2]	211	*Stenotrophomonas maltophilia*	HQ238831	100.0	Sp2
3	15	212	*Burkholderia cepacia*	GU048852	100.0	Sp3
4	5	563	*Achromobacter xylosoxidans*	AF531768	99.1	Sp4
5	3	205	*Acinetobacter baumannii*	HQ844640	98.8	Sp5
6	6	370	*Serratia marcescens*	GQ981178	100.0	Sp6
7	5	216	*Enterococcus faecium*	AJ420800	100.0	Sp7
8	4	237	*Staphylococcus haemolyticus*	JF895185	100.0	Sp8
9	1	238	*Staphylococcus capitis*	FJ357580	99.9	-
10	14	235	*Staphylococcus aureus*	FJ899095	100.0	Sp9
11	2	541	*Streptococcus gordonii*	AY281077	99.8	-
12	12	577	*Streptococcus pseudopneumoniae*	FJ823145	99.8	-
13	9	574	*Streptococcus mitis*	AF003929	99.5	Sp10
12	4	577	*Streptococcus pneumoniae*	AY525788	99.5	Sp11
14	2	578	*Streptococcus sanguinis*	NR_024841	99.4	Sp12
15	4	579	*Streptococcus oralis*	GU045379	99.9	-
16	28	581	*Streptococcus salivarius*	AY188352	99.9	-
17	1	175	*Micrococcus luteus*	AJ536198	100.0	-

Table 3.1: Identification of the 155 bacterial isolates obtained during this study. The T-RF group, abundance and size are presented alongside the closest match taxonomic identity, GenBank accession number and sequence identity (Seq.ID) of the reference and unknown sequences. For strains which were matched to more than one reference strain, Sp. ID indicates the abbreviated identifier given to these strains when reported in experiments below.

[2] Obtained from the HPA Southampton as described in 3.2

3.4 **Discussion**

The aim of this preparatory work was to isolate and identify a diverse set of clinically derived bacterial species from CF sputum samples. It was determined, through pilot studies, that the number of bacterial species required for the following experiments was at least 12.

The isolation of these bacterial species was conducted using non-selective solid media, BA and MH agar, in aerobic conditions with approximately 1x 10^7 viable cells detected. This number of viable bacterial cells is consistent with previous studies (Pye *et al.,* 1995; Stressmann *et al.,* 2011b). From these viable cells, a collection of 155 isolates were randomly selected and yielded isolates of 18 genetic groups of 16S rRNA gene sequences. Of these isolates, *P. aeruginosa, B. cepacia, A. xylosoxidans,* and *S. aureus* are routinely cultured from CF sputum samples (Lyczak *et al.,* 2002; LiPuma, 2010). With one exception (discussed below) the species isolated and identified have all been associated with human respiratory disease (Maeda *et al.,* 2011). The exception is *M. luteus.* Although this bacterial species has been linked to human infection (Hirata *et al.,* 2009), it has never, to the best of knowledge, been implicated as an aetiological agent of respiratory disease.

As found previously in a number of studies (e.g. Coenye *et al.,* 2002; Sibley *et al.,* 2011), direct non-selective culturing of bacteria from sputum samples can isolate reasonable diversity from CF patients. One bacterial species that was not cultured but has been regarded as a key pathogen in CF airway disease was *S. maltophilia* (Lyczak *et al.,* 2002). In order for the experimental assemblages (introduced in later Chapters) to be able to study the ecology of CF related bacteria better, it was resolved to add this species to the collection prior to use in BEF experiments.

This study encountered some difficulty when using T-RFLP to distinguish bacterial species as both *S. pneumoniae* and *S. pseudopneumoniae* were found to share the same T-RF length of 577 bases. This issue of identification of microorganisms using T-RFLP profiling alone has been previously discussed in Dickie and FitzJohn (2007). They recommended that the T-RFs were used as an initial guide to the diversity present with species identities confirmed by 16S rRNA gene sequencing.

Chapter 3b

3. b. Optimisation of the respiration measurement system

3.1 Introduction

Many previous BEF experiments have used a measure of productivity as the ecosystem function upon which the biodiversity impacts. With plants, a common measure of productivity is biomass (Hector *et al.,* 1999). Depending on the experimental system chosen the measure of the biomass of bacteria can be difficult to measure without jeopardising the integrity of the sample. One commonly used alternative is respired CO_2 (Bell *et al.,* 2005; Christen *et al.,* 2008; Salles *et al.,* 2009; Lawrence *et al.,* 2012). Bacteria produce CO_2 from aerobic respiration and as such CO_2 production has been shown to be closely linked to metabolic activity (Bell *et al.,* 2005; Schimel *et al.,* 2007).

In pilot experiments, CO_2 production was measured using titration-based methods. In this, CO_2 generated by bacterial activity dissolved in aqueous sodium hydroxide to form sodium bicarbonate. The concentration of sodium hydroxide at the end of the experiment was quantified by titration using hydrochloric acid. This proved to be labour-intensive and not well suited for the large number of microcosms planned. An alternative assay was required. One such assay for CO_2, referred to as the MicroResp™ Soil Respiration System (Campbell *et al.,* 2003) was investigated as a potentially more efficient method. Originally, this system was designed to monitor the respiration of soil bacterial communities. This system had been used for almost a decade for this purpose with over 60 journal articles published (*www.microresp.com/Publications.html*). Moreover, in one recent study, the MicroResp™ system has been applied successfully to a BEF experiment (Lawrence *et al.,* 2012). For the studies presented here on clinically-derived species, it was important first to establish the system and assess its effectiveness for this purpose. This included assessing the reproducibility, the sensitivity and CO_2 detection limits.

In the MicroResp™ system, bacterial cultures forming the microcosm mixes were inoculated into the deep-well plate at the point corresponding to "Substrate and bacterial assemblage" (Figure 3.1). During the experiment, CO_2 was generated by the growing bacterial assemblages. The CO_2 formed passed through the hole in the seal and dissolved into the indicator gel in a "Detection well". The amount of CO_2 generated was

then recorded by means of a colour change, red to yellow, by a pH sensitive dye within the gel. Before applying the MicroResp™ tool, a set of tests and adaptations were needed.

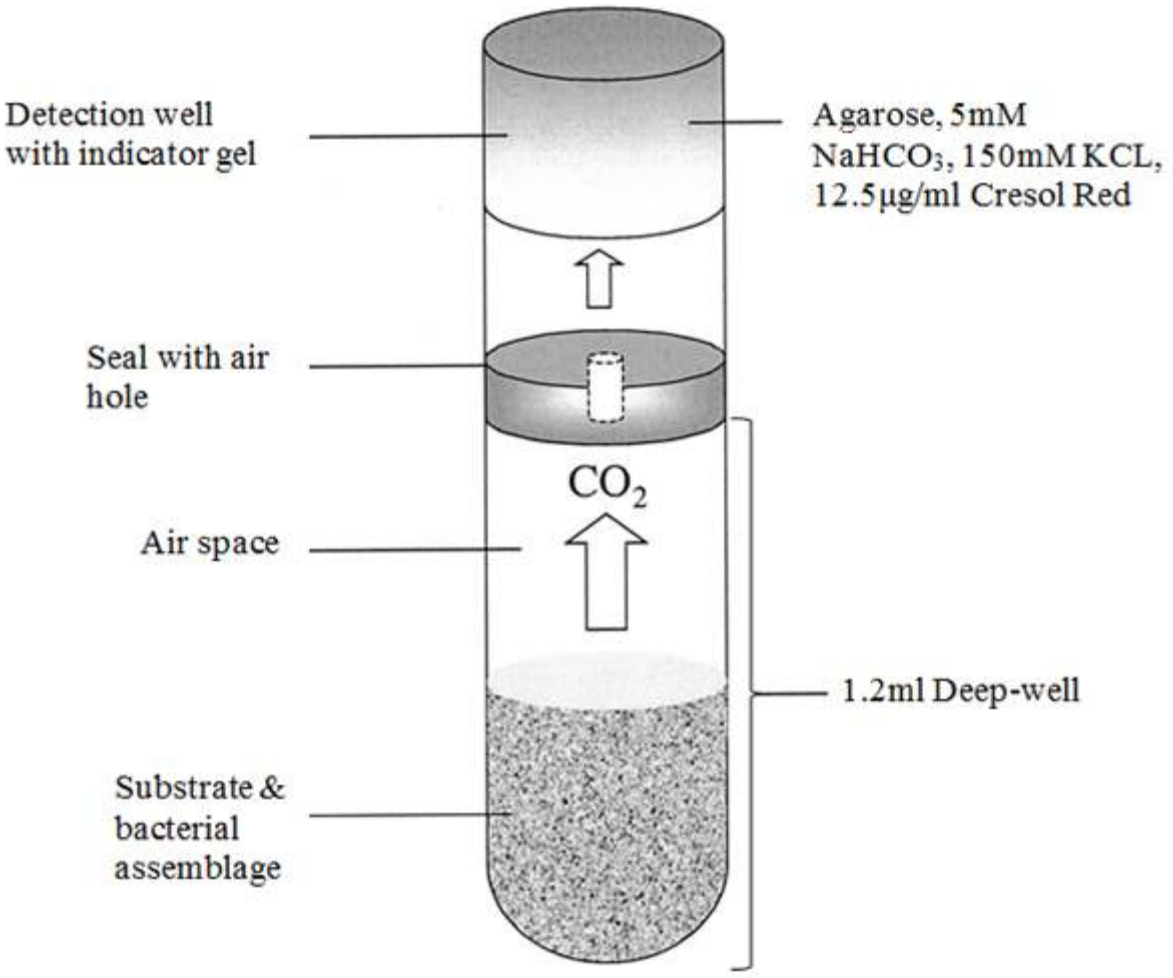

Figure 3.1: Schematic diagram of the key features of the MicroResp™ assay system. Adapted from Campbell *et al.* (2003), this diagram shows the positioning of an upper well from the indicator plate in relation to a lower reaction chamber well with bacterial assemblage.

3.2 Methods

Assembly of the MicroResp™ Soil Respiration System

The MicroResp™ system used two plates; one a conventional 96 well plate (Sterilin Ltd., Newport, UK) and a 1.2 ml deep-96-well (Sterilin Ltd.) plate. The 96-well plate contained the Cresol Red indicator gel (see below). The plates were overlaid and sealed together using specifically designed rubber seals and clamps (Macaulay Scientific Consulting Ltd.) such that a set of 96 extended wells were created (Figure 3.1).

Cresol Red indicator gel

Two protocols were employed to prepare the gel in which the pH indicator was located. The first was based on manufacturer's instructions with the second prepared following a period of experimentation.

To set up the assay, 150 µl of the Cresol Red (VWR International) indicator gel was set into each well of a 96-well microtitre plate (Sterilin Ltd.). The gel was prepared following the manufacturer's recommendation and contained 2.5 mM $NaHCO_3$ (Sigma-Aldrich), 150 mM KCl (Sigma-Aldrich), 12.5 µg/ml Cresol Red (VWR International) and 1% (w/v) agarose (Bioline) in sterile deionised water. After initial experimentation, a second gel was prepared with the concentration of $NaHCO_3$ increased to 5 mM. All other components remained constant.

The indicator gel was prepared by first dissolving Cresol Red, KCl and $NaHCO_3$ in the sterile deionised water to create the indicator solution, and heated to and held at 65°C. A 3% (w/v) agarose- gel prepared by heating until dissolved in sterile deionised water with the liquid gel then cooled to 65°C. When both components of the indicator gel were at 65°C, the indicator solution was decanted into the gel and mixed thoroughly. From here, 150 µl aliquots were transferred to all the wells in the 96-well plate and left to cool to room temperature. To preserve the longevity of the indicator gel, plates containing the indicator gel, once set, were stored in the dark in moist conditions for up to 72 hours. The storage of plates in atmospheric conditions is in contrast with the MicroResp™ technical manual which states plates should be stored in CO_2 deficient conditions. This difference in storage conditions did not however, significantly affect the stability of the indicator as shown by time zero absorbance readings (mean of the differences = 0.014 A572nm; $t_6 = 2.16$, $p = 0.074$).

Bacterial culture and assays of respiration

Assessments of the MicroResp™ system were made using a single strain namely *Pseudomonas aeruginosa* NCTC 10332. Bacterial cells were grown overnight in sterile MH broth (Oxoid Ltd.) at 37°C with shaking at 110 rpm. This overnight culture was used to inoculate 20 ml fresh sterile MH broth (1:19 dilution culture: sterile MH broth). The fresh culture was incubated at 37°C for 1.5 hours with shaking at 110 rpm. After incubation, bacterial cells were pelleted by centrifugation at 5,000 x *g* for 5 minutes and the supernatant discarded. The pellet was resuspended in sterile 1x M9 (Oxoid Ltd.). This bacterial cell suspension was adjusted to an OD_{600} of 0.1; approximately $1x10^4$ cfu/ ml. A 100 µl portion of the bacterial suspension was used to inoculate wells containing 400 µl sterile MH broth prior to sealing the chamber with the indicator plate.

The system was then incubated at 37°C with shaking at 110 rpm. Four of the bacteria-containing wells were unsealed at each of six time points up to 24 hours (t = 0, 2, 4, 6, 8 and 24). The absorbance, at a wavelength of 572 nm (A572nm), of each of the unsealed wells was recorded using a SpectraMax 190 spectrophotometer (Molecular Devises). Each time point was repeated twice and independently replicated twice.

CO_2 measurement

The Cresol Red indicator gel monitored the presence of CO_2 within the system by responding to the change in acidity caused by dissolved CO_2. The production of acid was in equilibrium with the concentration of CO_2 present (eq.2).

$$CO_{2(gas)} + H_2O + HCO_3^- \leftrightarrow 2CO_3^{2-} + 3H^+ \quad [2]$$

Increasing H^+ ion levels in the gel cause the Cresol Red indicator gel to change colour from red to yellow, with this quantified as above. As the pH of the indicator gel decreased, causing the Cresol Red indicator to turn yellow, the absorbance reading reduced.

Calibration

Calibration was achieved by producing different levels of CO_2 (eq.3). The $\%CO_2$ present was measured and this correlated against the absorbance of the indicator gel.

$$HCl + NaHCO_3 \rightarrow H_2O + NaCl + CO_2 \quad [3]$$

Ten concentrations of $NaHCO_3$ (Sigma-Aldrich) diluted as a serial 1: 2 dilution in sterile deionised water starting with a stock concentration of 500 mM (dilutions were: 250 mM, 125 mM, 62.5 mM, 31.25 mM, 15.6 mM, 7.8 mM, 3.9 mM and 0 mM) were prepared. A 1 ml volume of each concentration of $NaHCO_3$ was added to an equal volume of 1 M HCl (Sigma-Aldrich) in an airtight 40 ml serum bottle (Smith Scientific Ltd., Edenbridge, UK) containing three wells of the indicator gel. The reaction was run to completion over 18 hours at 37°C.

After this time, gas samples from within the serum bottle were analysed by gas chromatography (GC) using a thermal conductivity detector (Shimadzu UK Ltd., Milton Keynes, UK performed by Dr. Ioannis Vyrides of Imperial College, London). This gave the gaseous percentage composition for both CO_2 and nitrogen. For the same exposed wells, the absorbance at 572 nm (A572nm) was measured using a SpectraMax 190 spectrophotometer (Molecular Devices). This calibration process was independently replicated three times.

Statistical analysis

All statistical analyses were performed using *R* (v.2.12.2 - v.2.13.0).

3.3 **Results**

Detection limits

The range of absorbance values over which the indicator was informative were determined. Here, increased CO_2 levels correspond to a decrease in pH and absorbance values. The indicator gel recommended by the manufacturers became saturated at a mean absorbance of 0.246 A572nm (SD = 0.012, n = 7). The lower limit of CO_2 detection was any value below the mean negative control absorbance. This was established as 0.757 A572nm (SD = 0.050, n = 16) for the original indicator gel.

Bacterial respiration

To assess whether the maximum amount of CO_2 measurable (A572nm $\geq$ 0.246) was appropriate for the experiments to follow, a *P. aeruginosa* culture was grown within the system in MH broth. The result indicated that after 26 hours incubation, the bacterial growth had begun to reach stationary phase (data not shown) and absorbance of the indicator gel had fallen to 0.387 A572nm (SD = 0.029, n = 6). Despite this being within the tolerance limits, the MicroResp™ manual and Campbell *et al.,* (2003) indicated that this absorbance was approaching a non-linear relationship for CO_2 measurement. To ensure that the upper detection limit of the indicator was not likely to be reached in later experiments, the decision was taken that the indicator needed to be adapted.

Detection limits of the adapted Cresol Red indicator gel

To increase the linear range of the indicator gel in order to measure more acidic conditions the concentration of $NaHCO_3$ in the indicator gel was increased from the manufacturer's specification of 2.5 mM to 5 mM. The detection limits of the new indicator gel were tested as before. These results showed that the adapted indicator gel became saturated at 0.190 A572nm (SD = 0.023, n = 4). A full calibration was then performed on this indicator gel combining CO_2 measurements based on both absorbance values and Gas Chromatography data.

Calibration of Cresol Red indicator gel

The calibration was performed independently three times. Within each of these replications, the A572nm was recorded as the mean of three wells containing the indicator gel. The CO_2 within the system was determined by the chemical reaction

presented in eq.3. No-bicarbonate negative controls were also analysed with each of the replicates. The gas chromatograph (GC) determined CO_2 (measured as a percentage of the total gas volume) values ranged from 0.111 to 5.756 $\%CO_2$. These tests showed that the lower limit of detection of $\%CO_2$ for the GC was 0.1% (v/v). The GC results showed that as the concentration of $NaHCO_3$ increased, there was an increase in the $\%CO_2$ detected in the sample. The calibration also found that the indicator was sensitive to a level of 0.01 $\%CO_2$ as shown in relation to the change in indicator absorbance values. No significant differences were observed between the replicates ($t_5 < -1.41$, $p > 0.196$). To establish set boundaries for the detection limits of the indicator gel, the limits of the GC were used. As such, the indicator was calibrated to measure between 0.11 and 5.76 $\%CO_2$ reliably.

When the $\%CO_2$ measurements were plotted against the mean absorbance values, the relationship was found to be an exponential one (Figure 2a) with an $R^2 = 0.928$. To be able to use this calibration curve to assess the CO_2 within a system using the A572nm, both axes were transformed using natural logarithms (base = *e*; eq.4; Figure 2b)..

$$\log(absorbance_{572nm}) = -0.266 * \log(\%CO_2) - 0.804 \quad [4]$$

This equation was re-arranged in order to be able to calculate $\%CO_2$ from the A572nm values (eq.5).

$$\%CO_2 = e\text{^}\frac{(\log(absorbance_{572nm}) + 0.804)}{-0.266} \quad [5]$$

Units of Respired CO_2 in the Text

As the headspace in the BEF experiments was constant, the $\%CO_2$ (% v/v) was taken as the measure of respired CO_2. For convenience, this is presented throughout the text as units of CO_2 (UCO_2). To account for variation in the negative control wells between samples, the mean A572nm reading was subtracted from the measurements of the experimental microcosms prior to conversion to UCO_2.

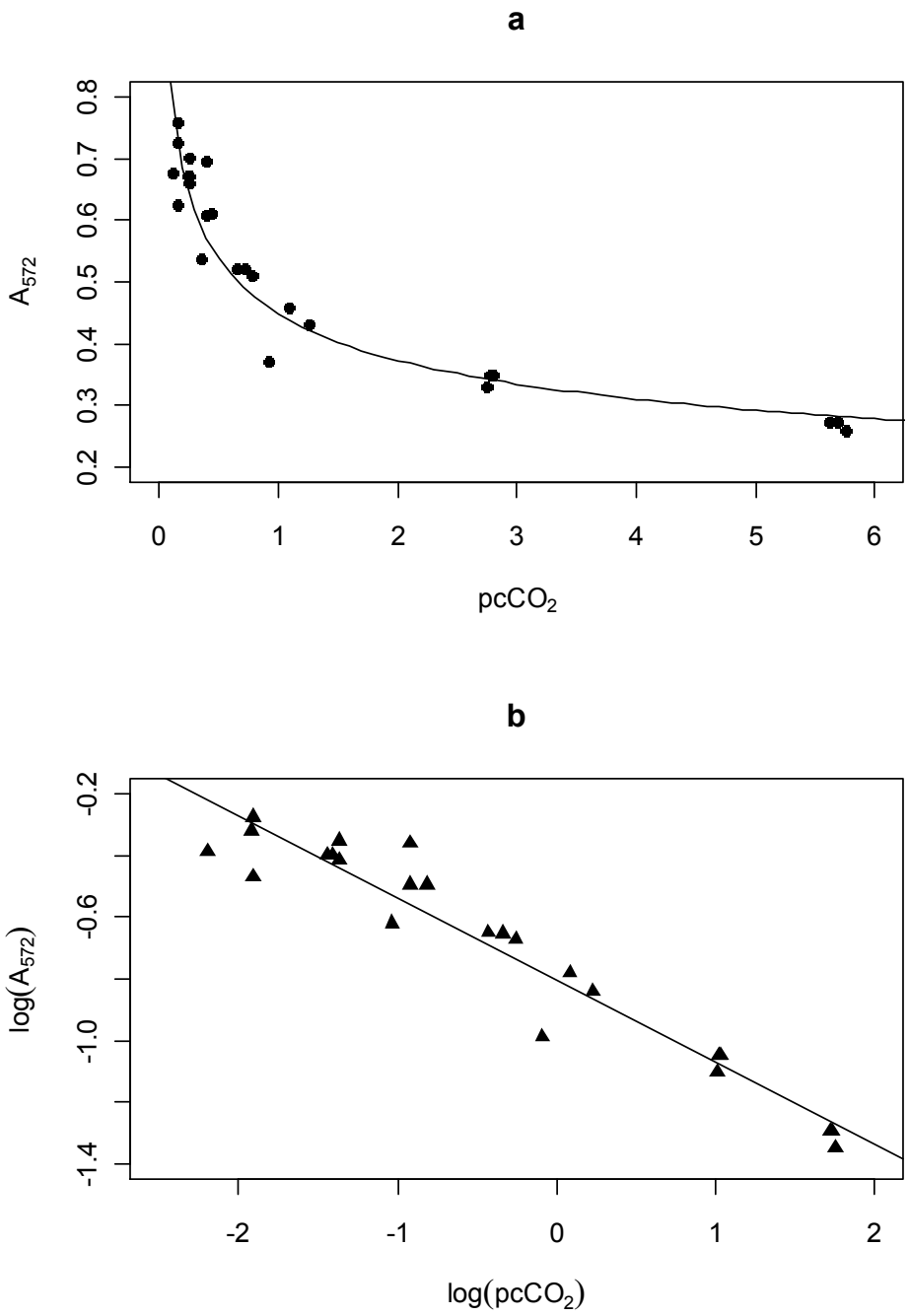

Figure 3.2: Calibration of the absorbance (λ = 572 nm) of the indicator gel as a function of $\%CO_2$. The calibration is shown with absorbance, measured at a wavelength of 572 nm, plotted, against the percentage of CO_2 measured in the closed system by gas chromatography. Figure 2a shows the non-transformed relationship. Figure 2b shows the relationships after both axes were transformed using the natural logarithm.

3.4 **Conclusions**

The aim of this study was to develop a method that would reproducibly and robustly calibrate the respiration of a BEF experiment. The method developed was based on the MicroResp™ Soil Respiration System (Campbell *et al.,* 2003) with this forming the basis of the measure of system function used here. The results produced by the testing of the detection limits of the manufacturer's indicator gel were consistent with previous calibration assays (Campbell *et al.,* 2003; MicroResp™ Technical Manual, 2007). This study also demonstrated the ability of this system to monitor the changes in CO_2.

As shown in previous studies assessing CO_2 respiration, the MicroResp™ system was also shown here to produce reproducible results. A modification of the indicator dye extended the linear detection range that was needed for future use in BEF studies. In addition to the robust calibration over this linear range, the high-throughput nature of the methodology was also seen as a clear advantage for the analysis of the many microcosms planned here. In short, the fully calibrated pH-indicator detection system allowed the measurement of CO_2 between 0.1 and 6% (v/v) at an accuracy of 0.01%. This also facilitated simple calculations to convert absorbance readings to units of respired CO_2. Importantly, over the planned experimental time period of 24 hours, the respiration of the bacterial assemblages was not likely to saturate the indicator.

Chapter 3c

3. c. Introduction of the experimental design and general linear model analysis

3.1 Introduction

Traditionally, the only experimental designs which could unambiguously separate linear (species) richness and species composition effects on ecosystem function were considered to be the fully factorial designs. In fully factorial designs, each species is present in every combination with all the other species. If a fully factorial design was employed for an experiment involving 12 species, there would be over 4000 experimental units, excluding replicates. Bell *et al.,* (2005; 2009b) however, produced the Random Partitions Design (RPD) as an alternative to fully factorial designs. In these papers, the RPD was shown to allow meaningful, practical manipulation and analysis of species in experimental microcosms. These studies analysed the experimentally derived differences in ecosystem function by using a General Linear Model (GLM). A feature of this approach is that key experimental factors were shown to be orthogonal (below) and thus less ambiguous to analyse.

3.2 The random partitions design

In the RPD, an experimental Partition is created. A Partition is a set of distinct microcosms in which each species is randomly allocated once to a microcosm mix (assemblage) at each of the richness levels tested. The choice of richness levels (*R*) is based upon the numerical factors of the total number of species (N) included in the experiment.

For example (Figure 3.3), for 12 species in an experiment there would be six factors (1, 2, 3, 4, 6 and 12) and thus six richness levels (*R* = 6):

1. a **one** species level with **12** microcosms with one species each,
2. a **two** species level with **six** microcosms with two species each,
3. a **three** species level with **four** microcosms with three species each,
4. a **four** species level with **three** microcosms with four species each,
5. a **six** species level with **two** microcosms with six species each and
6. a **12** species level with **one** microcosm with 12 species.

In this example, each of the 12 species was incorporated once at each species richness level. The RPD does not require all available species richness levels (factors of N) to be included, although a loss of explanatory power would result by doing so (Bell *et al.,* 2009b). Throughout this thesis, all factorial levels will therefore be included (Figure 3.3).

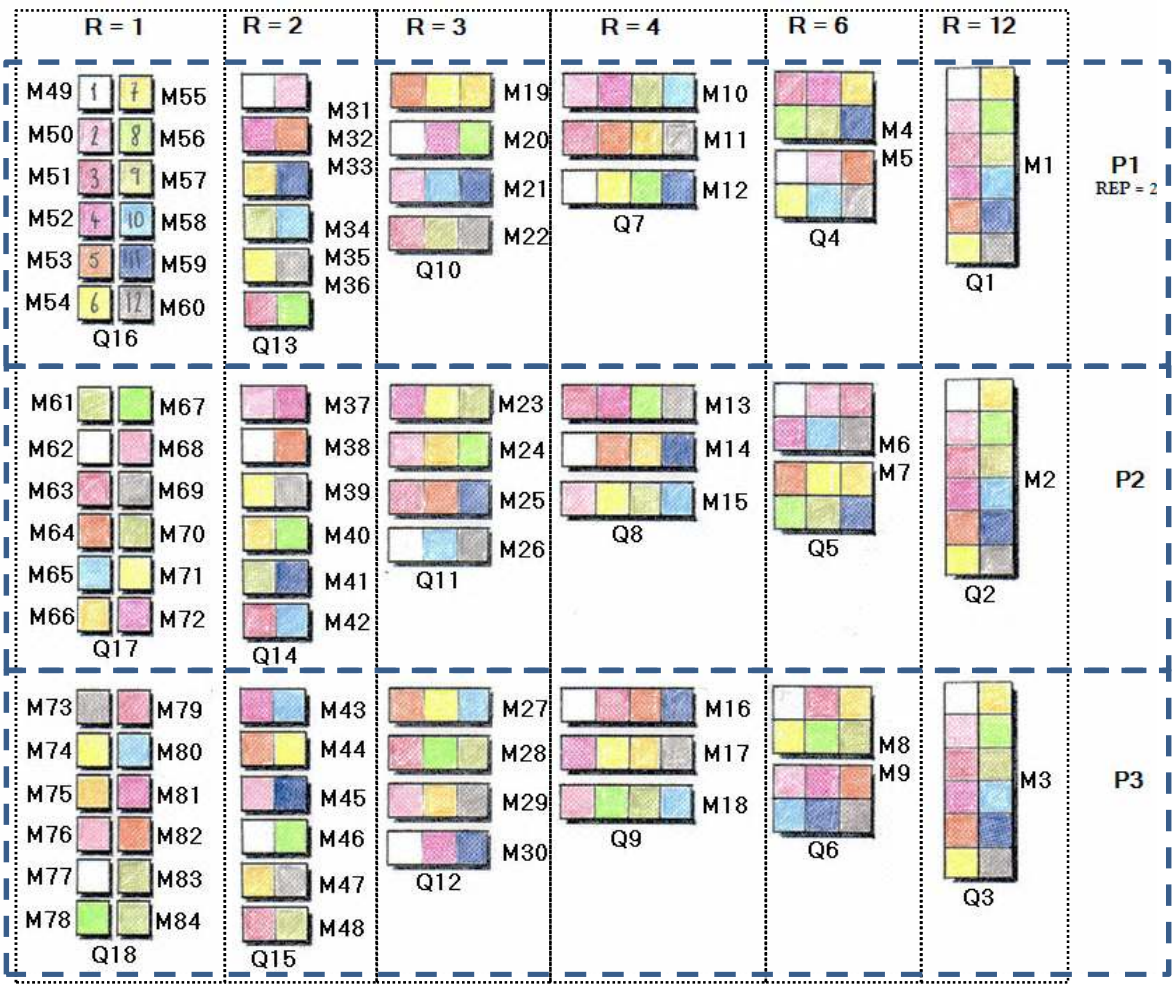

Figure 3.3: A diagrammatical representation of a single RPD with 12 species. Each square represents a single species, with each colour indicating one of 12 species and each block of squares represents a microcosm. These three Partitions comprised of 28 microcosms each, all replicated twice.

3.3 An example of the RPD analysis using experimental data

An example of the analysis will now be presented using 12 species. With 12 species, each Partition required 28 microcosms (not including replication) and tested species richness at six levels (1, 2, 3, 4, 6 and 12). A fully factorial design at these six levels would require 1718 experimental microcosms, not including independent replicates.

The data presented here were derived from three distinct Partitions with assays of cumulative respiration over 24 hours in Tryptone Water. With the three Partitions each replicated twice a total of 168 microcosms were analysed. The identification and choice of bacterial species, the details of experimental method, tests of leverage, homogeneity of variance and normality of residuals and a more thorough account of these experiments with detailed analysis are given in Chapters 3 (a and b) and 4.

The data (Figure 3.4) were shown to have a general trend of increasing respiration with increased species richness. Respiration was measured as units of respired CO_2 (UCO_2; Chapter 2) and ranged from 0.294 UCO_2 to 3.183 UCO_2.

The general linear model used in this analysis (eq.6) was created by the sequential addition of explanatory variables. The response variable (y) is the measure of respiration. The data were analysed in a specific manner. To preserve orthogonality, the linear model here required the linear richness term to be added first (discussed later). Each term was then analysed sequentially. This allowed each term in the model to analyse only the variation within the data that has not been accounted for by the preceding terms. The data were analysed using the equation with each of the five terms presented separately.

$$y = a + \beta_{LR}x_R + (\sum_1^N \beta_C x_C) + \beta_{NLR}x_R + \beta_Q x_Q + \beta_M x_M + e \qquad [6]$$

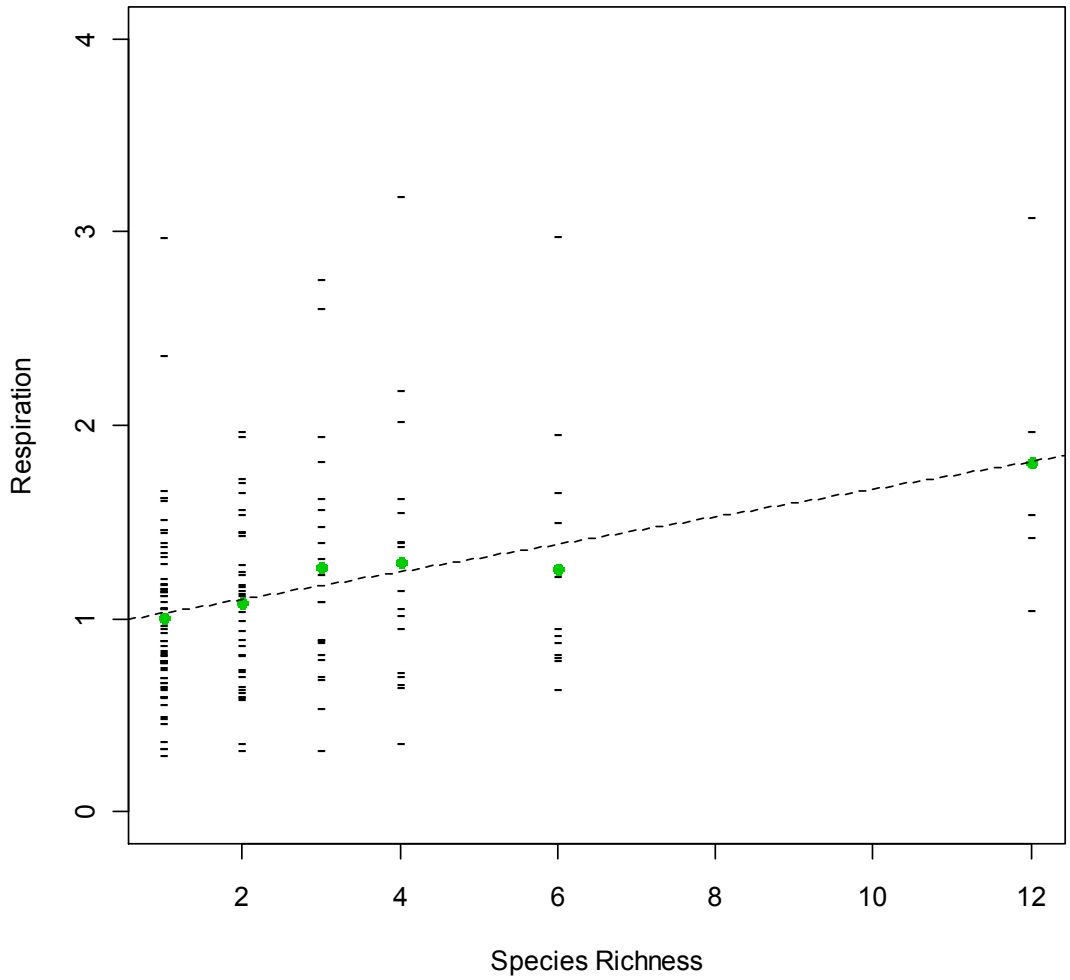

Figure 3.4: Respiration increased with increasing species richness. Respiration (y-axis) for each microcosm (n = 168) is represented by a single data point (-). These microcosms were taken from three Partitions and each replicated twice. The microcosms were assembled using 12 different species in mixtures of: one, two, three, four, six or 12 species (x-axis). The mean respiration values (•) are shown for each richness level. The dotted line shows the linear regression line through the points (R^2 = 0.094).

First term: Linear richness effect

The first explanatory variable entered into the model was the effect of linear richness (β_{LR}). The linear richness term, like the other terms used in this analysis have been previously defined by Bell *et al.* (2009b), has been considered to account for the additive effects of increasing the number of species within an assemblage. This term is analysed using a linear regression model (eq.7).

$$y = a + \beta_{LR} x_R + e \qquad [7]$$

The regression analysis found that $\beta_{LR} = 0.071$ (± 0.017 1se, $n = 168$). This slope of the linear regression was positive and significant ($F_{1,166} = 17.26$, $p < 0.001$), confirming the trend for respiration to increase as species richness (x_R) increased ($R^2 = 0.094$). Linear (species) richness is treated here as a continuous explanatory variable.

Second term: Species contributions

Species composition ($\sum_1^{12} \beta_C x_C$) was the second term entered into the model. The index C indicates a specific species (1 to 12) and the β_C terms are the estimated respiration for each species based on the average residual respiration associated with all the microcosms in which it was present (denoted by a binary presence/ absence matrix and added into the model as x_C). These were then entered into the model (eq.8)

$$y = a + \beta_{LR} x + (\sum_1^{12} \beta_C x_C) + e \qquad [8]$$

A significant ($F_{11,55} = 7.30$, $p < 0.001$) species composition term was found. When the species were analysed separately, it was observed that five (species 2, 3, 6, 7 and 10) of the 12 species had a significant effect ($F_{1,55} > 4.16$, $p < 0.046$).

The model coefficients indicate the magnitude of the effect each species had on the microcosms in which it was present (Figure 3.5). The sign, positive or negative, indicates whether a species is associated with a mean respiration that was greater than or less than that modelled by linear regression.

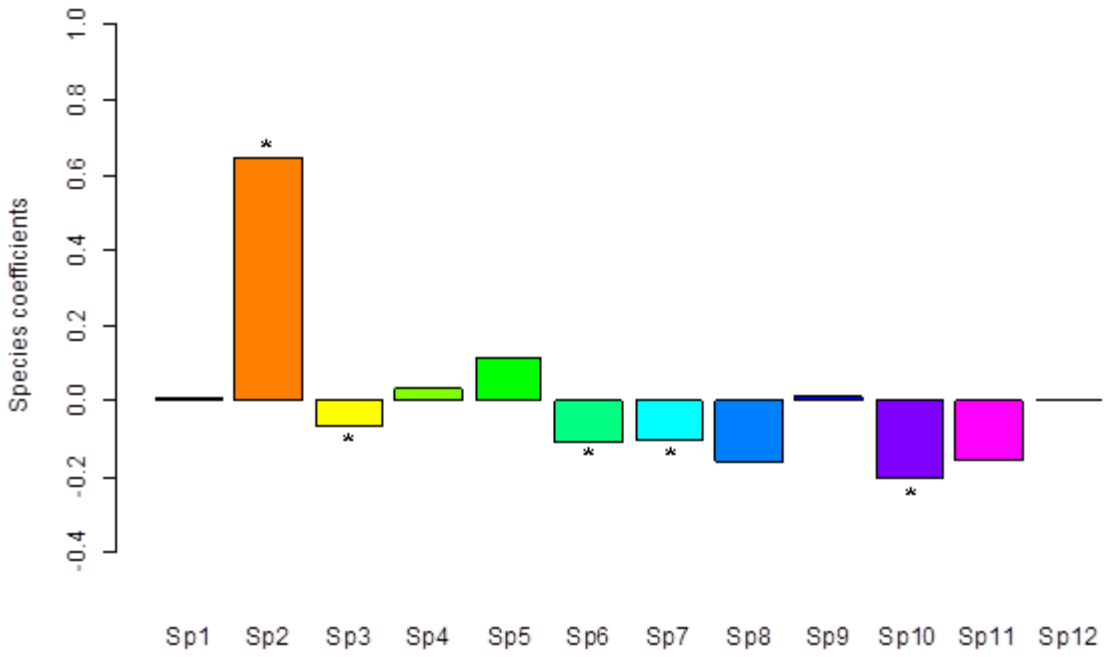

Figure 3.5: The linear model coefficients for each of the individual species. Linear model coefficients for each species were created using the mean residual values of all the microcosms in which a given species was present. The residuals values were calculated after the variance associated with the linear richness were removed. Significant differences were detected between some of the species.

Third term: Non-linear richness effects

The third explanatory variable is the non-linear richness term (β_{NLR}). Here the species richness (x_R) term is entered as an ANOVA-like factorial variable as opposed to the first continuous (regression-like β_{LR}) term. The β_{NLR} term accounts for the variance in the data that is associated with the species richness, but has not been previously accounted for by the linear richness term or composition term. This has been considered to represent the "interactions" between the species at each species richness level. The non-linear richness term was added to the model after the composition term (eq.9):

$$y = a + \beta_{LR}x_R + (\sum_1^{12} \beta_C x_C) + \beta_{NLR}x_R + e \qquad [9]$$

The sign of the coefficients indicates the direction of the effect the interaction term has on the microcosms at a given species richness level after linear richness has been fitted (Figure 3.6). Thus the negative values at six and 12 species indicated a correction in the model where the linear regression over estimated respiration in the higher species richness assemblages.

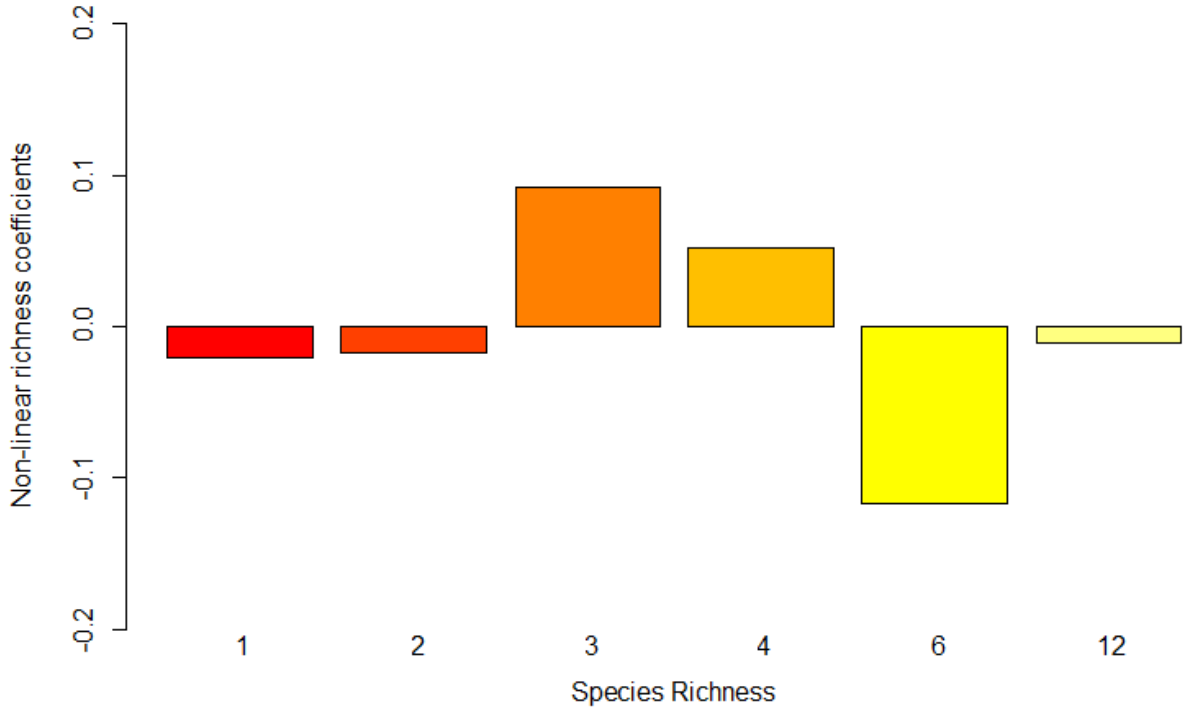

Figure 3.6: The linear model coefficients for the non-linear richness term. Calculated using the mean residuals of the GLM after the variance associated with linear richness and the individual species were accounted for. The coefficients showed that the mean residuals were different between each species richness level.

Fourth and fifth terms: Introduction to M and Q

The fourth and fifth terms in the model (eq.6) were $\beta_Q x_Q + \beta_M x_M$. To this point, the results relating to the biological effects of increasing species number (as continuous and factorial variables) and species identity have been described. Two further terms do however, influence the *F*-test and subsequent significance values, *Q* and *M*.

Each *Q* term within a Partition represents the distinct ordering (partitioned species pool) of the 12 species at a given species richness level (as illustrated in Figure 3c.1). In RPD experiments, there are *P* x *R* partitioned species pools where *P* is the number of partitions and *R* is the number of richness levels tested. In the case here, these are three and six respectively, giving 18 pools and 18 *Q* values or levels. In each of these, *Q* sums the respiration from all 12 species but each time in a different set of microcosm mixes. *Q* therefore accounts for the variance associated with the re-apportioned species pools (i.e. each distinct set of microcosms at each species richness level). This source of variance is important because it is the denominator for the *F*-test of both richness terms (Table 3.2). As these two terms are used as the *F*-test denominator in the ANOVA they are added at the end to form the final model (eq.6). The order of these two terms is also important. As *Q* and *M* are not orthogonal, *Q* must be entered first into the equation due to overlapping variance with *M*.

$$y = a + \beta_{LR} x_R + (\sum_1^N \beta_C x_C) + \beta_{NLR} x_R + \beta_Q x_Q + \beta_M x_M + e \qquad [6]$$

Each *M* value within an RPD experiment is associated with a distinct microcosm mix. In each Partition here, there are 28 distinct microcosm compositions, each replicated twice. *M* attributes the variance between each of the two replicates within each microcosm (as in Figure 3.3). This source of variance becomes the denominator from the *F*-test of the species composition term (Table 3.2).

Both *Q* and *M* were numbered here with levels which were reciprocal to the species richness (i.e. at the highest species richness levels the levels of *Q* and *M* were at the lowest). They were both entered as factorial variables in the analysis.

These two terms are fundamental to the model. These act as *F*- ratio denominators in the final ANOVA. This is because the variables that have been discussed above have over-lapping variance with one of these two terms. Therefore, to obtain an accurate significance value, the *F*-test must be performed using either *Q* or *M* as the denominator. The selection of the denominator for each explanatory variable is dependent on from which of the two terms, Q or M, the variance is taken (Table 3c.1).

This over-lapping of variance is demonstrated in Table 3.3 using a series of models, each with a different set or order of variables. By showing the resulting Sums of Squares (SS) for each model, it was possible to observe how the SS move between variables. Another facet of this analysis is that the species composition and the non-linear richness terms are orthogonal once the linear richness term has been entered into the model. In Table 3.2 this appears not to be an absolute orthogonality as there has been a "leakage" of 0.16 SS. If the non-linear richness and species composition terms are swapped in the analysis, leaving linear richness as the first term (data not shown) this "leakage" is once again shown. Though these terms were not perfectly orthogonal, they approached a good fit. The total SS accounted for by the analysis (SS = 32.78) is identical in both Models D & E (Table 3.2).

For the analysis of significance of Q and M, these are treated as variables in a standard ANOVA with the F-test denominator being the residual SS. All reported F- and p-values were calculated using the appropriate F-test denominator.

The results for Q and M showed that Q accounted for a significant amount of the variance ($F_{10,82} = 3.14$, $p = 0.002$), with M also highly significant ($F_{55,82} = 1.91$, $p = 0.004$).

Variables in the GLM	**df**	***F*d**	**A**	**B**	**C**	**D**	**E**
Species richness							
Linear richness (SS_{LR})	1	*Q*	-	4.60	4.60	4.60	0.29
Non-linear richness (SS_{NLR})	*R*-2	*Q*	-	-	0.49	0.49	0.65
Partitioned species pool (SS_Q)	*Q*-*R*	*r*	12.80	8.20	7.71	7.49	7.49
Species compositions (SS_C)	N-1	*M*	-	-	-	12.12	16.27
Species combinations (SS_M)	*M*-*Q*-N-1	*r*	19.43	19.43	19.43	8.08	8.08
Residuals (SS_r)			16.59	16.59	16.59	16.04	16.04

Table 3.2: Sums of Squares attributed to the terms in the analysis and their degree of overlap. The sums of squares (SS) associated with each variable in the GLM together with the degrees of freedom and the *F*-test denominator (*F*d) needed for the final ANOVA. The model C $SS_{\mathrm{Q}} = \sum(SS_{LR} + SS_{NLR} + SS_Q)$ equals the partition of the model A SS_{Q}. Therefore to have the two richness terms in the model they must be entered before *Q*. Likewise model D $SS_M = \sum(SS_C + SS_M)$ equals the partition of variance for the model C SS_M . The SS_{LR} is also shown to have overlaps with SS_Q and SS_{NLR}. By entering the linear richness term into the model first the non-linear richness and species identity terms are effectively orthogonal.

Model A: $y = a + \beta_Q + \beta_M$

Model B: $y = a + \beta_{LR} + \beta_Q + \beta_M$

Model C: $y = a + \beta_{LR} + \beta_{NLR} + \beta_Q + \beta_M$

Model D: $y = a + \beta_{LR} + \beta_{NLR} + (\sum_1^{12} \beta_C) + \beta_Q + \beta_M$

Model E: $y = a + (\sum_1^{12} \beta_C) + \beta_{LR} + \beta_{NLR} + \beta_Q + \beta_M$

Adaptation of the model

The model previously discussed was published by Bell *et al.,* (2009b). In the subsequent chapters, an adapted model was used. This adapted model added two further experimental terms, preceding both the biological (linear and non-linear richness and species composition) and statistical (*Q* and *M*) terms. These terms attributed the variance in the data to the replicate (β_{REP}) and the Partitions (β_P), and were entered at the beginning of the model, as factorial variables. Entered here, these terms removed the variance available to the effects of biodiversity that was associated with experimental error. By accounting for (removing) variance associated with experimental variation at the start of the modelling sequence, the Partitions were effectively normalised allowing the analysis to focus on the variance associated with species richness and composition.

To ensure that the correct denominator was used for the *F*-tests, it was shown that β_{REP} took variance from the residual SS only (Table 3.3). It was therefore orthogonal to all the other terms entered into the model. This term used the residual SS as the *F*-test denominator (as did *Q* and *M*). The β_p term was shown to overlap with the variance associated with *Q*. This implied that the Partitions were linked to the richness terms. Therefore, in the final ANOVA analysis, the Partition term used *Q* as the *F*-denominator. The inclusion of these terms gave a final GLM that was used for subsequent analysis (eq.10).

$$y = a + \beta_{REP}x_{REP} + \beta_P x_P + \beta_{LR}x_R + (\sum_1^N \beta_C x_C) + \beta_{NLR}x_R + \beta_Q x_Q + \beta_M x_M + e \qquad [10]$$

Variance not accounted for by the model or predicting microcosms

Eight models, based on the terms present in eq.10, were sequentially fitted to the three Partition dataset. The observed respiration was plotted against the predicted values from the six models (Figure 3.7). In each successive model, an additional term was added resulting in an improved fit to the data observed. Variables were added until the final model (Figure 3.7h) which had all the variables added including the residual term, therefore making it an exact fit of the model.

Variables in the GLM	**df**	***F*d**	**F**	**G**
Experimental				
Replicate (SS_{REP})	*REP*-1	*r*	-	10.43
Partition (SS_P)	*P*-1	*Q*	-	5.43
Linear richness (SS_{LR})	1	*Q*	4.60	4.60
Non-linear richness (SS_{NLR})	Δ-2	*Q*	0.65	0.57
Partitioned species pool (SS_Q)	*Q*-*P*-Δ	*r*	7.49	2.24
Species compositions (SS_C)	N-1	*M*	11.96	12.26
Species combinations (SS_M)	*M*-*Q*-N-1	*r*	8.08	7.76
Residuals (SS_r)			16.04	6.09

Table 3.3: The degree of overlap of the Sums of Squares for each term in the adapted analysis. The sums of squares (SS) associated with each variable in the adapted GLM together with their degrees of freedom and the *F*-test denominator (*F*d) needed for the final ANOVA. These results demonstrated that SS_{REP} has overlapping variance with SS_r and therefore the residuals were used as the *F*-test denominator for the replicate term. The Partition term was shown to have overlapping variance with SS_Q therefore, like the richness terms, Q was the *F*-test denominator for the Partition term.

Model F: $y = a + \beta_{LR} + (\sum_1^{12} \beta_C) + \beta_{NLR} + \beta_Q + \beta_M$

Model G: $y = a + \beta_{REP} + \beta_P + \beta_{LR} + (\sum_1^{12} \beta_C) + \beta_{NLR} + \beta_Q + \beta_M$

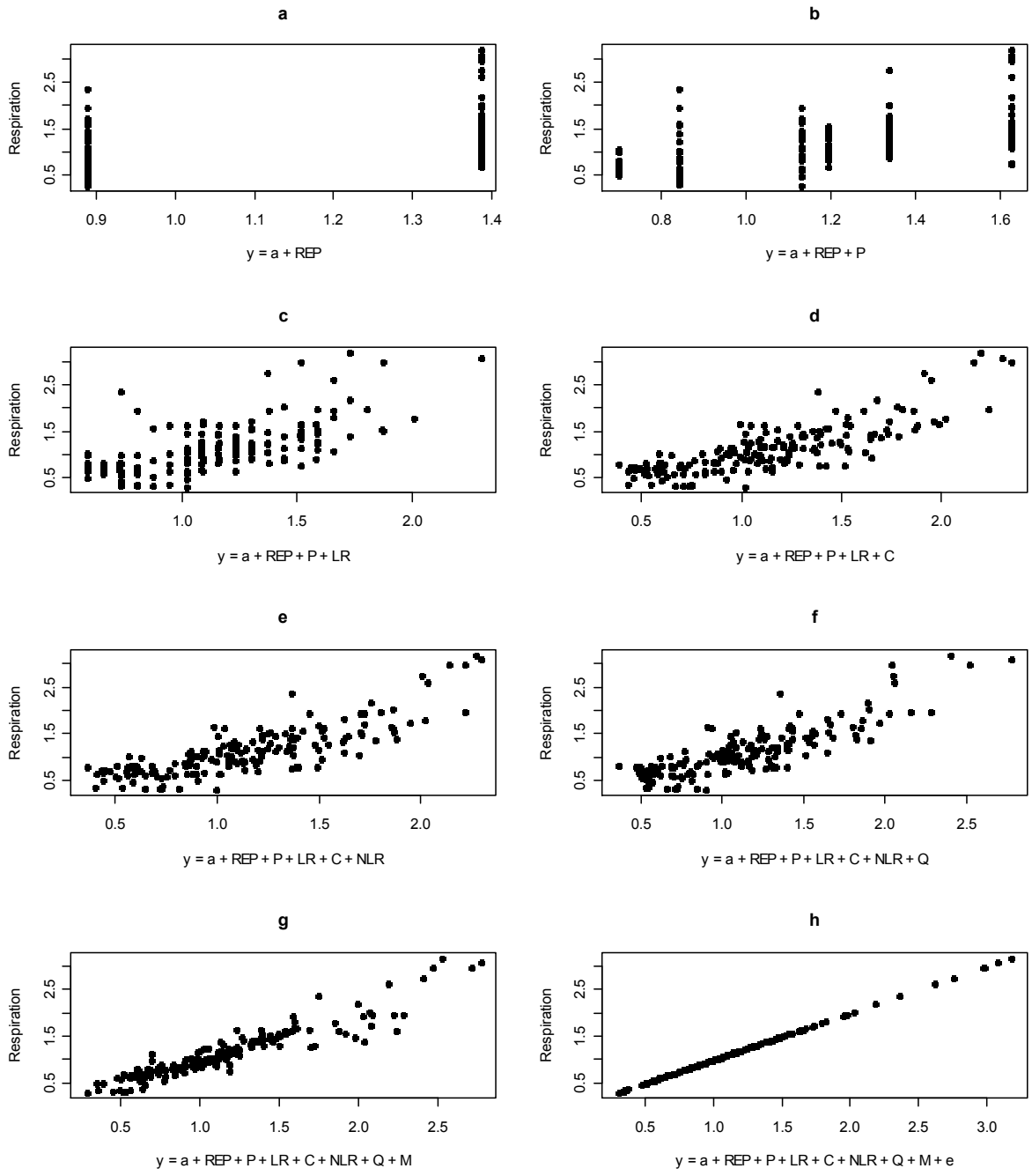

Figure 3.7: Plot of observed respiration against predicted respiration for eight linear models. For each graph, the observed respiration is plotted against the respiration predicted by the models stated below each graph. Each successive model includes an additional term, given in the Figure.

3.4 Conclusions

Increases in species richness resulted in increased respiration. However, at all richness levels a wide and similar range of respiration was observed. It is apparent that this result has not reached an asymptote and that further increases in diversity are likely to result in increased respiration.

This analysis has shown that the only variable that did not have a significant effect on the variation within the data was the non-linear richness term. This suggests that the increase in biodiversity and the composition of the species are the main biological drivers of respiration by the bacterial assemblages in the microcosms.

This chapter has also outlined the analytical procedure that will be implemented throughout this study. Although not illustrated here, the general approach taken to the fitting of linear models has been to seek iterative simplification and a minimal adequate model (Crawley, 2007, Grafen and Hails, 2002). In some situations, terms which have not been found to be significant have been retained in models to permit their comparison with other experiments.

The adaptation of the GLM has also shown that careful analysis of the SS was needed to ensure the correct analysis of the data and to overcome the overlapping nature of many of the factors. This analysis adapted an established design and statistical tool for use in investigating the effects of three aspects of biodiversity (linear addition of species, the selection effect within composition and the non-linear "interaction" term) on ecosystem function.

Chapter 4

4. The impact of biodiversity on respiration and its mediation by environmental characteristics

4.1 Introduction

A previous study investigated the effect of bacterial biodiversity, with species derived from tree-holes, on total community respiration (Bell *et al.,* 2005). It found a positive but decelerating relationship between the increasing number of bacterial species present in microcosms and respiration. In the present study, a similar experimental design was employed on bacterial species isolated from CF sputum samples.

Bacterial diversity was manipulated in a set of assemblages of increasing species diversity. A balanced biodiversity-ecosystem functioning (BEF) experiment was implemented so that bacterial isolates were manipulated according to the Random Partitions Design (RPD; Chapter 3c), into assemblages of increasing species diversity (Bell *et al.,* 2009b). This approach allowed bacterial activity, measured as respiration (the dependent variable), to be analysed by using a general linear model (GLM) with three explanatory (independent) variables; i) the number of species in any given microcosm mix (linear species richness), ii) the "interaction" term, which equated to the variance associated with the species richness, but not accounted for by the linear species richness term (non-linear species richness), and iii) the identities of the species present in a given microcosm mix (species composition). The assemblages were subsequently added to four experimental conditions. These had either Tryptone water or Defibrinated Horse Blood (DHB) as sources of contrasting nutrients (Chapter 2.2) as both a liquid and a more viscous medium. This allowed the effect of the environment on the impact of biodiversity to be tested.

Selecting an appropriate measurement for the system function and corresponding assay are vital for meaningful BEF experiments. The measure of function chosen here was respiration (cumulative units of CO_2 respired at 24hrs); a proxy for carbon turnover and activity (Chapter 3b). A suitable assay for biodiversity is also required for BEF experiments.

In the following experiment, 12 bacterial species were chosen randomly from a species pool of 18 isolates cultivated from the sputum of CF patients. The measure of

biodiversity was based upon the 16S rRNA gene sequence. Each species was identified by sequencing part of their 16S rRNA gene and matching to reference sequences in the databases (Chapter 3a).

Aims and Objectives

The aim of this study was to investigate the effects of bacterial biodiversity on total respiration. The biodiversity in this instance was created by manipulating the number of bacterial species present within an assemblage. Secondly the study investigated whether the impact of biodiversity on total respiration was mediated by the environment by testing the effect of nutrient source and viscosity. The potential use of this in medical microbiological research is also discussed.

4.2 Materials & Methods

Bacterial isolates

Bacterial species used in this study and the methods used to isolate and identify them are given in Chapters 2 and 3a.

Microcosm experiments

The experimental microcosm set up has been previously outlined in Chapter 2.8. The manipulated assemblages were mixed prior to inoculation of the experimental system. The amount of mixed culture used for inoculation was kept constant by dilution at the equivalent of $OD_{600} = 0.1$. Microcosm mixtures (henceforth mixes) were created by each species being randomly selected from the species pool without replacement for each species richness level. This resulted in each species being present once at all species richness levels. For example, at the three species level there were four mixes each with three of the 12 species present. To maximise the amount of information about the effect of biodiversity on the amount of CO_2 respired in this study, every factor level of the maximum richness level (1, 2, 3, 4, 6, and 12 species) was analysed (Bell *et al.,* 2009b). This approach gives the largest number of mixes possible where all of the species can be present at each level only once. One set of richness levels (henceforth a Partition) consisted of 28 microcosms with 12 monoculture mixes, six mixes with two species, four with three, three with four, two with six and finally one with 12. Three Partitions were created giving a total of 168 microcosm mixes. Each Partition was independently replicated twice.

Microcosm growth substrates

Two commercially available carbon substrates were used; Tryptone Water and Defibrinated Horse Blood. These media, their choice and preparation were as described in Chapter 2.2.

Measurement of Respiration

Full details of the respired CO_2 measurements and calibration can be found in Chapter 3b. The percentage of CO_2 dissolved in the indicator gel was measured using the MicroResp™ System. As the headspace in the BEF experiments was constant, the $\%CO_2$ (% v/v) was taken as the measure of respired CO_2. For convenience, this is presented as units of CO2 (UCO_2).

T-RFLP profiling

T-RFLP profiling was carried out as described in Chapter 2.6. Within this system, each of the 12 bacterial species had a specific terminal fragment length as described in Chapter 3a. Relative species richness values were taken from the relative band fluorescence data from each microcosm at the conclusion of the experiment. T-RFLP profiling detected a band when it represented more than 0.1% of the total band intensity. Whenever a bacterial species "dropped-out" of the experiment (had a signal intensity of <0.1%) it was assigned a nominal weighting in the analysis of 0.1. If it was not possible to obtain a T-RFLP profile for a particular microcosm, the species were weighted using their starting richness abundance.

Statistical analysis

The differences between the bacterial community respiration in four different environments were investigated. All analyses were performed in R (v.2.12.2 – v.2.13.0). This investigation was analysed using an adapted statistical method (eq.10; detailed in Chapter 3c).

$$y = a + \beta_{REP}x_{REP} + \beta_P x_P + \beta_{LR}x_R + (\sum_1^{12} \beta_C x_C) + \beta_{NLR}x_R + \beta_Q x_Q + \beta_M x_M + e \qquad [10]$$

All assumptions of normality and homogeneity of variance were assessed using the Shapiro-Wilk and Fligner-Killeen tests respectively, and confirmed by visual methods. The general linear model conformed to all assumptions required after *M* was added into the model. All comparisons of means were carried out using a paired Student's t-test unless otherwise stated. All R^2 values quoted are multiple R^2. These were calculated as the fraction of total variance that was accounted for by the variables in the model i.e. $\frac{SS_{Variable}}{SS_{Total}}$.

4.3 Results

Bacterial assemblages of varying diversity were grown using two different nutrient sources, Tryptone Water and Defibrinated Horse Blood (DHB), with the cumulative respiration measured in UCO_2 recorded at 24 hours. Results will also be presented regarding the effect of environmental viscosity on respiration. Three Partitions were created and each represented a unique set of 28 microcosms, with each independently replicated twice (see Appendix, Table A1, for the species combinations).

The respiration measured varied between nutrient source and viscosity.

Tryptone Water: Description of the data

Initial investigation of the Tryptone Water Partitions found that each of the three analysed had significantly different overall respiration means ($t_{55} > 2.33$, $p < 0.023$; Table 1). In addition, more respiration was observed in data from the constrained environment microcosms (mean = 1.26 UCO_2, SD = 0.71, n = 168) than the mixed environment microcosms (mean = 1.14 UCO_2, SD = 0.54, n = 168). A Student's t-test confirmed that this difference was significant ($t_{167} = -3.19$, $p = 0.002$).

Respiration tended to increase with increasing species richness (Figure 4.1). The linear regression gradients of respiration with species richness (R) were all positive and statistically significant ($p < 0.001$) for each of the three Partitions analysed in the constrained environment (Table 4.1). In the mixed environment, the gradients of the linear regression lines were all found to be positive but not all were statistically significant. Gradients in the constrained environment (mean = 0.13 UCO_2/R) were greater than in the mixed environment (mean = 0.07 UCO_2/R). These differences were however, not statistically significant ($t_2 = -2.27$, $p = 0.152$).

The data were assessed for leverage effects by outstanding data points. This identified the threshold, determined to be 0.01, over which data points would be considered "highly influential" (Crawley, 2007). In the mixed environment, 22 highly influential data points were observed with a further 32 highly influential data points observed in the constrained environment. To assess whether these highly influential points affected the gradient, they were removed from the analysis. The slopes of the regression analysis remained positive and statistically significant and as such these data points were retained in the analysis.

Defibrinated Horse Blood: Description of the data

Initial investigation of the DHB Partitions found no statistically significant differences between the respiration in any of the Partitions in the mixed environment ($t_{55} < 0.39$, $p > 0.698$). In the constrained environment however, significant differences were observed between Partition 3 and Partitions 1 and 2 ($t_{55} > 2.25$, $p < 0.028$) but not between Partition 1 and Partition 2 ($t_{55} = 1.04$, $p = 0.302$). The results also indicated that the constrained environment had a greater mean respiration (mean = 0.33 UCO_2, SD = 0.13, $n = 168$) than the mixed environment (mean = 0.21 UCO_2, SD = 0.078, n =168). A Student's t-test confirmed this difference was significant ($t_{167} = -12.49$, $p < 0.001$).

The linear regression gradients of respiration with species richness (R) were all positive and statistically significant ($p < 0.001$) for each of the three Partitions analysed in the constrained DHB environment (Table 1). Respiration increased with increasing species richness in both the mixed ($b = 0.013$ UCO_2/R) and constrained ($b = 0.015$ UCO_2/R) environments.

Comparison between nutrient sources

The respiration measurements of microcosm mixes grown in DHB were found to be more consistent across the Partitions than those in Tryptone Water (Table 4.1). Respiration from microcosms grown in the DHB (mean = 0.27 UCO_2, SD = 0.12, n = 336) was significantly ($t_{335} = -28.25$, $p < 0.001$) lower than in the Tryptone Water (mean = 1.20 UCO_2, SD = 0.634, $n = 336$). Further comparisons of the regression gradients found the increase in respiration per species increase was statistically significantly lower in both the mixed ($t_{167} = 3.95$, $p < 0.001$) and constrained ($t_{167} = 6.50$, $p < 0.001$) DHB environments than in the Tryptone Water.

Tryptone Water					
Mixed					
Partition	**Mean UCO_2**	**Minimum UCO_2**	**Maximum UCO_2**	**Regression intercept**	**Regression gradient**
1	1.38	0.29	3.18	1.03*	0.13*
2	1.09	0.32	2.75	0.94*	0.06
3	0.95	0.50	1.55	0.89*	0.02
All	1.14	0.29	3.18	0.95*	0.07*
Constrained					
Partition	**Mean UCO_2**	**Minimum UCO_2**	**Maximum UCO_2**	**Regression intercept**	**Regression gradient**
1	1.42	0.50	3.03	1.07*	0.14*
2	1.29	0.12	3.64	0.88*	0.16*
3	1.06	0.43	2.81	0.83*	0.09*
All	1.26	0.12	3.64	0.92*	0.13*
DHB					
Mixed					
Partition	**Mean UCO_2**	**Minimum UCO_2**	**Maximum UCO_2**	**Regression intercept**	**Regression gradient**
1	0.22	0.09	0.45	0.19*	0.01*
2	0.21	0.09	0.36	0.18*	0.01*
3	0.21	0.12	0.42	0.18*	0.01*
All	0.21	0.09	0.45	0.18*	0.01*
Constrained					
Partition	**Mean UCO_2**	**Minimum UCO_2**	**Maximum UCO_2**	**Regression intercept**	**Regression gradient**
1	0.27	0.14	0.54	0.25*	0.01
2	0.33	0.14	0.73	0.27*	0.02*
3	0.38	0.25	0.68	0.34*	0.02*
All	0.33	0.14	0.73	0.29*	0.01*

Table 4.1: Means, ranges and linear regressions (against species richness) for Respiration in microcosms in three Partitions grown in Tryptone Water or DHB, in mixed and viscous environments. An asterisk denotes significance ($p < 0.05$).

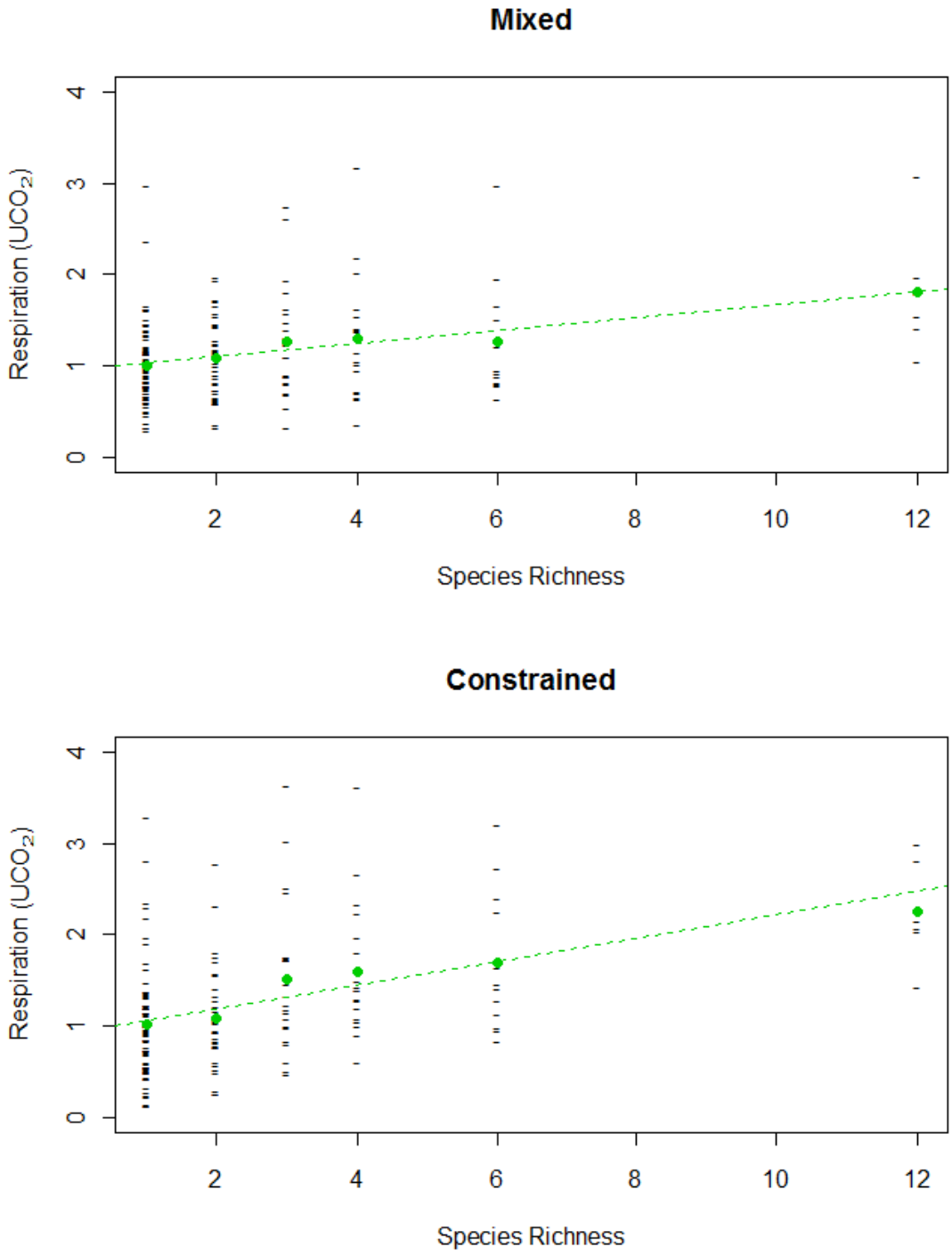

Figure 4.1: Respiration plotted against species richness in both mixed and constrained Tryptone Water based environments. The respiration, in units of CO_2 respired in 24 hours (y-axis), from each individual microcosm (-) are plotted against the species richness (x-axis) of each microcosm. These are shown to have a large variance around the means (•). The regression line shows the positive relationship between respiration and species richness.

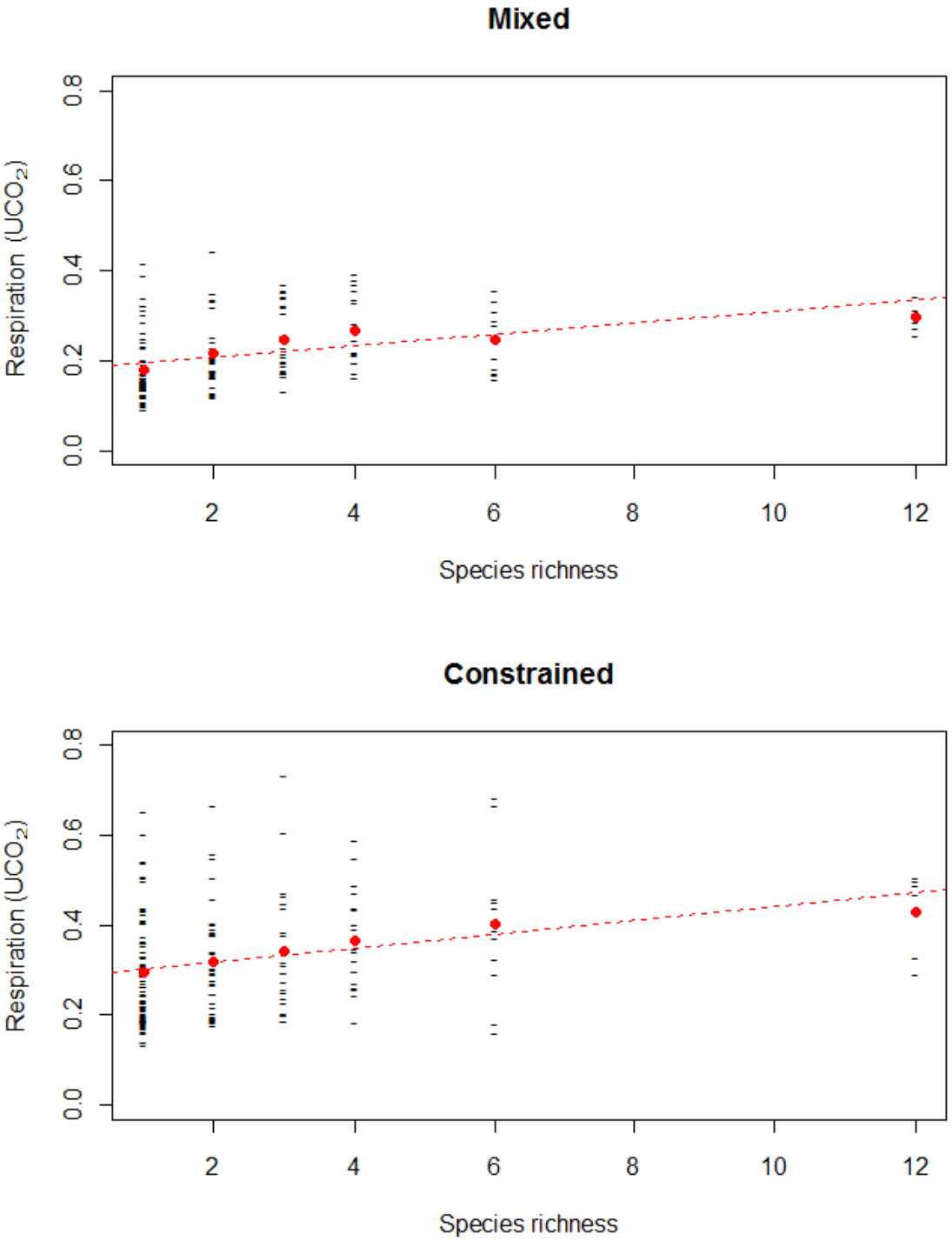

Figure 4.2: Respiration plotted against species richness in both mixed and constrained DHB based environments. The respiration, in units of CO_2 respired in 24 hours (y-axis), from each individual microcosm (-) are plotted against the species richness (x-axis) of each microcosm. These are shown to have a large variance around the means (•). The regression line shows the positive relationship between respiration and species richness.

Analysis using GLM showed that variation within the data set could only be accounted for by multiple variables.

In the results presented above, there was a positive relationship between mean community respiration and species richness (Figures 4.1 & 4.2). The data also showed that there were large variances around the mean respiration values for each of the species richness levels. Bell *et al.* (2009b) reported the following GLM for analysis of data obtained using the Random Partitions Design analysis (eq.6).

$$y = a + \beta_{LR}x_R + (\sum_1^N \beta_C x_C) + \beta_{NLR}x_R + \beta_Q x_Q + \beta_M x_M + e \qquad [6]$$

However, to account for the large variances around the species richness level means the GLM used in this study was extended as described in Chapter 3c (eq.10).

$$y = a + \beta_{REP}x_{REP} + \beta_P x_P + \beta_{LR}x_R + (\sum_1^N \beta_C x_C) + \beta_{NLR}x_R + \beta_Q x_Q + \beta_M x_M + e \qquad [10]$$

This analysis incorporated, in order, the variation in the replicates (β_{REP}), Partitions (β_P), the number of species as a continuous variable (β_{LR}), the composition of the species present in each microcosm (β_C), the number of species as a factorial variable (β_{NLR}) and finally the variances associated with the partitioned species pool (β_Q) and between the replicated microcosms (β_M).

In the model (eq.10) species richness was taken as being the number of species introduced into each microcosm. This has been standard practice in all previous applications of the RPD and other BEF experiments. It was however, hypothesised that some species may not increase in all combinations and that they will, most likely, not grow equally. It was resolved therefore, to use T-RFLP profiling to estimate the relative species proportions at the end of the experimental period. The analysis presented here has been modified by weighting the individual species with their relative abundance data for the microcosms at the end of the experiment.

The data presented here were investigated to assess whether the model conformed to the assumptions required for parametric statistical analysis. This found that the data possessed homogeneous variance (Fligner-Killeen test of homogeneity of variances; $X^2 < 0.96$, $p > 0.327$) in all environments tested. After analysis with the full model (eq.10) all the environments were however, shown to have errors which were non-normal as observed by failure of the Shapiro-Wilk normality test ($W < 0.96$, $p < 0.001$). No single transformation was found which would make all of the environments conform to the assumptions of parametric analysis. That is, to transform the data to be

suitable for analysis with parametric statistics each data set would require a different transformation. This would cause the comparison of the analysis to be non-intuitive. Analyses were therefore conducted using five sequential Kruskal-Wallis tests. There was no difference between the significance of the variables in the parametric and nonparametric testing (data not shown) and so a linear model was selected for the analysis.

Fitting the GLM 1: Environmental variation and linear richness affected the total microcosm mix respiration.

Tryptone Water: fitting replicates, Partitions and linear richness in the GLM

The different stages of the GLM (eq.10) analysis are presented in Table 2 with the fit of the models compared by R^2 and Akaike Information Criterion (AIC) and the ANOVA results (*F*-tests and *p*-values) for each variable. The ANOVA results of the experimental variables (the replicates and the Partitions) showed that both of these variables had a statistically significant effect on assemblage respiration. In the mixed environment, the mean respiration values associated with the first replicate (0.89 UCO_2, SD = 0.43, n = 84) were lower than the second replicate (1.39 UCO_2, SD = 0.53, n = 84). Similarly in the constrained environment, the first replicate (1.02 UCO_2, SD = 0.59, n = 84) was lower than the second (1.49 UCO_2, SD = 0.75, n = 84). As discussed later, these differences between the replicates were shown to be relevant in accounting for the variance observed.

The variance associated with the replicates (*REP)* was shown to be highly significant in both the mixed and constrained environments ($F_{1,82}$ = 141.93, $p < 0.001$ and $F_{1,82}$ = 53.81, $p < 0.001$ respectively; Table 4.1). The second step in the ANOVA found the effect of the Partitions (*P*) on the respiration was also highly statistically significant in both the mixed and constrained environments ($F_{2,10}$ = 11.01, p = 0.003 and $F_{2,10}$ = 9.88, p = 0.004 respectively). Significant differences were also observed between the two independent replicates (data not shown) within the Partitions (Table 4.2).

The effect of linear richness (*LR*) was shown to account for a significant amount of the variance associated with the residuals (the variance remaining after that associated with the replicates and Partitions had been removed) in both the mixed ($F_{1,10}$ = 125.29, $p < 0.001$) and constrained environments ($F_{1,10}$ = 34.89, $p < 0.001$).

After these three variables were entered into the analysis, the model accounted for less than half of the variance within the data in both the mixed (R^2 = 0.42) and constrained (R^2 = 0.33) environments.

Defibrinated Horse Blood: fitting replicates, Partitions and linear richness in the GLM

This GLM analysis was repeated on the respiration data measured from the microcosms grown in DHB (Table 4.1). The ANOVA found that neither the replicate (*REP*) nor Partition (*P*) term had a significant effect on the variation within the data in either environment (Table 4.3). The mean respiration in the mixed environment for the first (0.22 UCO_2, SD = 0.08, n = 84) and second (0.21 UCO_2, SD = 0.08, n = 84) replicates was observed to be similar. In addition, in the constrained environment, the mean respiration of first (0.33 UCO_2, SD = 0.13, n = 84) and second (0.32 UCO_2, SD = 0.12, n = 84) replicates was also observed to be similar.

In the mixed environment, the effects of the replicate and Partitions on the total respiration were found to be statistically non-significant ($F_{1,82}$ = 1.29, p = 0.259 and $F_{2,10}$ = 0.12, p = 0.887 respectively; Table 4.1). In the constrained environment, the effects of the replicate and the Partition were also found to be non-significant ($F_{1,82}$ = 1.11, p = 0.295 and $F_{2,10}$ = 0.07, p = 0.934 respectively).

The remaining variance was analysed in the next step of the ANOVA. The linear richness (*LR*) term was shown to be highly significant in the mixed ($F_{1,10}$ = 30.82, $p < 0.001$) and constrained environments ($F_{1,10}$ = 17.58, p = 0.002). With these three variables entered into the model, the variance accounted for was much lower in DHB than in Tryptone Water for both the mixed (R^2 = 0.15) and constrained (R^2 = 0.20) environments.

Comparison between nutrient sources

The results in DHB (Table 4.3) for these variables were in contrast to the analysis of the respiration from the microcosms grown in Tryptone Water (Table 4.2). The GLM analysis identified a significant effect associated with the replicate and Partition in Tryptone Water but not in DHB.

The fit of the models to the data showed that in all four environment combinations, the model so far accounted for less than 50% of the total variance. To investigate the mechanisms which caused the data set to vary away from the linear regression line, two further aspects of biodiversity were investigated; species composition and non-linear richness. The non-linear richness term used the species

richness as a factorial variable and was entered after the species composition term. The species composition term assessed the contribution of each species to the total respiration in each of the microcosms in which it was present.

Mixed

Step	Model	R^2	AIC	Res.Df	RSS	*F*	*p*
1	Respiration ~ 1	-	-	167	77.65	-	-
2	**1** + REP	0.21	234.75	166	67.22	141.93	<0.001
3	**2** + P	0.32	213.14	164	61.79	11.01	0.003
4	**3** + LR	0.42	189.89	163	30.9	125.29	<0.001
5	**4** + C	0.62	141.44	152	19.12	5.93	<0.001
6	**5** + NLR	0.63	145.04	148	18.42	0.71	0.602
7	**6** + Q	0.68	140.26	138	15.95	3.35	0.001
8	**7** + M	0.89	88.10	83	6.03	2.45	<0.001

Constrained

Step	Model	R^2	AIC	Res.Df	RSS	*F*	*p*
1	Respiration ~ 1	-	-	167	84.77	-	-
2	**1** + REP	0.11	348.92	166	75.74	53.81	<0.001
3	**2** + P	0.15	344.21	164	71.91	9.88	0.004
4	**3** + LR	0.33	306.80	163	65.16	34.89	<0.001
5	**4** + C	0.65	220.03	152	31.58	12.14	<0.001
6	**5** + NLR	0.67	217.95	148	29.52	2.66	0.096
7	**6** + Q	0.70	225.62	138	27.59	1.15	0.334
8	**7** + M	0.89	187.77	83	13.76	1.50	0.047

Table 4.2: The sequential fit of the GLM for respiration from mixed and constrained microcosms grown in Tryptone Water. The significance and fit of each new step of the model to the residuals remaining after the previous steps are fitted are presented. Values for R^2, Akaike Information Criterion (AIC) and the ANOVA results (*F*-tests and *p*-values) for each variable are given. The residual degrees of freedom (Res.Df) and residual Sums of Squares (RSS) indicated the total variance which still remains in the data. The appropriate degrees of freedom for each *F*-test are those described earlier (Chapter 3c; Table 3.3).

Mixed

Step	Model	R^2	AIC	*Res.Df*	RSS	F	*p*
1	Respiration ~ 1	-	-	167	1.03		-
2	**1** + REP	0.00	-375.71	166	1.03	1.29	0.259
3	**2** + P	0.00	-371.80	164	1.03	0.12	0.887
4	**3** + LR	0.15	-396.25	163	0.96	30.82	<0.001
5	**4** + C	0.45	-446.76	152	0.69	3.28	0.001
6	**5** + NLR	0.47	-444.63	148	0.55	15.77	<0.001
7	**6** + Q	0.49	-432.34	138	0.53	1.53	0.143
8	**7** + M	0.93	-644.86	83	0.12	5.28	<0.001

Constrained

Step	Model	R^2	AIC	*Res.Df*	RSS	F	*p*
1	Respiration ~ 1	-	-	167	1.02		-
2	**1** + REP	0.00	-209.70	166	1.01	1.11	0.295
3	**2** + P	0.12	-227.47	164	1.01	0.07	0.934
4	**3** + LR	0.20	-241.39	163	0.95	17.58	0.002
5	**4** + C	0.50	-298.11	152	0.44	12.51	<0.001
6	**5** + NLR	0.51	-293.46	148	0.38	4.18	0.03
7	**6** + Q	0.56	-291.92	138	0.34	2.31	0.019
8	**7** + M	0.91	-418.89	83	0.13	2.26	<0.001

Table 4.3: The sequential fit of the GLM for respiration from mixed and constrained microcosms grown in DHB. The significance and fit of each new step of the model to the residuals remaining after the previous steps are fitted are given. Values for R^2, Akaike Information Criterion (AIC) and ANOVA *F*-test and *p*-values for each variable are given. The residual degrees of freedom (Res.Df) and residual Sums of Squares (RSS) indicated the total variance which still remains in the data.

Terminal Restriction Fragment Length Polymorphism (T-RFLP) profiling showed the change in species richness over time.

In this study, the relative abundance of each species at the end of the experiment was measured. These were used to approximate the average contribution a species gave to the respiration of each microcosm mix in which it was present. The relative abundances of each species were assayed by T-RFLP profiling for microcosms initiated with two or more species. Monocultures were not profiled and the species allocated 100% of the respiration associated with their microcosm (Table 4.4).

Tryptone Water: T-RFLP profiling

The number of bacterial species identified in the constrained (mean number of species detected = 19.5, SD = 6.99, n = 12) environment was not statistically greater than those detected in the mixed (mean = 19.2, SD = 7.43, n = 12) environment (t_{11} = -0.30, p = 0.772). A Kruskal-Wallis rank sum test however found significant differences between the species present in Tryptone Water (X^2_{11} = 21, p = 0.033). Subsequent Wilcoxon rank sum tests showed that *S. maltophilia* (median = 28) was present significantly more than any other (median = 16.5) species (W = 1.50, p = 0.036, n = 24). Conversely *S. marcescens* and *S. mitis* (medians = 10.5) were absent more frequently than any other species (median = 19.5) with this difference approaching statistical significance (W = 41, p = 0.052, n = 24). Further, there was a strong correlation (r = 0.86) between whether the species was detected in both environmental viscosities in Tryptone Water.

Defibrinated Horse Blood: T-RFLP profiling

The results of the end-point T-RFLP profiling showed that there was a significantly (t_{11} = -5.22, p < 0.001) higher number of species present in the constrained (mean = 21.8, SD = 5.47, n = 12) than in the mixed (mean = 18.1, SD = 6.50, n = 12) environment. A Kruskal-Wallis test indicated that there were significant (X^2_{11} = 20.4, p = 0.040) differences between the species present in the two DHB environments. Wilcoxon tests found again that *S. maltophilia* (median = 29) was significantly (W = 2, p = 0.041) more present than any other species (median = 18.5). Also, in concurrence with the Tryptone Water results, *S. mitis* (median = 11) was absent more frequently than any other species (median = 21) with this difference approaching statistical significance

(W = 41, p = 0.053). A very strong correlation (r = 0.93) was observed between the species detected in both environmental viscosities in DHB.

Comparison between the nutrient sources

A strong correlation between the presence of each species in the two nutrient sources (r = 0.83) was found when data from both environmental viscosities were pooled. No significant difference (t_{23} = -0.77, p = 0.452) was found between the mean numbers of species present in Tryptone Water (mean = 19.33) and DHB (mean = 19.96).

	Tryptone Water			DHB		
Species	**Mixed**	**Const'd**	**Mean**	**Mixed**	**Const'd**	**Mean**
(1) ***Pseudomonas aeruginosa***	28	26	27.0	24	27	25.5
(2) ***Stenotrophomonas maltophilia***	28	29	28.5	29	29	29.0
(3) ***Burkholderia cepacia***	28	25	26.5	20	24	22.0
(4) ***Achromobacter xylosoxidans***	27	22	24.5	23	30	26.5
(5) ***Acinetobacter baumannii***	24	28	26.0	25	25	25.0
(6) ***Serratia marcescens***	9	12	10.5	10	18	14.0
(7) ***Enterococcus faecium***	17	25	21.0	18	23	20.5
(8) ***Staphylococcus haemolyticus***	17	15	16.0	18	22	20.0
(9) ***Staphylococcus aureus***	15	10	12.5	18	19	18.5
(10) ***Streptococcus mitis***	9	12	10.5	9	13	11.0
(11) ***Streptococcus pneumoniae***	13	14	13.5	12	16	14.0
(12) ***Streptococcus sanguinis***	15	16	15.5	11	16	13.5
Mean	19.2	19.5	-	18.1	21.8	-

Table 4.4: The numbers of microcosm mixes in which each of the bacterial species was detected, from a maximum of 30, by T-RFLP end-point profiling. The data from the T-RFLP profiling of the microcosms (n = 30) at the end of the 24 hours experiment indicated the composition of the community (by way of relative abundance). These results indicated that there was a greater number of non-detectable species in the mixed environment than in the constrained.

Fitting the GLM 2: species contributions and interactions are environmentally dependent sources of experimental variance.

The next two variables entered into the GLM analysis were the species composition and the non-linear richness terms. For the species composition term, the relative abundance of each species in each microcosm was estimated by using end-point T-RFLP profiling. These values were then used as weightings to approximate the contribution of each species to respiration. In the non-linear richness term, species richness was entered as an ANOVA-like factorial variable as opposed to the continuous linear richness regression term. Non-linear richness is associated with the variance in the data that has not been accounted for by the linear richness term or composition term and is considered to be related to the "interactions" between the species at each species richness level (Hector *et al.,* 2009).

Tryptone Water: adding the composition and non-linear richness terms to the GLM

The species were weighted with their final relative abundance and individual species effects were observed in both Tryptone Water environments (Figure 4.3). Six species had significant ($F_{1,55} > 4.40$, $p < 0.041$) effects on community respiration in the mixed environment, with eight found to be significant ($F_{1,55} > 4.22$, $p < 0.045$; Figure 4.3) in the constrained environment. The effects of the individual species summed together to give a significant composition term (Table 4.2). This significance was observed in both the mixed ($F_{11,55} = 5.93$, $p < 0.001$) and constrained ($F_{11,55} = 12.14$, $p < 0.001$) environments. After the composition term was added to the model, the new model accounted for over 60% of the overall variation in both the mixed ($R^2 = 0.62$) and constrained ($R^2 = 0.65$) environments. The addition of the composition terms produced the greatest improvement in the fit of the model to the variance within the data.

Further investigation of the composition term indicated that *S. maltophilia* was the only species to have positive, and significant, effects on respiration in both environments ($F_{1,55} > 30.5$, $p < 0.001$; Figure 4.3). The only other species to have a positive coefficient, in the mixed environment alone, was S. *sanguinis* ($F_{1,55} = 0.13$, $p = 0.719$). In both environments, there were a number of species that had negative, statistically significant effects on the respiration; in the mixed environment these species were *P. aeruginosa, B. cepacia, S. marcescens, S. haemolyticus* and *S. aureus* ($F_{1,55} > 4.22$, $p < 0.045$). In the constrained environment these species were *B. cepacia, A. xylosoxidans, A. baumannii, S. marcescens, S. haemolyticus, S. aureus* and *S. mitis*

($F_{1,55} > 4.40$, $p < 0.041$). The non-linear richness term in this model was shown to be close to significance in the constrained environment ($F_{4,10} = 2.66$, $p = 0.096$) but highly non-significant in the mixed ($F_{4,10} = 0.71$, $p = 0.602$) environment (Table 4.2).

Defibrinated Horse Blood: adding the composition and non-linear richness terms to the GLM

The species were weighted with their final relative abundance and individual species effects were observed in both DHB environments (Figure 4.4). In DHB, the species coefficients (Figure 4.4) in the mixed environment were typically 100-fold lower than those in the constrained environment. *S. maltophilia* had the most positive coefficient in both environments. *S. maltophilia* was also found to be the only species with a significant effect in the mixed environment ($F_{1,55} = 25.16$, $p < 0.001$). The other species shown to have positive coefficients were *P. aeruginosa* and *S. pneumoniae* (in the mixed environment only), *E. faecium* (in the constrained environment only) and *S. mitis* (both environments). In the constrained environment, most of the species were shown to have significant effects on community respiration. The only exceptions were *S. haemolyticus* ($F_{1,55} = 3.45$, $p = 0.068$) and *S. pneumoniae* ($F_{1,55} = 0$, $p = 0.999$) which were not shown to have significant effects in the constrained DHB environment.

The sum of the variance accounted for by the individual species gave significant species composition effects in both the mixed ($F_{11,55} = 3.28$, $p = 0.001$) and constrained ($F_{11,55} = 12.51$, $p < 0.001$) environments (Table 4.3). As with the results from the Tryptone Water, the addition of the composition term to the model gave the greatest improvement, thus far, in the fit of the model in both the mixed ($R^2 = 0.45$) and constrained ($R^2 = 0.50$) environments.

The non-linear richness term was found to be significant in both the mixed ($F_{4,10} = 15.77$, $p < 0.001$) and constrained ($F_{4,10} = 4.18$, $p = 0.030$) environments (Table 4.3). The non-linear richness effect increased the fit of the total model to $R^2 = 0.47$ and $R^2 = 0.51$ in the mixed and constrained environments respectively.

Comparison between the nutrient sources

In comparison to the analysis from the Tryptone Water (Figure 4.3), in DHB other species were also shown to have positive coefficients. The values for the coefficients were much lower in the DHB media than in the Tryptone Water. In Tryptone Water there was a modest correlation (using Spearman's Rho) between species presence and species coefficients (Spearman's $\rho = 0.46$) in constrained

microcosms. This indicated that bacterial species associated with higher coefficients were detected more often at the end of the experiment than those with lower coefficients. In the mixed environment, this relationship was weaker with a lower correlation (Spearman's ρ = 0.34). In the mixed DHB environment, the correlation between the presence of species and their coefficients (Spearman's ρ = 0.31) was found to be similar but weaker than that found in Tryptone Water. In the constrained environment however, the correlation (Spearman's ρ = 0.18) was much lower than in the Tryptone Water.

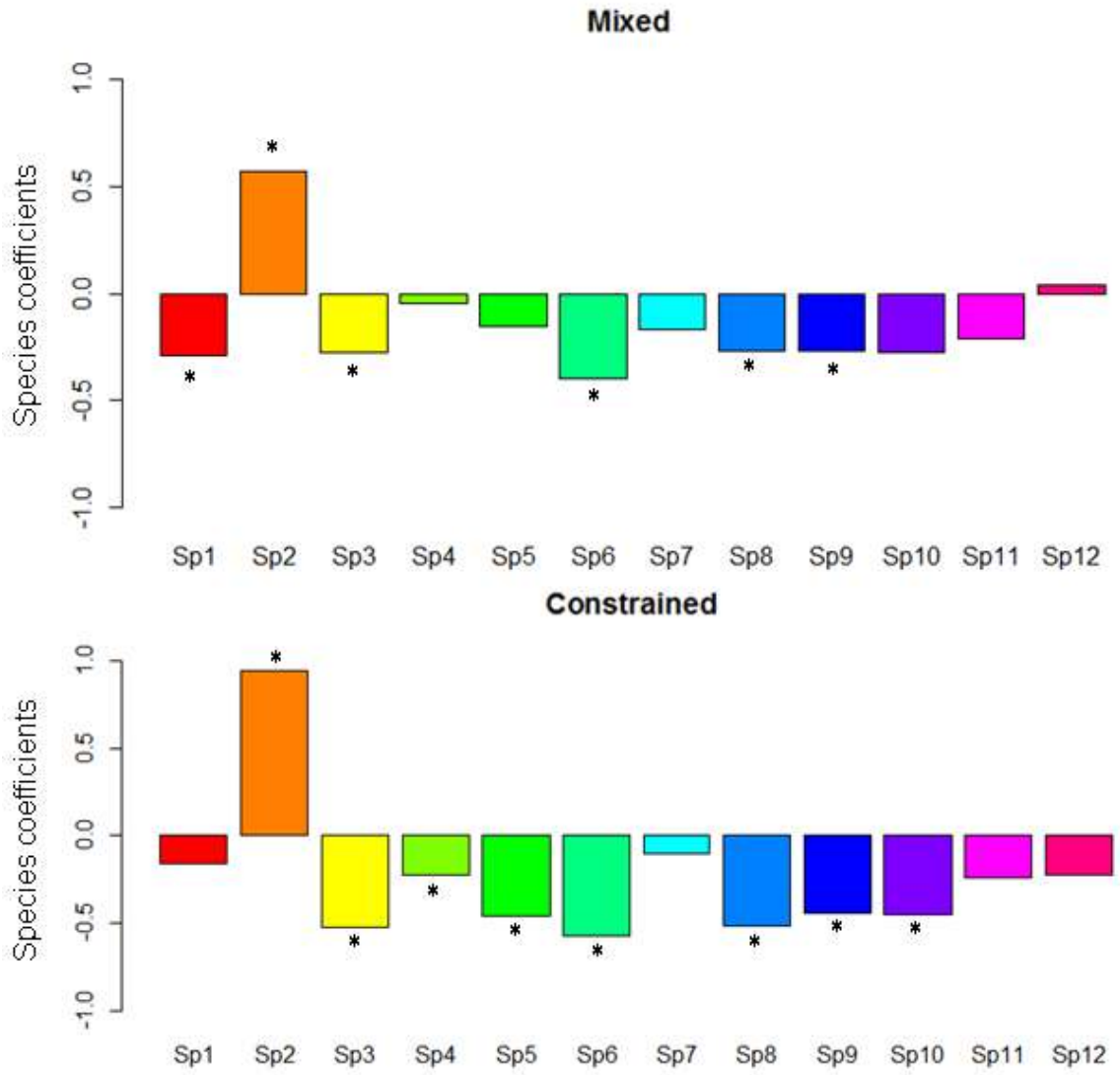

Figure 4.3: The species coefficients for each of the weighted individual species grown in mixed and constrained Tryptone Water environments. Species coefficients, calculated from the general linear model (y-axis), show the mean contribution of each species (x-axis) towards total respiration. Asterisks denote significant effects ($p < 0.05$). Values for species coefficients are presented in Appendix Table A2.

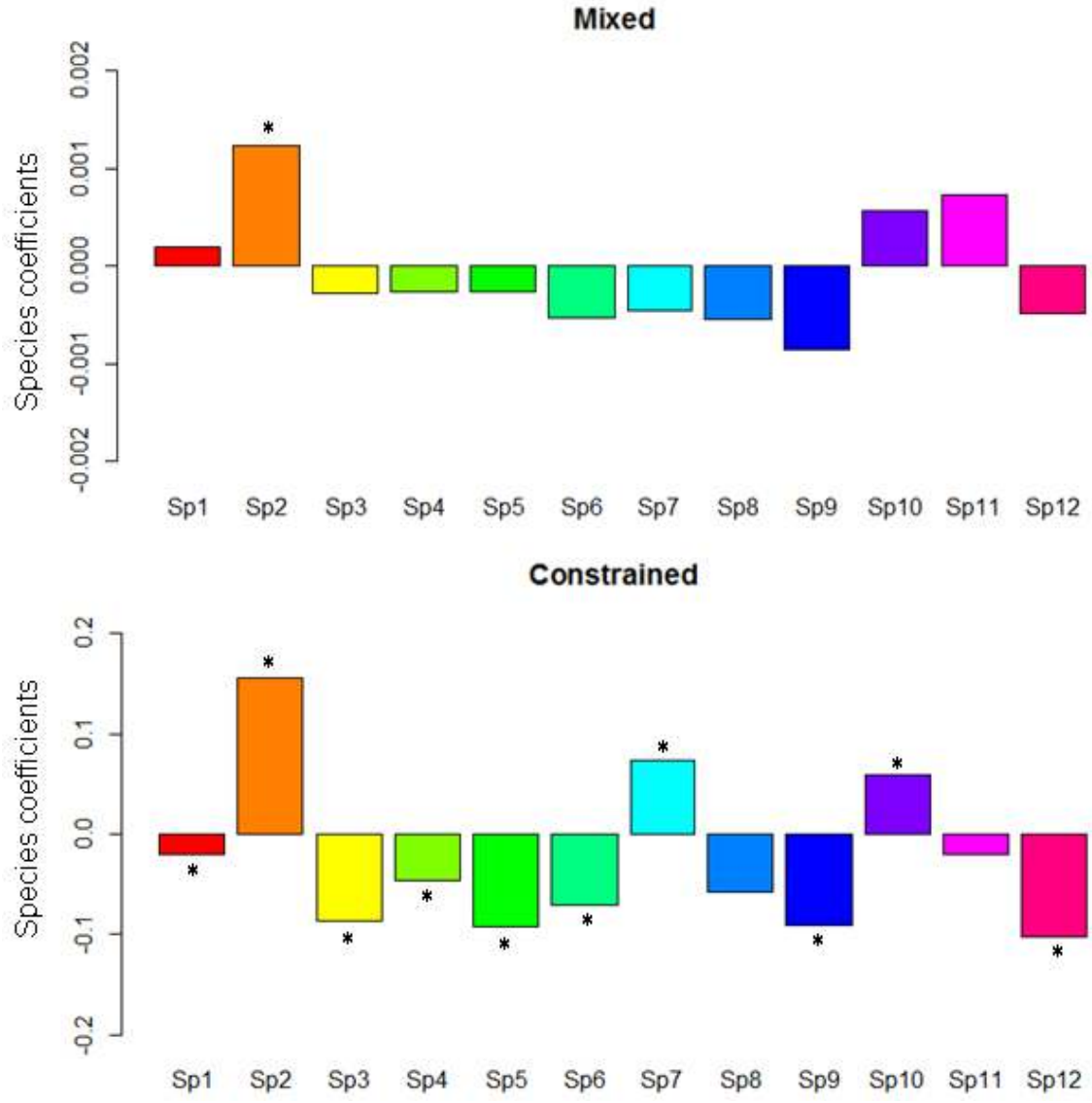

Figure 4.4: The species coefficients for the DHB media in both mixed and constrained environments. Species coefficients, calculated from the general linear model (y-axis), show the mean contribution of each species (x-axis) towards total respiration. Asterisks denote significant effects ($p < 0.05$). Values for species coefficients are presented in Appendix Table A3.

Fitting the GLM 3: the completion of the model by the addition of the statistically relevant terms.

The analysis of the *Q* and *M* terms were required for the analysis of the significance of all the variables reported previously. The significance of the *Q* and *M* terms therefore does not inform on the effects of biodiversity as they overlap with the experimental and biological variables (as shown in Chapter 3c). For completeness, the results associated with these terms will be presented.

Tryptone Water: adding Q and *M to the GLM*

In the mixed Tryptone Water environment, both the *Q* and M variables accounted for a significant proportion of the variance ($F_{10,82} = 3.35$, $p = 0.001$ and $F_{55,82} = 2.45$, $p < 0.001$ respectively). In the constrained environment however, *Q* was found to be non-significant whilst *M* remained significant ($F_{10,82} = 1.15$, $p = 0.334$ and $F_{55,82} = 1.50$, $p = 0.047$ respectively) After *Q* and *M* were included in the model, 89% of the variance ($R^2 = 0.89$) was accounted for in both environments.

Defibrinated Horse Blood: adding Q and *M to the GLM*

The *Q* term was found to be significant in the mixed environment only ($F_{10,82} = 2.31$, $p = 0.019$). This was in contrast to the *M* term that was found to be significant in both the mixed and constrained environments ($F_{55,82} > 2.26$, $p < 0.001$). The addition of the final two terms greatly increased the fit of the model (Table 4.3). After the addition of *Q* and *M* the model fitness increased to over 90% in both the mixed ($R^2 = 0.93$) and constrained ($R^2 = 0.91$) environments.

Comparison between the nutrient sources

The differences between the two nutrients, with respect to these variables, are shown in the significance of *Q*. As above, the *Q* term was found to be statistically significant in the mixed Tryptone Water and constrained DHB environments, but non-significant in the other two environments. The *M* term was significant in all the environments.

The impact on the model by using end-point T-RFLP profiling

This study was the first investigation to define experimentally the composition of the community at the end of the analysis. By doing this, this study found that the weighting of the species altered all of the terms entered into the GLM after the linear richness (data not shown). Comparisons were made between the species coefficients presented in this Chapter and those whose presence absence was binary (Chapter 3c). The coefficients calculated presented different patterns dependent on whether the species were weighted or not (Figure 4.3 Mixed). This, for reasons outlined in Chapter 3c, subsequently affected the terms entered later in the analysis (discussed further below). The fit of the model to the data was shown to be greater with the inclusion of the relative abundances than using un-weighted, binary contributions. Using the binary contributions, the model then accounted for 56% and 53% in the mixed and constrained environments respectively; much lower than the weighted model fitness presented here ($R^2 = 0.89$).

The relative importance of the biodiversity effects on CO_2 production.

This analysis has shown that the relative effects of three aspects of biodiversity (linear richness, species composition and non-linear richness) are affected by the environment. The predictive power of these aspects can be assessed by substituting the appropriate coefficients from the weighted values into a simplified model (eq.11):

$$y = \beta_o + \beta_{LR}x_R + \begin{bmatrix} \beta_{Sp1} \\ \vdots \\ \beta_{Sp12} \end{bmatrix} x_C + \begin{bmatrix} \beta_{NLR1} \\ \vdots \\ \beta_{NLR12} \end{bmatrix} x_R \qquad [11]$$

The respiration values predicted by the model were plotted against the actual observed values. The resulting coefficients of determinations (R^2) were calculated. The step with the greatest increase in R^2 was regarded as the most influential in describing the effect of biodiversity on the system.

From the four experimental environments tested (two nutrient sources x two viscosities), the fit of the simplified model had a mean $R^2 = 0.40$ (SD = 0.09, $n = 4$). The majority of the variance within the data was not accounted for using these terms. The results do however, also show that there were conserved trends within the amount of variance each of the terms accounted for. The first observation was that the intercept alone accounted for a negligible amount of the variance. This was anticipated but the intercept value was important for making the other coefficients meaningful in the

prediction of respiration. The fit of the model up to the linear richness term (the second term in eq.11) was found to have a mean $R^2 = 0.124$ (SD = 0.05, $n = 4$). In all of the environments, the addition of the species composition term increased the fit of the predicted values to the observed mean $R^2 = 0.368$ (SD = 0.07, $n = 4$). Finally the non-linear richness term was added to the predicted equation completing the model.

The R^2 values showed that the species composition has a greater predictive power when entered into the model than either the linear or non-linear richness terms. This increase in predictive power of the model was only observed after the linear richness term was entered into the analysis.

Predicted respiration was calculated by fitting the model (eq.11) for each of the four environmental combinations. These predicted values were plotted against the observed respiration (Figure 4.5). Four linear regressions were fitted and any data points that lay outside two standard deviations (i.e. significantly larger or smaller than predicted) were considered of interest. There were a total of 40 outlying data points across all four environments. Of these, 31 had significantly higher observed values than predicted and were termed "overachievers". Of these overachievers, six were found in the mixed Tryptone, eight in the constrained Tryptone, nine in both the mixed constrained DHB environments. The remaining nine showed observed values significantly lower than those predicted and were termed "underachievers". There was a significantly greater number of overachievers than underachievers ($t_3 = 4.16$, $p = 0.025$). Of these underachievers, three were found in the mixed Tryptone, four in the constrained Tryptone and one each in the mixed and constrained DHB environments. When the species were investigated in these outstanding microcosm mixes, a number of observations were made. The first of these was that *S. maltophilia* was present as overachievers in all environments, except the constrained DHB. The three species combination of *B. cepacia, A. baumannii* and *S. pneumoniae* was also an overachieving combination in all environments. *B. cepacia* was found to be present, in eight different species combinations, in 11 of 31 overachievers. Of the underachievers, the combination of *S. marcescens* and *S. sanguinis* was present in five of nine underachieving microcosms. This combination was present only once, as part of a 12 species community, as an overachiever.

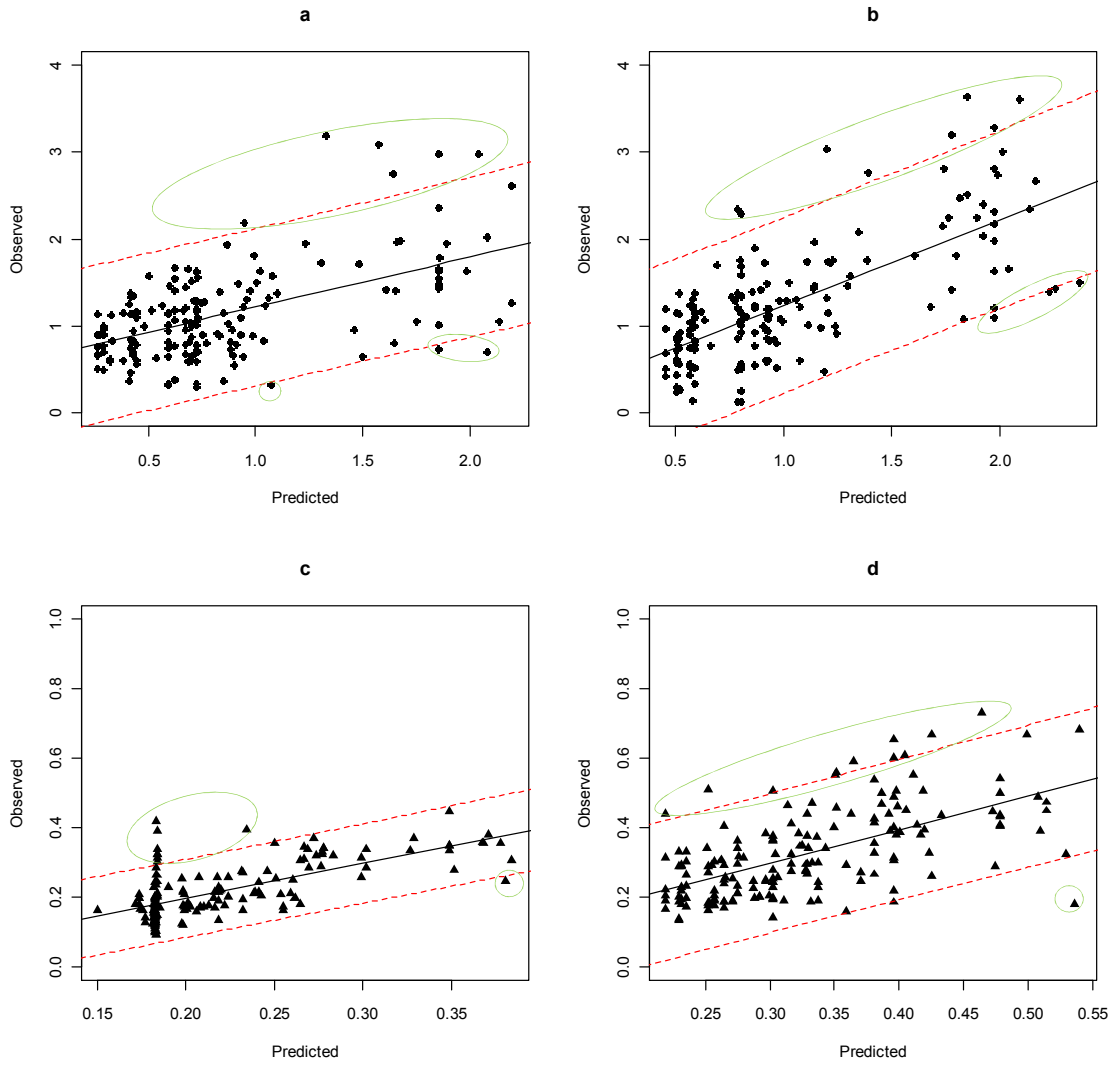

Figure 4.5: Observed respiration plotted against predicted respiration. Predicted respiration values were calculated by substituting the linear model coefficients into the simplified model (eq.11). The observed respiration (UCO_2) is plotted against the predicted respiration (UCO_2) for measurements taken in the mixed (a) and constrained (b) Tryptone environments and the mixed (c) and constrained (d) DHB environments. The regression line (solid black) showed the positive relationship between the predicted and observed respiration. Any data points outside (encircled in green) two standard deviations (dotted red) from the linear regression line were called an outlier and the composition analysed.

4.4 Discussion

The aim of this study was to investigate how changes in biodiversity affected the total activity, measured by respiration, of bacterial assemblages. In addition, this study aimed to observe how the nutrient content and viscosity of the environment altered the effect of biodiversity on respiration. The four different environments were; i) Tryptone Water as a liquid, ii) Defibrinated Horse Blood (DHB) as a liquid, iii) viscous Tryptone Water and iv) viscous DHB. Units of respired CO_2 (UCO_2) were measured after a 24 hour incubation period and the results analysed using a GLM. Overall, bacterial assemblages tended to respire more in Tryptone Water and constrained environments than in DHB or mixed environments.

The four growth conditions all affected the statistical significance of the experimental and biological variables.

Growth environments mediated the significance of both the experimental (replicates and Partition) and the biological (linear richness, species composition and non-linear richness) variables. Generally, the significance of the experimental variables was altered only across the nutrient sources. The change in the significance of the biological variables was generally affected by both nutrient content and viscosity.

The significance of the experimental variables was associated with a change in nutrient.

When microcosm growth on the two nutrient sources were compared there was significantly greater respiration generated by assemblages when grown in Tryptone Water than in DHB (Table 4.1). This difference in overall respiration suggested that the Tryptone Water was richer and more readily utilisable as a nutrient source than DHB as had been hypothesised prior to the start of the experiment. The variance within the respiration measurements was also greater in Tryptone Water than in DHB. Upon analysis using the GLM, it was found that the experimental variables were significant in the Tryptone Water but not in the DHB media (Tables 4.2 & 4.3).

The replicate (REP) variable

Significant differences between the paired replicates were identified. Generally the first replicate had less respiration than the second replicate for microcosms grown in Tryptone Water. This discrepancy between the replicates was not however observed in microcosms grown in DHB. This could have been a product of the reduced respiration observed in DHB. In contrast, the increased range of respiration values produced by the

bacterial assemblages in Tryptone Water made it more likely that differences would be observed. Expanding on this, the discussion of the replicate variable will therefore focus on the respiration measured in the Tryptone Water environments.

It is possible that the state of the bacterial cells as they were inoculated into the microcosms could affect the overall respiration values. The initial conditions of, and early events within, a system have been considered to influence the resulting organisation and by extension the outcome. These influences were termed "founder effects" by Cohen (1999). These may have been important here and a detail of establishment of the experiment should be considered. In establishing the microcosm mixes, there was c. 20 minutes between initiating the processing of the first and second sets of replicates. This additional time may have altered the state of the bacterial cells entering the experiment.

If bacteria were initially in different growth states then their response to the new experimental environments would possibly be quite different. This response could take the form, for example of variation in lag phases or profiles of genes expressed (Schimel *et al.,* 2007). Despite best attempts to minimise this, the results show that these effects were highly significant in the Tryptone Water environments. That is, small differences between replicates resulted in significantly less respiration in the second set of replicates.

This provides some insight into the difficulties that can be experienced in predicting outcomes in natural systems. Despite the variation that may occur in the colonisation of a microcosm or lung, ecological mechanisms can be discerned.

The Partition (P) variable

The Partition term was analysed after the variance accounted for by the replicates was removed from the data. As such the Partition terms analysed the variance within the remaining residuals. Partitions were found, as with the replicate term, to have significantly different respiration from microcosm mixes in Tryptone Water but not in DHB.

This may again be related to the typically greater respiration in Tryptone Water, where the respiration has greater "freedom to vary" between the Partitions (i.e. the differences among the DHB partitions) if any, would be smaller and harder to detect. An important question arises as to where the differences between the paired Partitions resulted from. These differences could result from the combinations of species or alternatively the founder effects may have been important. The influence of founder

effects was tested by inspection of the respiration of the single and 12 species microcosm mixes. These were included in all Partitions. Therefore, if the variation between the Partitions was based primarily on the combinations of species, the respiration measured from these assemblages should be highly similar. This was not found to be the case. This suggests that the variation present within the data were more likely to be a function of a founder effect (i.e. experimental variation).

Interestingly, these founder effects have not been reported before. However, recognising these effects is important to understanding, and accounting for, the variance within the data and potentially in other studies. If these effects were not taken into account by the model at this stage, the variation present would have been attributed to other, biological, variables (in particular the richness terms) within the model, thus misleading the interpretation of data.

The significances of biological variables are generally affected by changes in both nutrient content and viscosities.

The biological variables tested, in order of model entry, were; i) linear species richness, ii) species composition and iii) non-linear species richness. These variables were able to account for the remaining variance (Bell *et al.,* 2009b; Chapter 3c). As such, the variance accounted for by the experimental variables was not available to any of the biological variables, thus providing a more conservative analysis of the effects of biodiversity.

The linear species richness (LR) variable

A significant and positive relationship was found between species richness and respiration (Figures 4.1 & 4.2). This result has also been established by previous BEF experimental studies, the majority of which being in plant-based systems (e.g. Tilman *et al.,* 1996; Loreau & Hector, 2001 and reviewed in Duffy, *et al.,* 2002). More recently however, similar findings have also been reported for bacterial BEF experimental studies (Wohl *et al.,* 2004; Bell *et al.,* 2005). Although this is the "classical" BEF experiment result, the design of the experiment is important in order to be able to analyse these findings more thoroughly.

Here, an adapted RPD and analysis was performed. In this, the positive relationships between the species richness and respiration were found to be significant even after the variance accounted for by all the other experimental variables was removed. The linear richness term was also found to be significant in all of the environments tested.

The effect of linear richness on respiration supports the central hypothesis of this study, and indeed thesis. The significance values identified for the linear richness terms show that bacterial biodiversity affected the average respiration of the assemblages, at the given level of diversity. For this reason, the linear richness term is entered as the first biological term in the model. The other two biological variables in the model are present in order to better understand the relationship mechanistically and account for the remaining variance. With the variance accounted for by the experimental and linear richness terms, over half of the variation within the data still remained unassigned. Therefore, although linear richness was significant, at this point other mechanisms causing this variance need to be investigated.

These mechanisms are described as the species composition and the non-linear richness or "interaction" terms. These two biological variables investigate the presence of the selection and complementarity effects (Loreau & Hector, 2001; Fox, 2005), and encompass the processes by which biodiversity is thought to affect system function.

The species composition (C) variable

The selection and complementarity effects are two of the most important phenomena in BEF research beyond linear richness (Becker *et al.,* 2012) and are fundamental to the study of biodiversity and ecosystem function. Complementarity effects are discussed below. The selection effects can be described as the processes of particular species being able to affect the system function to a greater degree than other species present. In this study, the species composition term indicated the degree to which the selection effect was present within this system. This term tested whether any species had significant effects on the total respiration of the microcosms in which it was present.

It was found that the species composition had highly significant effects in all of the environments tested. That is the composition of species in a microcosm mix had a significant influence on its respiration. At least one of the species coefficients was significantly different in each environment. While the overall species composition term was found to be significant in all environments, individual species were found to have differing coefficients which were dependent on the environment.

Generally, the coefficients in Tryptone Water were dominated by *S. maltophilia*. This species possessed the only significantly positive coefficient out of the 12 tested. Three species were shown to have significant negative effects in both Tryptone Water environments; *B. cepacia, S. marcescens* and *S. haemolyticus*. This could suggest that

these species were exhibiting competitive interactions with the other species (Foster & Bell, 2012).

The coefficients in DHB were generally more evenly distributed with more positive coefficients. This suggested that the nutrients in DHB in this microcosm system created fewer opportunities for one or more bacterial species to facilitate an assemblage. This may suggest that the various assemblages generally worked together to utilise the nutrient source, without strong effects conferred by specific species. Even so, *S. maltophilia* was again found to be the only bacterial species with significantly positive coefficients in both viscosities.

The coefficients also altered with the change in viscosity. In the constrained environment, a greater number of species had significant effects on respiration (Figures 4.3 & 4.4). The constrained environment was included in this study to increase the number of "local" neighbourhood based interactions (Kuemmerli *et al.,* 2009; Elias & Banin, 2012). This could potentially lead to, or augment, the expression of genes at local levels and as such this forms a direction for future research. It is also interesting to note that growth in biofilm (an example of a clinically relevant constrained system (Elias & Banin, 2012)) is associated with changes in gene expression, phenotype and activity (Whiteley *et al.,* 2001).

After the species composition terms were entered into the model over half of the total variance had been taken into account. The species composition effects accounted for the greatest amount of variance within the data.

The non-linear richness (NLR) variable

The non-linear richness term is considered to represent the interactions between species within the richness levels. These can also be thought of as complementarity effects where these effects have been defined as "*reflecting niche differences and /or facilitative interactions among species*" (Fox, 2005). This term therefore accounts for the variation that is associated with species richness but not accounted for by the linear richness term. As such, this variable becomes the deviation of the residuals from the linear relationship based on the number of species present. The most likely cause of this variation, after the species number and the species composition have been accounted for is the interactions between the species at each richness level.

This was the biological variable most affected by the change in environmental conditions. In Tryptone Water, the non-linear richness term was non-significant in the mixed environment indicating no significant interactions present within the richness

levels. This supports both hypotheses that Tryptone Water would serve as a "broad spectrum" nutrient source and that species in well mixed communities would have a lower degree of interactions (Kuemmerli *et al.,* 2009). Interestingly, increasing viscosity increased the significance of the non-linear richness terms (Table 4.3). Similarly, previous studies have reported that the more viscous the environment, the more prevalent interactions are (Kuemmerli *et al.,* 2009; Elias & Banin, 2012). Also similar to results presented later (Chapter 6), they also hypothesise that the number of synergistic interactions, outnumber any antagonistic interactions (Elias & Banin, 2012).

In the DHB, with the increase in viscosity came a reduction in significance of the non-linear richness term. In both environments, interactions were significant. This suggests that the utilisation of DHB was likely to require a greater degree of indirect cell interactions between the bacterial species. This would be supportive of Pishchany and Skaar (2012) who presented the processes involved in bacterial disruption of erythrocytes and release of haemoglobin to be costly. Possibly, in the constrained environment, the spatial restriction of the bacterial species limited the scope for and significance of these interactions.

The addition of the non-linear richness term to the model increased the proportion of variance accounted for only marginally in all environments. Even though there was only a small improvement in the model fit, the non-linear richness term was retained. This term, evaluating the contribution of the between species interactions, was a crucial part of assessing the ecological mechanisms within this system.

The statistical variables, Q *and* M

The variables *Q* and *M* are applied here as statistical terms. This places them in a third category of variables outside the previously discussed experimental or biological terms. As such, these two variables were used as *F*-test denominators because the variance accounted for by these terms overlapped with one or more of the experimental or biological variables (as shown in Chapter 3c).

How was the model suited to the analysis?

Here, variation within the experimental procedure was removed from the analysis prior to the analysis of biodiversity. Within the field of bacterial BEF research this has not, to the best of my knowledge, been published before.

Generally, this model has shown that biodiversity has a significant impact on respiration. The model was also able to differentiate between the species. Further to this, the model has informed on the general inclusion of interaction-like effects that

cause increased variance within the system. This model has also been able to partition and remove variance that would otherwise have affected the analysis through the inclusion of the replicate and Partition terms. As seen by comparison of the analysis reported here, Table 4.2, and that reported in Chapter 3c, the inclusion of these terms can alter the effect that many of the variables have (specifically the non-linear richness term).

From the observed against predicted respiration graphs (Figure 4.5) the model coefficients have been shown to account for ~40% of the variation in the data. This is a substantial achievement which still leaves an important quantity available for further elaboration of the causes of the BEF relationship. The remaining variation however is likely to be a function of phenomena such as the founder effects, and thus not able to be fully accounted for.

The addition of T-RFLP increased the fit of the model.

This study reported the first implementation of the RPD method that accounted for variations within the microcosm mixes arising over the course of the experiment. This was performed using T-RFLP profiling and required piloting to ensure the species that could be distinguished by T-RF band size. The resulting relative abundances from the profiling allowed each species to be weighted in the GLM analysis. This weighting assigned the total respiration to each constituent species based on their presence at the end of the experiment. Whilst not based on a RPD GLM, a similar approach to weighting has been recently taken by Lawrence *et al.* (2012) who used culture-based methods to assess the presence of the mixed environmental bacterial species at the end of their experiment.

The weighting of the species using only their relative abundance at the end-point of the experiment could not integrate the dynamics of the species within an assemblage. This method was an approximation as to how the assemblage had changed over the 24 hours incubation period. It is notable that when the species were weighted in the analysis, this altered which species coefficients were significant without losing any model fitness.

Despite these results providing the realised species richness, the GLM analysis was always performed on the initial (i.e. manipulated) species richness. This is standard practice in direct manipulation experiments (Loreau & Hector, 2001; Becker *et al.,* 2012). If a species was not detected at the endpoint, this does not mean that it was no longer present or did not contribute to the respiration observed. If however the zero

values were used in the analysis, the overall effect of linear richness did not change (data not shown); this would have negated the balanced design.

The choice of nutrient caused the mediation of significant variables.

The choice of nutrient to be used was based on a lot of detailed testing (Chapter 2.2). Key requirements were that the media used were reproducible with low batch variation, were clinically relevant and that they should produce contrasting degrees of growth. Both Tryptone Water and DHB were commercially available for use in diagnostic laboratories and so filled the first two criteria. They were also nutrient sources that the bacterial species could encounter in a human context. One further practical advantage with these as liquid nutrient sources was that to alter the viscosity of the media, the addition of a gelling agent was all that was required. Even after the addition of the gelling agent it was possible to aliquot accurately the small volumes required in these experiments.

A previous bacterial BEF experiment has also indicated that the alteration of nutrient sources can mediate the effects of biodiversity on ecosystem function. Langenheder *et al.* (2010) investigated how the impact of biodiversity in environmentally derived bacterial assemblages when the substrates were altered from three single substrates to combinations of two and three.

The increase in viscosity showed variation of significant effects and potential mechanisms occurring in the CF lung.

The results presented and discussed here show that the viscosity of the system modulates the impact of biodiversity on total respiration. In particular, the viscosity affected the species coefficients and non-linear richness terms with more coefficients significant across all the species when constrained environments were compared to mixed. This suggests a reduced ability for a single species to dominate the assemblage. The non-linear richness term was shown to increase in significance in the Tryptone Water and decrease in significance in DHB. These changes in significant coefficients indicated that there are distinct differences in the biological effects. This suggests that the environment within the colonised CF airways may possess ecologically relevant mechanisms that would not be observed in liquid, planktonic growth, but have been reported in this study.

The difference between a mixed and constrained environment, as reported here, has potential implications to the microbial diversity detected in the airways of paediatric

and adult CF patients. The results from the T-RFLP profiling indicate that there are more "drop-out" events occurring in the less viscous, mixed environments. It might have been expected that the increased growth in constrained Tryptone Water would have led to more loss of slow-growing populations. This result does however, indicate the contrary that less inter-species competition occurs in the constrained rather than in the mixed environments. This kind of protective or stabilising effect in biofilm and other viscous environments is well attested to in the literature (e.g. Elias & Banin, 2012).

Conclusions

This study found that increases in species richness were associated with increased respiration and that biodiversity was shown to have a significant impact on respiration by assemblages. The analysis also found that initial experimental variations (founder effects) cause significant changes in respiration, although this can be compensated for in the GLM analysis. Using the adapted GLM, it was also found that species composition was an important variable in accounting for the spread of assemblage respiration around the linear regression. The composition term singularly accounted for the largest part of the variance in the data around the linear regression at each richness level. This therefore indicates a driver for substantial differences between microcosm-associated respiration. The composition term does little however to explain how an increase in diversity leads to an increase in respiration. The non-linear richness term indicated that there were different community dynamics which affected the respiration depending on the viscosity and nutrient content of the environment studied here.

This chapter has shown positive relationships between species diversity and respiration. The potential mechanisms that support this will be explored later. Extending from this chapter however were questions on the degree to which the data obtained were influenced by the use of a single isolate of a species.

Chapter 5

5. The effect of phenotypic biodiversity on respiration with ecotypes of *Pseudomonas aeruginosa*

5.1 Introduction

The previous chapter reported the effect of increasing the number of species on the respiration of an assemblage. So far, differences between species in assemblages have been reported in the context of different nutrition sources and environmental viscosities. Variation within each of the species has not as yet been considered. The central aim of this chapter is to determine whether differences in respiration would be observed in assemblages of increasing diversity of isolates of a single species.

Whilst CF airways can be infected by range of bacterial species, colonisation with *Pseudomonas aeruginosa* causes particular clinical concern (Emerson *et al.,* 2002; Rogers *et al.,* 2003; Foweraker *et al.,* 2005; LiPuma, 2010; Greally *et al.,* 2012). This bacterial species can be cultured in the early stages of pulmonary infections (i.e. in patients below 10 years of age and in much older ones; Goss & Burn, 2007). Colonisation of the CF lung by *P. aeruginosa* correlates with a decrease in well-being, an increase in disease severity and an increased need for antibiotic treatment (FitzSimmons, 1993; Emerson *et al.,* 2002). *P. aeruginosa* can often become a chronic airway infection by early adulthood in individuals with CF (Rosenfeld *et al.,* 2001). Over the course of the chronic infection, *P. aeruginosa* has been shown to "shed" genetic information with lines of this species arising that show differing genomic content (Smith *et al.,* 2006; Huse *et al.,* 2010).

Because of the clinical significance of *P. aeruginosa,* previous studies have investigated the extent of variation between clinical isolates (Burns *et al.,* 2001). These previous studies have indicated that different *P. aeruginosa* isolates can often have different phenotypic characteristics (Drenkard & Ausubel, 2002). These include metabolic profiles (Rogers *et al.,* submitted manuscript), morphologies (Häußler *et al.,* 1999; Emerson *et al.,* 2002) and antibiotic susceptibility profiles (Emerson *et al.,* 2002; Foweraker *et al.,* 2005).

As a result of its clinical importance, the ability to treat infections by this species has been seen as particularly important. As such, many methods have been developed to

assess the efficacy of antimicrobial compounds on *P. aeruginosa* and other bacterial species. Among these methods, the broth dilution (Das *et al.,* 2010) and the Kirby-Bauer disk diffusion assay (Bauer *et al.,* 1966; Koeth *et al.,* 2000; Andrews, 2008) are two of the most commonly used, reproducible susceptibility tests (Das *et al.,* 2010).

Aims and Hypotheses

This chapter will investigate the impact of within-species diversity on total assemblage respiration. This will be accomplished using 12 *Pseudomonas aeruginosa* isolates shown to be phenotypically different. Here, isolates will be manipulated into three Partitions of microcosm mixes (as reported in Chapter 4). These microcosms were used to inoculate media containing two types of nutrient source (Tryptone Water and Defibrinated Horse Blood), with the production of CO_2 measured.

5.2 Material and Methods

Isolation and identification of clinical Pseudomonas aeruginosa

Isolation of the bacterial isolates, and their characterisation, was carried out as previously described (Chapter 2.1).

Selection of isolates and phenotypic assays

Previously, a single *P. aeruginosa* isolate was used in the biodiversity studies reported in the previous chapter. This isolate, dwr1, was used again in this current chapter to assess how it performed in an assemblage consisting only of clinical *P. aeruginosa* isolates. Eleven additional isolates, identified as *P. aeruginosa* by 16S rRNA gene sequence analysis were randomly selected from the pool of 40 isolates (as described in Chapter 3a).

Phenotypic characteristics assayed were; i) the presence of a blue-green pigment ii) the ability to grow in conditions of elevated NaCl concentration and iii) antibiotic resistance profiles. All phenotypic assessments were independently replicated three times. These assays are described in turn. Isolates were cultured aerobically overnight at 33°C on *Pseudomonas* selective agar. The agar was prepared following manufacturer's instructions using *Pseudomonas* agar base (Oxoid Ltd.) with *Pseudomonas* CN selective supplement (Oxoid Ltd.) that selectively isolated *P. aeruginosa*. As above, all isolates were confirmed as *P. aeruginosa* by means of 16S rRNA gene sequence analysis. For the phenotypic characterisation, the culture of isolates on *Pseudomonas* selective agar allowed the visual identification of pigmentation and was scored as present or absent (after Laine *et al.,* 2008).

The ability of the isolates to tolerate an elevated NaCl (Sigma-Aldrich) levels in broth was assayed. Sterile MH supplemented with an additional 3% (w/v) NaCl was inoculated with 100 µl of ~1 x10^4 culture of each isolate of *P. aeruginosa* grown under aerobic conditions in sterile MH broth overnight at 37°C in an orbital incubator with shaking at 110 rpm. Increased salt assays were incubated at 33°C aerobically for 18 ± 2 hours, with a score for positive growth recorded for cultures that had a turbidity reading of $OD_{600} > 0.5$ after this time.

Antibiotic resistance profiles were determined using an adapted Kirby-Bauer disk diffusion assay (Bauer *et al.,* 1966; Koeth *et al.,* 2000). Each isolate of *P. aeruginosa* tested here was prepared as described above prior to the start of the assay. A 200 µl portion of a culture of one of the 12 clinical *P. aeruginosa* isolates was placed

in the base of a sterile 90 mm Petri dish (Sterilin). To this, 20 ml of molten sterile MH broth at 50°C supplemented with 2% w/v technical agar was added and mixed to obtain an even distribution of bacteria within the agar.

Sterile filter paper disks (diameter = 6 mm) were soaked with ceftazidime (2 µg), ciprofloxacin (1 µg) or tobramycin (1.5 µg). The other six antibiotics used in this study (Table 5.1) were purchased as pre-infused disks from Oxoid Ltd. The disks were placed onto the surface of agar and incubated at 37°C for 18 ± 2 hours to allow bacterial growth. After incubation, the diameter (in millimetres including the 6mm disk) of any zone of inhibition formed around the disks was measured. A zone of inhibition greater than 7 mm diameter was categorised as a negative result (susceptible), with a zone of 7 mm or less recorded as a positive result (resistant). From this, a binary matrix showing resistance profile was generated.

Assembly of experimental microcosms

Microcosms used in this study were assembled using the same partitions and nutrient conditions, in a mixed environment, as previously described (Chapter 2.8).

Measurement of CO_2 production

The measurements of CO_2 produced by the microcosms were assayed using the method described (Chapter 2.9).

EcoPlate™ community level phenotypic profiling

The EcoPlate™ system was used to analyse the sole carbon source utilisation for isolates that could not be separated by the above tests. Assays were performed as previously described (Chapter 2.10).

Statistical analysis

Analysis using the general linear model was executed as previously described. All t-tests are paired unless otherwise stated. For measurements recorded using the EcoPlate™ system; prior to any analysis of the data the OD_{590} reading of the control well was subtracted from the raw OD_{590} from each substrate within that set of 31 (Garland & Mills, 1991; Garland, 1996). Next, any value, after subtraction of the control well OD_{590}, which was found to have an $OD_{590} < 0.06$ was deemed to be below the detection threshold of the system and amended to zero (San Miguel *et al.,* 2007). Remaining values were given a score of 1 indicating that growth had occurred.

Antibiotic	Class of antibiotic	Concentration
ceftazidime	Cephalosporins (3rd generation)	2 µg
chloramphenicol	Chloramphenicol	10 µg
ciprofloxacin	Quinolones	1 µg
erythromycin	Macrolides	10 µg
penicillin g	Penicillins	10 µg
streptomycin	Aminoglycosides	1.5 mg
sulfisoxazole	Sulphonamides	100 µg
tetracycline	Tetracyclines	10 µg
tobramycin	Aminoglycosides	1.5 µg

Table 5.1: The antibiotic challenges used to assess the phenotypic variation within the *P. aeruginosa* isolates tested. The resistance of the *P. aeruginosa* isolates to nine antibiotics was tested. The nine antibiotics came from eight different classes of antibiotics. The concentrations of the antibiotics were the same as those that were infused into the disks, prior to assessment.

5.3 Results

Of the 155 bacterial isolates cultured from three sputum samples expectorated from two CF patients, previously described in Chapter 2 and 3a, 40 were identified as *P. aeruginosa*. A set of 12 *P. aeruginosa* isolates were selected at random and each was given an identifying code starting with the letters "dwr". Before using these 12 isolates in the BEF experiment in this chapter, a set of phenotypic assays were carried out. All phenotypic assays were independently replicated three times confirming these traits as reproducible.

Variation within clinical Pseudomonas aeruginosa *isolates*

The 16S rRNA gene sequences of the 12 *P. aeruginosa* isolates were compared. The results showed that there was a difference of only three base pairs between sequences generated from all 12 isolates when these were matched over the 550 bp region analysed. Thus, the sequences from all 12 isolates were shown to have over 99.8% similarity and as such were all confirmed as *P. aeruginosa*.

To investigate what phenotypic differences were present between the isolates, pigment production, growth in high sodium chloride concentration and resistance profile for nine antibiotics was assessed in three independent experiments. In more detail, the production of blue-green pigment was observed in three of the 12 isolates (dwrPa8, dwrPa9, dwrPa12) when grown on *Pseudomonas* selective agar. Nine isolates were able to grow in elevated salt conditions. With respect to the antibiotics tested a relatively complex pattern of susceptibility and resistance, as defined above, was identified (Table 5.2). It was found for example that 11 of the 12 isolates were resistant to penicillin G, although the isolate regarded as susceptible (dwrPa9) was shown to be resistant in this assay to tobramycin, ciprofloxacin, erythromycin, chloramphenicol and tetracycline. The most resistant isolate, dwrPa3, was found to be resistant to eight of the nine antibiotics tested and was susceptible again in terms of this assay only to erythromycin.

Of the 12 isolates tested, eight gave distinct phenotypic profiles; these were dwrPa2, dwrPa3, dwrPa5, dwrPa6, dwrPa8, dwrPa9, dwrPa10 and dwrPa12. Two pairs of indistinguishable phenotypic profiles were also formed. These were dwrPa4 & dwrPa11 and dwrPa7 & dwr1. As the aim was to distinguish phenotypically the isolates in this study, a further round of assays was carried out based on the ability of the isolates to use a range of single carbon substrates. In these assays, isolates dwr1 and dwrPa7 differed in their ability to grow on nine substrates (Tween 40, β-methyl-D-

glucoside, i-erythritol, D-mannitol, glucose-1-phosphate, α-ketobutyric acid, L-threonine, phenylethylamine and putrescine). Isolates dwrPa4 and dwrPa11 were found to differ in their ability to grow on two substrates (Tween 40 and putrescine).

Thus a combination of these phenotypic assays above was able to phenotypically distinguish every isolate in this collection. These phenotypically distinct isolates from CF sputum will henceforth be termed *P. aeruginosa* ecotypes (as Cohen, 2002)

Identity	Pigment	NaCl	ceftazidine	chloramphenicol	ciprofloxacin	erythromycin	penicillin G	streptomycin	sulfisoxazole	tetracycline	tobramycin
dwr1[a]	0	1	1	0	0	0	1	1	1	0	0
dwrPa2	0	1	1	1	1	1	1	1	1	0	0
dwrPa3	0	0	1	1	1	0	1	1	1	1	1
dwrPa4[b]	0	1	0	1	0	1	1	1	1	1	0
dwrPa5	0	0	1	1	0	0	1	1	0	1	0
dwrPa6	0	1	0	0	0	0	1	1	1	1	0
dwrPa7[a]	0	1	1	0	0	0	1	1	1	0	0
dwrPa8	1	1	1	1	0	0	1	0	1	1	0
dwrPa9	1	1	0	1	1	1	0	0	0	1	1
dwrPa10	0	1	0	0	1	0	1	1	1	0	1
dwrPa11[b]	0	1	0	1	0	1	1	1	1	1	0
dwrPa12	1	0	1	0	0	0	1	1	1	0	0

Table 5.2: The binary matrix for the phenotypic traits observed for the 12 *P. aeruginosa* ecotypes used in this study. Each of 12 *P. aeruginosa* isolates tested was given a strain identifier. Eleven phenotypic traits were observed, and a binary matrix created. Traits were scored present or absent. In the case of antibiotics a "positive" score indicated resistance. "a" & "b" indicate the two isolates that were indistinguishable using these methods.

Intra-species diversity had no significant effect on assemblage respiration.

In this study, the microcosms were established in mixed environments only. Assemblage respiration was recorded as previously described in Chapter 3b. Again, 168 microcosms were formed comprising six isolate richness levels within three Partitions, which were independently replicated twice.

Tryptone Water

The mean assemblage respiration (UCO_2) for this environment was found to be 0.94 UCO_2 (SD = 0.08, n = 168), with a minimum respiration of 0.71 UCO_2 and a maximum respiration of 1.07 UCO_2. The relationship between respiration and the number of *P. aeruginosa* ecotypes present in an assemblage (isolate richness) was investigated. The data were plotted as respiration against isolate richness (Figure 5.1).

To assess the influence of biodiversity on respiration, the data were analysed by means of a GLM (eq.10), as before. The results of the analysis are presented alongside R^2 and AIC values, two measures of model fitness, in Table 5.3.

$$y = a + \beta_{REP}x_{REP} + \beta_P x_P + \beta_{LR}x_R + (\sum_1^N \beta_C x_C) + \beta_{NLR}x_R + \beta_Q x_Q + \beta_M x_M + e \qquad [10]$$

The first model term that was analysed accounted for the variance associated with the experimental replicates. There was no significant difference (t_{83} = -1.31, p = 0.194) between mean respiration in the first replicate, 0.93 UCO_2 (SD = 0.09, n = 84), and the second, 0.94 UCO_2 (SD = 0.08, n = 840). The analysis using the GLM showed that the replicates did not account for a significant proportion of the variance within the data ($F_{1,82}$ = 1.70, p = 0.196; see Table 2).

The second model term investigated the effect of the three Partitions on the assemblage respiration. The mean assemblage respiration was shown to be 0.98 UCO_2 (SD = 0.02, n = 56), 0.85 UCO_2 (SD = 0.08, n = 56) and 0.99 UCO_2 (SD = 0.04, n = 56) for Partitions 1 – 3 respectively. The effect of the Partitions on the respiration was found to be highly significant ($F_{2,10}$ = 124.77, $p < 0.001$).

After the variance associated with the replicates and Partitions were accounted for, the residuals were analysed against the linear richness term. This analysis showed that there was no significant effect of linear richness on the residual variance ($F_{1,10}$ = 1.35, p = 0.272).

Defibrinated Horse Blood

The mean respiration in this environment was found to be 0.29 (SD = 0.10, n = 168). The respiration was plotted against isolate richness. The relationship between the mean respiration and richness and demonstrates a neutral effect (Figure 5.1).

The mean respiration recorded for each of the replicates was 0.30 UCO_2 (SD = 0.10, n = 84) and 0.28 UCO_2 (SD = 0.09, n = 84). The replicate term was found to be highly significant ($F_{1,82}$ = 17.23, $p < 0.001$). The Partitions recorded mean respiration values of 0.37 UCO_2 (SD = 0.05, n = 56), 0.17 UCO_2 (SD = 0.04, n = 56) and 0.32 UCO_2 (SD = 0.04, n = 56) with the effect of the Partitions on the respiration observed was found to be highly significant ($F_{2,10}$ = 289.86, $p < 0.001$). The linear richness term was shown to have no significant ($F_{1,10}$ = 0.00, p = 0.995) effect on the variance.

Comparison between nutrient sources

The overall mean respiration produced in DHB was observed to be lower than the mean respiration observed in Tryptone Water. This difference (0.65 UCO_2) was found to be highly significant (t_{167} = 108.98, $p < 0.001$).

In both nutrient sources, the Partitions had a significant effect on assemblage respiration. The next stage of the analysis showed that the effect of linear richness was non-significant in both environments. It therefore followed that further aspects of biodiversity needed to be investigated to account for the variation in the dataset.

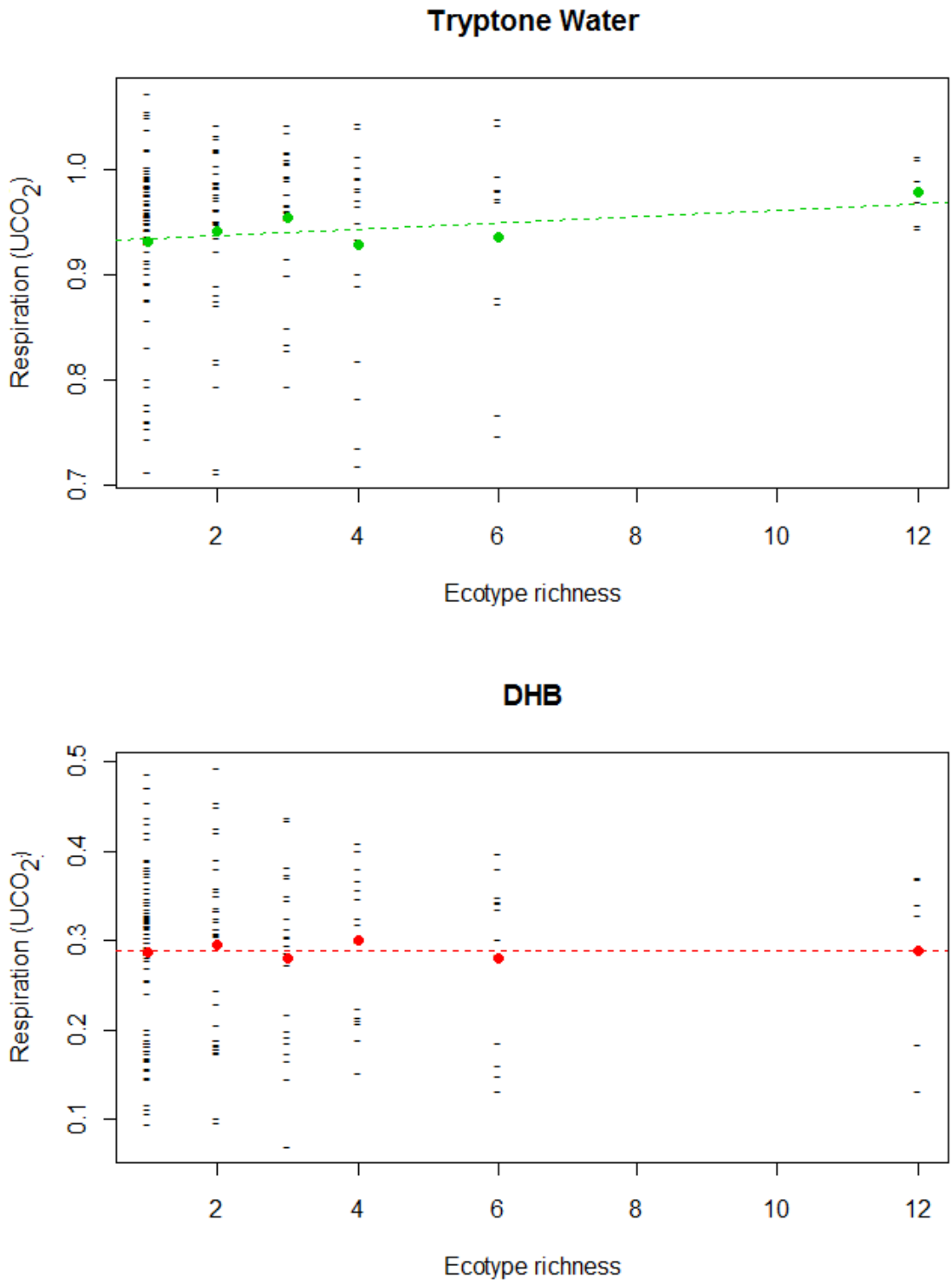

Figure 5.1: The relationship between increasing numbers of ecotypes and respiration in two distinct nutrient environments. The respiration, in UCO_2, (y-axis) for each of the microcosm mixes (-) are shown at each of the ecotype richness levels (x-axis). Mean respiration (•) did not match with increasing ecotype richness in both the Tryptone Water and DHB environments.

Tryptone Water

Step	Model	R^2	AIC	Res.Df	RSS	*F*	*p*
1	Respiration ~ 1	-	-	167	1.15	-	-
2	**1** + REP	0.00	-354.8	166	1.15	1.70	0.196
3	**2** + P	0.61	-508.5	164	0.45	124.77	<0.001
4	**3** + LR	0.62	-509.6	163	0.45	1.35	0.272
5	**4** + C	0.70	-526.3	152	0.36	2.72	0.006
6	**5** + NLR	0.71	-523.7	148	0.34	1.46	0.285
7	**6** + Q	0.73	-516.6	138	0.31	1.53	0.145
8	**7** + M	0.88	-540.5	83	0.15	1.61	0.025

DHB

Step	Model	R^2	AIC	Res.Df	RSS	*F*	*p*
1	Respiration ~ 1	-	-	167	1.54	-	-
2	**1** + REP	0.01	-308.4	166	1.52	17.23	<0.001
3	**2** + P	0.82	-589.8	164	0.28	289.86	<0.001
4	**3** + LR	0.82	-587.8	163	0.28	0.00	0.995
5	**4** + C	0.86	-601.7	152	0.23	2.46	0.012
6	**5** + NLR	0.86	-597.8	148	0.22	0.89	0.505
7	**6** + Q	0.87	-592.6	138	0.20	1.74	0.085
8	**7** + M	0.94	-598.0	83	0.10	1.46	0.058

Table 5.3: The ANOVA table for the analysis of the respiration produced by the 12 *P. aeruginosa* ecotypes in Tryptone Water and DHB. For each step in the analysis a new variable was entered. The fit of the model (R^2 and AIC) were calculated. The residual degrees of freedom (Res.Df) and residual Sums of Squares (RSS) are a measure of the variation left within the data. The results of the ANOVA (*F*-test and *p*-value) were also calculated for each variable based on the significant amount of variation accounted for.

The contributions of the individual* Pseudomonas aeruginosa *ecotypes were affected by the nutrient source.

In this study, the impact of phenotypic variation between ecotypes that were the same species was examined. As previously shown these were almost indistinguishable at the 16S rRNA gene level and as such had the same T-RF length. It was therefore not possible to detect these individual ecotypes at the end of the experiment by T-RFLP profiling. For this reason, each of the *P. aeruginosa* ecotypes was given the same weighting and a binary matrix was used to code presence or absence in the microcosms.

Tryptone Water

The isolate composition term was calculated by using the sum of the SS accounted for by each isolate in the analysis. In this way, the isolate composition term becomes the amount of variance accounted for by the mix of ecotypes as a whole. Isolate composition was shown to have a significant impact on respiration ($F_{11,55} = 2.72$, $p = 0.006$). The isolate coefficients were calculated by taking the mean residuals associated with the microcosm mixes in which each isolate was present (Figure 5.2). These ranged from -0.032 to 0.036 coefficient units (CU) and were a mixture of four positive and seven negative coefficients. Four *P. aeruginosa* ecotypes, dwr1, dwrPa2, dwrPa10 and dwrPa11, showed significant effects in relation to the extent of respiration recorded in the microcosms in which they were present ($F_{1,55} > 4.11$, $p < 0.048$).

The coefficients for the non-linear richness term were found to range from -0.016 to 0.022 CU though no significant effect on respiration was identified ($F_{4,10} = 1.46$, $p = 0.285$). Overall, the model with the non-linear richness term entered accounted for the majority of the variance within the data ($R^2 = 0.71$).

Defibrinated Horse Blood

Here, again, it was found that isolate composition had a significant effect on the assemblage respiration ($F_{4,10} = 2.46$, $p = 0.012$) with the individual isolate coefficients ranging from -0.02 to 0.02 CU (Figure 5.2). These coefficients were shown to be evenly distributed with six positive, dwrPa2 - 7, and six negative isolate coefficients. Of these, two ecotypes were found to have negative and significant effects on assemblage respiration ($F_{4,10} > 4.65$, $p < 0.036$).

The non-linear richness levels had coefficients which ranged from -0.008 to 0.014 CU. This variable was found to have no significant effect on the variance within

the data ($F_{4,10}$ = 0.89, p = 0.505). Overall, the full model accounts for the majority of the variance within the data (R^2 = 0.86).

Comparison between nutrient sources

In these analyses, neither of the richness terms (linear or non-linear) was significant. In both media however, the Composition term was significant, and the ecotype contributions were mixed. With the exception of one isolate (dwrPa3), all of the ecotypes changed the sign of their coefficients with the change in nutrient source.

Once the full model was assembled, the majority of the variance in the dataset was accounted for (R^2_T = 0.88; R^2_D = 0.94) despite linear richness not having a significant effect.

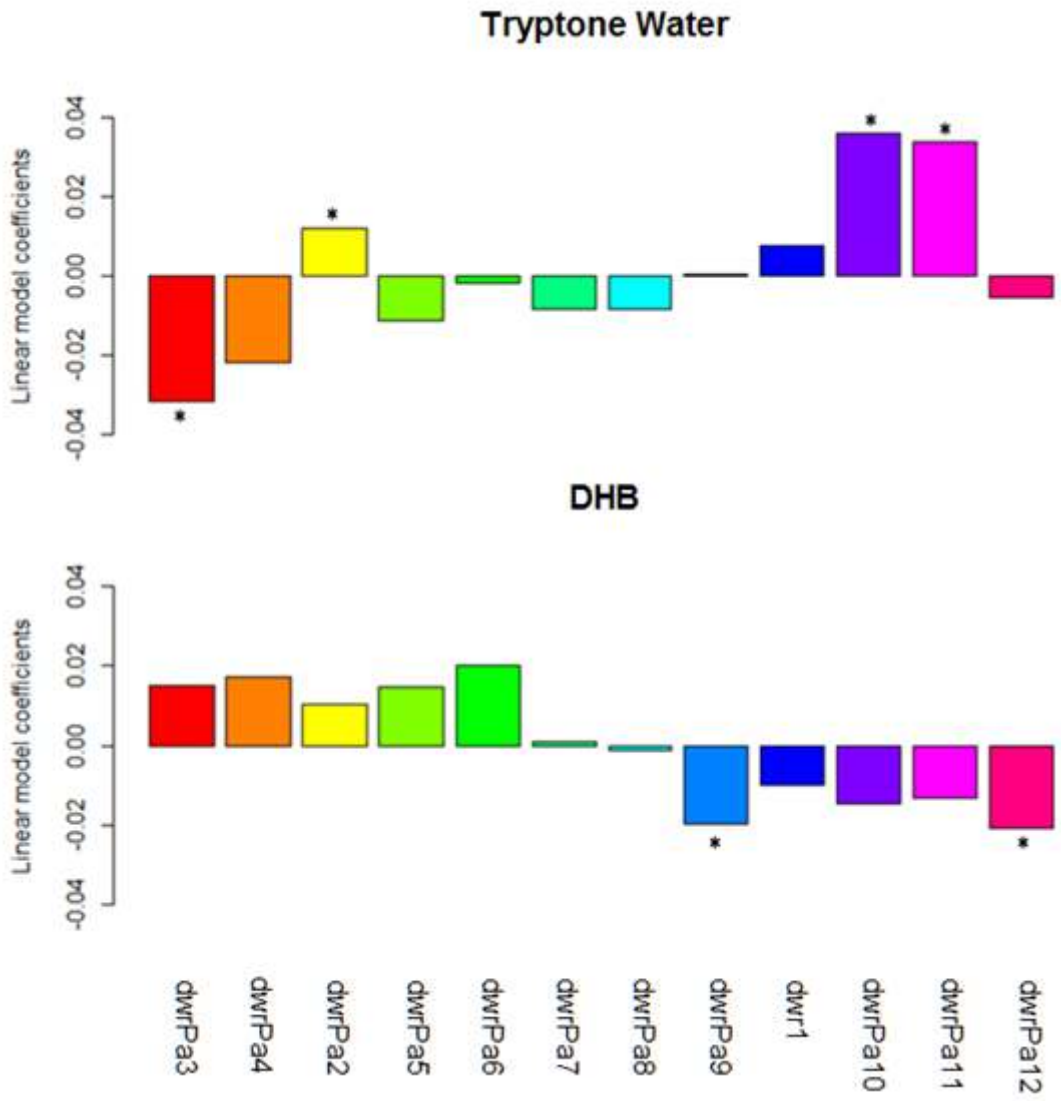

Figure 5.2: The individual ecotype coefficients in both media. The coefficients for the 12 *P. aeruginosa* ecotypes in both the Tryptone water and the DHB show that, with the exceptions of dwrPa3, dwrPa8 and dwrPa12, all the ecotypes behaved differently in different environments. This result also indicated that there was variation between the ecotypes as to how they contributed to the total activity of the assemblage. The full isolate coefficients are presented in Table A4.

5.4 **Discussion**

This Chapter presented results from an experiment investigating the effects of biodiversity on respiration using ecotypes of a single bacterial species. The study reported here made use of the "microdiversity" hypothesised to be present within bacterial species and reported specifically for *P. aeruginosa* in the context of CF airway infection with this species (Moore *et al.,* 1998; Smith *et al*,. 2006). Microdiversity can be envisaged here as the phenotypic variation present within a species. 12 phenotypically-distinct ecotypes, confirmed as *Pseudomonas aeruginosa,* were obtained. The degree of genomic similarity between these ecotypes is not known, but the phenotypic differences identified support some degree of genetic divergence. These ecotypes were assembled into microcosm mixes and the total respiration measured, as previously described (Chapter 3b).

No significant linear richness effect on respiration.

No significant effect on respiration was found by increasing the number of ecotypes in the assemblages as shown by the linear regression. This was in contrast to the results in the previous chapter for multispecies assemblages and was, to some extent, unanticipated. This result – a neutral effect of biodiversity in ecotype assemblages – has however, been supported by the findings of Zhang *et al.* (2009) in which an increase in the number of phenotypically distinct *Pseudomonas fluorescens* isolates did not affect ecosystem function. Both studies suggest that the ecotypes of the species in question do not contribute in an additive manner.

The observed lack of obvious additive effect may have a number of explanations. Genomic differences within *P. aeruginosa* have been shown to occur over the course of a chronic infection. Smith *et al.* (2006) showed that a single isolate of *P. aeruginosa* can produce many radiated mutants over a 96 month chronic infection. Between the different lineages of a species phenotypic, and genotypic, variation has been shown to be present (Flynn *et al.,* 2011). These phenotypic differences may also confer advantages over other lines of that species or over other species, for example in niche exploitation (Latta *et al.,* 2011). This process can explain why different species have an additive component (as described in the previous Chapter), as the species are able to use different aspects of the nutrient (such as a raw carbon source, or a downstream digestion product). This is made possible by the large different metabolic potential "metagenome" present in the species. For this reason, the species are rarely in outright competition. This study, however, used ecotypes from the same genetic species,

where the difference in traits may stem from mutation or transfer events in the same parental lineage (Smith *et al.,* 2006). These isolates are derived from a common ancestor and therefore their ability to utilise substrates can be expected to be similar.

It has been postulated that the presence of microdiversity is present in many microbial systems (Jaspers & Overmann, 2004). This microdiversity may also confer enhanced fitness for a species over a broader range of conditions than more phenotypically-homogenous populations (Moore *et al.,* 1998).

Variation driven by selection effects

The results above have shown that there is no significant effect of isolate richness on mean respiration. The data still show a large amount of variation. This variation may be explained by the ecotype composition term or the selection effects. This is most easily demonstrated by the ecotype coefficients (Figure 4). These show a general trend of each of the ecotypes "polarising" their coefficients between the nutrient sources. This may indicate that two sub-populations of *P. aeruginosa* were present within these ecotypes. The results showed that only a single isolate, dwrPa3, had positive coefficients in both environments. The other ecotypes were split five to six as having positive coefficients in the DHB and Tryptone Water respectively. This explanation for the polarised coefficients, which is an example of niche exploitation, is supported by work from Jaspers & Overmann (2004) who suggest that differential growth rates between isolates of *Brevundimonas* were due to differential utilisation of carbon sources.

Phenotypic variation

In this study, a number of assays were used to determine a range of phenotypes for a collection of *P. aeruginosa* ecotypes. Previous studies have used colonial morphological differences to phenotypically-distinguish lines of *P. fluorescens* in BEF experiments (Zhang *et al*., 2009). Whilst such morphological variation has been shown in isolates of *P. aeruginosa* grown from CF sputum previously, these characteristics were insufficiently detailed to separate the ecotypes studied here. As such, other phenotypic differences were required. The assays that were selected exploited traits that were considered distinct from the processes controlling the utilisation of the Tryptone Water and DHB nutrient sources. Despite the colonial morphological similarities shown by this collection of ecotypes, it was found that only a limited number of phenotypic assays were required to differentiate the ecotypes primarily through their

antibiotic susceptibility profile. Phenotypic variation within genetically similar isolates has been shown frequently in clinical *P. aeruginosa* isolates (e.g. Foweraker *et al.,* 2005) and in aquatic bacterial species (Moore *et al.,* 1998; Jaspers & Overmann, 2004).

The results indicated that there was no significant effect of non-linear richness on respiration. This was consistent with the results that indicated a strong role for the isolate composition term. If the driver of variation is the selection of the isolate(s) within a microcosm, then the degree to which there is an interaction may become less relevant as there is increased scope for the occurrence of overachieving ecotypes. To investigate further the possibility of subpopulations, the mean monoculture respiration for each of the ecotypes was ranked for both of the nutrient sources. In both media, the highest ranking *P. aeruginosa* ecotypes were the same ones that were shown to have positive isolate coefficients. This further supported that microdiversity existed and was capable of affecting the within richness level respiration.

Conclusions

This study has identified and investigated an aspect of the previous BEF experiment that remained unexplored, namely the impact of using phenotypically-distinct isolates of the same species. With respect to the effect of biodiversity on respiration, only the composition and species effects were significant in the manipulated assemblages. The number of different ecotypes present within the assemblages had no significant positive or negative effect on the respiration. Evidence was found to support their being distinct sub-populations of P. aeruginosa *ecotypes in this study. This may be important in the ability of species such as* P. aeruginosa *being able to utilise nutrients.*

Chapter 6

6. Cooperation between bacterial species increases in higher richness levels on single carbon substrates.

6.1 Introduction

In previous chapters, the individual species and interaction terms were found to be mediated by the environment. It was therefore resolved to study their relative contribution in greater detail. This study did so by assaying a set of assemblages with a series of single carbon substrates. The BIOLOG system used here has previously been published as a means to assess bacterial community phenotypes, but not in BEF experimental studies.

The BIOLOG assay system monitors the respiration of bacterial cells using a redox reaction indicator dye, tetrazolium violet (Garland & Mills, 1991; Bi *et al.,* 2011). Initial development and results using the BIOLOG system were reported by Garland in a series of publications (Garland & Mills, 1991; Garland 1996; 1997). These studies showed the robustness and reproducibility of the system to elucidate phenotypic differences between bacterial communities. These phenotypic differences were assayed by testing for bacterial respiration on a series of single carbon sources (SCSs) arranged in a 96-well plate system. This system was later developed to assay phenotypic traits in whole samples from communities. Of the different types of BIOLOG plates devised, one has been designed for bacterial ecologists with three replicated sets of SCSs. This plate is called the EcoPlate™ and consists of 31 SCSs plus one negative, no-substrate, control per replicate. The 31 substrates present in the EcoPlate™ can be split into seven types; amines, amino acids, carbohydrates, carboxylic acids, phosphorylates, an ester and polymeric substrates (Garland & Mills, 1991; Yang C. *et al.,* 2011).

The previous chapters have also introduced and applied the Random Partition Design (RPD) to consider aspects of biodiversity. If the experiments however, were re-designed to be fully factorial (i.e. all the species present will be with the other species in every combination) then the RPD method would not be needed. As there would be no partitions as all combinations would be included and as each species would be present more than once at each richness level, the design would no longer be balanced.

Previous studies have successfully used a fully factorial design to investigate ecological mechanisms between small numbers of organisms or variables (Harrison *et al.,* 2007; Wießhaupt *et al.,* 2012). In this study, the number of bacterial species used was restricted to six to make a fully factorial experiment practicable. Four levels of richness (1, 2, 3 and 6 species) were established. Each species or species combination was tested for their ability to utilise one or more of the 31 SCSs. A general linear model, adapted from eq.10, and other statistical methods were used to analyse the data.

6.2 Materials & Methods

Bacterial culturing and identification

Bacterial species used in this study were isolated and identified as previously described (Chapter 2.1 and 3a).

Bacterial preparation and EcoPlate™ community level phenotypic profiling

Bacterial culture preparation and EcoPlate™ community level phenotypic profiling was performed as previously described (Chapter 2.10). Respiration measurements (see below) were taken at both 24 and 48 hours post inoculation with the values at 48 hours used in the further analysis.

Microcosm assembly

Bacterial species were assembled into microcosm mixes as previously described (Chapter 2.7). For this experiment a fully factorial design was used to create assemblages with one, two, three and six species. Species richness levels four and five were excluded. This resulted in a total of 42 separate microcosms (six at one species, 15 at two species, 20 at three species and one at six species) excluding independent replication.

Statistical analysis

Prior to any statistical analysis the OD_{590} measurements were corrected as previously described (Chapter 5.2). For assessing phenotypic variation between the microcosms, these OD_{590} values were converted into binary scores, i.e. all values above the detection threshold were deemed "positive" and scored 1. These binary scores were converted into a distance matrix of Jaccard's distance coefficients (eq.1):

$$d_{Jac} = 1 - \frac{a}{a+b+c} \qquad [1]$$

where: a = the number of "positives" shared between two microcosms, b = number of positives unique to the first microcosm, and c = number of positives unique to the second microcosm. The resulting tree was drawn using the hierarchical clustering complete linkage method to link similar clusters within this distance matrix in R (v.2.12.2 – v.2.13.0).

The effects of the explanatory variables (time, species richness) were analysed by using a two-way ANOVA. All assumptions for parametric statistics were assessed using visual analysis and confirmed using the Shapiro-Wilk normality test and the Fligner-Killeen test of homogeneity of variances. In the event of the models used failing these assumptions, non-parametric statistics (Kruskal-Wallis and Wilcoxon rank sum test) were used in preference to parametric tests. Transformations were not performed on these data (Chapter 4); with non-normal errors not undermining the model or allowing simple comparisons between the environments tested.

A general linear model (GLM) was also used in this study. The model was altered from the one previously described (eq.10; Chapter 3c) due to the fully factorial design of the experiment. This model (eq.12) entered variables of the replicates, linear and non-linear richness and species composition only.

$$y = \beta_0 + \beta_{REP}x_{REP} + \beta_{LR}x_R + \sum_1^6(\beta_c x_c) + \beta_{NLR}x_R + e \qquad [12]$$

Due to the omission of the *Q* and *M* terms, the *F*-test denominator chosen for all the variables was the final residual sums of squares. Orthogonality of the variables was tested to dictate the order the variables were entered into the model.

6.3 **Results**

Selection of bacterial species

The phenotypic variation between 12 species used in BEF experiments (Chapter 3a) was measured through their ability to grow on 31 different single carbon sources ranging from single amino acids (i.e. Serine) to long polymeric molecules with pentose rings (i.e. Tween 80; Table A5). Table A6 (Appendix) shows a binary matrix of the usage of the single carbon sources (SCSs). The data in this matrix were converted by the complete linkage method to a tree (Figure 6.1) that shows the 12 species in clusters based on overall patterns of utilisation of SCSs. Certain species were found to have similar overall patterns of utilisation and therefore clustered together. Here two groups with indistinguishable profiles were observed, *S. maltophilia, B. cepacia* with *S. haemolyticus* and *A. baumannii* with *S. sanguinis*. These species within the clusters had a mean Jaccard's distance of 0.00. Conversely, species with very different patterns of utilisation were also identified. The most phenotypically dissimilar isolates in this assay were *S. pneumoniae* and *P. aeruginosa* which had a Jaccard's distance of 0.91. Two broad clusters were found with a single outlier, *S. pneumoniae*. The first cluster contained *S. mitis, A. xylosoxidans, S. marcescens, A. baumannii* and *S. sanguinis*. The second cluster was comprised of *P. aeruginosa, S. aureus, E. faecium, S. haemolyticus, S. maltophilia* and *B. cepacia*.

For the purpose of experimentation, it was important that reproducible patterns of SCSs utilisation were detected for these species. As such, these assays were repeated independently twice with indistinguishable results obtained. Six species were chosen for inclusion in this study with SCS utilisation patterns distinct from one another. The species chosen on this basis were *A. baumannii, A. xylosoxidans, P. aeruginosa, S. aureus, S. maltophilia* and *S. pneumoniae.* The mean Jaccard's distance between these species was 0.44 (SD = 0.25, n = 66).

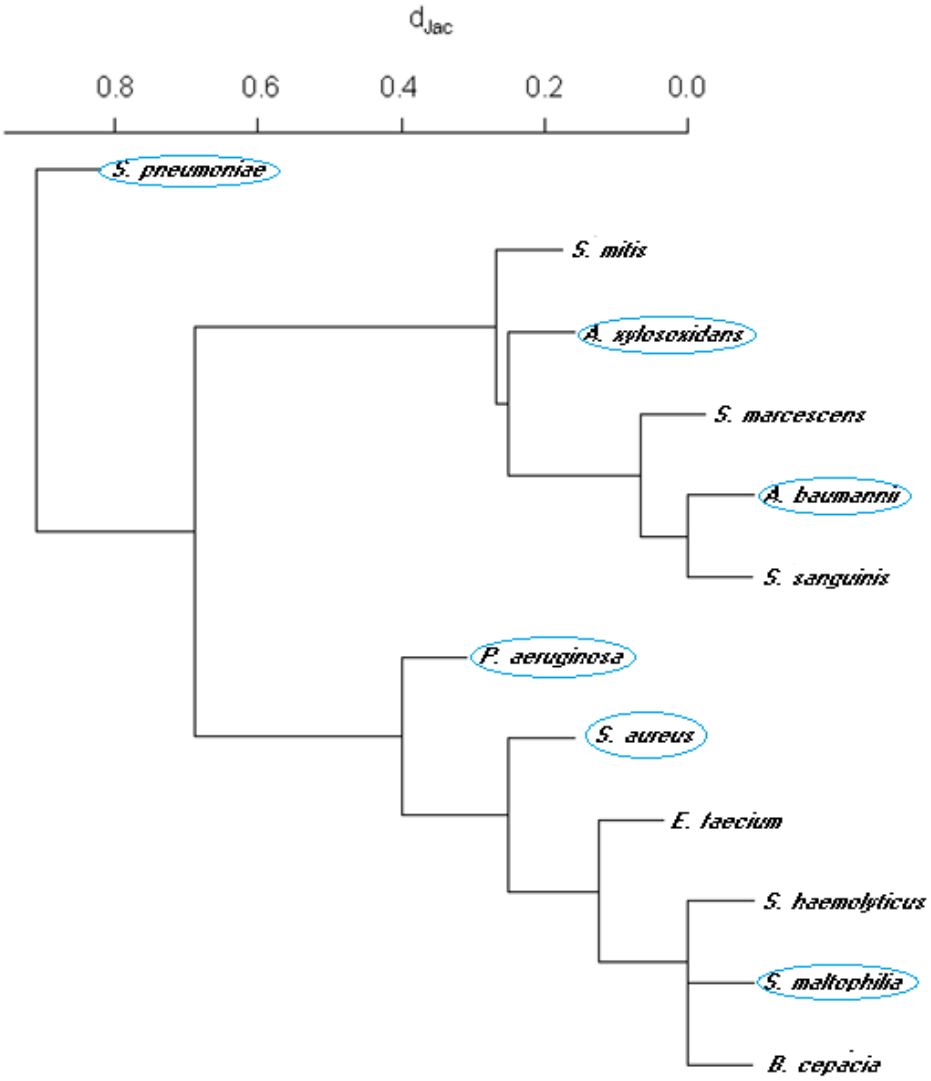

Figure 6.1: Tree based on the binary EcoPlate™ SCS utilisation profiles of the 12 species used in BEF experiments. 12 bacterial species were assessed for their ability to utilise 31 carbons sources in an EcoPlate™. In this diagram, the Jaccard's distances are approximated by the physical length between the bacterial nodes (cophenetic correlation coefficient = 0.964). The full distance matrix from which this tree was drawn can be found in the Appendix (Table A6).

The effect of time and species richness was significant on overall community respiration.

Assemblages were set up containing one, two, three and all six of the species above. As such, a total of 42 assemblages were tested in the BIOLOG system with all assays independently replicated twice. OD_{590} values were recorded as a measure of the respiration of the bacterial assemblages at 24 and 48 hours post incubation. Each assay included a no-substrate control blank that was used to determine values regarded as positive as previously stated in the materials and methods.

The assemblage respiration recorded after 24 hours had a mean of 0.68 OD_{590} (SD = 1.05, n = 1376). This was lower than the mean assemblage respiration recorded after 48 hours (mean = 1.00 OD_{590}, SD = 1.25, n = 1376). This difference was shown to be statistically significant (V = 45739, $p < 0.001$) by using a paired Wilcoxon ranked sum test.

The rates of respiration over the two time periods were also assessed. These rates were determined by using linear regression analysis and specifically examining the gradients associated with the species richness (shown graphically in the appendix; Figure A7). These analyses showed that all of the gradients were positive over both periods of time and for all richness levels (range 0.010 to 0.046). For monocultures collectively, the gradient over the first 24 hour period was found to be steeper than for the second 24 hour period; the gradient decreased from 0.019 OD_{590}/ hour (± 0.007 1se, n = 192) for the first 24 hours, to 0.010 OD_{590}/ hour (± 0.011 1se, n = 192) for the second 24 hour period. This trend was observed for all of the species richness levels tested. Accordingly, assemblages comprising of two species showed the same trend; with the gradient for the first 24 hours, 0.020 OD_{590}/ hour (± 0.004 1se, n = 240) decreasing to 0.010 OD_{590}/ hour (± 0.007 1se, n = 240) over the second 24 hours. Assemblages with three species showed a decrease in gradient from 0.035 OD_{590}/ hour (± 0.004 1se, n = 320) over the first 24 hours to 0.015 OD_{590}/ hour (± 0.006 1se, n = 320) over the second 24 hour period. The same trend was observed in assemblages with six species with a decrease from 0.046 OD_{590}/ hour (± 0.004 1se, n = 16) over the first 24 hours to 0.023 OD_{590}/ hour (± 0.004 1se, n = 16) in the second 24 hours.

When the gradients were compared further, it was found that the increase in respiration was significantly greater in assemblages with six species than all of the other richness levels ($t_{340} > -2.14$, $p < 0.001$). A significantly greater level of respiration was found in assemblages with three species than those in monoculture over the first 24

hours (t_{340} = -24.83, $p < 0.001$) but not over the second 24 hours (t_{340} = -0.87, p = 0.999). There were no significant differences found between the increase in respiration in monocultures and assemblages with two species ($t_{340} < -0.5$, $p > 0.900$).

A single time point of 48 hours incubation was chosen for further focused analysis to maximise the cumulative respiration signal. This time period also maximised SCS utilisation; although three of the SCSs (D-malic acid, γ-hydroxy butyric acid and itaconic acid) did not support the growth of bacteria in any microcosm after 24 hours, these did support growth after 48 hours.

Assessment of carbon sources for the support of bacteria assemblages.

After 48 hours, no growth was observed at any richness level, for 11 of the 31 carbon sources tested. These carbon sources were glucose-1-phosphate, D,L-α-glycerol phosphate, α-cyclodextrin, glycogen, α-D-lactose, β-methyl-D-glucoside, i-erythritol, D-mannitol, D-glucosamic acid, D-galacturonic acid and L-threonine. These SCSs were omitted from further study. Growth on a further four carbon sources (2-hydroxy benzoic acid, glycyl-L-glutamic acid, D-galactonic acid γ-lactone and phenylethylamine) was detected, but was only found in the presence one of the six species selected. As their impact on the experiment was limited to these species, these were also no longer studied.

Sixteen SCSs remained in the analysis. These were 4-hydroxy benzoic acid, α-ketobutyric acid, L-arginine, L-asparagine, D-cellobiose, D-malic acid, γ-hydroxybutyric acid, itaconic acid, N-acetyl-α-glucosamine, pyruvic acid methyl ester, L-phenylalanine, putrescine, L-serine, Tween 40, Tween 80 and D-xylose.

Although these SCSs were found to support only a limited number of bacterial species in monoculture, in higher richness levels the utilisation of the SCSs did not rely on specific bacterial species. All of these 16 SCSs could be utilised by the six species when combined. This utilisation became a function of biodiversity. Therefore these SCS were included in the further analytical steps. For the six species in monoculture, the most widely used SCSs were pyruvic acid methyl ester, Tween 40 & Tween 80 with four of the species able to utilise this substrate. The next most widely utilised SCSs were 4-hydroxy benzoic acid, L-arginine, L-asparagine, N-acetyl-D-glucosamine, L-phenylalanine, and D-xylose which could be utilised by three species. A-ketobutyric acid and L-serine were utilised by two species with D-cellobiose, γ-hydroxybutyric acid and itaconic acid used by one species in monoculture. D-malic acid could not be used by any of the species in monoculture.

Increasing linear richness and species composition have significant effects on community respiration.

For all of the 16 informative SCSs investigated in this study, a GLM (eq.12) was used to analyse the effect that biodiversity (linear richness, species composition and non-linear richness) had on the respiration detected.

$$y = \beta_0 + \beta_{REP}x_{REP} + \beta_{LR}x_R + \sum_1^6(\beta_c x_c) + \beta_{NLR}x_R + e \qquad [12]$$

Partitioning the variance of these explanatory variables found that orthogonality was conserved between the replicate (β_{REP}), species composition (β_C) and non-linear richness (β_{NLR}) terms regardless of the inclusion of the linear richness term (data not shown). As the order in which the variables were entered into the model was shown not to affect the variation they accounted for, the terms were entered as previously described (Chapter 3c).

The data (shown graphically in the appendix; Figure A8) were analysed using eq.12 and the outputs of the ANOVAs (*F*-test and *p*-values) are presented in Table 6.1. The ANOVA found that the replicate term had a significant effect for seven of the 16 SCSs tested ($F_{1,74} > 4.25$, $p < 0.043$). This term however, accounted for a mean model fitness of less than 2% (mean $R^2 = 0.02$, SD = 0.02, $n = 16$).

The results also showed that linear richness ($F_{1,74} > 8.49$, $p < 0.005$) and species composition ($F_{1,74} > 3.96$, $p < 0.001$) were highly significant in all of the SCS tested, with the exception of γ-hydroxybutyric acid. For γ-hydroxybutyric acid, the species composition had a significant effect ($F_{5,74} = 7.62$, $p < 0.001$) but the linear richness term was found to be non-significant ($F_{1,74} = 2.65$, $p = 0.108$). The fitness of the model was tested after the linear richness term was entered. The analysis found that 11% (mean $R^2 = 0.11$, SD = 0.04, $n = 16$) of the mean variance was accounted for. After the species composition had been entered, the mean model fitness increased to over 50% of the variance being accounted for (mean $R^2 = 0.57$ SD = 0.14, $n = 16$).

The species coefficients were calculated as the mean residuals for all the microcosms in which a given species was present. The coefficients are shown for all the SCSs tested in Figure 6.2. These results indicated that *A. baumannii* and *A. xylosoxidans* were the species with the most number of positive coefficients (ten and eleven from 16 SCSs respectively). The results also found that in the six SCSs where *A. baumannii* was shown to have negative coefficients, *S. maltophilia* was found to have a positive coefficient.

Further to the linear richness and species composition, the significance of the non-linear richness term was also analysed. The non-linear richness terms were found to be significant in only two of the 16 SCS tested. These two substrates were Tween 40 ($F_{2,74} = 3.82$, $p = 0.026$) and L-asparagine ($F_{2,74} = 3.38$, $p = 0.039$). The remaining non-linear richness terms for each of the substrates were found to be statistically non-significant ($F_{2,74} < 2.63$, $p > 0.079$). The fitness of the model after the inclusion of the non-linear richness term was shown to be only 2% greater than the previous model (mean $R^2 = 0.59$, SD = 0.14, $n = 16$).

		Pyruvic acid methyl ester		Xylose		Tween 40		Tween 80	
	df	***F***	***p***	***F***	***p***	***F***	***p***	***F***	***p***
REP	1	2.56	0.110	2.90	0.093	2.92	0.091	7.98	0.006
LR	1	25.78	<0.001	20.61	<0.001	19.07	<0.001	14.16	<0.001
Comp	5	15.99	<0.001	16.29	<0.001	14.17	<0.001	15.44	<0.001
NLR	2	2.23	0.120	2.63	1.000	3.82	0.026	2.42	0.096
		Serine		**Phenylalanine**		**Arginine**		**Asparagine**	
	df	***F***	***p***	***F***	***p***	***F***	***p***	***F***	***p***
REP	1	8.12	0.006	4.42	0.039	4.25	0.043	5.12	0.027
LR	1	30.37	<0.001	15.03	<0.001	19.74	<0.001	9.32	0.003
Comp	5	22.60	<0.001	23.92	<0.001	23.21	<0.001	18.57	<0.001
NLR	2	1.17	0.316	1.97	0.147	3.38	0.039	1.97	0.146
		N-aceyl D-glucosamine		**Putrescine**		**Cellobiose**		**4-hydroxy benzoic acid**	
	df	***F***	***p***	***F***	***p***	***F***	***p***	***F***	***p***
REP	1	2.29	0.135	9.23	0.003	2.39	0.130	4.58	0.036
LR	1	29.36	<0.001	8.49	0.005	47.32	<0.001	19.60	<0.001
Comp	5	57.24	<0.001	15.60	<0.001	50.97	<0.001	24.67	<0.001
NLR	2	0.76	0.473	1.00	0.374	0.00	1.000	2.38	0.100
		Γ-hydroxybutyric acid		**Itaconic acid**		**D-malic acid**		**A-ketobutyric acid**	
	df	***F***	***p***	***F***	***p***	***F***	***p***	***F***	***p***
REP	1	0.83	0.364	0.03	0.873	0.55	0.459	0.16	0.690
LR	1	2.65	0.108	11.75	0.001	19.42	<0.001	8.62	0.004
Comp	5	7.62	<0.001	6.77	<0.001	3.96	0.001	12.13	<0.001
NLR	2	0.66	0.521	1.03	0.362	1.07	0.349	0.49	0.612

Table 6.1: The effect of biodiversity on respiration of the microcosm mixes grown in each of the nutrient sources when analysed by ANOVA. From the GLM analysis, the significance of the effects of the replicates (REP), linear richness (LR), species composition (Comp) and non-linear richness (NLR) on the respiration was assessed by ANOVA. This analysis was performed on all 84 microcosms in each of the 16 SCSs tested.

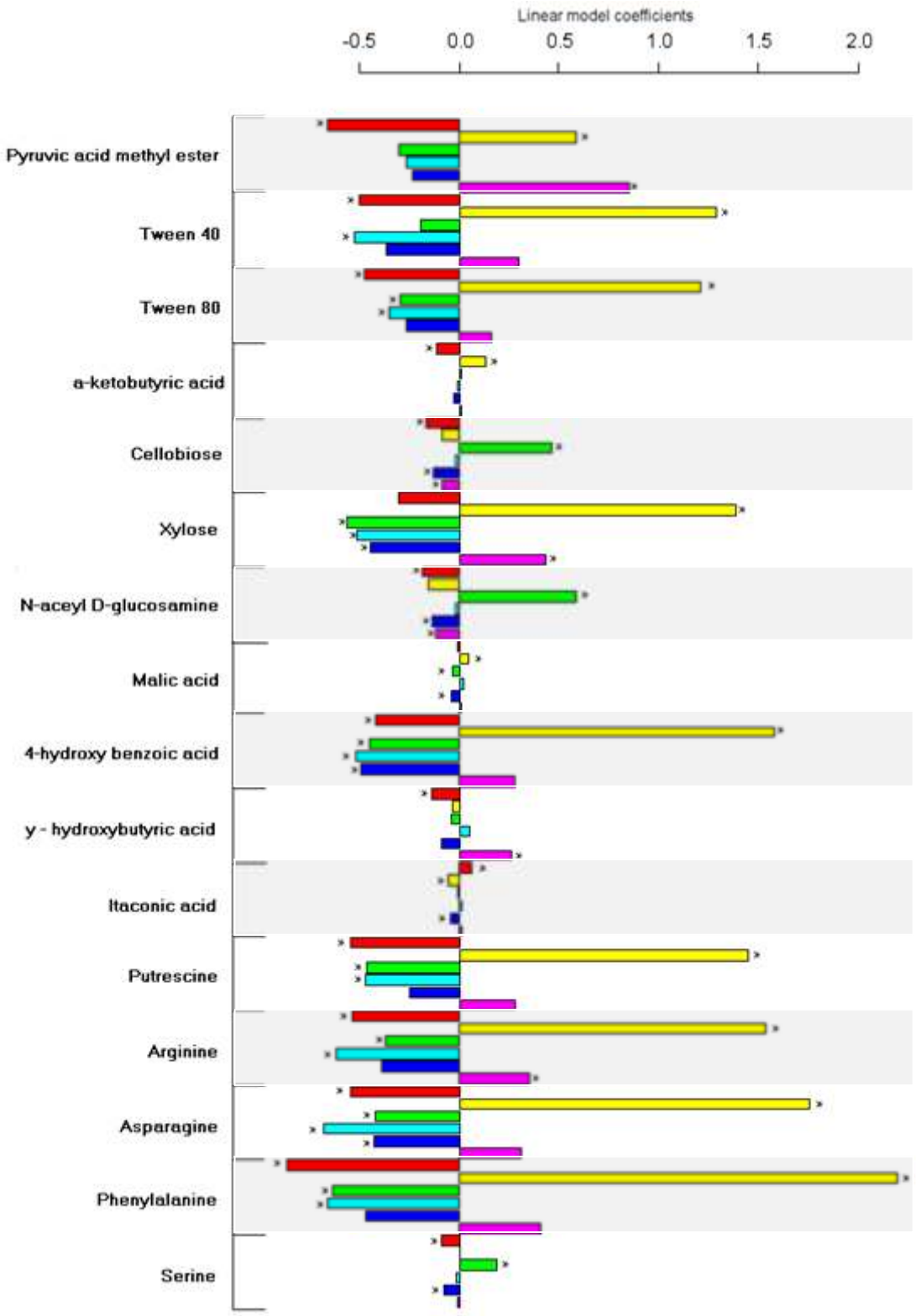

Figure 6.2: Species coefficients for each of the species in grown on each SCS. The species coefficients for all species (*P. aeruginosa*, *A. baumannii*, *S. maltophilia*, *S. pneumoniae*, *S. aureus* and *A. xylosoxidans*) in the experiment are shown. Asterisks denote significance ($p < 0.05$).

The number of microcosms that indicated positive interaction events increased with increasing biodiversity.

The analysis presented here has shown that for the majority of substrates, the species richness and species composition have statistically significant effects on the respiration of the microcosms. From this result and the nature of the fully factorial design (that every species is in every combination with all the species), the hypothesis that all the contributions were additive was tested.

If the monocultures were able to "predict" the respiration of the higher complexity microcosm mixes, then this result would suggest that the species were indeed additive. To test this, a predicted data set was calculated for each SCS by taking the mean of the monoculture respiration for each species present in a given microcosm. These results showed that all of the SCSs tested had positive relationships (Figure 6.3) between the observed and predicted values. This however was not possible with D-malic acid utilisation as this SCS was shown not to support growth of any of monocultures. As such, all of the predicted values for D-malic acid equalled zero and therefore no regression line could be calculated.

By subtraction of the predicted from the observed values, the differences in the respiration were obtained. These differences in respiration, termed differentials, were ranked in order of magnitude and are shown as plotted in Figures 6.4 – 6.6. These results showed that the number of positive differentials increased as the species richness increased.

In Figure 6.4, the mean differential was 0.31 (SD = 0.77, n = 240) for the 240 two species microcosm mixes. The microcosm mixes with three species are shown in Figure 6.5. The mean differential here was 0.56 (SD = 0.67, n = 320). Using a Wilcoxon rank sum test, the median differentials of the three species richness level microcosms (median = 0.06, 0.29 and 0.49 for richness levels 2, 3 and 6 respectively) were found to be statistically significantly greater than those of the two species mixes ($W = 29358$, $p < 0.001$). In the 16 microcosm mixes with six species present (Figure 6.6), the mean differential was 0.63 (SD = 0.49, n = 16). Using a Wilcoxon rank sum test, it was found that there was no statistically significant difference between the mean differential of the six species microcosm mixes and that of the three species mixes ($W = 2933$, $p = 0.326$).

The differentials were calculated to be positive, negative or neutral (differentials = 0.00). Of the differentials calculated for the two species microcosm mixes, 30% (72)

showed negative values, 12.9% (31) were neutral and the remaining microcosm mixes (137) had positive differentials. The number of negative and neutral differentials reduced in the 320 three species microcosm mixes with only 11.6% (38) negative and 4.4% (13) recording a zero difference. The remaining 269 microcosm mixes were found to have positive differentials. For the 16 six species assemblages, the differentials increased so there were no negative or zero values observed. The significance of the effect of increasing species richness on the differences in respiration was tested using linear regression. This result found that species richness significantly ($F_{1,574} = 52.60$, $p < 0.001$) increased the differentials by 0.269 (± 0.037 1se, $n = 576$) for each increase in richness.

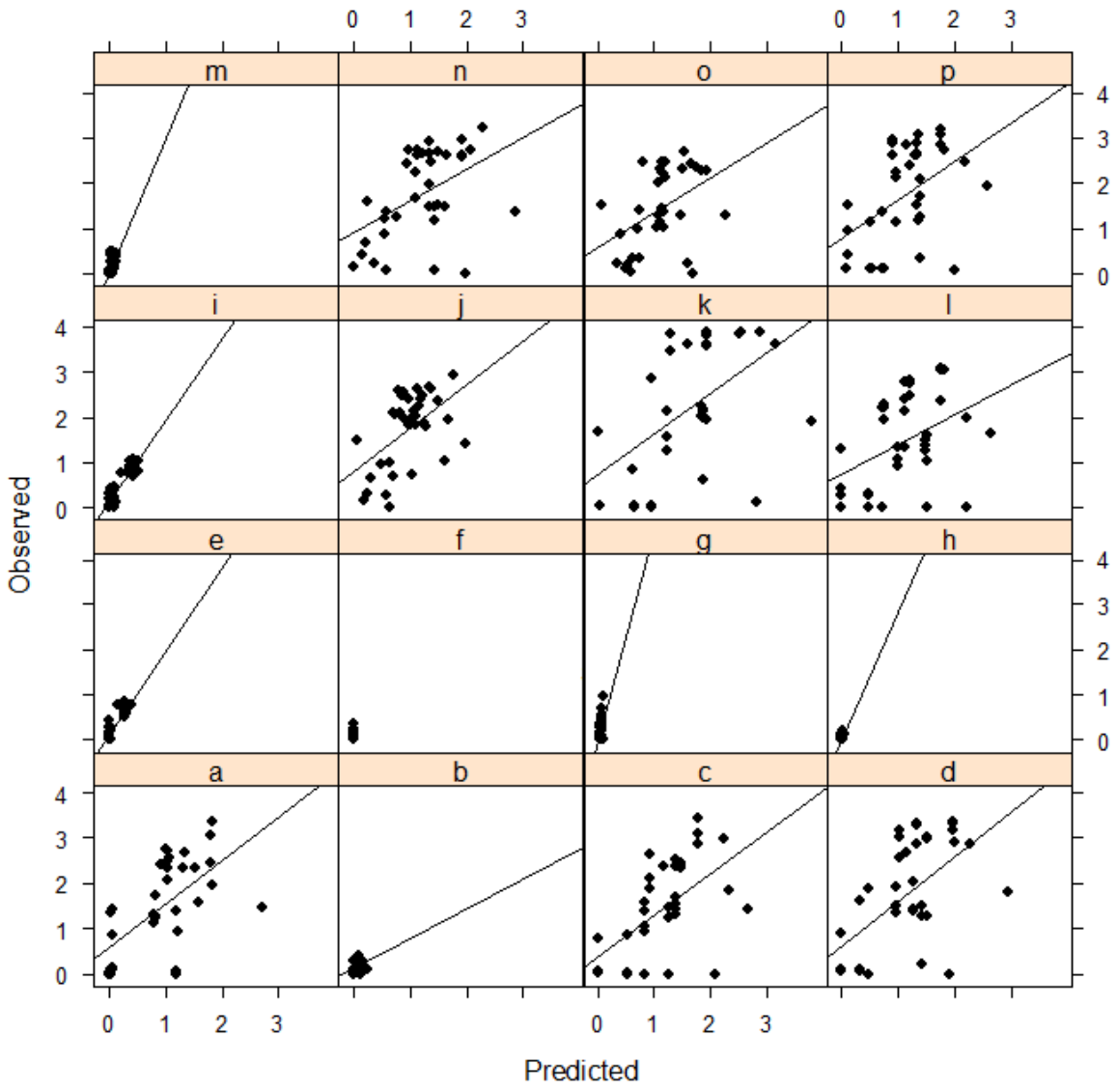

Figure 6.3: Correlations between the observed and predicted respiration values when grown on each SCS. Predicted respiration values (x-axis), calculated from the mean monoculture measurements, were plotted against the observed respiration values (y-axis). Linear regression lines were fitted to the plots. Each of the 16 SCS used were plotted individually: a = 4-hydroxy benzoic acid, b = α-ketobutyric acid, c = arginine, d = asparagine, e = D-cellobiose, f = D-malic acid, g = γ-hydroxy butyric acid, h = itatonic acid, I = N-aceyl-D-glucosamine, j = pyruvic acid methyl ester, k = phenylalanine, l = putrescine, m = serine, n = Tween 40, o = Tween 80, p = D-xylose.

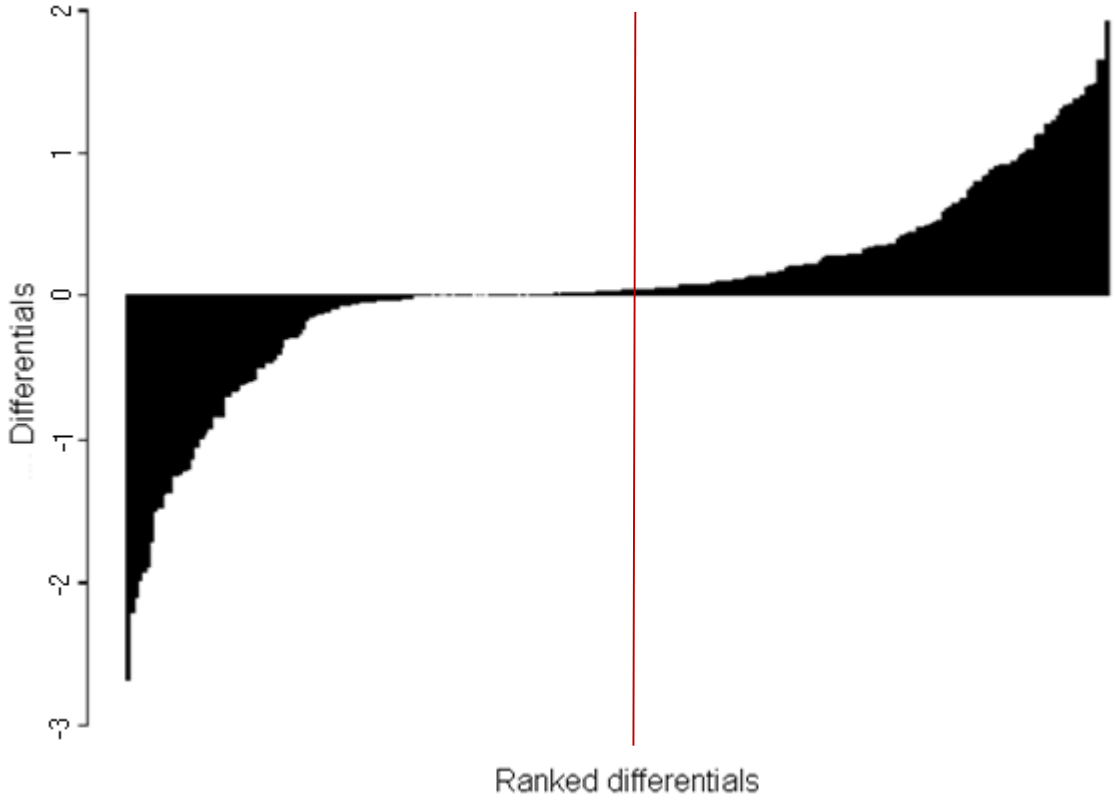

Figure 6.4: The difference in respiration between the observed and predicted values in microcosms with two species present. By subtraction of the predicted from the observed values, the differences in the respiration were obtained. These differences in respiration, termed differentials, were ranked in order of magnitude. The magnitudes of the differentials were plotted on the y-axis. Along the x-axis, the differentials were ranked in order of their magnitude. Each differential represents one of 240 assemblages of two bacterial species grown on a distinct SCS. The red line represents the 120th ranked assemblage.

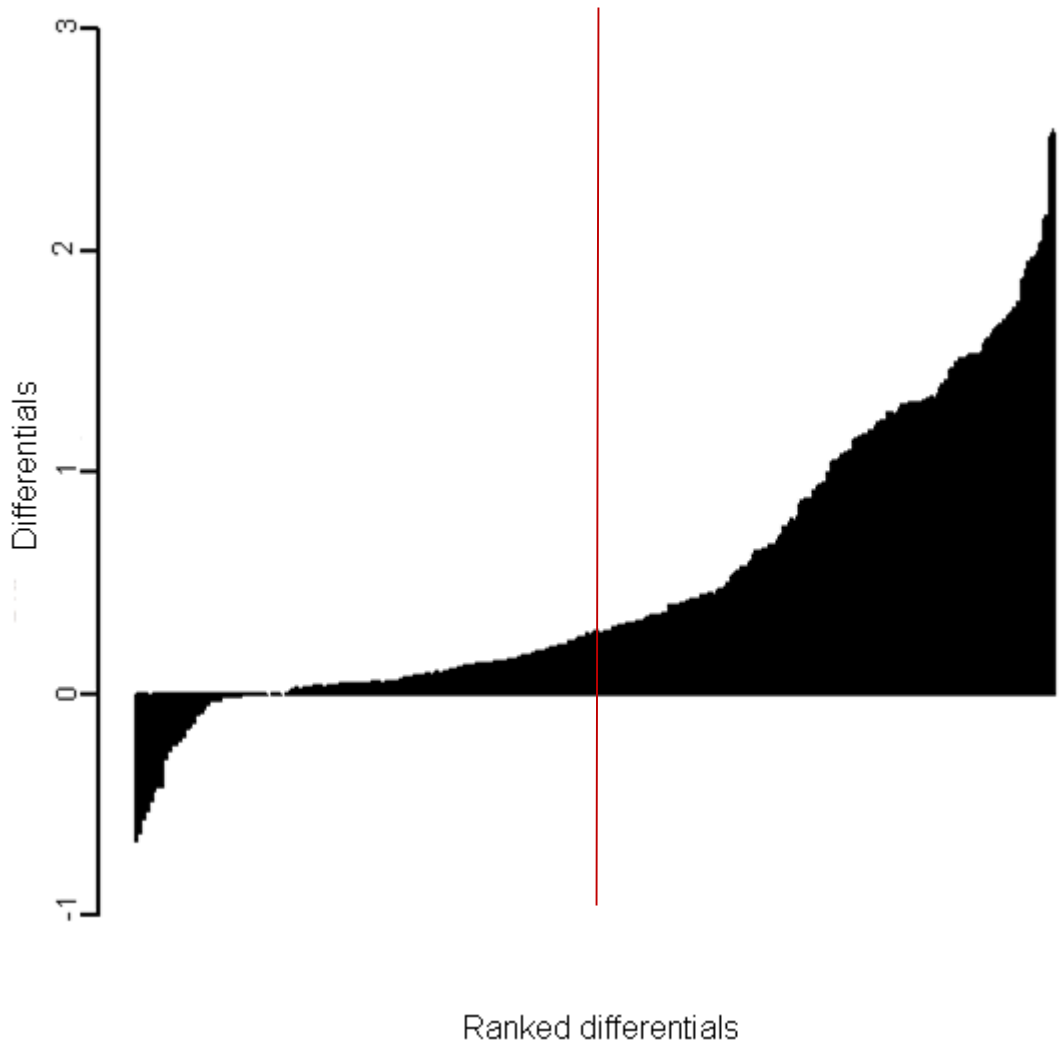

Figure 6.5: Respiration from three species assemblages showed that the observed values were higher than predicted in the majority of cases. By subtraction of the predicted from the observed values, the differences in the respiration were obtained. These differences in respiration, termed differentials, were ranked in order of magnitude. The magnitudes of the differentials were plotted on the y-axis. Along the x-axis, the differentials were ranked in order of their magnitude. Each differential represents one of 320 assemblages of three bacterial species grown on a distinct SCS. The red line represents the 160th ranked assemblage.

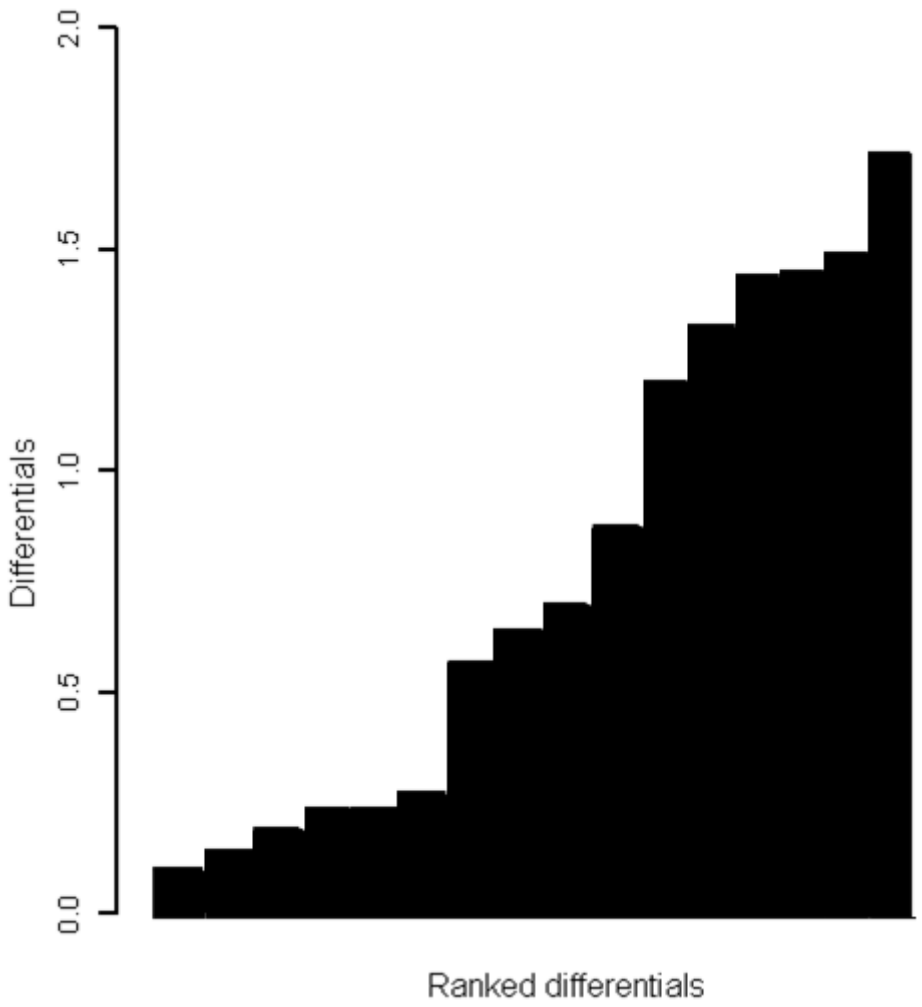

Figure 6.6: The respiration measured in six species microcosms showed increased respiration in the observed values than predicted by additive monoculture values. By subtraction of the predicted from the observed values, the differences in the respiration were obtained. These differences in respiration, termed differentials, were ranked in order of magnitude. The magnitudes of the differentials were plotted on the y-axis. Along the x-axis, the differentials were ranked in order of their magnitude. Each differential represents one of 16 assemblages of six bacterial species grown on a distinct SCS.

6.4 Discussion

In this chapter, the aims were to investigate the influence of a variety of simple carbon sources on the relationship between biodiversity and respiration. This was implemented using a fully factorial design at four species richness levels (1, 2, 3, and 6) with six bacterial species. The overall conclusion from this chapter is that, in general, the higher the species richness levels the more likely that the respiration obtained surpassed the additive effect of the microcosm mixes.

Linear richness effects are generally significant across all nutrient sources

In this study, all 16 SCSs analysed were associated with significantly positive linear richness gradients. The selection of SCSs for the analysis was however, based on the ability for the majority of the species to utilise the substrate thus allowing the effect of biodiversity on respiration to be observed. The presence of significant linear richness effects were supported by the findings presented in Chapter 4, albeit with individual substrates rather than the previous nutrient systems. This result also showed that the respiration of the system was not tied to the ability of the species to respire in monoculture. To date, no previous study has used a BIOLOG system to investigate the impact of biodiversity on community respiration.

The higher species richness produced a higher level of synergistic cooperation

In this study, a set of predicted values was created using the respiration recorded by the monocultures. These were the respiration that was predicted if the species were simply additive in effect. By subtracting the predicted from the observed respiration values, it was possible to identify how the microcosms differed from the additive monocultures (Loreau & Hector, 2001). These results showed that as the diversity of the assemblage increased, then the number of observed positive outcomes also increased (Figure 6.4 – 6.6). This indicated that more diverse assemblages were more likely to produce a greater than predicted respiration.

Two mechanisms in BEF experiments are key when attempting to account for this variation. These are the selection and complementarity effects (Loreau & Hector, 2001; Fox, 2005). The importance of these effects when analysed in the GLM is discussed below. First, the discussion will focus on how the increase in synergistic interaction with increasing species richness is apparent from the results.

To have higher than predicted respiration suggested that the species present were able to interact synergistically, or exhibit overyielding (Hector *et al.,* 2009) and respire

at a level greater than that predicted by the monoculture measurements. To achieve this, the species present must be able to interact in a way that increases the productivity of the mixture above that of the monoculture.

This increase in synergistic interactions with increasing species number informs on the nature of these bacterial communities. At the lower two species richness level, about a fifth of the microcosms had respiration levels lower than that predicted by the monocultures (Figure 6.4). This number of negative residual respiration values reduced to just over 10% in the three species assemblages (Figure 6.5). No negative values were found in the six species assemblages (Figure 6.6). This may indicate that negative interactions are only observed in assemblages where there are a lower number of species. As the number of species increases, then positive synergistic interactions may become more likely to dominate. This synthesis of the results is in contrast to Becker *et al.* (2012) who found that as the number of genotypes increases from a single species; the complementarity decreases. This thesis has already shown (Chapter 5) that *P. aeruginosa* isolate assemblages behave differently than mixed species ones. The difference between results from Becker *et al.* (2012) and this present study may be a function of how the data were collected. Had the final abundances of the assemblages in this study been recorded as in Chapter 4, it would have been possible to re-analyse the data in the same way as Becker *et al.* (2012), using the additive partition design (Loreau & Hector, 2001), to more directly compare the findings.

It is interesting to ask why synergistic interactions are more prevalent at higher species numbers. This increased prevalence of synergistic interactions could theoretically be due to a number of different reasons. Firstly, if two species display a highly negative interaction in a two species microcosm, as the number of bacterial species present increases this negative interaction may be diluted. This dilution effect could serve to reduce the opportunity for these species to directly interact and any interactions become increasingly less likely to be exclusively between the two species in question. Such an effect could of course be studied by a set of experiments that manipulate the environmental viscosity at the same time. Secondly, with an increased number of species there is a greater chance for one or more of the species to be, by chance, better at utilising the given SCS. This is the selection effect as discussed in the previous chapters. Lastly, with an increase in the species number there is a better chance that there is one or more species that will cooperate and "decide" to expend energy and cleave bonds in the carbon source.

This result was in part unexpected. This was because of the limited scope (compound targets) for each of bacterial species to utilise the SCSs differentially. These observed enhancements in respiration, perceived to be synergies, could arise from a number of processes including the utilisation of neighbours waste products which otherwise could have been inhibitive.

The significant effect of time on the respiration of the microcosm mixes.

In this study, respiration was measured at two time points; after 24 hours and 48 hours post inoculation. Although respiration increased over both time intervals from 24 to 48 hours the rate of increase was less than that over the first 24 hours and higher diversity assemblages respired at a higher rate than less diverse assemblages over both time points. This decreasing relationship between time and respiration has previously been described (Langenheder *et al.,* 2010; Yang L. *et al.,* 2011). In mixed species assemblages cooperation has been shown to enhance the productivity of the assemblage (Elias & Banin, 2012). This cooperation can however, also lead to the presence of cheaters that use less energetically challenging substrates or metabolic pathways (Hibbing *et al.,* 2010). The presence of cheaters overwhelming the microcosm could account for the reduction of respiration. Alternatively after 24 hours the species may have become predominant, thus reducing the respiration over the second time period.

Species composition is the most important variable but selection effects seen

The species composition effect, in this study, has been shown to drive variation in addition to the linear richness term. The species coefficients (Figure 6.2) showed that there were typically only three species, *A. baumannii, A. xylosoxidans* and *S. maltophilia* that produced positive coefficients. The GLM analysis confirmed that the species composition had a significant effect on the respiration (Table 6.1).

This study was especially suited to identifying the selection effect in assemblages due to the fully factorial design. This is because the species are in all possible combinations, therefore a greater amount of data are generated. The use of SCSs however narrows the niches available for these species to occupy. This therefore produces a greater selection pressure on the species present depending on whether or not they can successfully compete for the substrate, its cleavage by-products or waste from other species (Flynn *et al.,* 2011).

Non-linear richness was rarely significant

In this study, the interactions were investigated by both the non-linear richness term and by comparing the observed respiration against the additive predicted values. The comparison between the observed and predicted values has yielded some interesting conclusions about the drivers of the biodiversity effect; however the non-linear richness term was shown to be significant in only two of the sixteen SCSs tested. The species coefficients may mean that more specific interactions were occurring, as illustrated by alternating positive coefficients between *A. baumannii* and *S. maltophilia*, than indicated by the GLM analysis. This suggests these species were able to exploit contrasting niches (Foster & Bell, 2012). Further evidence for this was also observed by the positive coefficients of both *A. baumannii* and *A. xylosoxidans* being present together for many of the SCSs. This suggests that these two species were able to synergistically interact.

In this study, the GLM could be underplaying the importance of the non-linear richness term due to its apparent non-significance. In the previous chapters however, the non-linear effect was primarily associated with the deviation of the mean species richness from the linear regression line. These studies had 12 species present whereas this current chapter used six species. The reduced number of species may have resulted in the deviation of the means not being significant. Gaining an improved understanding of interactions in BEF is an important direction for future research in both environmental and clinical scenarios. It is proposed that the general increase in respiration above that predicted by the monocultures is due to interactions. Further study into the expression profiles of the bacteria is required to investigate the physiological and biochemical mechanisms behind the synergies to elucidate how these interactions occur within the assemblages. As far as it does, this study produced evidence that interactions within assemblages are important in terms of biodiversity effects on respiration.

Conclusions

This chapter has investigated the ability of an assemblage of bacterial species to utilise and respire one of 16 single carbon sources. Through GLM analysis, it has been shown that both the species richness and the species composition have significant effects on the respiration recorded. It was also found that respiration of higher diversity assemblages was not dictated by the respiration recorded in the monocultures. The implications of this and the other results in this thesis will now be discussed in detail here.

Chapter 7

7. Discussion

The primary cause of mortality in individuals with CF is respiratory failure resulting from chronic bacterial infections. A greater understanding of the polybacterial nature of this disease is therefore needed. The focus of this study was on the ecological mechanisms at work within these bacterial communities (Harrison, 2007, Yang L. *et al.,* 2011) as these have the potential to provide fresh insight into this condition. This thesis has been based on applying experimental methodologies to understanding the link between bacterial biodiversity and ecosystem function.

The aim of these studies was to identify the significance of a range of ecological mechanisms in relation to their impact on the productivity of bacterial assemblages. This was realised using clinically relevant bacterial species isolated from sputum samples from individuals with CF. In this Discussion, the results generated will be considered in relation to the ecological mechanisms occurring between bacteria in CF associated airway disease.

Previous reports have investigated the structure of the bacterial communities in CF associated airway infections (Harrison, 2007; Stressmann *et al.,* 2012) and have stressed the need to understand the ecology of these communities. Harrison (2007), in her review of the microbial ecology of CF respiratory infections, speculated that there are interactions between species that increase the pathogenicity of the community. Although the work in this thesis cannot directly comment on the pathogenicity of the assemblages, these studies have provided evidence that synergistic interactions occur and the species richness and selection effects are ecologically significant.

7.1 A brief summary of results from mixed species and *P. aeruginosa* ecotypes BEF experiments

The results from Chapter 4, using 12 different species, and Chapter 5 using 12 *P. aeruginosa* ecotypes are reviewed in Table 7.1. Briefly summarising these results:

In the case of the 12 different species, the experimental variables, replicate and Partition, were significant in Tryptone Water but not in DHB. Two of the biological variables, linear richness and composition, were significant in both Tryptone Water and DHB. The composition term made the greatest contribution to the fit of the model, accounting for ~30% of the variance followed by linear richness accounting for ~12%

of the variance. The third biological variable, non-linear richness was significant only in DHB however it only accounted for ~2% of the variance.

In the experiments using *P. aeruginosa* ecotypes, the Partition term was significant in both Tryptone Water and DHB, while the replicate term was only significant in DHB. The only significant biological variable was composition which accounted for 8% and 4% of the variance in Tryptone Water and DHB respectively.

The complete GLMs for the mixed species and *P. aeruginosa* ecotype experiments accounted for similar proportions of the variance within the data (>80%). For mixed species, the coefficients of the three biological terms were used to calculate predicted values of respiration which were plotted against the observed values. The fit of these simplified models had a mean $R^2 = 0.40$ (SD = 0.09, $n = 4$).

In all cases there was significantly greater respiration in Tryptone Water than in DHB with greater respiration in constrained environments than in mixed. The viscosity of the system also affected the impact of biodiversity on respiration. In particular, the viscosity affected the non-linear richness terms and the positioning of the species coefficients. Nearly all *P. aeruginosa* ecotypes changed the sign of their coefficients between Tryptone Water and DHB.

Type of term	Variable	Tryptone Water		DHB		Species
		Mixed	Const'd	Mixed	Const'd	
Experimental	**REP**	+++	+++	-	-	Mixed
	P	+++	+++	-	-	
Biological	**LR**	+++	+++	+++	+++	
	C	+++	+++	+++	+++	
	NLR	-	+	+++	++	
Experimental	**REP**	-		+++		*P. aeruginosa*
	P	+++		+++		
Biological	**LR**	-		-		
	C	+++		++		
	NLR	-		-		

Table 7.1: Outline of the significance of the model terms to the respiration data for mixed species and *P. aeruginosa* ecotypes on four growth substrates. The four substrates were Tryptone Water as mixed and constrained, and Defibrinated Horse Blood (DHB) also as mixed and constrained environments. The experimental variables are labelled REP (replicates) and P (partitions). The biological variables are labelled LR (Linear Richness), C (Composition) and NLR (Non-Linear Richness). +++ = $p < 0.005$, ++ = $p < 0.05$, + = $p \leq 0.1$, - = $p > 0.1$

Comparing mixed species and mixed P. aeruginosa ecotype BEF experiments.

The results presented in Chapters 4 and 5 will now be contrasted in further detail (Figure 7.1). For microcosms in Tryptone Water, the respiration values for the species and the *P. aeruginosa* ecotypes in monocultures were not significantly different. Significant differences were observed between the linear regression lines. To explore this further, the mixed species in Chapter 4 were considered to be phylogenetically distinct with the *P. aeruginosa* ecotypes considered to be phylogenetically similar. Interestingly, the variance within the data may be a factor of the relatedness of the species. The assemblages of *P. aeruginosa* alone were shown to be much more similar in their ability to respire on Tryptone Water than mixed species assemblages. These results also suggest that the between bacterial interactions are likely to be more significant in the mixed species assemblages than those with *P. aeruginosa* alone.

In DHB, there was no statistical difference between the mean monoculture respiration produced by the species and ecotype mixes. There was however, a significant difference between the gradients of the regression lines calculated from the relationship between respiration and richness. These results show that there was a given level of respiration that the *P. aeruginosa* ecotypes achieved when grown on DHB that did not differ at any richness level. This level of respiration was only "bettered" by the mixed species assemblages when all 12 were present. These results have found that the monoculture means for either species or ecotypes had no significant differences. Thus the difference in the regression lines was related to the species assemblages' utilisation of the wider functional capacities that mixed species growth afforded through interaction.

Overall, these results suggested that the greater the genetic differences between the members in a BEF experiment, the more likely they are to interact synergistically in an assemblage. This suggestion has been supported by previous studies (Flynn *et al.,* 2011; Latta *et al.,* 2011; Elias & Banin, 2012) and raised questions on the types of interactions that were occurring within the growing species assemblages.

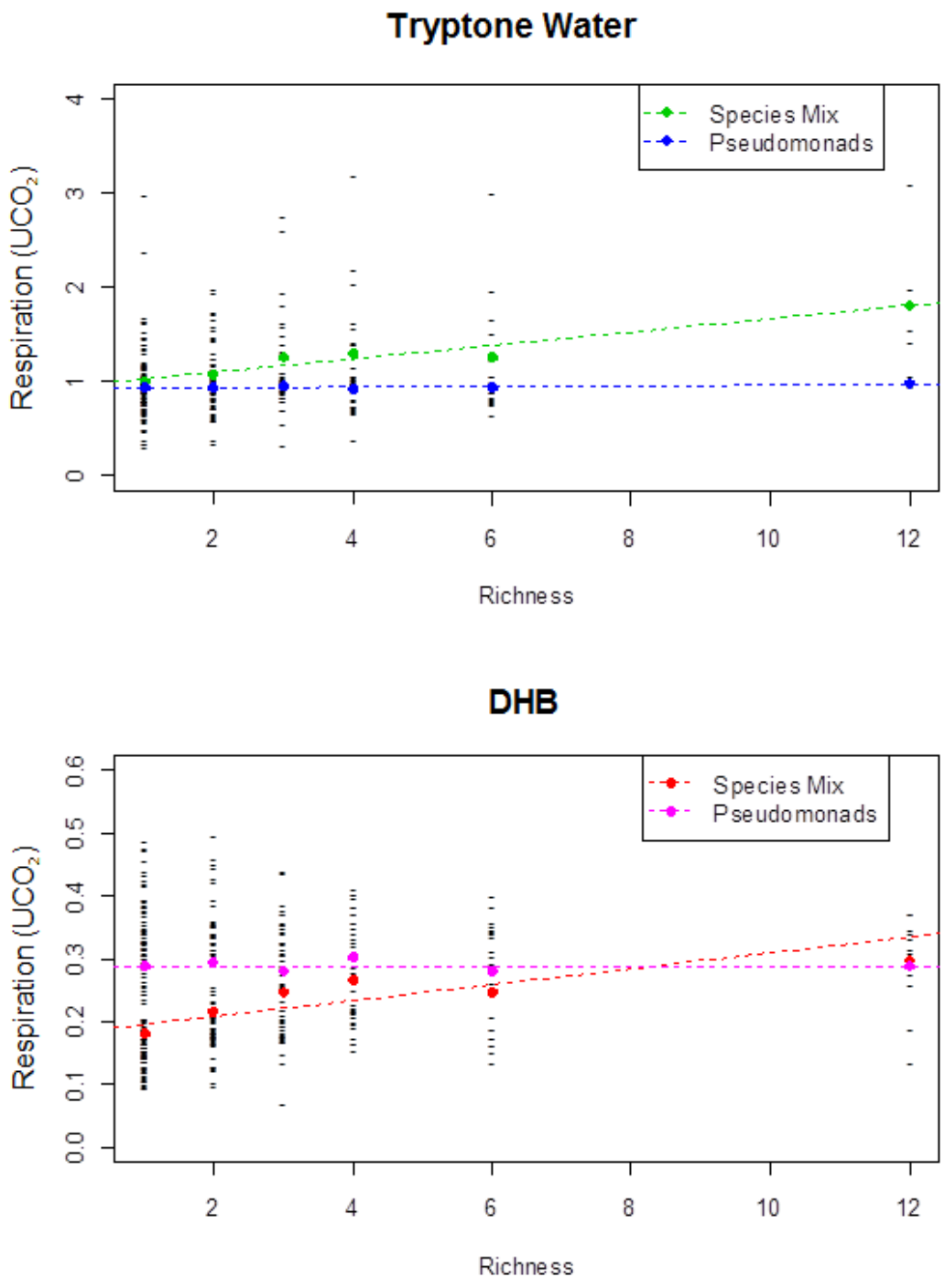

Figure 7.1: The respiration measured at each richness level for both mixed and *P. aeruginosa* ecotype assemblages. The respiration, in UCO_2, (y-axis) for each of the microcosm mixes (-) are shown at each of the richness levels (x-axis). The data from both the mixed species and single species microcosms have been included. Mean respiration (•) did not match with increasing isolate richness in both the Tryptone Water and DHB environments.

7.2 A brief summary of results from BEF experiments on single carbon substrates

In Chapter 6, the relationship between diversity and respiration of six species growing on sixteen simple carbon sources was studied. This was executed using a fully factorial design with four species richness levels (1, 2, 3 and 6) and six bacterial species.

Similar to the 12 species BEF experiments in Chapter 4, it was found that both species richness and species composition had significant effects on respiration. Species composition was the most important variable on SCSs, which was the same for both the 12 species and *P. aeruginosa* ecotype BEF studies. In contrast, non-linear richness was very rarely significant on SCSs.

A notable observation on SCSs was that at the higher species richness levels, the respiration surpassed the additive effect of the microcosm mixes. This indicated the six species were synergistically interacting, or overyielding. Given the limited metabolic cleavage potential for each of the SCS substrates it may be expected that there would be limited benefit from most increases in species richness. In practice, it has been observed that that up to the limit of the six species there is a strong tendency for respiration to increase with species richness.

The changes in nutrient substrate or viscosity in Chapters 4 and 5 produced marked changes in the in the BEF relationship. Notably in Chapter 6, for the 16 substrates the BEF relationship was relatively conserved

7.3 Decisions made affecting the experimental design

Before any BEF experiments could be fully implemented, key decisions regarding the choice of bacteria and the assay of ecosystem function were made. The bacterial species were isolated from adult CF patients on non-selective media in aerobic conditions. To be included in the BEF experiments, all species needed to be aerotolerant and to respire producing CO_2. Thus, some commonly reported obligate anaerobic CF associated species, such as those from within the genus *Prevotella*, were not included or isolated. This method of cultivation was similar to other BEF studies that have manipulated the biodiversity of bacteria and monitored respiration (e.g. Lawrence *et al.,* 2012). Plate colony isolation yielded a broad range of species, 18 in total, from a collection of 155 isolates. Isolation of a similarly wide diversity of bacterial species from CF sputum samples using non-selective media has also been reported by Coeyne *et al.* (2002). Each of the species isolated had been reported as

present in CF respiratory secretions in previous studies (Coeyne *et al.,* 2002; Flayhart *et al.,* 2004; Guss *et al.,* 2011; Sibley *et al.,* 2011; Filkins *et al.,* 2012; Madan *et al.,* 2012) with one exception. This exception, *M. luteus*, a Gram-positive coccus found in these CF samples, has previously been reported as associated with human infections, but not previously in relation to the CF airway (Hirata *et al.,* 2009).

By adulthood, patients are colonised by a higher diversity of bacterial species than paediatric patients (Cox *et al.,* 2010). Of these species *P. aeruginosa* is one of the most prolific colonisers (Lyzack *et al.,* 2002). Along with other species, *P. aeruginosa* have been shown to persist for years in the CF airways (Smith *et al.,* 2006; Stressmann *et al.,* 2012). Previous studies have also reported a positive correlation between the severity of the airway disease and bacterial species richness (Delhaes *et al.,* 2012). The choice of examining sputa from clinically stable (not on IV antibiotics) adult patients therefore, gave a strong probability of obtaining a diverse collection of bacteria that have been part of, and adapting to, the airway community over a prolonged period of time (Yang L. *et al.,* 2011a)

To monitor accurately the activity of the isolates, the measure of ecosystem function needed to be resolved. Respiration (CO_2 production) is a commonly used measure in bacterial BEF experiments of ecosystem function (Bell *et al.,* 2005; Salles *et al.,* 2009; Lawrence *et al.,* 2012). This measure has been used as it is an assay that does not rely on a single metabolic pathway; instead CO_2 is generated through metabolic activity (Schimel *et al.,* 2007; Salles *et al.,* 2009). This measure was particularly useful in this study as the choices of nutrient sources and the altering of viscosity meant that other commonly used methods such as protein production or cell density would have been more difficult to assess due to interference resulting from components of the media. The choice of the MicroResp™ system to facilitate these measurements was driven by the need for a higher-throughput method than the titration method used in the pilot studies. The original titration methodology had proved too slow to produce accurate data.

Tryptone Water and Defibrinated Horse Blood (DHB), were used as two contrasting nutrient sources; one broad in relation to "accessible" nutrient resources, the other more recalcitrant. It should be stressed that many different nutrient sources were considered and rejected for the reasons outlined in Chapter 2.2. In addition, the levels of the Tryptone Water and DHB used were also iteratively altered so the growth of all species was supported and the carbon dioxide levels in the BEF study were detectable.

The statistical design and analysis of the data posed another key decision. For its ability to discriminate the three aspects of biodiversity of interest the RPD GLM was chosen. The RPD was able to assess the significance of the species number, composition and the interaction effects and was suitable for bacterial BEF experiments. Other methods of analysis were considered along with the RPD, these included the additive partitions design (Loreau & Hector, 2001) and the diversity-interaction model (Kirwan *et al.,* 2009). The additive partitions design was developed in order to determine the selection and complementarity effects present within a community. It does not, however, allow the assessment of individual species. The diversity-interaction model allows the assessment of individual species and their scope for interactions, but does not allow assessment of selection effects or the increase in species richness. As the RPD included all these aspects, it was an appropriate means of analysis. The disadvantage to the RPD analysis was that the biodiversity effects are investigated in broad terms which are not so amenable to detailed scrutiny but this was deemed necessary to investigate all the mechanisms desired.

The adaptation of the model was carried out after the data had been generated. Initial investigation of the data showed large discrepancies between the replicates and high significance values of the variables studied. Many different approaches, such as separating the replicates and the Partitions and averaging the replicates, were applied to the analysis of the data (data not shown). However, the most informative analysis was the adapted RPD presented here. Interestingly, differences in the replicate values presented here were less extreme than those taken from a previously published study (Bell *et al.,* 2005). The implication of this has potentially wider importance to the field of ecology.

7.4 The relationship of the current studies to CF microbiology

The motivation behind this study was to investigate ecological mechanisms that can exist within bacterial assemblages in CF associated airway disease. Having a polybacterial system means that many species are present, even with the heavy use of antibiotics throughout the life of a CF patient, the interaction between the species remains high (Rogers *et al.,* 2004; Harrison, 2007).

Although species diversity has been shown to be high in the sputum samples examined in this study, a recent study has reported evidence from ex-planted lungs that the bacterial species diversity can be as low as one or two species (Goddard *et al.,* 2012). In support of these results, Rudkjøbing *et al.* (2011; 2012) observed that a low

diversity of aerobic species was present in a further five ex-planted CF lungs. With this low level of diversity reported in ex-planted lungs, the potential for synergistic interactions between the bacterial species within the lung tissue would be greatly reduced. These results might also imply that the selection effect was highly significant in creating a restricted habitat with a single dominant species.

A major difference between lung and sputum samples is that both Goddard *et al.,* (2012) and Rudkjøbing *et al.* (2011) report findings from "end-stage" patients (i.e. samples are from lungs that have been removed from patients). These studies make it clear that the low diversity could be a factor of the end-stage disease. In contrast, Rudkjøbing *et al.* (2012) reported results indicating that in sputum samples from non-end-stage patients, a more diverse species load was present. Typically, sputum samples are taken from adult CF patients who are not in need of lung transplantation. From these results, it could be postulated that as the disease progresses and the lung function declines, the biodiversity of the system also decreases. Indeed, this has been supported by recent studies (Delhaes *et al.,* 2012; Stressmann *et al.,* 2012). Goddard *et al.* (2012) also reported data showing an apparent discrepancy between the bacterial component of the microbiota characterised in the lung and corresponding expectorated sputum samples. Here, the number of species present in the sputum samples was greater than those found in the lung samples. Despite this, the dominant species, typically *P. aeruginosa,* found within sputum samples was similar, in the majority of the samples, to that in the corresponding lung sample (Goddard *et al.,* 2012).

In the studies investigating ex-planted lungs suggest the focus was on a specific time-point in the progression of the disease and therefore cannot be generalised to all stages of CF associated pulmonary infections. This reinforces the need for greater understanding of the ecology of these systems and, specifically, the mechanisms that lead to dominant species.

Although this debate about the "true" microbiota colonising the CF lung is on-going it is clear that sputum samples from CF patients harbour a diverse range of bacterial species (Sibley *et al.,* 2009). Rogers *et al.* (2006) goes further to show sputum bacteria not as contaminants but as part of the airway community. Species present in the lungs, the oral cavity and the upper airways may form a single pool (Rogers *et al.,* 2006) that may vary in membership over time and stage of disease, by movement, acquisition or even dominance of species. This compounds the need to understand the ecological mechanisms between the species in this pool and how they adapt over time as assemblages or communities.

7.5 Reported results and other BEF studies.

As demonstrated in Chapters 4 and 6 on 18 different nutrient sources, when the biodiversity of a system was altered by increasing the number of species present there was a matched increase in respiration. In contrast to these positive relationships, Chapter 5 found that when the biodiversity of the system was altered by increasing the number of *P. aeruginosa* ecotypes, there was no increase in respiration.

Of the eight studies presented in Table 1.1, all of those using mixed species assemblages report positive relationships between biodiversity and ecosystem function (Wohl *et al.,* 2005; Bell *et al.,* 2005; Salles *et al.,* 2009; Langenheder *et al.,* 2010; Lawrence *et al.,* 2012; Tan *et al.,* 2012). Those that use single species assemblages report either a neutral (Zhang *et al.,* 2009) or a negative (Becker *et al.,* 2012) relationship. The results in this thesis parallel these findings using species from CF sputum, a habitat much less diverse in species than natural habitats such as soil or water.

Due to the potential link between the relatedness of the isolates and the BEF relationship, the results of this thesis suggest that the similarities in the 16S rRNA gene between the isolates should be taken into account. This is supported by the work of Flynn *et al.* (2011) and Latta *et al.* (2011) who discuss the role of phylogenetic distance in bacterial biodiversity effects on ecosystem function.

7.6 The effect of biodiversity within CF airway infections

The contribution of these BEF relationships to CF microbiology and the understanding of the bacterial communities will now be considered.

The relationship between bacterial biodiversity and patient lung function has been reported to be potentially positive (van der Gast *et al.,* 2011; Delhaes *et al.,* 2012). The results in Chapters 4 and 6 suggest that the effect of biodiversity on activity has a similar relationship. It is not clear however whether the respiration of an assemblage can be correlated in a meaningful way to a loss of lung function. The results presented do suggest that as the number of bacterial species increases within an assemblage the more complementary the microcosm mixes become. This could suggest that increasing species richness increases the complementarity within the assemblage, allowing the community of bacteria to use more recalcitrant and other nutrients more efficiently or remove more inhibitive waste products (Elias & Banin, 2012). A balance of these activities could enable stable communities to persistence in the CF airway potentially causing a transformation where more nutrients become available, due to damage, thereby allowing a single species to start to dominate. This could be a mechanism by

which to arrive at the conditions reported by Goddard *et al.* (2012) and Rudkjøbing *et al.* (2012).

Potentially, bacterial species at different stages in this process could behave differently in BEF experiments. For example, had the bacterial species studied here not had a period of adaptation e.g. were isolated from neonatal or paediatric CF respiratory samples, the interactions between them, therefore their effect on system function, could be different from those presented. Alternatively, the *P. aeruginosa* isolates could begin to behave as distinct species if they were cultivated from depauperate, end-stage samples. Both of these queries would form interesting further studies and depending on the results from these this may have the potential to identify a "shift" in the behaviour of the species related to disease progression.

7.7 Viscosity alters ecological behaviour

The results in Chapter 4 clearly demonstrated that, regardless of the nutrient source, the effect of increasing environmental viscosity is to increase respiration. This was discussed as the result of increased interactions as bacterial cells were "coerced" into close physical proximity and thus interactions. As a large number of species enter the airways, there will be a selection pressure, at a local level, that may favour assemblages of species that are able over a short period of time to synergistically interact (Harrison, 2007). This coercion is a phenomenon that Kuemmerli *et al.* (2009) measured experimentally, with *P. aeruginosa* isolates and siderophore production, and Xavier (2011) has reviewed. This increase in interactions within a constrained space can also be detected in nutritionally limited environments by an increase in the significance of the interaction variables. These results are those presented in Chapter 6, where synergistic interactions increase with richness on single carbon sources.

Compared to healthy individuals and paediatric CF patients, the mucus in adult CF airways displays raised viscosity (Baconnais *et al.,* 1999; Bals *et al.,* 1999), broad nutrient content (Palmer *et al.* 2007) and high bacterial diversity (Sibley *et al.,* 2009). The data in this study suggest that species in this environment can be highly synergistically active.

Current treatments that aim to rehydrate, and therefore soften, the mucus (e.g. osmotic agents; Minasian *et al.,* 2009) may have effects on the bacterial assemblages that have not yet been fully realised. One recent study that has investigated the effect of an inhaled osmotic agent, mannitol, and the corresponding sputum microbiology was published by Aitken *et al.* (2012). This study reported that the diversity of bacterial

species was similar in both the treated and untreated groups, but the forced expired volume in one second, a standard measure of lung function, in those inhaling mannitol was significantly increased. This softening of the mucus could disrupt some of the interactions present between the bacterial species present. For example, any direct interaction that may have been occurring due to the constrained position of the cells could potentially be reduced as the environment would no longer be "fixing" the bacteria in place. The implications of the mucus softening treatments on the ecology within the bacterial assemblages are unknown, but the effect of the treatment was positive for the patients (Minasian *et al.,* 2009; Aitken *et al.,* 2012).

Interactions within the CF airways may take many forms. An example of interactions present in the CF airway has been shown between *Stenotrophomonas maltophilia* and *P. aeruginosa* in the form of diffusible signalling factors (Ryan *et al.,* 2008). If the environment becomes such that the rate of diffusion reduces (i.e. thickening) then advantageous interactions will be more likely to be present between bacteria in defined localities. This has begun to be investigated in CF by Rudkjøbing *et al.* (2012) using *in situ* hybridisation. It would be a useful avenue of future study to investigate how the interactions altered during the process of rehydration, as this could directly inform the management of CF respiratory health. Results from this and Kuemmerli *et al.* (2009), suggest that this process could disrupt assemblages of bacteria by changing the spatial environment and the types of interactions possible. This may therefore give another reason for the upturn in patient well-being alone or in combination with antibiotic therapy.

An interesting area of future research therefore, could be to concentrate on the biochemical mechanisms, by way of "–omics" approaches, that underpin these broad processes. This may allow more precise identification of the synergies between the species (Frossard *et al.,* 2012).

7.8 The importance of *Stenotrophomonas maltophilia* to respiration

Previous studies have presented data that proposed *S. maltophilia* should be considered a major driver in CF related airway infections, particularly in those patients presenting with more severe disease symptoms (Goss *et al.,* 2004; Rudkjøbing *et al.,* 2012). This thesis reports evidence that *S. maltophilia* may be highly influential in increasing the activity of the community however, it has been suggested that the presence of *S. maltophilia* does not directly correlate to a worsening of the disease (Goss *et al.,* 2004).

At this time, the evidence that *S. maltophilia* is highly influential is best arrived at through direct manipulation of the bacterial species. To evaluate how this result could be important to CF patients, two scenarios can be postulated. First, an increase in respiration is detrimental to the host. In this scenario, *S. maltophilia* has been shown to be an "aggressor" species in the community and potentially a target for antibiotic therapy for eradication prior to the destabilisation of the community. Conversely, the second scenario is that an increase in respiration is in fact beneficial to the host, e.g. increase synergies between the bacteria equate to a more stable community and therefore stable lung function. Here, antibiotic therapy targeting this species may be less desirable.

7.9 Bacteria cooperate in mixed culture

A recent study by Foster and Bell (2012) investigated the cooperation between species in pair-wise comparisons. Their findings were that two species bacterial assemblages were less productive than the sum of their monocultures. The results presented in Chapter 6, indicate that less productive two species assemblages do occur but there is a much greater proportion (~60%) of more positively productive assemblages. Chapter 6 goes on to show that at higher species richness levels the number of less productive assemblages decreases until none are found.

The findings demonstrated that multi-species groups were typically more productive than less diverse assemblages. A similar observation has been made by many studies investigating the ecological (Naeem *et al.,* 1994; Bell *et al.,* 2005) and the clinical effects (Harrison, 2007; Elias & Banin, 2012) of increased bacterial biodiversity. There are however reports that indicate that higher bacterial diversity produces more antagonistic interactions (Jiang *et al.,* 2008; Becker *et al.,* 2012) or that, in a clinical setting, a single bacterial species is capable of dominating an entire ecosystem (Goddard *et al.,* 2012).

7.10 Presence of sub-populations in *P. aeruginosa* ecotypes

The methods applied in this thesis did not explicitly test the precise mechanisms that drove the BEF relationships. Evidence has been presented however, that show an increase in synergistic interactions in assemblages of higher species complexity. These results raise the question as to how a species, in particular *P. aeruginosa*, is able to dominate crises in the CF lung. The species coefficients, in Chapter 5, show that there was generally a divide between those *P. aeruginosa* ecotypes that positively affected the

respiration of the assemblages when grown in Tryptone Water and those in DHB. This suggests the presence of sub-populations within the *P. aeruginosa* species. Although there are potentially sub-populations of *P. aeruginosa* ecotypes, data in this thesis have shown that there are unlikely to be any interaction events between them. With this lack of complementarity, the *P. aeruginosa* assemblages cannot be more productive than a mixed species assemblage with high diversity and therefore should not be able to out-compete the mixed species.

Results similar to this have been previously reported when discussing the presence of diversity within species. Moore *et al.* (1998) and Jaspers & Overmann (2004) postulated that within species variation is important for the survival of the species in challenging habitats. The increase in survival is potentially due to niche differentiation allowing a single species to increase its functionality to closer to that of a mixed species assemblage (Moore *et al.*, 1998; Jaspers & Overmann, 2004).

7.11 **Founder effects are significant in the majority of experiments.**

Thus far, the discussion has been concerned with the differences between the assemblages consisting of mixed species and those of *P. aeruginosa* ecotypes. One similarity however that was present throughout the experiments analysed was the significance of the founder effects. These effects were tested using the adapted GLM in order to account for these effects and therefore "normalise" the data. This prevented the loss of significance between the replicates, or the biological terms be more significant than they actually were. This "alternative" approach could have fundamental implications in these results. Therefore further discussion and testing is needed to establish whether these founder effects are inherent to ecological experiments. How these effects change the biology of the bacterial species present in these experiments is as yet unknown.

7.12 **Conclusions**

This thesis describes and reports three studies in which the ecological mechanisms of bacterial diversity affecting total respiration were investigated. The studies combined to produce a central hypothesis testing result. That is, generally, the assemblage of bacteria was able to respire more when a greater number of species are present.

The experiments reported in this thesis show that experimental micro-ecology can elucidate ecological processes that may occur between the bacteria present in polymicrobial infections. The next step in this field of research is to begin to create more complex *in vitro,* or potentially *in vivo,* models to assess which biodiversity effects are conserved and continue the cross-over between empirical research and medicine.

Conclusions that can be drawn from this thesis are as follows:

- Mixed species assemblages had a general trend to increase respiration with species number but this trend was not observed in assemblages of *P. aeruginosa* ecotypes alone.
- With respect to BEF experimentation, subpopulations were observed in assemblages of *P. aeruginosa* ecotypes alone.
- Species composition was the term which contributed the most to the fit of the model in every experiment.
- These bacteria worked more synergistically in high diversity communities.
- A limitation in nutrient source resulted in an increase in interactions.
- Founder effects were responsible for significant variation within the data and therefore should be taken into account before assessing biological variables.
- The methods used throughout this thesis allowed relevant and robust conclusions to be made and were therefore shown to be fit for purpose.

The direct significance of these findings to CF airway infections is as yet unclear, but these results have shown that ecology can provide insights which are relevant to medical science.

Appendix

Appendix

Richness Level	Partition Number		
	1	**2**	**3**
2	1,2	1,5	1,8
2	3,8	2,4	2,11
2	4,5	3,10	3,9
2	6,12	6,12	4,10
2	7,11	7,8	5,6
2	9,10	9,11	7,12
3	1,4,8	1,10,12	1,4,11
3	2,10,11	2,7,9	2,7,12
3	3,9,12	3,5,11	3,8,9
3	5,6,7	4,6,8	5,6,10
4	1,6,8,11	1,5,7,11	1,3,5,11
4	2,4,9,10	2,6,9,10	2,8,9,10
4	3,5,7,12	3,4,8,12	4,6,7,12
6	1,2,5,6,10,12	1,2,3,4,10,12	1,3,5,6,8,9
6	3,4,7,8,9,11	5,6,7,8,9,11	2,4,8,10,11,12

Table A1: The species present in each microcosm mix in all three Partitions used. The combinations of species at species richness levels 2, 3, 4 and 6 used in each of the three Partitions in Chapters 4 & 5.

Species	Mixed			Constrained		
	SC	F	p	SC	F	p
(1) *Pseudomonas aeruginosa*	-0.29	4.74	0.034	-0.16	1.37	0.247
(2) *Stenotrophomonas maltophilia*	0.57	30.5	<0.001	0.94	60.02	<0.001
(3) *Burkholderia cepacia*	-0.27	6.9	0.011	-0.52	19.85	<0.001
(4) *Achromobacter xylosoxidans*	-0.05	0.37	0.544	-0.23	4.4	0.041
(5) *Acinetobacter baumannii*	-0.15	2.3	0.135	-0.46	13.75	<0.001
(6) *Serratia marcescens*	-0.4	5.51	0.023	-0.57	8.82	0.004
(7) *Enterococcus faecium*	-0.17	1.66	0.203	-0.1	0.74	0.393
(8) *Staphylococcus haemolyticus*	-0.27	4.22	0.045	-0.51	8.54	0.005
(9) *Staphylococcus aureus*	-0.27	4.28	0.043	-0.44	7.2	0.01
(10) *Streptococcus mitis*	-0.28	2.94	0.092	-0.45	5.2	0.026
(11) *Streptococcus pneumoniae*	-0.21	1.73	0.194	-0.24	1.64	0.205
(12) *Streptococcus sanguinis*	0.04	0.13	0.719	-0.23	1.95	0.168

Table A2: The linear model coefficients and associated ANOVA result for each individual species in the experiment at both Tryptone Water environments. The species coefficients (SC) are given for each of the species (species number, 1 – 12) in Tryptone Water. The ANOVA results (*F*-test and *p*-value) are also presented.

Species	Mixed			Constrained		
	SC	F	p	SC	F	p
(1) ***Pseudomonas aeruginosa***	0.0002	0.47	0.497	-0.020	4.69	0.035
(2) ***Stenotrophomonas maltophilia***	0.0012	25.16	<0.001	0.156	60.52	<0.001
(3) ***Burkholderia cepacia***	-0.0003	1.49	0.228	-0.087	9.88	0.003
(4) ***Achromobacter xylosoxidans***	-0.0003	1.48	0.229	-0.047	5.53	0.022
(5) ***Acinetobacter baumannii***	-0.0003	0.93	0.339	-0.092	9.55	0.003
(6) ***Serratia marcescens***	-0.0005	1.74	0.192	-0.07	10.79	0.002
(7) ***Enterococcus faecium***	-0.0005	1.55	0.218	0.074	8.4	0.005
(8) ***Staphylococcus haemolyticus***	-0.0005	0.63	0.432	-0.058	3.45	0.068
(9) ***Staphylococcus aureus***	-0.0009	1.23	0.273	-0.091	4.81	0.033
(10) ***Streptococcus mitis***	0.0006	0.33	0.57	0.059	12.39	0.001
(11) ***Streptococcus pneumoniae***	0.0007	0.72	0.398	-0.02	0.00	0.999
(12) ***Streptococcus sanguinis***	-0.0005	0.30	0.585	-0.103	7.59	0.008

Table A3: Coefficients and ANOVA for each individual species grown on the DHB media. The species coefficients (SC) are given for each of the species (species number, 1 – 12) in DHB. The ANOVA results (*F*-test and *p*-value) are also presented.

Isolate	Tryptone Water			DHB		
	EC	F	p	EC	F	p
dwrPa3	-0.032	4.33	0.042	0.015	2.69	0.107
dwrPa4	-0.022	0.01	0.922	0.017	0.10	0.754
dwrPa2	0.012	4.11	0.048	0.010	0.05	0.825
dwrPa5	-0.011	0.14	0.709	0.015	0.12	0.729
dwrPa6	-0.002	0.05	0.823	0.020	2.74	0.103
dwrPa7	-0.008	0.10	0.748	0.001	2.52	0.118
dwrPa8	-0.008	0.07	0.788	-0.001	2.32	0.133
dwrPa9	0.000	0.10	0.759	-0.020	4.65	0.036
dwr1	0.008	2.09	0.154	-0.010	1.49	0.227
dwrPa10	0.036	9.02	0.004	-0.015	3.81	0.056
dwrPa11	0.034	9.66	0.003	-0.013	1.47	0.231
dwrPa12	-0.006	0.22	0.641	-0.021	5.15	0.027

Table A4: Individual ecotype coefficients and the corresponding ANOVA results
The ecotype coefficients (EC) are given for each of the isolates in both Tryptone Water and DHB. The ANOVA results (*F*-test and *p*-value) are also presented.

Type of carbon source	Carbon source	Number of monocultures able to utilise substrate
Negative	Negative	0
Ester	**Pyruvic acid methyl ester**	**4**
Phosphorylates	Glucose-1-phosphate	0
	D,L-α-glycerol phosphate	0
Polymer	**Tween 40**	**4**
	Tween 80	**4**
	α-cyclodextrin	0
	Glycogen	0
Carbohydrate	**D-cellobiose**	**1**
	α-D-lactose	0
	β-methyl-D-glucoside	0
	D-xylose	**3**
	I-erythritol	0
	D-mannitol	0
	N-aceyl-D-glucosamine	**3**
Carboxylic acid	D-glucosaminic acid	0
	D-galactonic acid γ-lactone	0
	D-galacturonic acid	0
	2-hydroxy benzoic acid	3
	4-hydroxy benzoic acid	**3**
	γ-hydroxybutyric acid	**1**
	Itaconic acid	**1**
	α-ketobutyric acid	**2**
	D-malic acid	**0**
Amino acid	**L-arginine**	**3**
	L-asparagine	**3**
	L-phenylalanine	**3**
	L-serine	**2**
	L-threonine	0
	Glycyl-L-glutamic acid	1
Amine	Phenlethylamine	1
	Putrescine	**3**

Table A5: Single carbon sources present in the EcoPlate™. A list of the single carbon sources present in the EcoPlate™ and the number of monocultures that were able to utilise them.

	P. aeruginosa	*S. maltophilia*	*B. cepacia*	*A. xylosoxidans*	*A. baumannii*	*S. marcescens*	*E. faecium*	*S. haemolyticus*	*S. aureus*	*S. mitis*	*S. pneumoniae*
S. maltophilia	0.30										
B. cepacia	0.30	0.00									
A. xylosoxidans	0.56	0.63	0.63								
A. baumannii	0.50	0.47	0.47	0.19							
S. marcescens	0.56	0.53	0.53	0.25	0.07						
E. faecium	0.22	0.13	0.13	0.60	0.53	0.60					
S. haemolyticus	0.30	0.00	0.00	0.63	0.47	0.53	0.13				
S. aureus	0.40	0.13	0.13	0.69	0.53	0.50	0.25	0.13			
S. mitis	0.50	0.46	0.46	0.27	0.20	0.27	0.42	0.46	0.54		
S. pneumoniae	0.91	0.78	0.78	0.87	0.80	0.79	0.89	0.78	0.75	0.85	
S. sanguinis	0.50	0.47	0.47	0.19	0.00	0.07	0.53	0.47	0.53	0.20	0.80

Table A6: Distance matrix based on utilisation of each of the species in monoculture. A distance matrix calculated using Jaccard's distance coefficients based on the binary utilisation profiles of the 12 species in monoculture on the 31 singles carbon sources in Table A5.

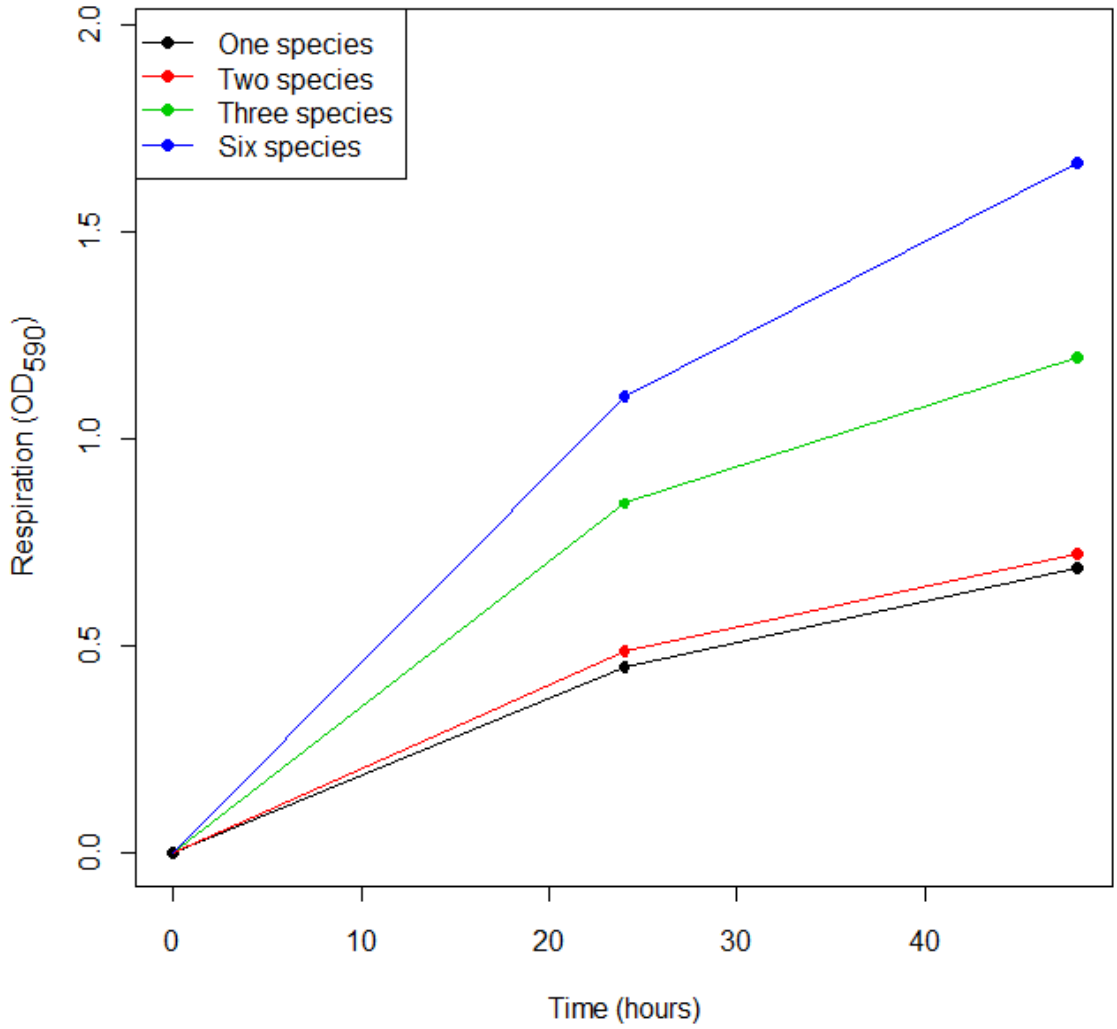

Figure A7: The mean respiration of the different richness levels at 24 and 48 hours post inoculation. The mean respiration (OD_{590}) measured at 24 and 48 hours post inoculation at each species richness level.

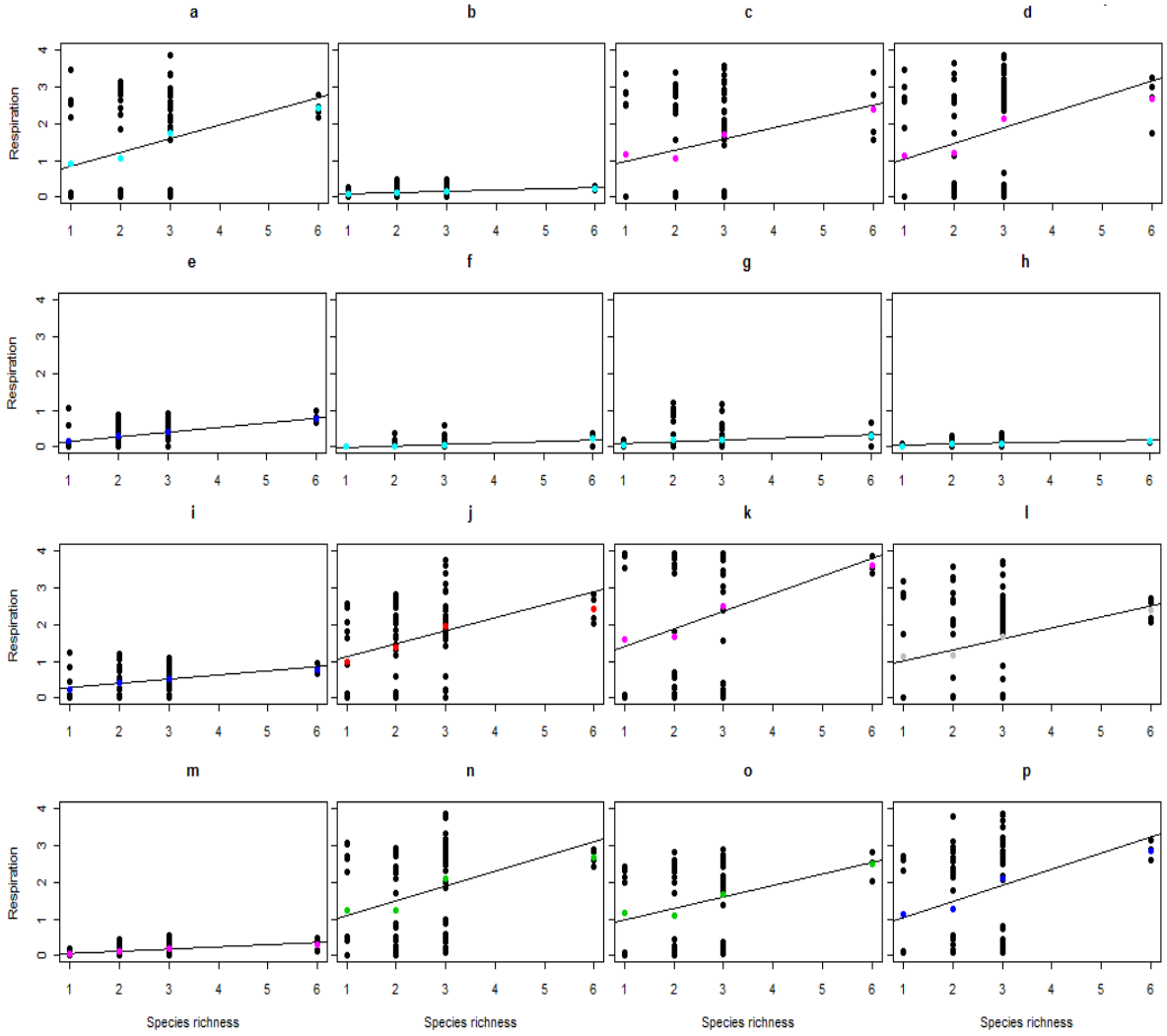

Figure A8: The respiration of all the microcosms at each richness level and single carbon source. The respiration (OD_{590}; y-axis) for each microcosm at all four richness levels (x-axis). Each plot represents a different single carbon source. The black dots represent the individual microcosm; the coloured dots represent the mean respiration at that richness level. The linear regression line (solid black line) is shown for each single carbon source: a = 4-hydroxy benzoic acid, b = α-ketobutyric acid, c = arginine, d = asparagine, e = D-cellobiose, f = D-malic acid, g = γ-hydroxy butyric acid, h = itatonic acid, I = N-aceyl-D-glucosamine, j = pyruvic acid methyl ester, k = phenylalanine, l = putrescine, m = serine, n = Tween 40, o = Tween 80, p = D-xylose.

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"I've been reading Fifty Shades of Grey*; this is way more complicated…"*

Anon.