University of Dundee

DOCTOR OF PHILOSOPHY

Deciphering the contribution of root hairs in barley to the structure and function of the rhizosphere bacterial microbiota

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Senga Robertson-Albertyn

University of Dundee

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Declaration

The results presented here are of investigations conducted by me. Work other than my own is clearly identified with references to relevant researchers and/or their publications. I hereby declare that the work presented here is my own and has not been submitted in any form for any degree at this or any other University.

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µl	microlitre
µm	micrometre
16S	ribosomal subunit (S = Svedberg sedimentation coefficient = 10-13 s)
18S	ribosomal subunit (S = Svedberg sedimentation coefficient = 10-13 s)
3D	3 dimensional
A	adenine
AA	amino Acid
ABC	ATP-binding cassette
ACC	1-aminocyclopropane-1- carboxylate
AcN	acetonitrile
AMF	arbuscular mycorrhizal fungi
ANOVA	analysis of variance
ASV	amplicon sequence variants
ATP	adenosine triphosphate
BC	before Christ (time)
BC	Bray-Curtis (statistical analysis)
BH	Benjamini-Hochberg Procedure
Bi/bi	bacterial isolate
bp	base pairs
c	Class
C	Cytosine
Ca	Calcium
Ca (NO3)$_2$	calcium nitrate
CAP	canonical analysis of principal coordinates
CFU	colony forming unit
cM	centimorgan
cm	centimetres
CO$_2$	carbon dioxide
CoCl$_2$	cobalt chloride
CuSO$_4$	copper sulphate
DEN	Density
DES	DNA elution solution (ultra-pure water)

dH2O	distilled water
DNA	deoxyribo nucleic acid
dNTP	deoxy-nucleoside triphosphate
e.g.	*exempli gratia* "for example"
EC	Enzyme Commission
EI	electron impact
EMS	ethylmethane sulfonate
et al	*et alia* "and others"
EtOH	ethanol
EXU	exudates
f	family
FBA	flux balance analysis
FDR	false discovery rate
Fe III	iron +3
Fe-EDTA	Ferric Ethylenediaminetetraacetic acid
Fw.	forward (primer)
g	genus
G	guanine
g	grams (measurement)
GC Plot	guanine-cytosine Plot
GC-MS	gas chromatography mass spectrometry
gDNA	genomic DNA
GO	gene ortholog
h	hour
H_3BO_3	boric acid
HCN	hydrogen cyanide
Hv	*Hordeum vulgare*
i.e.	*id est* "in other words"
ID	identity
ISR	induced systemic resistance
ITS	internal transcribed spaces
JHI	James Hutton Institute
k	kingdom
KEGG	Kyoto encyclopaedia of genes and genomes

KH_2PO_4	potassium phosphate
KNO_3	potassium nitrate
KO	KEGG ortholog
l	litre
Lab	laboratory
LCO	lipo-chitooligomer
m	metre
M	molarity
MAMP	microbe associated molecular pattern
max	maximum
mbar	millibar
MBDSTFA	N-methyl-N-tert-butyldimethylsilyl-trifluoroacetamide
mbp	mega base pairs
$MgSO_4$	magnesium sulphate
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
$MnCl_2$	manganese chloride
MNU	N-methyl-N-nitrosourea
MRS	Man, Rogosa & Sharpe
N	nitrogen
N	number of
N, S, E, W	North, South, East, West
Na_2MoO_4	sodium molybdate
NGS	next generation sequencing
ng	nanogram
NH_4	ammonium
NH_4Cl	ammonium chloride
NO_3	nitrate
NTC	no template control
Nut	nutrient
O	order
^{o}C	degrees Celsius

OTU	operational taxonomic unit
P	phosphate
PBS	phosphate buffered saline
PCA	principle components analysis
PCoA	principle coordinates analysis
PCR	polymerase chain reaction
PE	paired-end
PERMANOVA	permutational multivariate analysis of variance
PGP	plant growth promoting
PGPR	plant growth promoting rhizobacteria
PHRED score	'Phil's read editor', quality score for bp in FASTQ files
PICRUSt	phylogenetic investigation of communities by reconstruction of unobserved states
pM	picomolar
Po	organic phosphate
pp	polypeptone
ppm	parts per million
PQQ	pyrroloquinoline quinone
PSB	phosphate solubilising bacteria
QF	Quarryfield
QIIME	quantitative insights into microbial ecology
qPCR	quantitative polymerase chain reaction (real-time PCR)
Quast	QUality ASsesment Tool
R2A	Reasoners two agar
RAST	rapid annotation of microbial genomes using subsystems technology
RHL	*rhl1.a*
RHM	root hair mutant
RHP	*rhp1.b*
RNA	ribonucleic acid
rpm	rotations per minute
rRNA	ribosomal RNA
Rv.	reverse (primer)
s	second
SAR	systemic acquired resistance

SDW	sterilised deionised water
SEM	scanning electron microscopy
SOM	soil organic matter
Sp	"species" known genus, unknown species
Spp	"species", specifically all species in a given genus
SRCT	synchrotron radiation computed tomography
SynComs	synthetic communities
T	thymine
TCA cycle	tricarboxylic acid cycle (also known as the citric acid/ Krebs cycle)
TEA	triethylamine
TP	Tayport
TransAAP	TransportDB's Transporter Automatic Annotation Pipeline
TukeyHSD	Tukey Honest Significant Differences
U	uracil
UK	United Kingdom
UoD	University of Dundee
v	volume
VIGS	virus induced gene silencing
WGS	whole genome sequencing
WT	wild type
wUniFrac/	
WU	weighted UniFrac
$ZnCl_2$	zinc chloride
µmol	micromole

SUMMARY

The thin layer of soil surrounding and influenced by plant roots, termed the rhizosphere, defines a distinct and selective microhabitat compared to that of the surrounding soil: the bulk soil. The microbial populations that reside in the rhizosphere, commonly referred to as the rhizosphere microbiota, participate in a variety of interactions with their host plant ranging from parasitic to mutualistic relationships. Due to the contribution of the microbiota to pathogen protection and nutrient uptake the rhizosphere microbiota has emerged as a determinant of crop yield. Consequently, a better understanding of plant-microbiota interactions in the rhizosphere can pave the way for novel applications enhancing sustainable crop yield and global food security. Experimental evidence indicates that the host plant is the driver of, at least in part, the rhizosphere microbiota, but genetic relationships underpinning these interactions are not fully understood. Filling this knowledge gap will allow plant breeders to select novel crops which better interact with the soil biota and, at the same time, biotechnologists can profit from microbial genetic information to develop a new generation of inoculants for agriculture.

In this PhD project barley (*Hordeum vulgare* L.), the world's 4th and UK's 2nd most cultivated cereal, was used as an experimental model to dissect plant-bacteria interactions in the rhizosphere. The hypothesis that root hairs: the tubular outgrowths of the root epidermis, modulate the physical and chemical environment in the rhizosphere to facilitate the colonisation of members of the microbiota implicated in mineral uptake was tested. To test this hypothesis, three interconnected experimental approaches were developed:

By using 16S rRNA gene amplicon sequencing, it was demonstrated that the presence and development of root hairs are a determinant of nearly one fifth of the barley rhizosphere microbiota, with a bias for members of the order Actinomycetales, Burkholderiales, Rhizobiales, Sphingomonadales, and Xanthomonadales.

In order to gain further insights into the molecular basis of this differential recruitment, the project went on to investigate both the physical and chemical environment conditioned by root hairs. A pilot investigation of the rhizodeposition profiles using GC-MS of wild type and root hair mutants revealed that plant-

genotype dependent patterns among the 69 amino and organic acids were detected and a further 23 sugars and sugar alcohols detected, pointing at root secretion as an additional selective layer in the barley rhizosphere. Additionally, an amplicon sequencing survey of soil cores with different density, mimicking presence/absence of plant root hairs, revealed that this physical parameter is capable of triggering the differential enrichment of bacteria associated with the orders Bacillales, Burkholderiales and Xanthomonadales but not Actinomycetales. Thus, the physical perturbation of the soil environment alone cannot be the sole recruitment cue for the barley microbiota. However, it does indicate that soil density contributes, in part, to microbial recruitment.

Finally, to discern the full genetic potential of plant-associated bacteria, an indexed bacterial collection of the barley microbiota was constructed by isolating, on synthetic media, individual rhizosphere bacteria. A total of 85 isolates were further selected for full genome sequencing including members of the orders Actinomycetales, Flavobacteriales and Xanthomonadales. A comparative genomic approach was deployed to identify plant-growth promoting traits among 53 of these isolates.

This experimental work was complemented with a critical appraisal of efforts to communicate to and increase the awareness of the general public on the importance of the plant microbiota for global food security.

In the long term, the scientific outputs of this project can be deployed to devise novel strategies aimed at enhancing sustainable crop production in the UK and to globally and increase the awareness of the general public to global food security, particularly with the potential of the bacterial isolate library to be used in collaboration with multiple research groups investigating a range of microbial approaches to improve crop sustainability.

CHAPTER 1

INTRODUCTION

1.1 The plant microbiota as an extended host phenotype

The plant host and its associated microorganisms, termed the plant microbiota are considered to constitute a holobiont whose combined genes are often described as a hologenome (Rosenberg and Zilber-Rosenberg, 2018). This holobiont combines the adaptability and metabolic diversity of the heterotrophic microbiota with the complex cellular machinery of the autotrophic eukaryotic host (Bordenstein and Theis, 2015).

Laboratory-based studies have shown that the plant host can interact in a beneficial manner with individual members of the microbiota, commonly described as plant growth promoting rhizobacteria (PGPRs) (Beneduzi et al., 2012). PGPRs have been shown to perform some important biological functions including indirect pathogen protection resulting from microbial competition, microbial secretion of compounds that induce systemic acquired resistance (SAR) and induced systemic resistance (ISR) plus enhanced nutrient acquisition due to microbial metabolic activities such as nitrogen fixation and organic phosphate solubilisation (Friesen et al., 2011, Loon, 2007, Pii, 2015).

With this in mind, the beneficial services that can be conferred by the microbiota in response to host mediated cues can be considered to be an extension of plant traits and as such translates as a research area that can be used as a tool in the management and improvement of crops.

To date, the key mechanisms driving the composition of the microbiota and the molecular interplay that occurs with plants remains to be fully elucidated experimentally (Rodriguez et al., 2019). Understanding the factors that dictate the assembly, dynamics and function of the microbiota is a research priority in order to harness the potential for applications in improving crop yields and reducing agrochemical inputs (Trivedi et al., 2017, Schlaeppi and Bulgarelli, 2015).

Considering the vast biodiversity, complexity and array of interactions that take place in the soil, achieving this will be a multidisciplinary and challenging task.

However, the advances in high-throughput sequencing technologies combined with increased computational resources, collectively referred to as metagenomics has been chief in pushing the limits of this field of research (Knief, 2014, Sharpton, 2014). By harnessing the potential of both classical and advanced microbial techniques such as flux balance analysis (FBA) and stable isotope probing (SIP) in combination with metagenomics, we are now rapidly pushing back the boundaries of our ability to unravel and harness plant-microbe interactions for crop sustainability (Thommes et al., 2019, Radajewski et al., 2000).

This chapter will introduce the main techniques that can be used to dissect plant-microbiota interactions at the root-soil interface. It will go on to discuss the recent findings obtained by the applications of these approaches as well as the knowledge gaps of this research field. It will conclude by introducing barley, the model plant used in this experimental work, and the aims and objectives of this project.

1.2.   How to study the plant microbiota?

The development of culture-independent techniques, combined with advancements in metagenomics, has allowed for the phylogenetic characterisation of microbial communities across a variety of environments with an unprecedented level of detail (Handelsman, 2004). Since the initial creation of these techniques in the late 1970s, where functionally annotating a whole genome sequence required in depth manual computational interpretation by teams of experts, the technical difficulties related to the analysis of specific phylogenetic markers have gradually declined, allowing this process to expand (Woese and Fox, 1977, Kyrpides, 2009).

In the current day, automated gene annotation tools can be used to functionally annotate genomes by using homology to existing annotations or by identifying conserved domains (Medigue and Moszer, 2007). Many of these tools use a single pipeline where new genome annotations are constructed based on existing data and then incorporated into the gene database to be used as a template for future gene annotations (Kulski, 2016).

Although this technique is a highly efficient way of functionally annotating whole genome sequence data it has some draw backs, the primary one being the ever

increasing number of annotated genomes that include a large proportion of mis-annotations (Schnoes et al., 2009). However, the improved development and accessibility of public repositories, where sequence information can be annotated at both a taxonomic and functional level coupled with the development of open source tools for data analysis have massively contributed to the expansion of this research field making this process a common 'lab practice' in the present day (Kulski, 2016).

The various methodological approaches that can be implemented to examine the rhizosphere microbiota will be discussed in this section, specifically, approaches based on the sequencing and analysis of microbial DNA.

Sequencing-based microbiota studies typically fall into two categories: (1) targeted amplicon sequencing, which focusses on selecting specific target genes of a given microbial community or (2) whole (meta) genome approaches, such as shotgun sequencing.

These approaches can provide not only a comprehensive overview of the potential functional roles of microbes in specific environments but also accurate taxonomic assignment of members of the microbiota and uncategorized bacteria isolated from soil (Oniciuc et al., 2018). By combining these highly useful analytical approaches a detailed picture of plant-microbe interactions in a variety of environments can be obtained.

1.2.1 Targeted amplicon sequencing

Targeted amplicon sequencing is a common approach to microbiota studies, this is conducted using sets of known phylogenetic markers to group organisms based on how phylogenetically similar they are to one another and organisms that have already been identified using public repositories (Bybee et al., 2011). A widely accepted approach (mainly due to the availability of large *in silico* datasets, which contain an ever increasing number of reference sequences) is to target the 16S rRNA (ribosomal RNA) gene, which contains 9 hypervariable regions (V1-V9) as a phylogenetic marker for bacteria and archaea (Mizrahi-Man et al., 2013) (**Figure 1.1**).
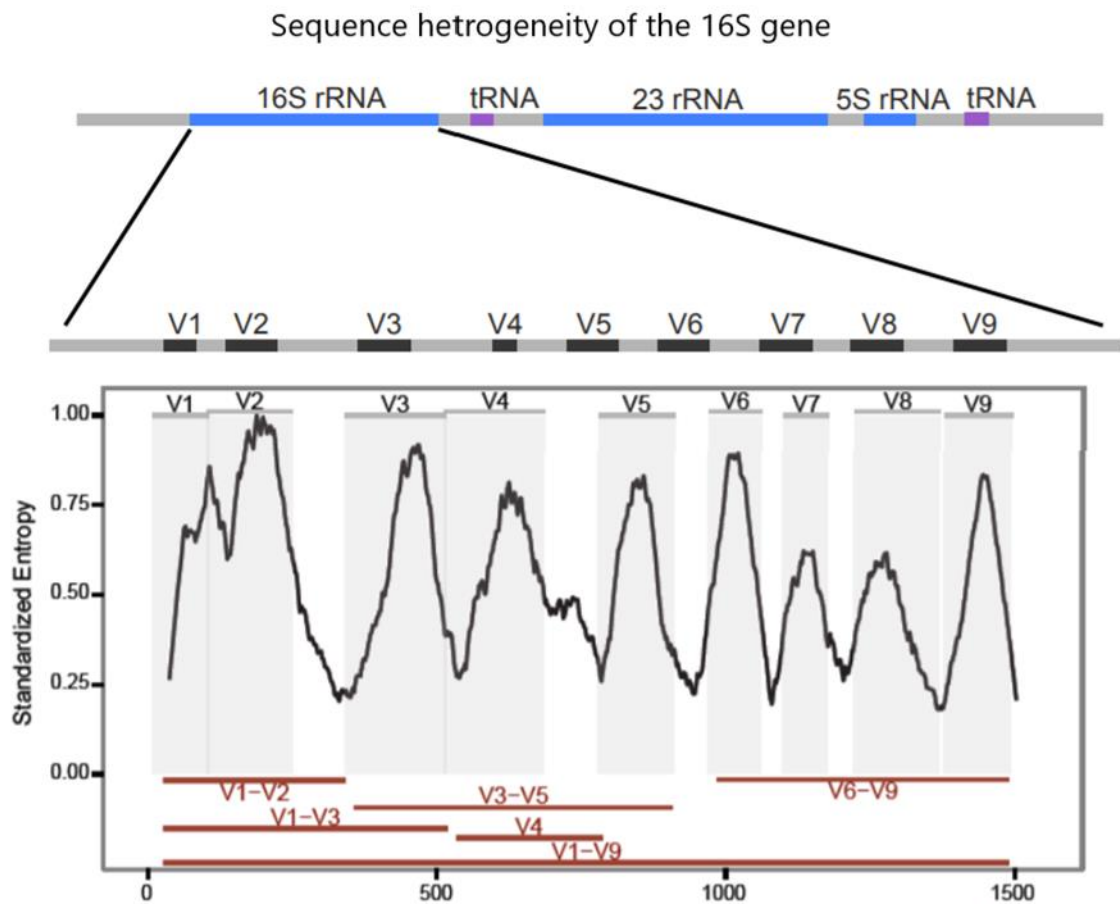
Figure 1.1 Hypervariable regions V1-V9 and conserved primer binding regions of the 16S rRNA gene. *The nine hypervariable regions of the 16S gene including Shannon entropy calculated from nucleotide variation at each base position along the 16S gene. Commonly sequenced regions are depicted by red lines. (Johnson. J, 2017)*

Sequence regions in ribosomal genes evolve very slowly, meaning that the majority remain largely conserved between species, allowing for the use of broad spectrum primers that will be able to capture the 16S region of almost all bacteria and archaea from a given environment (Clarridge, 2004). Importantly, the hypervariable regions V1-V9 are highly differentiated between species and contain rapidly evolving DNA, this provides a foundation for identifying organisms at a detailed enough taxonomic resolution to identify organisms up to genus level (Chakravorty et al., 2007). It is important to state that no single region can differentiate among all bacteria, as a result of this and where possible, analysis of at least two regions is advisable to increase data accuracy (Chakravorty et al., 2007).

The 16S rRNA gene sequencing approach is specific to bacteria and archaea and cannot be used for the community profile analysis of eukaryotic members of

the microbiota (Doudna and Rath, 2002). Instead, the 18S rRNA sequencing approach is typically employed; this targets the 18S rRNA gene of the small 40S eukaryote ribosomal subunit and its differentiation between species is based on the same principle as that of bacterial analysis (Hogan et al., 1984). If the target of a microbiome study is a fungal community internal transcribed spacer (ITS), phylogenetic marker profiling is typically employed (Bengtsson-Palme et al., 2013). The small ribosomal subunit of fungi is highly conserved, containing a very small proportion of non-conserved sequence regions between species (Raja et al., 2017). This makes taxonomic definition very challenging. Fortunately, the DNA sequences found between the genes encoding the ribosomal subunits of fungi display similar proportions of synonymous sequencing between species and a region of high variability for identification of organisms at more detailed taxonomic levels. (Schoch et al., 2012)

Irrespective of the kingdom that is targeted during a study, amplicon sequence data must then be analysed further *in silico* in order to remove artefacts from PCR and sequencing, to assign sequences to specific samples (this is typically carried out by the use of barcoded primers during amplification that can later be linked back to the source sample computationally), to cluster sequencing data into operational taxonomic units (OTUs) based on sequence similarity and to remove non-prokaryote OTUs typically derived from plant and invertebrate mitochondria or chloroplasts which are often amplified due to the sequence similarity with the 16S gene due to symbiotic coevolution (Beckers et al., 2016). OTUs are considered to be the 'building blocks' of the bioinformatics analysis of the microbiota and are used to reconstruct its taxonomic composition (Jünemann et al., 2017). However, alternative approaches based on unique sequencing variants, designated amplicon sequence variants (ASVs), are gaining momentum in microbiota studies. ASVs infer the biological sequences in the sample prior amplification/sequencing errors and distinguish sequences differing by as little as one nucleotide. ASVs define unique sequences reproducible/comparable across studies (Callahan et al., 2017)

Historically the database "Greengenes" was the most commonly used taxonomic repository for these studies. However, its use is becoming increasingly obsolete as it was last updated in 2013 (Balvočiūtė and Huson, 2017). Instead, the SILVA database is now more commonly used for OTU taxonomic assignment as it is

regularly maintained and sequence data is quality checked (deNBI, 2019). Following OTU assignment, their properties can be investigated (e.g., presence/absence, relative abundance, relatedness) then subjected to further ecological and statistical analyses which are aimed at identifying the ways in which environmental, chemical, physical and biological factors influence the composition of the bacterial communities in question. The selection of statistical analyses and comparison tools is a vital part of the construction of a bioinformatics pipeline and must be based on the objectives of the study. Ideally before setting up a study it is advisable to consider how it is planned in order to analyse it and how much data or samples will be required to make the study statistically robust.

1.2.2 Single genome and metagenomics sequencing

Elucidating the functionality of an entire microbial community such as the rhizosphere microbiota can be challenging due to the large variety of phylotypes in a given sample and the inherent sequencing bias towards the dominant organisms in a particular environment (Suchodolski, 2013). It is possible to take a simplified approach to the analysis of functional genes within a biological system by using a targeted approach to the analysis, by targeting amplicon sequences to genes of known, specific functions (functional markers) in a technique commonly referred to as gene-targeted metagenomics. This method is essentially the same as the technique outlined previously that is used to study the taxonomy of a microbial community but instead amplifies genes of known function and relies heavily on dedicated gene sequence databases (Kim et al., 2013). This approach means that genes of interest may not be yet available in these databases and does not allow for the identification of new functional genes, it also requires individual amplification and analysis of each gene of interest, arguably making this a relatively inefficient approach to take, particularly in large scale studies.

An alternative to gene targeted metagenomics is shotgun sequencing (shotgun metagenomics) which allows the entirety of the DNA collected in an environment to be sequenced (Quince et al., 2017). Following sequencing, bioinformatics are used to align lengths of sequence data (contigs) by layering based on homogeneity, ultimately building a picture of the community detailing both

taxonomy and functionality (Sharpton, 2014). This approach can also be used for individual members of the microbiota that have been isolated using traditional microbiological techniques and in a culture independent manner to examine entire communities. Shotgun metagenomics, also referred to as whole genome sequencing (WGS) is becoming increasingly popular, not only due to the ever reducing cost of the process and increase in available information in reference databases but due to its rise in popularity in the scientific community (Muir et al., 2016, Zhou et al., 2018).

By using consensus approaches to data analysis, not only can studies conducted in different environments be easily compared with other research projects but it also allows continued improvement and growth of publicly available analytical resources. However, the use of this approach does come with caveats.

In the past, functionally annotating a whole genome sequence required in depth manual computational interpretation by teams of experts (Kyrpides, 2009). In the current day, automated gene annotation tools can be used to functionally annotate genomes by using homology to existing annotations or by identifying conserved domains (Medigue and Moszer, 2007). Many of these tools use a single pipeline where new genome annotations are constructed based on existing data and then incorporated into the gene database to be used as a template for future gene annotations. Although this technique is a highly efficient way of functionally annotating whole genome sequence data it has some draw backs, the primary one being the ever increasing number of annotated genomes that include a large proportion of misannotations (Schnoes et al., 2009). In addition to this 30 to 50% of genes in a bacterial genome are yet to have had any functional annotation assigned (Hanson et al., 2009).

There are an array of tools that can be used to investigate the functionality of whole sequence data. One of the most commonly used tools is the SEED in combination with the annotation tool RAST (Rapid Annotation of microbial genomes using Subsystems Technology), the SEED is a continually updated integration of genomic data with a genome database where sequence reads can be submitted using a web interface and annotations are constructed by RAST that can then be analysed in the SEED viewer (Overbeek et al., 2014). A benefit of using the SEED for analysis is that gene orthologs are grouped based on their

category, subcategory then subsystem combined with a brief description of the role thought to be performed. This allows the user to easily search for specific functions of interest and to export the data generated for computational statistical analysis and visualisation. One drawback of using this tool is that exported data is typically based on EC (Enzyme Commission) numbers making the output difficult to merge with data from other annotation tools (McDonald and Tipton, 2014).

Another popular annotation tool is KEGG (Kyoto encyclopaedia of genes and genomes) which is a collection of 18 databases containing information regarding genomes, biological pathways, diseases, drugs and chemical compounds which, combined and mapped together are referred to as the KEGG pathway (Manyam et al., 2015). It is designed to assist with understanding high-level functions and utilities of biological systems and provides a number of outputs including EC numbers, GO (gene ortholog) terms, KEGG orthologs and KEGGBRITE hierarchies (which describe the functional hierarchy of biological organisms), this allows KEGG outputs to be merged with data from some other tools. However, a drawback of KEGG based assembly is that the process is not as simple as that of using RAST; instead, the user must use a bioinformatics package such as eggNOG to extract the annotations.

Other popular annotation tools are EFICAz (Enzyme Function Inference by Combined Approach) and BRENDA (BRaunschweig ENzyme Database) (via BLAST(Basic Local Alignment Search Tool)) which have been demonstrated to produce less annotations than the tools described earlier, in addition to this BRENDA ortholog data is built primarily from *Escherichia coli K-12* substr. MG1655 (the most-used model organism in modern biology) which may result in lower resolution outputs for different bacterial genera (Griesemer et al., 2018).

In addition to metabolic annotations there are prediction tools such as TransAAP (TransportDB's Transporter Automatic Annotation Pipeline) which has been developed to predict membrane transport and their substrates that are not always apparent based on standard metabolic annotation tool outputs (Elbourne et al., 2017).

Although there are advantages and disadvantages of using particular annotation tools it has been shown that by combining these tools, the total number of genes

and reactions annotated can be remarkably increased, which is an advantage overall (Griesemer et al., 2018). Genome modelling of *Clostridium beijerinckii* using a combination of the SEED, KEGG and RefSeq resulted in almost double the number of unique annotations compared with using one of these tools alone (Milne et al., 2011).

Despite these considerations the use of predictive metagenomics is a highly efficient way in which to, at the very least, perform initial screenings of a given environment and/or formulate hypotheses to be tested with other or additional methodologies such as wet lab verification of functionality.

1.3 Back to the future: the rising of indexed bacterial collection to study the plant microbiota

One of the perceived barriers severely impairing the advancement creating indexed bacterial collections was the fact that the rhizosphere microbiota is represented by soil-dwelling bacteria (see below, paragraph 1.6) that, historically, have been considered recalcitrant to *in vitro* cultivation (Eichorst et al., 2007). For instance, scientists have routinely been able to isolate "beneficial" members of the plant microbiota, often referred to as plant growth promoting rhizobacteria (PGPR), yet the molecular manipulation of the plant microbiota in a 'community context' has remained an elusive task (Islam et al., 2016, Kloepper et al., 1989). This one-microbe-at-a-time approach simply cannot recapitulate interbacterial interactions occurring at the root-soil interface. This is further exacerbated by the relatively limited lack of a detailed understanding of the metabolic capacities of plant-interacting bacteria, as opposed to what has been achieved with gut bacteria (Ridaura et al., 2013). However, a breakthrough study revealed that the majority (~58%) of the *Arabidopsis thaliana* (hereafter Arabidopsis) root microbiota, identified in sequencing surveys, can be associated with individual bacterial isolates (Bai et al., 2015). The establishment of such indexed bacterial collections will allow scientists to combine isolated members of the microbiota into 'microbial consortia' of known composition, often referred to as synthetic communities (SynComs) (Herrera Paredes et al., 2018). These can be transplanted into germ-free plants and their impact on given plant phenotypes properly discerned. Combined with whole genome information, this a powerful tool to formulate testable hypotheses and gain novel insights into plant-microbe

interactions.

Perhaps not surprisingly, this experimental approach is taking centre stage in microbiota science. In Arabidopsis, microbial consortia have successfully been adopted to identify the host genetic determinants of the leaf-associated communities and demonstrate that components of the plant immune system shape the root microbiota (Bodenhausen et al., 2014, Hacquard et al., 2017). However, this approach has not yet been adopted for critical crops such as small grain cereals, primarily due to the lack of indexed bacterial collections for these species.

1.4 The microbiota thriving at the root-soil interface

Soil has been described as the most complex, diverse and important biomaterial on Earth (Young and Crawford, 2004). The complexity of soil arises from the fact that it is the interface between the hydrosphere, lithosphere, atmosphere and biosphere (Montgomery et al., 2000). Soil is considered to be a three-phase system consisting of solid minerals and organic matter; and a porous phase which consists of both the gas and liquid phase in the form of water (Totsche et al., 2018). Soil hetrogeneity is influenced by a number of factors including; the nature of the mineral substrate; the physical features such as structure, texture and density; and by biological interactions between and within groups of organisms such as bacteria, fungi, algae, plants, insects and small mammals (Cardoso et al., 2013). Soil is a hub of biodiversity proposed to house over 25% of all living species, and has been estimated that over one billion bacteria consisting of over 20,000 species are present in a single gram (Bach and Wall, 2017, Swami, 2019, Roesch et al., 2007). As a result of the high degree of the heterogeneity in the soil nutrients, water and other resources become compartmentalized (Fransen et al., 2001). Soil biodiversity is best described by the presence of a plethora of microhabitats varying in a great range of factors such as physicochemical properties, soil structure, energy sources, and carbon sources (Totsche et al., 2018).

One of the most peculiar of these microhabitats is represented by the rhizosphere. The rhizosphere was first described in 1904 by German agronomist Lorenz Hiltner originating from the Greek word "rhiza", meaning root (Hartmann et al., 2008). Hiltner described the rhizosphere as "the area around a plant root

that is inhabited by a unique population of microorganisms influenced by the chemicals released from plant roots" (Hartmann et al., 2008).

A number of studies have proposed that the bacterial populations thriving in the rhizosphere and their associations with plant roots are primarily derived from the soil and are modulated by the plant host (Hartman and Tringe, 2019, Bulgarelli et al., 2013) (**Figure 1.2**).
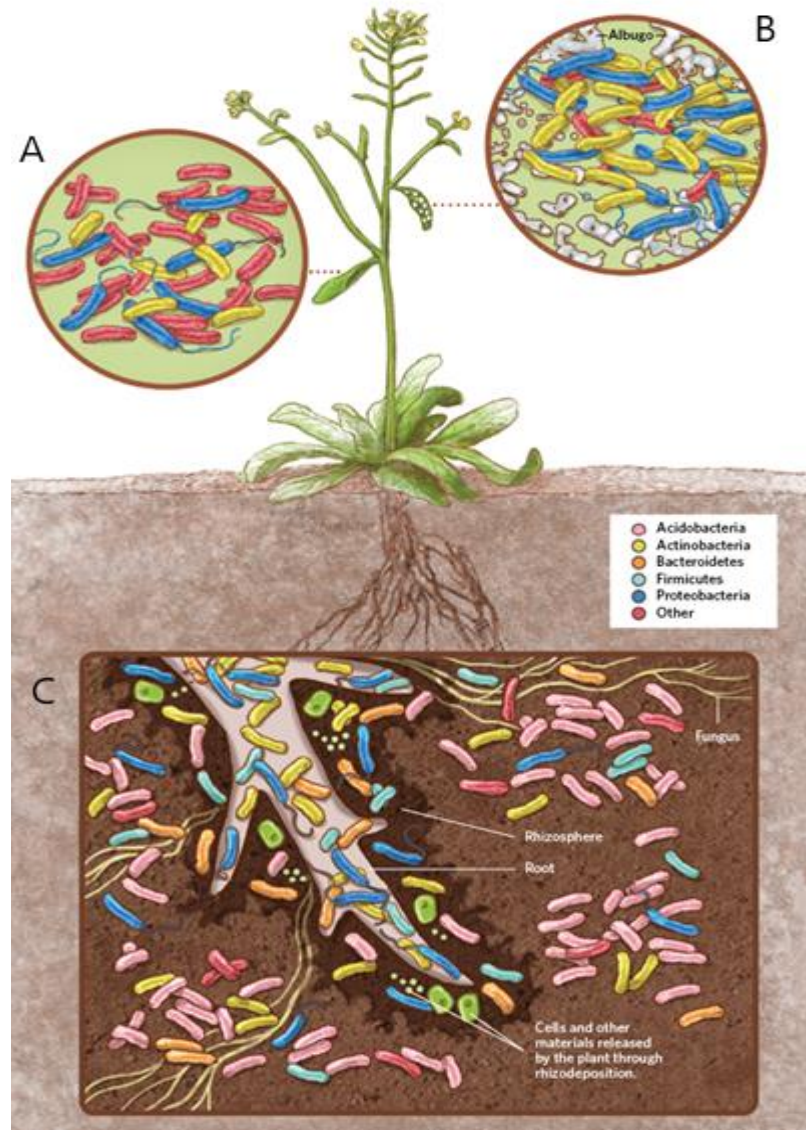
Figure 1.2 The plant microbiome. The microbiome of a plant contains microbial communities, including bacteria that can interact directly with the plant. Bacteria populate all regions of a plant from the leaf surface to the rhizosphere and even within the root in the form of endophytic bacteria. (A) the phyllosphere and its microbiota , (B) The phyllosphere microbiota with an oomycete pathogen infection, (C) the soil and rhizosphere microbiota (Bulgarelli. D, 2018)

An example of this is in a study using Arabidopsis as a host, which revealed that different soil types that were sampled across numerous sites spanning two

continents helped to shape the root associated bacterial microbiota in a qualitative and quantitative manner (Bulgarelli et al., 2012, Lundberg et al., 2012). To corroborate these findings, controls in the same studies using surface sterilized seeds in axenic conditions did not detect a root associated microbiota. Furthermore, it has been observed that rice seedlings that had been germinated under axenic conditions and appeared to be sterile can recover and recruit a distinct microbiota within just days of being transplanted to soil (Edwards et al., 2015).

It has also been suggested that the soil, at least partly, may be the origin of the microbiota colonising the above ground plant organs but it is widely accepted that environmental parameters have a far more profound impact on the above ground plant microbiome (Vorholt, 2012, Zarraonaindia et al., 2015, Wallace et al., 2018).

Considering these observations, it is clear that, to varying degrees, the soil is the starting inoculum for the whole plant microbiota. With this in mind it is important to consider what edaphic factors are drivers of the structure and function of the microbiota, known as the "soil effect".

1.5 Edaphic factors shaping the microbiota

There are a multitude of factors which dictate the quality of soil and, in turn the impact this has on the microbiota including (but not limited to) geography, cultivation practice, organic and inorganic soil content, compaction, chemical inputs and climate (Bünemann et al., 2018, Schreiter et al., 2014). A study of field grown lettuce (*Lactuca sativa*) in three different soil types that had been exposed for over a decade to the same climatic conditions and cropping history revealed distinct bacterial community compositions (Schreiter et al., 2014). However, despite differences to the structure of the microbiota, the rhizosphere microbial profiles were more alike than they were to their respective bulk soils. Furthermore, several of the bacterial genera that were significantly enriched (Pseudomonas, Rhizobium, Sphingomonas and Variovorax) were present in all three rhizospheric samples. This study indicates that the "soil effect" drives the initial source from which microbial recruitment is initiated but that this is then modulated by the activity of plant roots in the formation of the rhizosphere and its microbiota. A 2013 study investigating the impact of soil compaction on the

microbiota showed that compacted soil significantly reduced microbial abundance, increased diversity, and persistently altered the community structure of the microbiota (Hartmann et al., 2013).

Interestingly, it has been demonstrated that it is not only compaction which can dictate soil density, particularly at the root soil interface. In a study using synchrotron technology (a synchrotron accelerates electrons to extremely high energy and makes them change direction sporadically, these electrons produce X-rays as they circulate around the synchrotron) to investigate the impact of root hairs on both soil structure and gas and liquid exchange it was revealed that the rhizosphere of barley with no root hairs was far denser than that of barley with normal root hair development (Koebernick et al., 2017). This finding further compounds that although soil is the starting microbial inoculum, rhizospheric microbial recruitment is mediated by both the plant and the immediate local environment.

1.6 The 'rhizosphere effect' on the microbiota

It is commonly understood that a suite of organic compounds are secreted by plant roots in to the surrounding soil, they consist of low-molecular-weight organic compounds that are freely and passively released by the roots and mucilage such as sloughed off cells (Nguyen, 2009). This process is typically referred to as rhizodeposition and creates a distinct environment by modifying the physical, chemical and biological properties of the soil surrounding the roots (Jones et al., 2009) (**Figure 1.3**).

The ability of copiotrophs (fast growing microorganisms with higher nutritional requirements that thrive in carbon rich environments) to utilise the carbon released by the process of rhizodeposition may represent a competitive advantage which could enable them to outcompete oligotrophs (slow growing microorganisms with low nutritional requirements and typically low population density) (Fierer et al., 2007). Consistently, a shift in the composition of the rhizosphere microbiota is observed when there are changes to the availability of low molecular weight carbon compounds. For example, when three soils were altered using low molecular weight carbon substrates (representing a selection of common rhizodeposits such as glucose, glycine and citric acid) a proportional increase in the population density of Betaproteobacteria, Gammaproteobacteria

and, to a lesser extent, Alphaproteobacteria was observed (Eilers et al., 2010). The characteristics of the rhizosphere environment allow microorganisms to be either promoted or inhibited, ultimately contributing to the establishment of the rhizosphere microbiota (Mendes et al., 2013). Although rhizosphere ecology is primed by the process of rhizodeposition it is also shaped by proton and gas exchange combined with the physical structure of the soil and the impact that plant roots play in water and nutrient availability (Hartman and Tringe, 2019).
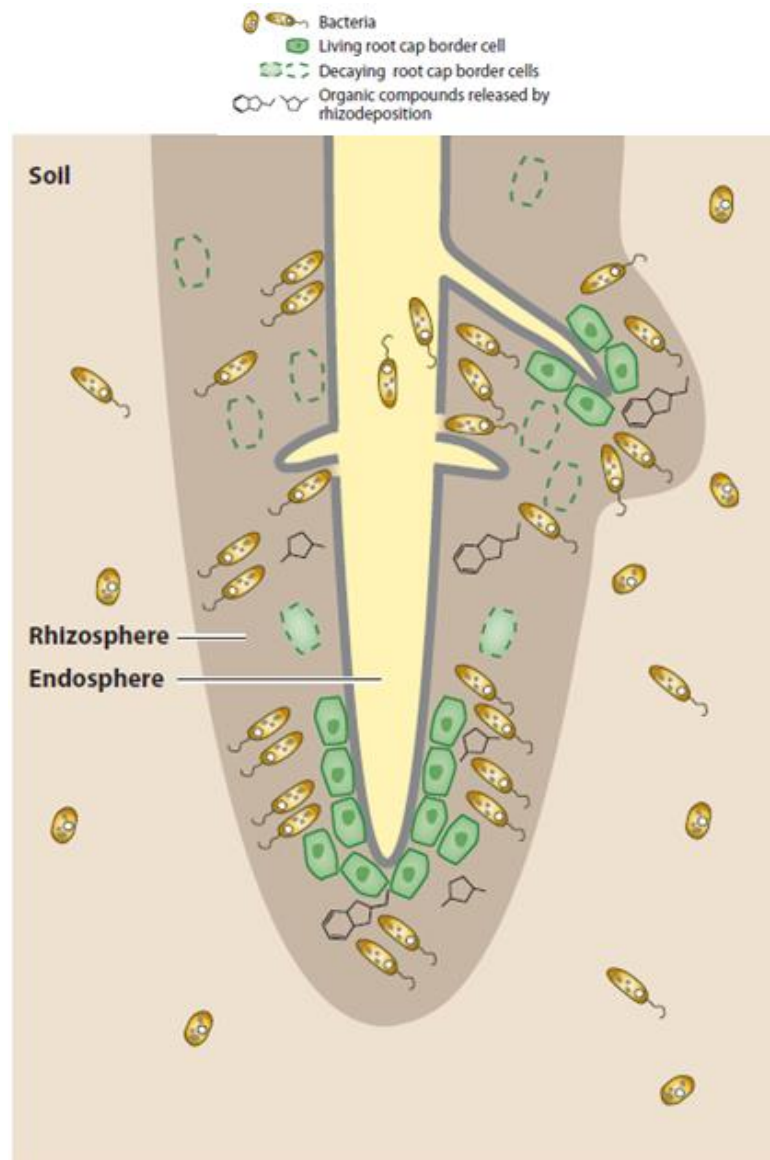
Figure 1.3 The rhizosphere. The rhizosphere is composed of plant derived organic compounds and decaying root cap border cells which provide a nutrient rich environment for the bacterial rhizosphere microbiota. (Bulgarelli et al., 2013)

Proton release to facilitate nutrient uptake can significantly impact the surrounding pH, this is important because pH plays a key role in microbial soil dynamics (Berg and Smalla, 2009). A primary characteristic of the rhizosphere is

the distinct composition of the microbiota which features an increase in microbial activity, density, genetic exchange and reduced biodiversity when compared to unplanted soil (Hacquard et al., 2015, DeAngelis et al., 2009, Kent and Triplett, 2002).

The microbial shifts that occur between the rhizosphere and soil have been widely reported, so much so that the term "rhizosphere effect" is commonly used terminology.

Across a variety of plant species including Arabidopsis, barley and wheat these shifts have been shown to affect nine bacterial phyla in particular; Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Firmicutes, Gemmatimonadetes, Proteobacteria and Verrucomicrobia dominate soil biomes (Alegria et al., 2016). However, the taxonomy of the root and rhizosphere microbiota is much narrower, specifically by a proportional increase of Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria, and a decrease in the abundance of other dominant members of the soil biota (Bulgarelli et al., 2013). Moreover, at a higher taxonomic resolution, further enrichment can be observed, the enrichment of members of the taxonomic families Comamonadaceae (Betaproteobacteria), Pseudomonadaceae (Gammaproteobacteria) and Streptomycetaceae (Actinobacteria) are a recurring and distinctive feature of the microbial communities thriving at the root-soil interface and are frequently observed to discriminate between plant- and soil-associated habitats (Hacquard et al., 2015).

The discrimination between plant- and soil- associated bacterial communities continues at lower taxonomic ranks (OTU) where microbial profiles continue to specialize further; this taxonomic variation starts to reveal where bacterial recruitment defines species-specificities and, within the same host species, 'host individualities' of the plant microbiota which begins to unravel this distinction between the microbial communities thriving in the rhizosphere of different plant hosts in a variety of environmental conditions.

The combination of these observations adds further weight to the proposition that the establishment and composition of the rhizosphere microbiota, which is distinct from that of other microbial habitats is mediated, at least in part, by the physical and chemical composition of the rhizosphere.

Therefore, elucidating plant determinants of the rhizosphere and governing plant microbe interactions in this environment is becoming an urgent task in both basic science and translational applications; for example, reducing the application of agro chemicals, which are both environmentally and economically expensive, or improving soil fertility to increase the availability of arable land.

1.7 Barley as a model to study plant-microbiota interactions in the rhizosphere

It is proposed that the domestication of crops started over 10,000 years BC when farmers began to save seeds from wild plants with preferred traits for the next generation (Badr et al., 2000). Over time this process increased yields, improved fertility, changed plant architecture to reduce lodging (near ground stem bending), changed the shape and size of seeds, prevented seed shattering and created adaptations to flowering times ultimately converting wild crops in to the domesticated crops that are relied on in the present day (Schmid et al., 2018, Zohary and Hopf, 1988). Barley (*Hordeum vulgare* L.*)*, wheat (*Triticum aestivum* L.*)* and rye (*Secale cereale* L.*)* belong to the Poaceae family, of which all grasses belonging to this family are believed to have evolved from the same common ancestor (Devos, 2005).

In the present day barley is the UK 2nd and World's 4th most cultivated cereal. It has considerable importance as a key component in animal feed and is becoming more popular in the health industry due to its beta glucan content (Delaney et al., 2003, Newton et al., 2011). In Scotland (United Kingdom) barley is of significant economic importance with the whisky industry generating £5.5 billion towards the UK economy.

At the same time, barley represents an excellent model for genetic research because of its diploid nature and the large collections of mutants, accessions and experimental populations (Dockter et al., 2014) . Furthermore, the barley genome recently became available to the scientific community which can also benefit from barley-tailored, cutting-edge experimental tools and resources such as genome editing and speed breeding protocols (Mascher et al., 2017, Lawrenson et al., 2015, Watson et al., 2018). It is of note that, given the similarities in the vegetative and reproductive biology as well as cultivation practices, barley is a better proxy

(compared to rice and maize) for other small grain cereals, such as the global staple crop wheat.

1.8 Root hairs shape the rhizosphere environment

Root hairs are long tubular outgrowths that are composed of root epidermal cells termed trichoblasts that emerge just behind the root elongation zone (Holz et al., 2018, Jungk, 2001). In cereals, including barley, 20 to 90 root hairs emerge per mm of root length and can project up to 1m in to the soil (Gahoonia et al., 1997, Brown et al., 2012b). Root hair formation is of vital importance to flowering plants: they aid water and nutrient uptake, increase root surface area increasing this uptake further and assist with soil anchorage (Czarnes et al., 1999, Gilroy and Jones, 2000, Grierson and Schiefelbein, 2002). Recent studies have also revealed that root hair morphology can have a direct impact on the density of the surrounding soil, affecting the size and frequency of pores and channels, in turn affecting water and gas availability in the rhizosphere (Koebernick et al., 2017). Moreover, root hairs are the main site of the exudation of small carbon-based compounds and have high cell turnover, as a result this process, described as rhizodeposition, enhances carbon enrichment in the rhizosphere (Holz et al., 2018).

During the 3.5-year period this project has been undertaken barley has been used as a model as a model to dissect the recruitment cues of the microbiota thriving in association with crop plants. These studies revealed that more than 94% of the annotated sequences retrieved from the barley rhizosphere indicate a dominant role that these microorganisms play at the root-soil interface (Bulgarelli et al., 2015). In addition, work conducted in the lab demonstrated that different barley genotypes recruit distinct microbiotas which clearly pointed at the host genetic components as a driver of the rhizosphere microbiota. However, at the inception of this project a precise understanding of the individual barley traits shaping the rhizosphere microbiota was poorly understood.

1.9 Aims and objectives

The overarching aim of this body of work was to gain novel insights into the molecular basis of plant-bacteria interactions at the root-soil interface and

elucidate the potential contribution of the rhizosphere microbiota to plant growth, development and health.

It was hypothesised that root hairs modulate the physical and chemical environment in the rhizosphere to facilitate the colonisation of members of the microbiota implicated in mineral uptake. To test this hypothesis, the following objectives were pursued:

1)   Comparing the bacterial microbiota associated with barley genotypes with fully developed root hairs and chemically induced root hair mutations using a next-generation sequencing approach;

2)   Determining the impact of soil density, an edaphic parameter influenced by root hair presence, on the structure and composition of the bacterial microbiota;

3) Investigating the composition of rhizodeposits associated with the aforementioned wild type and root hair mutant barley genotypes;

4)   Developing a 'barley indexed bacterial collection' by isolating, on artificial media, culturable members of the barley microbiota then subjecting them to whole-genome sequencing, followed by applying a comparative genomic approach to determine the metabolic capacities encoded by these bacteria and their potential contribution to barley growth.

In the long term, the scientific outputs of this body of work can be deployed to devise novel strategies aimed at enhancing sustainable crop production in the UK and globally through microbial treatment approaches such as seed inoculation with PGPRs.

CHAPTER 2

ROOT HAIR MUTATIONS DISPLACE THE RHIZOSPHERE MICROBIOTA

This chapter has been adapted from the publication "Root hair Mutations displace the bacterial rhizosphere microbiota", Robertson-Albertyn et al, Frontiers in Plant Science 2017

2.1. INTRODUCTION

2.1.1 Rhizodeposition

A plant-based mechanism that shapes the rhizosphere is the process of rhizodeposition whereby plants release, through passive and controlled mechanisms, an array of organic compounds in the area surrounding the roots encompassing all material that is lost from plant roots including water soluble exudates, insoluble materials, lysates, decaying fine roots and gases such as CO_2 (Nguyen, 2009, Cheng and Gershenson, 2007). Not only does this shape the chemical and physical rhizosphere environment but it is also considered to be a major driver of the rhizosphere microbiota community structure, as the organic substrates released in to the soil environment act as a source of nutrition for the microbiota, which in turn promotes bacterial proliferation (Dennis et al., 2010, Bulgarelli et al., 2013).

For example, variations in rhizodeposition patterns across differing barley genotypes has been shown to correlate with specific metabolic abilities performed by their respective microbiotas (Mwafulirwa et al., 2016). This serves as an example of passive host genetic-mediated control of rhizodeposition which influences the rhizosphere microbiota.

2.1.2 Root hairs

A critical role is played by root hairs, not only in rhizodeposition due to cell turn over, being the main site of exudation and increasing exudate distribution but also

in the acquisition of scarcely mobile soil minerals (Singh Gahoonia et al., 1997, Brown et al., 2012b, Holz et al., 2018). In cereals, root hairs have been shown to be a major determinant of rhizosphere formation, specifically the area of soil organically enriched by the roots, and the function of the rhizosphere, specifically the metabolic activity that takes place at the rhizoplane and within the rhizosphere region (George et al., 2014, Delhaize et al., 2015, Pausch et al., 2016, Holz et al., 2018).

Compellingly, root hairs have been implicated in defining an evolutionarily conserved site for bacterial colonization in grasses. For example, the bacterium 3F11, isolated from the surface sterilized seeds of *Zea nicaraguensis* and with 99% identity to *Enterobacter asburiae* has been shown to colonize the root hairs of the grass and significantly improve phosphate solubilisation (Shehata et al., 2017). In addition, bacterium *Pseudomonas* sp. DSMZ 13134 has not only been observed to efficiently colonize the root hairs of either soil- or quartz sand-grown barley seedlings, whilst in competition with resident *Pseudomonas spp,* but it was also identified as inducing plant growth promotion effects (Buddrus-Schiemann et al., 2010).

Despite a multitude of studies which link root hairs, the rhizosphere, plant fitness and the rhizosphere microbiota it is still primarily unclear if and how root hairs can shape the bacterial communities in the rhizosphere.

2.1.3 Investigating the impact of root hairs on the barley rhizosphere microbiota

In this study, barley was used as an experimental model in order to gain novel insights into the role that root hairs play in shaping the community composition of the rhizosphere microbiota. A comparison was conducted between the bacterial communities thriving at the root-soil interface of two barley varieties with fully developed root hairs, Karat and Dema, and their mutant lines, which had been backcrossed and inbred to be nearly isogenic, and were either completely devoid of root hairs, designated *rhl1.a*, or whose root hair formation was interrupted at an early developmental stage, shortly after bulge formation, designated *rhp1.b* (Janiak and Szarejko, 2007).

In addition, in order to gain insights into the relationships between the plant and soil-mediated recruitment triggers of the rhizosphere microbiota, studies were

conducted in two distinct Scottish agricultural soils representative of barley growing areas varying in soil organic matter (SOM). Using high-throughput 16S Illumina MiSeq sequencing and established computational approaches, we have demonstrated that root hairs are a determinant of the bacterial community inhabiting the rhizosphere and that perturbations in their development can significantly impact on the recruitment of individual members and orders of the microbiota.

2.2. MATERIAL AND METHODS

2.2.1. Root hair morphology imaging (performed by Agnieska Janiak et al)

The seedlings of parent and mutant barley varieties were grown in aeroponic conditions. Prior to this the seeds were surface-sterilized then left overnight at 4°C to start germination. The following day, the seeds were transferred to glass tubes containing wet cotton bungs then stuck to a second tube using parafilm.

After 5 to 7 days of growth, root hair zones were analysed with Stemi 2000-C (Zeiss) stereoscopic microscope and AxioVision LE (Carl Zeiss) software. For scanning electron microscopy (SEM), 1 cm segments were taken from the root differentiation zone of 7-day old seedlings to be analysed. These were fixed in 3% glutaraldehyde in a 0.1 M sodium phosphate buffer, pH 7.2 for 24 hours. They were then washed three times with the 0.1 M sodium phosphate buffer, pH 7.2 and post-fixed in 2 % osmium tetroxide in a phosphate buffer for 2 hours.

After subsequent triple washing with a 0.1 M sodium phosphate buffer, pH 7.2, samples were dehydrated through an ethyl alcohol series (50%, 60%, 70%, 80%, 90%, 95% and 100%, 10 minute duration for each step). The samples were dried in a Critical Point Pelco-CPD2 using carbon dioxide then mounted on aluminium stubs with double-sided adhesive tape and sputter coated with gold in a Pelco SC-6 sputter coater. The samples were subsequently viewed and photographed using a Tesla BS 340 scanning electron microscope at 20 kV. Fomapan Type 400/120 film was used to record the images.

2.2.2. Plant genotypes and growth conditions

The barley varieties Karat and Dema alongside their isogenic mutant lines *rhl1.a* and *rhp1.b* respectively were selected for this study. The mutant genotypes were originally generated by the chemical treatment of Dema and Karat with N-metylo-N-nitrosourea (MNU) or a double treatment with sodium azide and MNU by the Department of Genetics, University of Silesia and provided by Agnieska Janiak (Janiak and Szarejko, 2007).

Barley seeds were surface sterilized by being agitated with 96-100% EtOH and then rinsed 4 times with sterile distilled water, they were then germinated aeroponically on 0.5% sterile distilled water agar plates for 3-5 days. Seedlings with comparable rootlet development were selected and individually sown in 12 cm diameter pots which had been pre-filled with soil. Two Scottish agricultural soils were used as substrates for this protocol; 'Quarryfield', collected near the village of Kingoodie, Scotland, UK (56° 27' 5″ N, 3° 4' 29″ W) and 'Tayport' which was sampled near the village of Tayport, Scotland, UK (56° 25′ 40″ N, 2° 52′ 58″ W). Soil analysis was conducted as a service of Yara UK Ltd. (Grimsby, UK) to determine the physical and chemical properties of both Tayport and Quarryfield soils. Both soils are sandy silt loams with very similar silt, clay and sand content, they also share the same pH of 6.2 and a similar phosphorous content. Tayport soil was observed to have a significantly higher potassium content of 429 ppm vs 162 ppm in Quarryfield, it also had a higher magnesium content of 292 ppm vs 156 ppm. Conversely Quarryfield soil displayed a higher calcium content of 2661 ppm vs 1786 ppm. These are summarised in **Table 2.1**

Pots were arranged in a randomized design and grown under controlled glasshouse conditions (18/14 °C (day/night)) temperature regime with 16-hour day length supplemented with artificial lighting to maintain a minimum light intensity of 200 µmol quanta m^{-2} s^{-1}). Pots were watered every 2 days with the application of 50 ml of deionized water to each.

A total of 5 replicates for each genotype (i.e., 5 individual pots) were maintained alongside 5 unplanted pots containing the same soil substrates used as 'Bulk' soil controls. In addition, 5 plants derived from unsterilized seeds showing no obvious signs of infection were maintained in order to investigate the impact of seed sterilization on the recruitment of the rhizosphere microbiota. The seedlings

were grown for 4 weeks post-transplantation, by which point the genotypes had reached early stem elongation; Zadoks stages 30-35 (Tottman et al., 1979)

Table 2.1 Soil properties of Quarryfield and Tayport soil provided by YARA UK Ltd.

		Quarryfield	Tayport
Organic matter (%)		5.0	2.9
Soil particles (%)			
	Silt	47.39	39.95
	Clay	11.28	12.29
	Sand	41.33	48.36
Soil texture		Sandy Silt Loam	Sandy Silt Loam
pH		6.2	6.2
Mineral content (ppm)			
	Phosphorus	99	101
	Potassium	162	429
	Magnesium	156	292
	Calcium	2661	1786

2.2.3. Bulk soil and rhizosphere DNA preparation

The full methodology for this study is shown in **Figure 2.1.** Plants were excavated from the substrate once growth had reached early stem elongation phase. The roots were separated from the stems using tweezers and a scalpel both sterilised using 100% EtOH. The above ground portion of the plants were dried at 70 °C for 48 hours and the dry weight recorded. The roots were vigorously agitated by hand to remove loosely adhering soil particles, leaving the rhizosphere soil remaining on the roots. For each plant, the first 6 cm of the root system and its tightly adhering soil layer, which is operationally defined as the rhizosphere, was collected and placed in sterile 50 ml falcon tubes containing 15 ml sterile Phosphate-buffered saline (PBS) solution. The tubes were then vortexed for approximately 30 seconds in order to dislodge and suspend the rhizosphere soil from the root. Using sterile tweezers, the roots were then transferred to a second sterile 50 ml falcon containing 15 ml sterile PBS solution. These were again vortexed for approximately 30 seconds in order to maximize the removal of

rhizosphere soil from the roots.
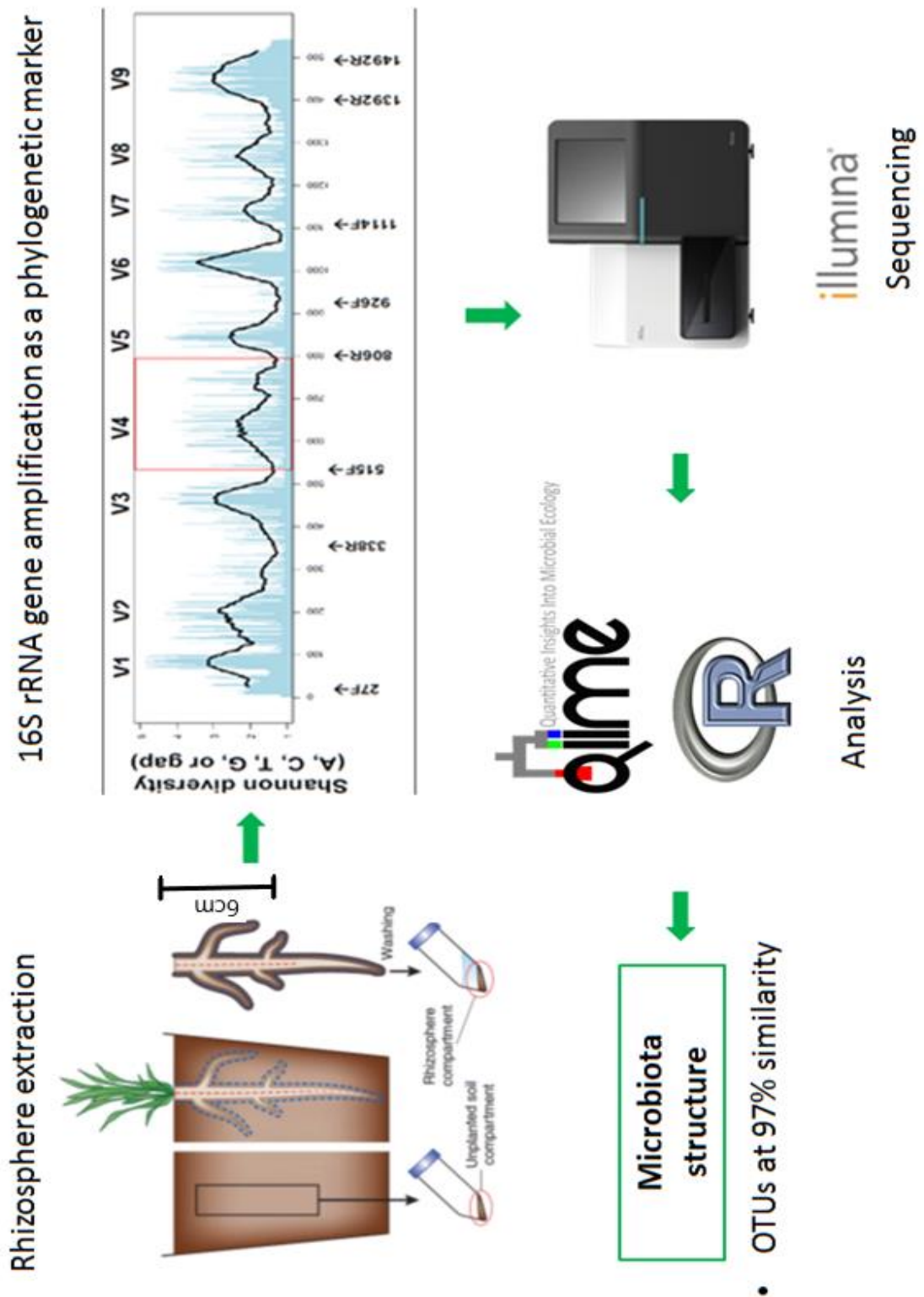
Figure 2.1 Experimental design of root hair mutant study. *The Different Barley genotypes were grown in 2 different Scottish agricultural soils in controlled conditions then sampled after 6 weeks (Hirsch and Mauchline, 2012). DNA was extracted using MP Biomedicals™ FastDNA™ SPIN Kit for Soil standard protocol then subjected to Illumina miseq sequencing following 16S PCR amplification of the V4 region(Lundberg et al., 2013) to form an extensive DNA library for analysis using Qiime and R.*

Subsequently, the roots were removed from the tube using sterile tweezers and the PBS buffers were pooled together into an individual falcon tube. The PBS/rhizosphere solution was then centrifuged at 1,500 g for 20 minutes to form a visible rhizosphere pellet. The supernatant was discarded, and the rhizosphere sample was frozen using liquid nitrogen then stored at -80 °C for future DNA extraction. The same process described above was used to process the bulk soil samples with the only variation being that a portion of the soil corresponding to the first 6 cm area explored by plant roots was excised using a sterile spatula.

The FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, USA) was used to extract DNA from individual samples following the manufacturer's instructions. Approximately 0.5 g of defrosted bulk and rhizosphere samples were resuspended in the Sodium Phosphate and MT buffers provided in the kit, transferred into the Lysis Matrix E tubes and homogenized using the Tissue Lyser II instrument (Qiagen, Hilden, Germany) at 20 s^{-1} rotations for 30 seconds. DNA samples were then eluted in 100 µl DES water and concentrations measured using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, USA). DNA samples were stored at -20 °C for future use.

2.2.4. 16S rRNA gene amplicon sequencing

Amplicon libraries were generated using selective PCR amplification of the hypervariable V4 region of the 16S rRNA gene using the PCR primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and
806R (5'-GGACTACHVGGGTWTCTAAT-3'). The PCR primer sequences were fused with Illumina flowcell adapter sequences at their 5' termini and the 806R primers contained 12-mer unique 'barcode' sequences in order to enable the multiplexed sequencing of multiple samples (Caporaso et al., 2011).

For each individual bulk and rhizosphere sample, 50 ng of DNA was subjected to PCR amplification using the Kapa HiFi HotStart PCR kit (Kapa Biosystems, Wilmington, USA).

The individual PCR reactions were performed in 20 µl final volume containing 4 µl 5X Kapa HiFI Buffer, 10 ng Bovine Serum Albumin (Roche, Mannheim, Germany), 0.6 µl 10 mM Kapa dNTPs solution, 0.6 µl of 10 µM solutions of the

515F and 806R PCR primers and 0.25 µl of Kapa HiFi polymerase. The reactions were executed in a G-Storm GS1 Thermal Cycler (Gene Technologies, Somerton, UK) using the following program: 94 °C (3 min), followed by 35 cycles of 98 °C (30 s) denaturing, 50 °C (30 s) annealing, 72 °C (1 min) elongation with a final elongation step of 72 °C (10 min). For each 515F-806R primers combination a no template control (NTC) was subjected to the same process. In order to minimize potential biases during the PCR amplifications, individual reactions were performed in triplicate. Furthermore, using the original DNA a minimum of 2 independent sets of triplicate reactions per primer combination were performed.

Aliquots of individual replicates and the corresponding NTCs were examined on a 1% agarose gel prior to purification. Only samples that displayed the expected amplicon size (380 bp) and whose corresponding NTCs were not detectable on agarose gel were taken forward to the purification step. Any samples not displaying the appropriate amplicon size or with contamination in their equivalent NTCs were discarded and the PCR amplification process was repeated.

Individual replicates of each sample were pooled together and purified using Agencourt AMPure XP kit (Beckman Coulter, Brea, USA) using a ratio of 0.7 µl AMPure XP beads to 1 µl of sample following the manufacturers guidelines. Following purification, the double stranded DNA concentration of 3 µl of each sample was quantified using Picogreen (ThermoFisher, UK) following the manufacturer's recommendations. Subsequently, individual barcoded samples were pooled in an equimolar ratio to create amplicon libraries for Illumina MiSeq sequence analysis.

All Quality Checking and processing was conducted by the Genome Technology group based at the James Hutton Institute, Invergowrie, Dundee as follows: Illumina-compatible library pools were quality checked using a Bioanalyzer (High Sensitivity DNA Chip; Agilent Technologies) and quantified using both Qubit and qPCR (Kapa Biosystems, Wilmington, USA). Denaturation and dilution were performed as recommended (Illumina guide 15039740 v01) using average (mean of Qubit & qPCR) concentration measurements.

Amplicon libraries were supplemented with 15% of 4 pM phiX solution. High-quality libraries were run at 10 pM final concentration on an Illumina MiSeq

system with paired-end 2 x 150 bp reads following established protocols for FASTQ file generation (Caporaso et al., 2012).

2.2.5. OTU Table and taxonomy matrices generation

QIIME, version 1.9.0 (Caporaso et al., 2010) was used to process the FASTQ files produced by the MiSeq analysis. Unless otherwise specified, the default parameters were used. Forward and reverse read files from individual libraries were decompressed and merged using the command join_paired_ends.py, with a minimum overlap of 5 bp between reads. Demultiplexing of overlapping paired-end (PE) reads and quality filtering was performed using the command split_libraries_fastq.py, setting a minimum PHRED score of 20. Only these high-quality PE reads were used to define Operational Taxonomic Units (OTUs) at 97% sequence identity.

OTUs were identified using the 'closed reference' approach against the chimera-checked Greengenes database (DeSantis et al., 2006) version 13_5. OTU-picking was conducted using the SortMeRNA algorithm (Kopylova et al., 2012). Single OTUs, i.e., OTUs with just one sequencing read in the entire dataset, were filtered in silico and discarded prior to further analysis. The OTU Tables generated from the two independent sequencing runs (each substrate was processed separately) were merged using the command merge_otu_Tables.py. The merged OTU Table was further processed *in silico* to remove OTUs assigned to chloroplasts and mitochondria, as these were likely to be the result of cross-amplification of host-derived DNA.

A taxonomy matrix, showing the number of reads assigned to individual bacterial phyla, was generated using the command summarize_taxa.py. The merged OTU Table and the taxonomy matrix were used in R for statistical analysis and figure preparation.

2.2.6. Data analysis

Statistical analysis was performed in R. Unless otherwise stated, the functions used were retrieved from the default installation of R or the R package Phyloseq (McMurdie and Holmes, 2013). For alpha diversity calculations, the OTU Table was rarefied at an even sequencing depth of 9,202 sequencing reads per sample which produced 6,083 unique OTUs. Observed OTUs and Shannon diversity

Index were calculated using the function "estimate richness". The function ggplot from the package ggplot2 was used for data visualisation.

The normality of data distribution was evaluated using the Shapiro-Wilk test for each dataset. For those whose Shapiro-Wilk test yielded a p value < 0.05 (the alpha level imposed to infer if the tested data was distributed normally), the impact of the soil effect was assessed using the function wilcox.test first. This was followed by non-parametric analyses of variance using the functions kruskal.test and the posthoc.kruskal.dunn.test from the package PMCMR (Pohlert, 2015). Conversely, an analysis of variance was adopted for datasets with a normal distribution.

To investigate the phyla differentially enriched between the bulk soil and the rhizosphere samples an analysis of the individual microbiome profiles was performed using phyla rarefied counts with the package ANCOM (Weiss et al., 2017).

For beta diversity calculations and in order to identify individual bacteria differentially enriched between microhabitats and genotypes, count data was not rarefied. OTUs present in low abundance (less than at least 5 counts in 20% of the samples used) were eliminated from the table. This process is a modification of an abundance threshold previously used with a comparable sequencing protocol for rice (Edwards et al., 2015). Adjusting this threshold to 20% of the samples allowed it to be possible to discard poorly reproducible OTUs, but still retain distinctive and unique features of the barley genotypes in the study. (i.e., the number of replicates per genotype in each soil type).

Operational taxonomic unit counts were transformed into relative abundances using the function transform_sample_counts for beta diversity calculations. Bray-Curtis, which is sensitive to OTU relative abundance and Weighted UniFrac, sensitive to both OTU relative abundance and phylogenetic assignment (Lozupone and Knight, 2005), distances were calculated with the "ordinate" function. The Adonis function from the package Vegan was used to partition distance matrices among sources of variation.

In order to identify individual bacteria differentially recruited between microhabitats (i.e., bulk and rhizosphere) and barley genotypes a differential analysis of the count data using negative binomial generalized linear models

using the package DESeq2 (Love et al., 2014) was implemented. Using the function DESeqDataSetFromMatrix raw OTU count data and sample information was converted into a DESeq object. The DESeq function was also used to perform differential analysis on separated OTU tables between soil types. The number of OTUs enriched in and discriminating between rhizosphere profiles and unplanted soil in each of the tested genotypes (designated, the 'rhizosphere effect') was initially determined for this analysis. Next, pair-wise comparisons between members of the same wild type-mutant pair (i.e., Karat versus *rhl1*.a and Dema versus *rhp1*.b, respectively; designated 'sample effect') were conducted.

In order to consider the effect of the barley genotype on the microbiota to be robust only an OTU that was identified as both a) significantly enriched in one genotype (or another) plus the pair-wise comparisons and b) significantly enriched in the rhizosphere of the same plant genotype compared to unplanted soil (i.e., the intersection of the 'sample effect' and 'rhizosphere effect').

The function count from the plyr package was used to determine the number of OTUs assigned to a given order in pair-wise comparisons between mutants and their corresponding wild type plants. The probability of randomly identifying 11 or more OTUs assigned to a specific order among bacteria differentially recruited between mutants and wild type plants was calculated using a cumulative hypergeometric probability. The function phyper was used for these calculations which takes into account the total number of OTUs assigned to a given order within the rhizosphere enriched OTUs (m), the number of rhizosphere enriched OTUs that are not assigned to that given order (n), and the total number of OTUs differentially recruited between genotypes (k). Venn diagrams were generated to visualise the differentially enriched OTUs using the package VennDiagram.

The scripts used to analyse the data and generate the figures for this study is available on GitHub at **https://github.com/BulgarelliD-Lab/Barley-RHM**. Data used for the analysis is available in **8.1.1. Appendix 1: Supplementary Database 1** and in the supplementary data of (Robertson-Albertyn et al., 2017)

2.3. RESULTS

2.3.1. Wild type and root hair mutant morphology

The *rhp1.b* genotype develops root hairs to the primordium stage only and tip growth is arrested after formation of the bulge. *rhl1.a* is completely devoid of root hairs and displays a disturbed pattern of root epidermis cells, with undistinguishable trichoblasts (**Figure 2.2**) (Marzec et al., 2013).
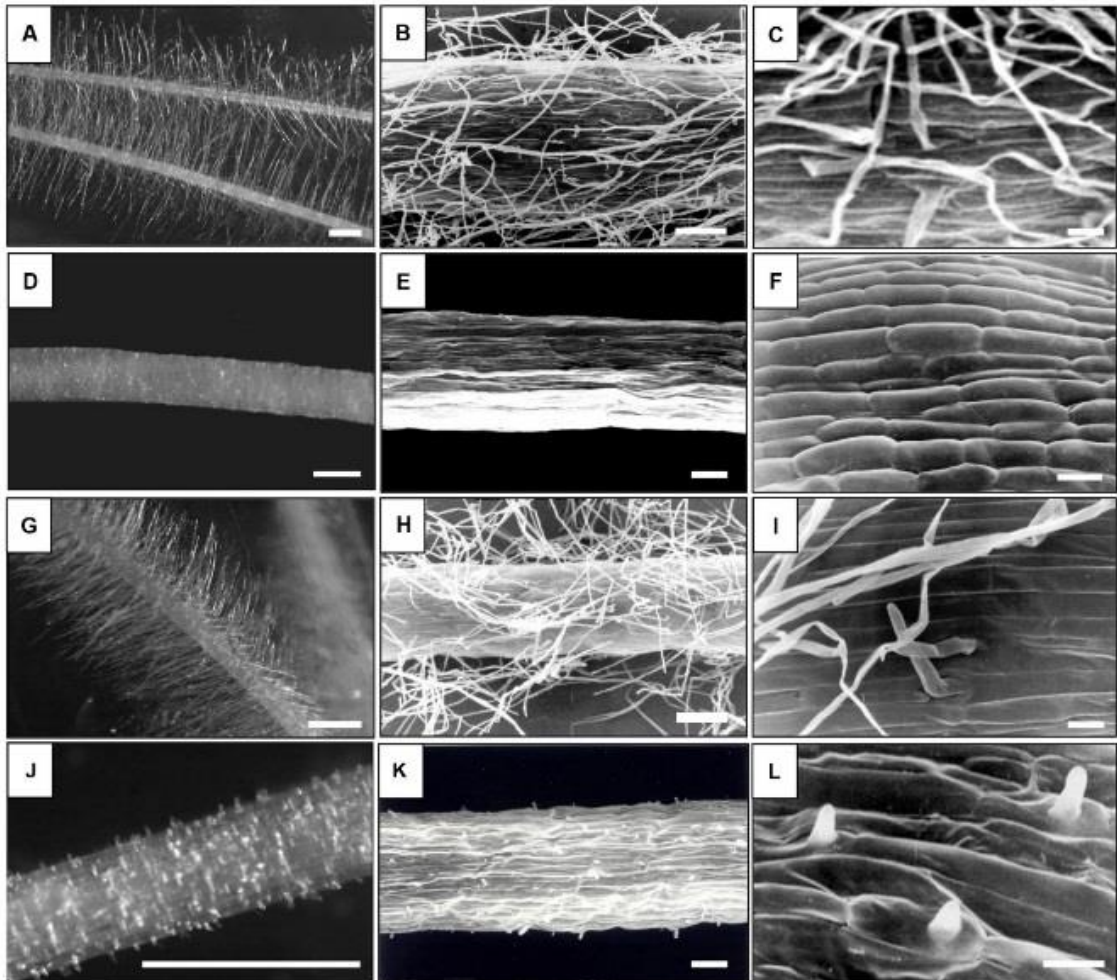
Figure 2.2. Root hair morphology of the genotypes used in this study. (A-C - 'Karat,')(D-F - *rhl1.a* mutant), (G-I -'Dema,') (J-L - *rhp1.b* mutant). Bars: (A,D,G,J) – 1 mm, (B,E,H,K) – 100 µm, (C,F,I,L) – 20 µm; (A,D,G,J) – Stereoscopic microscope images, (B,C,E,F,H,I,K,L) – Scanning Electron Microscope(SEM) images.

The mutations observed in *rhp1.b* and *rhl1.a* are recessive and have a monogenic inheritance. The genes responsible for the mutant phenotypes have been mapped on barley chromosomes 1H and 7H, respectively (Chmielewska et al., 2014). In this study mutant lines that were backcrossed twice with their corresponding wild type genotype and further self-pollinated 7 times were used.

2.3.2. Soil type impacts on DNA extractions, sequencing library properties and plant growth

Upon sampling it was apparent visually that there was a clear genotype effect on the plant fitness and rhizosphere formation, in particular, wild type plants had a larger rhizosphere than that of their isogenic mutants and longer root systems (**Figure 2.3**). The impact of soil type and the host genotype on the barley rhizosphere microbiota was investigated by examining the total amount of DNA recovered from the individual sample preparations, the proportion on DNA assigned to microbial OTUs and above ground dry weight.
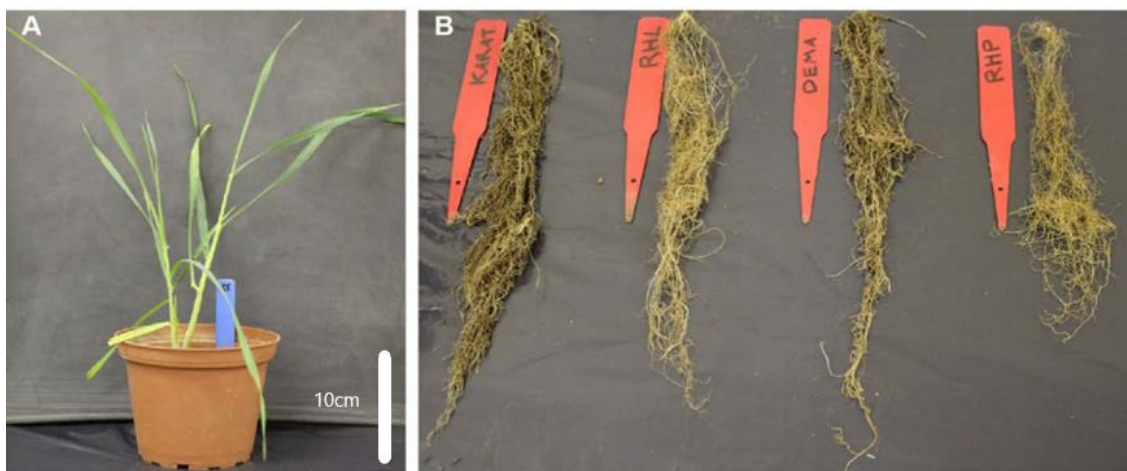
2.3 Representative above- and below-ground characteristics of the barley lines used in this study. *(A) Barley plants at the time of sampling (B) Whole root specimens of the indicated genotypes showing the adhered rhizosphere soil. Label width = 1.5 cm; Label length 13 cm.*

When examining total DNA recovered per sample it could be observed that a significantly larger yield of DNA was obtained from the Quarryfield preparations compared with samples obtained from Tayport soil (**Figure 2.4A**) (Mann-Whitney-Wilcoxon test, $P < 0.001$). However, when data was corrected for soil type, the barley host genotype displayed no impact on the total DNA isolated from the rhizosphere samples. A total of 5,847,887 high-quality sequencing reads were produced from amplicon sequencing of the 16S rRNA gene. After OTUs identified as being mitochondria or chloroplast derived were removed from the sequence data, the total number of high-quality sequencing reads reduced to 5,718,298, retaining almost 98% of the original high-quality reads for downstream analysis (mean per sample = reads; max = 830,673 reads; min = 9,202 reads) (**Figure 2.4B**)
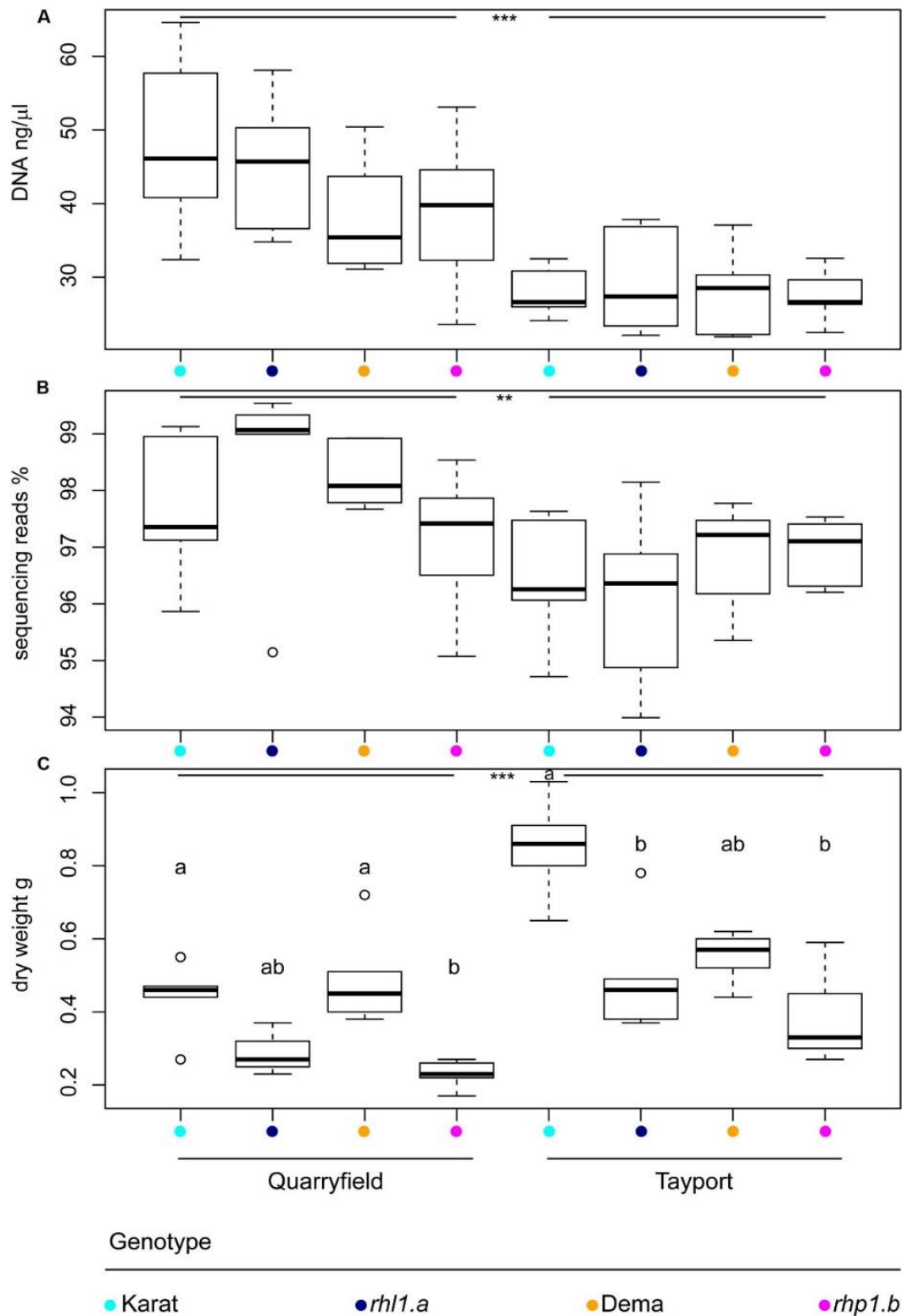
Figure 2.4. The soil type defines the properties of microbiota DNA and plant growth. *Average **(A)** DNA concentration of the rhizosphere samples, **(B)** proportion of sequencing reads assigned to microbial OTUs, and **(C)** the above ground biomass of the indicated plant genotypes grown in Quarryfield and Tayport soils. Higher and lower edges of the box plots represent the higher and lower quartiles, respectively. The bold line within the box denotes the median. Maximum and minimum observed values are represented by the whiskers. Dots denote outlier observations whose value are 3/2 times greater or smaller than the upper or lower quartiles, respectively. Asterisks denote statistically significant differences between soil types (***P < 0.001, **P < 0.01). In **(C)** different letters denote statistically significant differences between genotypes by Kruskal–Wallis non-parametric analysis of variance followed by Dunn's post hoc test (P < 0.05, BH corrected).*

Interestingly, it could be observed that there was a clear soil effect on the proportion of prokaryotic reads obtained from rhizosphere samples. The total DNA data of samples obtained from Quarryfield soil showed a significantly higher proportion of reads classified as bacteria and archaea compared to Tayport soil. However, also in this case, a host genotype effect was not detected (two-way ANOVA; soil effect $P < 0.01$, genotype effect $P = 0.77$, interaction term $P = 0.30$). In order to investigate if these observations mirrored the growth performance of the plants in the two soils the aboveground plant dry weight was measured at the time of sampling and was used as a proxy for plant growth. Interestingly, the average biomass of plants grown in Tayport soil was greater than that of the plants grown in Quarryfield soil which had a higher organic matter content (Mann-Whitney-Wilcoxon test, $P < 0$ .05). However, pair-wise comparisons between the tested genotypes failed to identify any coherent patterns between soils. For example, in Quarryfield soil, only the mutant *rhp1.b* displayed an above ground biomass significantly less than the Dema and Karat genotypes. Conversely, in Tayport soil, it was only the biomass of the Karat genotype that was significantly greater than the biomass of each of the mutant genotypes (**Figure 2.4C**) (Kruskal–Wallis and Dunn's post-hoc tests, $P < 0.05$, BH corrected).

2.3.3. Bacteroidetes and Proteobacteria dominate the wild type and root hair mutant rhizosphere microbiota

In total, 45 bacterial and archaeal phyla were identified in the sequence data, following rarefication at an even sequencing depth of 9,202 reads, 41 phyla were retained for downstream analysis (**8.1.1 Appendix 1: Supplementary Database 1**). However, the majority of these retained phyla had a relative abundance less than 1% with just 10 displaying a relative abundance greater than this.

Of these ten phyla, their cumulative mean relative abundance accounted for over 97% of the generated rarefied sequencing reads (**Figure 2.5**, **8.1.1 Supplementary Information 1: 8.2 Supplementary Database 1**). Irrespective of the barley genotype or soil type rhizosphere specimens were significantly enriched in members of the phyla Bacteroidetes and Proteobacteria versus unplanted bulk soil samples: the average relative abundance of these two phyla in the rhizosphere reached 15.3% and 56.9%, a three- and two-fold increase, respectively, compared to bulk soil samples (ANCOM analysis, $P < 0.05$, FDR

corrected) (**8.1.1. Appendix 1: Supplementary Database 1**).

Conversely, members of the phylum Acidobacteria appear to favour a bulk soil environment compared to rhizospheric: Acidobacteria average relative abundance declined from 20.2% in bulk soil to just 6.9% in rhizosphere samples (**8.1.1. Appendix 1: Supplementary Database 1**).

The proportions of the seven remaining dominant phyla showed very little fluctuation and remained stable across sample types (e.g., Actinobacteria average relative abundance shifted from 6.6 - 5.1% across bulk and rhizosphere samples, respectively) (**8.1.1. Appendix 1: Supplementary Database 1**), any fluctuations observed were not significant.

The observation of a clear rhizosphere effect compared to bulk soil is not unexpected, the rhizosphere has a lower pH than bulk soil. This is the result of plants releasing H ions to balance their charge due to the uptake of cations, in turn this contributes to shaping the chemical rhizosphere environment in which bacterial species can proliferate (Gahoonia and Nielsen, 1992).
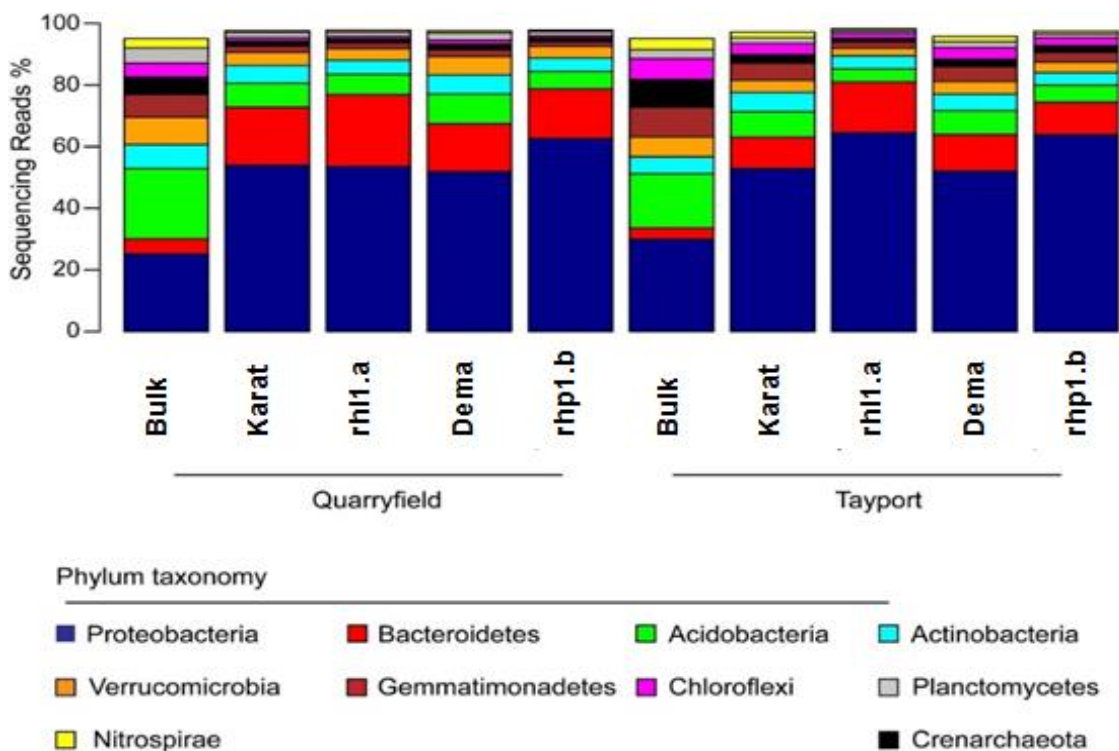
Figure 2.5. The barley rhizosphere microbiota is dominated by Bacteroidetes and Proteobacteria. Average relative abundance (% of sequencing reads) of the phyla identified in the indicated samples gathered from Quarryfield and Tayport soils. Only phyla displaying an average relative abundance > 1% in the whole sequencing dataset have been included.

2.3.4. Root hair mutants host a simplified and distinct rhizosphere microbiota

When alpha diversity, the diversity within samples, was analysed; at the OTU level, no significant effect of the soil type or the microhabitat was identified on either the number of observed OTUs, the Chao Index or the Shannon diversity Index, which are all considered proxies for bacterial richness and evenness, respectively (**Figure 2.6**) (Mann-Whitney-Wilcoxon, $P > 0.05$). The number of observed OTUs is based on the number of unique OTUs identified in each sample set and is not affected by the number of sequence reads assigned to each individual OTU. The Chao index is an estimation based on the abundance of individual OTUs and their relatedness to each other, the Shannon diversity index is based on the abundance and evenness of the OTUs in each sample set.

Nonetheless, there was a clear effect of root hair mutations irrespective of the soil environment; OTUs richness and evenness was significantly lower in the communities associated with *rhl1.a* and *rhp1.b* mutants than the corresponding bulk soil samples in all tests conducted (**Figure 2.6**) (Kruskal–Wallis and Dunns post-hoc tests, $P < 0.05$, BH corrected).
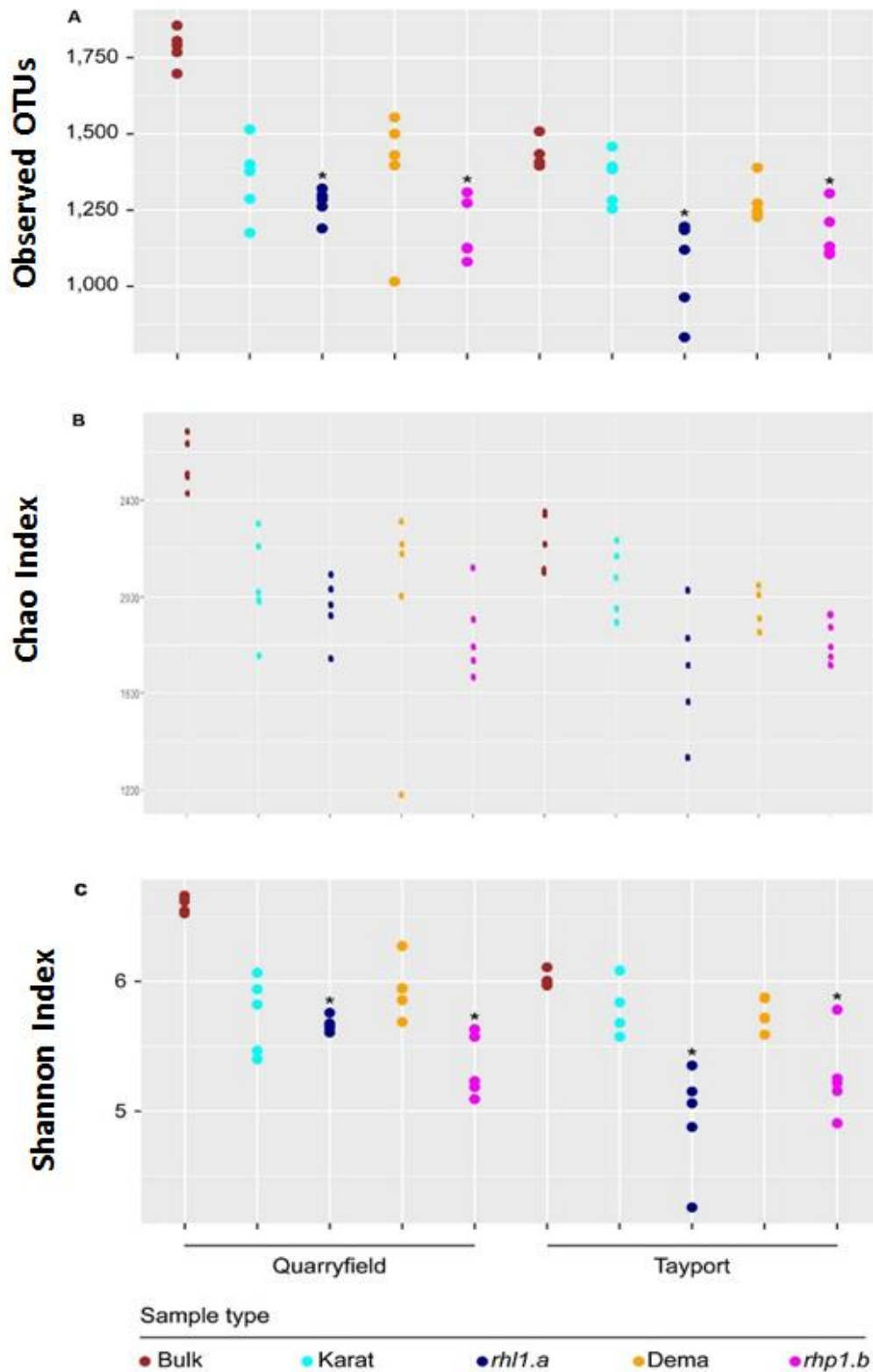
Figure 2.6 Root hair mutants host a 'reduced-complexity' community. *(A) Total number of observed OTUs and* ***(B)*** *Chao1 Index calculated for bulk and Rhizosphere samples. Circles depict individual biological replicates, Asterisks denote statistically significant differences between the indicated samples and bulk soil controls based on Kruskal–Wallis non-parametric analysis of variance followed by Dunn's* post hoc *test (P < 0.05, BH corrected).* ***(C)*** *Shannon's diversity index calculated for bulk and rhizosphere samples. Circles depict individual biological replicates. Asterisks denote statistically significant differences between the indicated samples and bulk soil controls based on Kruskal–Wallis non-parametric analysis of variance followed by Dunn's* post hoc *test (P < 0.05, BH corrected).*

Following this observation, the diversity between samples; Beta diversity, was characterised. In order to do this, it was necessary to set an abundance threshold to remove low count, poorly reproducible OTUs from the analysis. Following the application of the abundance threshold 1,993 OTUs of the initial 8,811 OTUs passed the parameters set. However, these retained OTUs accounted for 5,407,724 reads, which was greater than 94% of the total generated high-quality reads (**8.1.1. Appendix 1: Supplementary Database 1**). This data was used to generate Weighted UniFrac (WU) and Bray-Curtis (BC) distance matrices. Using the WU distance, which is sensitive to both OTU relative abundance and phylogenetic assignment, it was observed that bulk soil and rhizosphere communities clearly segregated along the axis accounting for the largest variation in a Principal Coordinates Analysis (the rhizosphere effect), in addition, the soil effect was well defined by the second axis (the soil effect) (**Figure 2.7A**).

This diversification was supported further by partitions of the distance matrix among sources of variation: the microhabitat (rhizosphere effect) had the strongest effect on variation in the weighted UniFrac distances, this was followed by the soil effect and by their interaction term (**Figure 2.7B**). Interestingly, when bulk soil samples were removed from the analysis and the calculations were repeated, there was a clear genotype dependent effect on the rhizosphere communities, particularly between wild type and mutant rhizosphere samples: the spatial separation between wild type and mutant associated communities observed in the PCoA (**Figure 2.7A**) was supported by a significant effect in the partitions of the variance (**Figure 2.7B**, Adonis test, genotype effect R2 = 0.18, *P* < 0.01). However, it is important to stress that this genotype dependant effect was significantly less than the impact of soil type on the rhizosphere bacterial communities. When the Bray-Curtis distance matrix, which is sensitive to OTU relative abundance only, was used, a more pronounced impact of the soil on the barley microbiota could be observed. Despite this marked soil effect, the effects of the microhabitat and, most importantly, the host genotype on the barley rhizosphere microbiota remained clear (**8.1.1. Appendix 1: Supplementary Database 1**).

A

B

Soil type * Microhabitat effect		
	R2	*P*
Soil	0.16	< 0.01
Microhabitat	0.54	< 0.01
Soil: Microhabitat	0.04	< 0.01

Soil type * Genotype effect		
	R2	*P*
Soil	0.41	< 0.01
Genotype	0.18	< 0.01
Soil: Genotype	0.08	< 0.01
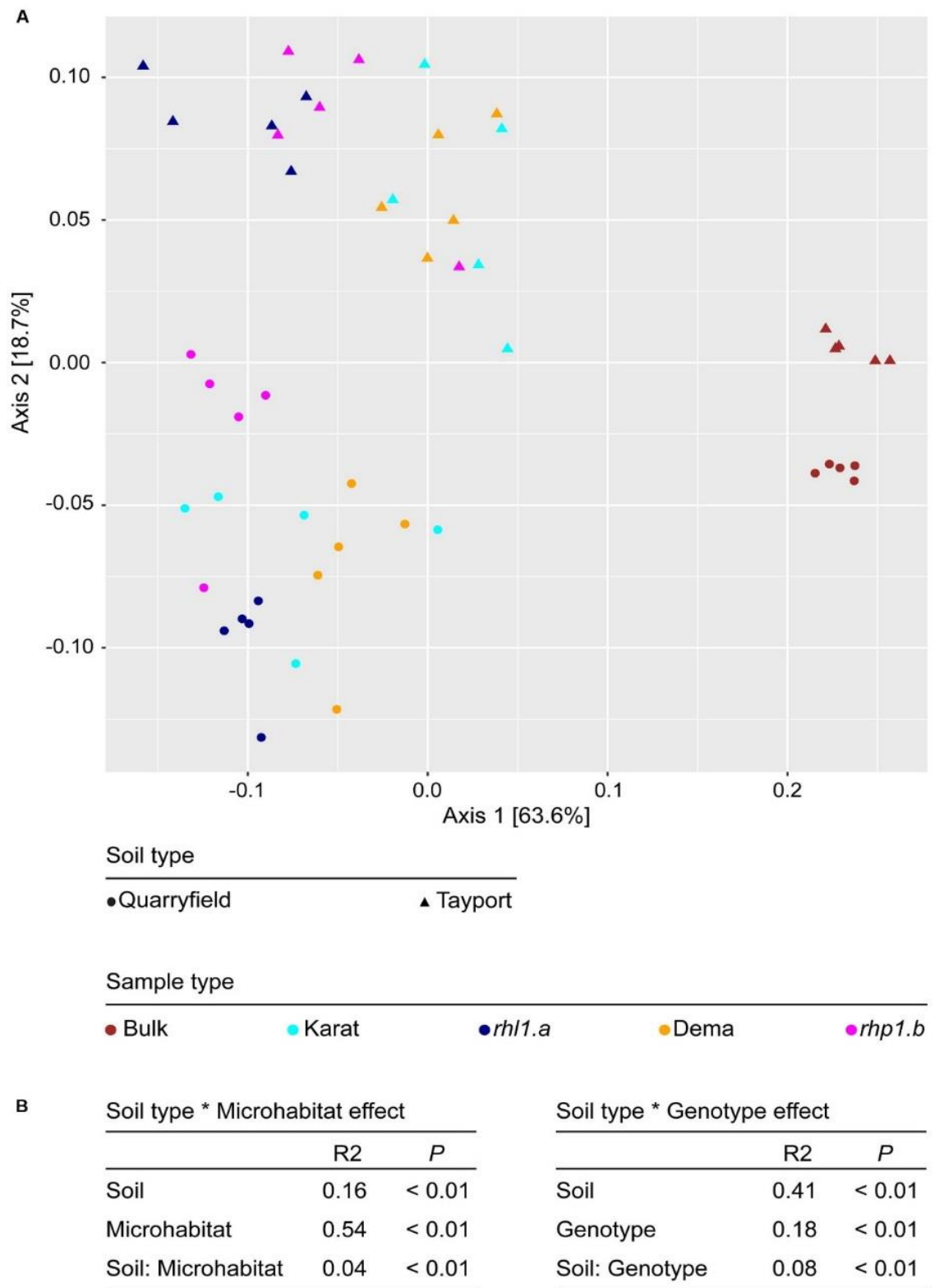
*Figure 2.7 Root hair mutations do not impair barleys ability to shape the rhizosphere microbiota. (A) PCoA calculated using the weighted UniFrac distance matrix (sensitive to both OTU relative abundances and taxonomic affiliation). Formula taken from (Schloss, 2008). Bulk and rhizosphere sample replicates are depicted by shapes whose spatial proximity reflects the degree of similarity of their microbiotas. (B) Permutational analysis of variances calculated using the UniFrac distance matrix for the indicated effects. The R^2 values indicate the proportion of variation in distances which can be explained by the specified grouping of samples. For the calculation of the soil type * genotype effect, bulk soil samples were excluded from the analysis. P-values were calculated for 5,000 permutations.*

2.3.5. Individual bacteria discriminate between the microbiotas of wild type and root hair mutants

To identify the bacteria whose presence and/or abundance caused the observed diversity in community composition between microhabitats (i.e., the 'rhizosphere effect' on the microbiota), the number of OTUs significantly enriched in and differentiating between rhizosphere samples from unplanted soil was calculated.

In Quarryfield soil, over 500 OTUs fulfilled these criteria in each of the genotypes tested (number of rhizosphere enriched OTUs in Karat *n* = 537; Dema n = 529; *rhl1*.a n = 520; *rhp1*.b n = 557. Wald test, *P* < 0.01, FDR corrected) (**8.1.1. Appendix 1: Supplementary Database 1**). Likewise, and in line with the deviation observed in the PCoA plots, Tayport soil subjected to the same analysis showed that mutant plants had more OTUs enrichment in their rhizosphere compared to wild type plants (number of rhizosphere enriched OTUs in Karat, n = 452; Dema n = 473; *rhl1*.a n = 568; *rhp1*.b n = 558. Wald test, *P* < 0.01, FDR corrected) (**8.1.1. Appendix 1: Supplementary Database 1**).

Not only did root hair mutants retain the capacity to shape the soil microbiota in a similar manner to that of the wild type plants but also in Tayport soil their distinct profiles are represented by a greater number of significantly enriched OTUs compared to unplanted soil. Following this observation, it was important to determine whether any of these rhizosphere enriched OTUs varied between barley genotypes, and if so, how many unique OTUs were assigned to a particular genotype. In Quarryfield soil, there were a total of 12 OTUs that were significantly differentially enriched in the comparison between Karat-*rhl1.a* genotypes, in the comparison Dema-*rhp1.b* 33 OTUs were identified as significantly differentially regulated. The distribution of these OTUs was not equally distributed between the pairwise genotype comparisons, rather, the majority of these OTUs, 7 and 29, respectively, were enriched in the mutant homologs *rhl1.a* and *rhp1.b*, respectively (Wald test, *P* < 0.01, FDR corrected) (**Figure 2.8A**, **8.1.1. Appendix 1: Supplementary Database 1**). Interestingly, Tayport soil, which has a smaller organic matter content than Quarryfield soil (**Table 2.1**), displayed a more pronounced genotype effect on the community profiles of the barley microbiota with significantly more OTUs differentiated between genotypes than those that were observed in Quarryfield.
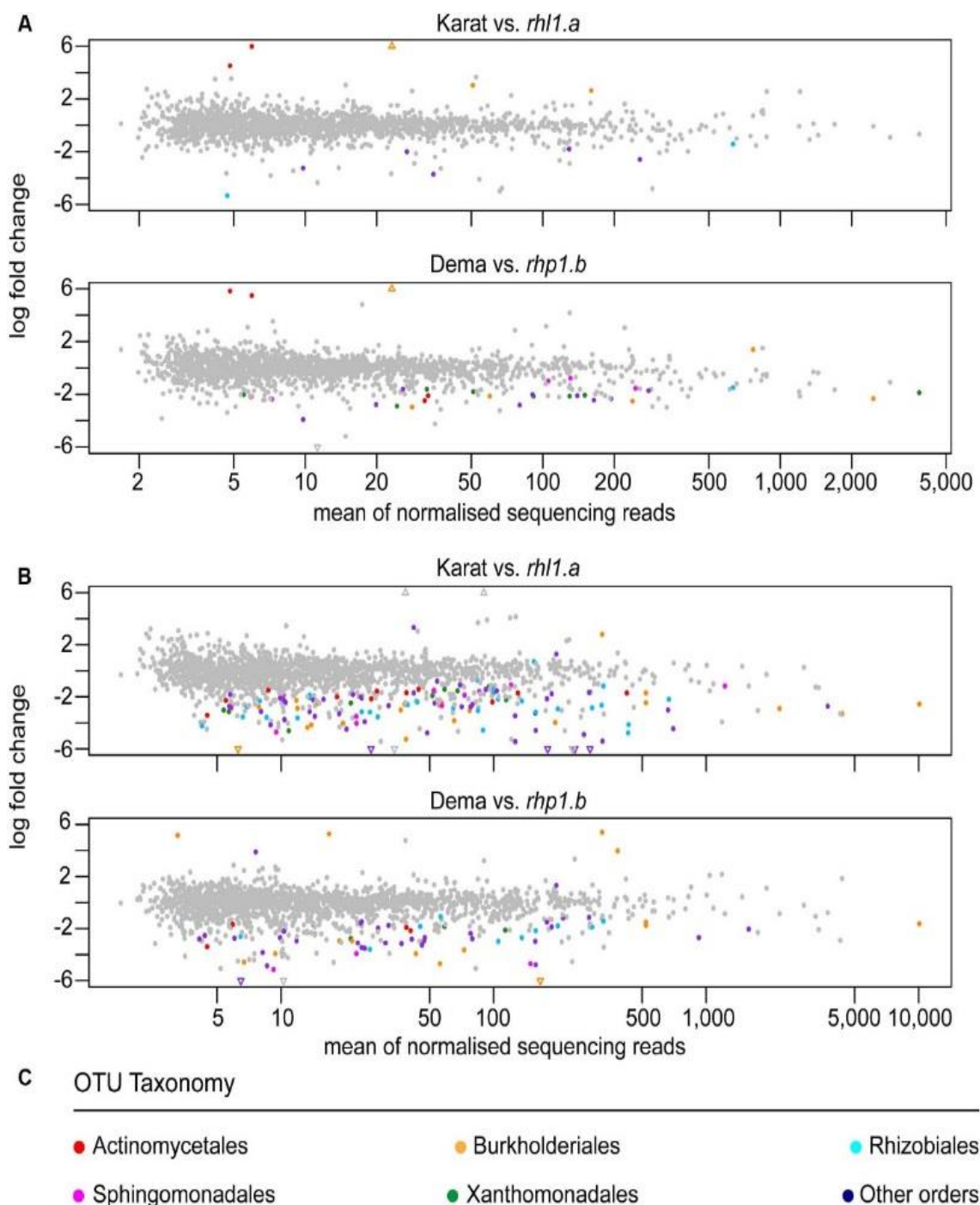
Figure 2.8. Individual members of the bacterial microbiota discriminate between the rhizosphere of wild-type and root hair mutants. *OTUs differentially enriched in the indicated pair-wise comparisons between genotypes grown in **(A)** Quarryfield soil or **(B)** Tayport soil. Individual OTUs are represented by circles whose x–y coordinates correspond with the mean abundance and the logarithmic fold change between wild-type and mutant genotype rhizosphere, respectively. Triangles depict OTUs whose fold change exceeds the scale on the y-axis. In all comparisons made the positive fold change is associated with the enrichment of an OTU in wild-type specimens. OTUs that are significantly enriched in a rhizosphere environment (Wald test, P < 0.01, FDR corrected) are represented by colours recapitulating **(C)** their taxonomic classification at order level. Grey circles represent OTUs which are not significantly enriched.*

For example, a total of 132 OTUs were differentially recruited in the comparison between the Karat and *rhl1.a* genotypes, furthermore in the comparison between Dema and *rhp1.b* community profiles 70 OTUs differentiated between the genotypes (Wald test, *P* < 0.01, FDR corrected) (**Figure 2.8B**, **8.1.1. Appendix 1: Supplementary Database 1**).

A striking observation in the differentiation of OTU recruitment between genotypes in Tayport soil was that the vast majority of these differentially recruited OTUs, 128 and 64, were significantly enriched in the mutant genotypes *rhl1.a* and *rhp1.b* compared to their cognate wild type background (Karat and Dema, respectively). Comparisons were also made between both wild type and both mutant genotypes and it was observed that the impact of root hair mutations on the rhizosphere microbiota exceeded the effect of other or additional host genetic traits differentiating the tested genotypes. In particular, only 1 and 22 OTUs were identified as differentially recruited between wild types Karat and Dema in Quarryfield and Tayport soils, respectively (Wald test, *P* < 0.01, FDR corrected) (**8.1.1. Appendix 1: Supplementary Database 1**). The taxonomic assignment of the OTUs that differentiated between genotypes was investigated and it was observed that members of the orders Actinomycetales, Burkholderiales, Rhizobiales, Sphingomonadales and Xanthomonadales occurred in more than 10 instances in at least one soil type (**Figure 2.8C**, **8.1.1. Appendix 1: Supplementary Database 1**).

Cumulative hypergeometric calculation identified 11 or more occurrences of members of these orders among bacteria differentially recruited between wild type and mutant plants have probabilities exceeding 0.75 (**8.1.1. Appendix 1: Supplementary Database 1**). Considering that Actinomycetales, Burkholderiales, Rhizobiales, Sphingomonadales and Xanthomonadales rank at the top in term of number of OTUs assigned among the 26 orders differentiating between wild type and root hair mutants, our data suggest that the host genotype acts predominantly on abundant members of the microbiota.

Compellingly, a consistent overlap between the numbers of OTUs differentially recruited in wild type and mutant plants in the two test soils was not observed (**Figure 2.9**). A notable exception to this general trend was represented by an

individual OTU, classified as *Janthinobacterium sp.*, enriched in the mutant *rhp1.b* in a soil-independent manner and representing 6 -12% of the entire rhizosphere community (**8.1.1. Appendix 1: Supplementary Database 1**).
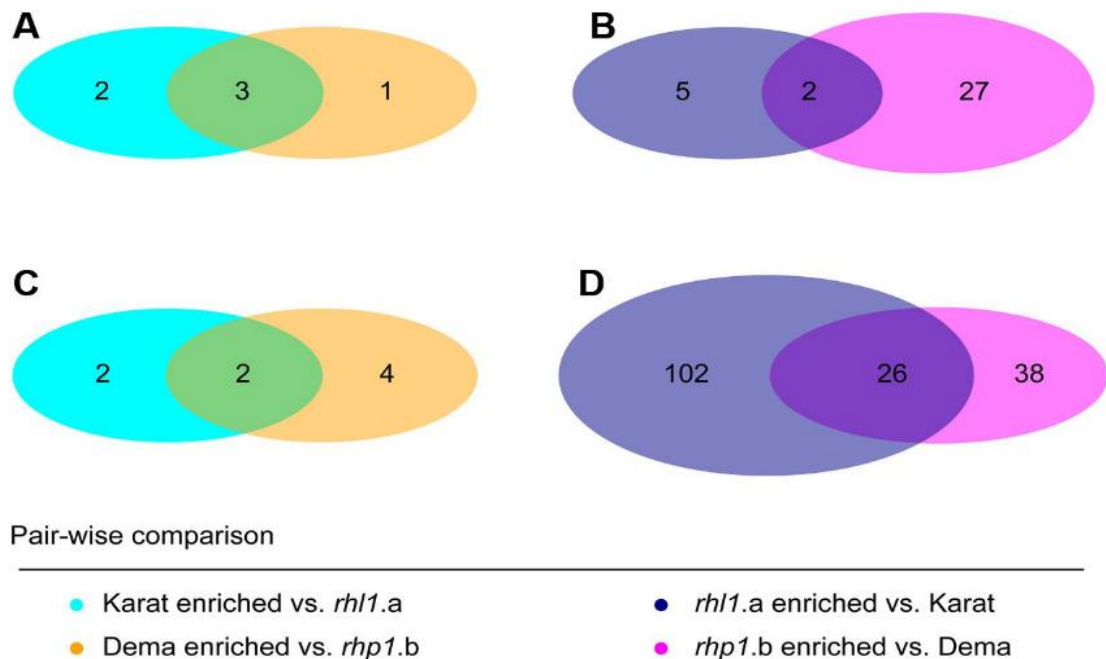
Figure 2.9 Soil type modulates the host genotype effect on the rhizosphere microbiota. *Number of OTUs differentially recruited between genotypes grown in (A, B) Quarryfield soil or (C, D) Tayport soil. Diagram color depicts the indicated pair-wise comparisons. Intersections of the diagrams highlight OTUs differentially recruited in a soil type-independent manner. (Wald test,* P < 0.01, *FDR corrected).*

2.4. DISCUSSION

Despite root hair mutations being observed to perturb rhizosphere formation (Brown et al., 2012b, George et al., 2014) this data indicates that it is not necessary for root hairs to be fully developed for microbial proliferation to occur at the barley root-soil interface (**Figure 2.3**) and suggests that soil type clearly exerts the strongest impact on microbial DNA concentration. This reinforces the concept that edaphic factors drive the bacterial microbiota thriving at the root–soil interface (Bulgarelli et al., 2013). However, it is important to note that edaphic factors can interfere with the efficiency of DNA, recovery per se, rather than directly influencing microbial activity and biomass. For example, specimens grown in Tayport soil were associated with a significantly smaller concentration

of microbial DNA compared with samples obtained from Quarryfield soil, whose organic matter content is almost double that of the former substrate (organic matter Tayport 2.9%; Quarryfield 5.5%, **Table 2.1**).

In addition, it was observed that edaphic factors impact the growth performance of the plants: Tayport samples yielded a significantly higher aboveground biomass compared to Quarryfield samples (**Figure 2.4C**). Based on these observations it could be speculated that both the quantitative and qualitative nature of the rhizosphere microbiota may make use of the photosynthetic resources of the plants to contribute to optimum growth of the host in each soil type. However, it is likely that should this be the case, it is likely to be a contributing factor in plant growth performance with the major influence likely to be the increased availability of the nutrients potassium and magnesium in Tayport soil which are essential elements for plant growth and root development (Fageria, 2001). It is also important to consider the morphological differences between root hairs of the wild type and mutant plants which may impede efficient nutrient acquisition.

In particular, the marked difference in the proportions of Bacteroidetes and Proteobacteria in rhizosphere samples compared to bulk soil (**Figure 2.5**) is reflective of the selective bacterial enrichment that has been observed in other cereal species, such as wheat , maize and rice (Edwards et al., 2015, Peiffer et al., 2013, Turner et al., 2013). Interestingly, these bacterial community profiles are comparable to those observed in wild and cultivated barley genotypes grown in a German agricultural soil (Bulgarelli et al., 2015), this could possibly represent a feature of a core barley microbiota which is conserved across soil types. Together, this data indicates that, at a higher taxonomic rank, the recruitment of a rhizosphere microbiota that is distinctive to that observed in soil is virtually unaffected by the root hair mutations explored in this study.

When the taxonomic resolution of the investigation was increased to the OTU level, clear signatures of root hair development on microbiota recruitment were observed. It is of note that, root hair mutants hosted bacterial microbiota of reduced-complexity when compared with their cognate wild-type parents in both soil types. This was demonstrated in a significant reduction of ecological indices summarising the richness and evenness of the communities (**Figure 2.6**).

Remarkably, the communities displaying a 'reduced-complexity' that inhabited the rhizosphere of root hair mutants were clearly distinct from both the corresponding wild-type and bulk soil profiles respectively (**Figure 2.7**), this suggests that these communities profiles are the likely result of a perturbation of the host recruitment signals as opposed to their colonisation being opportunistic. The distinctive characteristics of the root hair mutant microbiota was represented by the significant enrichment of individual bacteria, which ultimately dominated their rhizosphere profiles (**Figure 2.9**).

When examining these selective enrichments two striking features were observed. The first was the clear soil-dependency of this trait; plants grown in Tayport soil (which had the lowest organic content) yielded the highest number of differentially regulated OTUs compared to Quarryfield samples. In addition, the majority of these OTUs were not conserved between the two soil types (**Figure 2.10**). It was previously demonstrated that a cooperative action of both host–microbe and microbe–microbe interactions shape the barley rhizosphere microbiota (Bulgarelli et al., 2015) it can be speculated that the lower proportion of organic matter, and the reduced microbial density observed as a result of this in Tayport soil, has swung the balance of this cooperative relationship in favour of the hosts recruitment cues.

These results could also suggest that edaphic factors that differentiate between the two soil types may promote the establishment of a bacterial microbiota whose metabolic capacities are either required or better adapted to the needs of the host plant in a particular soil type. This could be the result of multiple factors such as changes in exudation patterns due to nutrient stress, the physical soil environment being favourable for particular bacterial species, previous crops grown in the soil enriching particular bacterial species or previous agricultural treatments. The second remarkable feature is that, in at least one of the soils tested, the mutant *rhp1.b* (featuring interrupted root hair development) was host to a more distinct microbial profile compared to that of the root hairless mutant *rhl1.a* and the wild-type plants with well-developed root hairs. (**Figure 2.7**). This observation suggests that the presence or length of root hairs alone is not sufficient to explain the microbial diversity recorded between the barley genotypes used in this study.

The genetic pairs Karat-*rhl1.a* and Dema-*rhp1.b* have previously been molecularly categorized, identifying a subset of proteins which were observed to be differentially accrued in these genotypes (Janiak et al., 2012) . This may be a direct connection to the recruitment cues of the barley microbiota shaped by root hair mutations. Of particular interest, several plant ATP-binding cassette (ABC) transporters were identified were identified in the previously referenced study. This may be of relevance as ABC transporters are known to play an imperative role in the secretion of phytochemicals during the process of rhizodeposition (Baetz and Martinoia, 2014).

Intriguingly, whilst the identification of the substrates transported by some ABC transporters is somewhat ambiguous due to some ABC transporters having the ability to transport multiple, structurally unrelated substrates or because the identification of their substrates has been performed indirectly without any flux measurement, many have been successfully identified (Lefèvre and Boutry, 2018).

It has been observed that disruption of some specific ABC transporters can alter both the exudate and microbiota profiles of some plants. For example, It has been recorded that a statistically significant reduction in the attraction of *Bacillus subtilis* is induced by using virus induced gene silencing (VIGS) to knock down the ABC transporter gene, ABC-G33 in tomato (Cox et al., 2019). It was also observed in Arabidopsis when the ABC transporter, ABC-G30 was disrupted, however this effect was only apparent in plants grown for two consecutive generations in the same soil (Badri and Vivanco, 2009).

It is important to note that in a multigenerational plant soil feedback study (PSF) in Arabidopsis it was observed that the second generation of Arabidopsis showed that the main effect on the rhizosphere microbiota was the enrichment, in the mutant plants of individual members of the microbiota which is remarkably similar to effect that root hairs were observed to have on the microbial community in this study (**Figure 2.9**) (Fitzpatrick et al., 2018). Therefore, it can be hypothesized that ABC transporter-mediated alterations to rhizodeposition represent, at least partially, the disruption of the host recruitment signals which shape and ultimately alter the rhizosphere microbiota in root hair mutants.

However, it is likely that a multitude of factors contribute to the microbial shifts

observed in the rhizosphere between the barley root hair genotypes encompassing many chemical, physical and biological influences.

The length and density of root hairs can physically alter the soil environment which, in turn, may impact the cross kingdom biological communities residing within it. Current research in to the impact of root hairs on soil structure and density using high resolution synchrotron imaging to study two barley genotypes, Optic wild type and an optic derived ethyl methanesulfonate (EMS) mutant line exhibiting no root hairs has revealed that root hairs significantly alter the connectivity and porosity of the detectable rhizosphere pore space, conversely the genotype with no root hairs showed the opposite with a significant decrease in the same factors (Koebernick et al., 2017).

This finding is highly relevant to this study as soil pores and channels govern important aspects of the soil environment: the movement of air, water, chemicals and other fluids meaning that shifts in soil density can alter the rhizospheric soil environment (Nimmo, 2013). Based on this it can be hypothesised that shifts in soil density shapes the physical soil environment which plays a role in shaping the microbiota based on their metabolic and aerobic/anaerobic abilities and requirements. Further studies aimed at deciphering the impact of root hairs (or the lack thereof) on both barley rhizodeposition and soil density will contribute to test these hypotheses.

Although the rhizosphere microbiota is clearly modulated by soil type at the highest resolution (i.e., the OTU level) the impact of root hair mutations is evident; a bias for members of the orders Actinomycetales, Burkholderiales, Rhizobiales, Sphingomonadales, and Xanthomonadales was identified among the bacteria differentially recruited between wild-type and root hair mutants, which also represent the dominant members of the community (**Figure 2.8**). This data provides a deeper level of understanding of the host modulated microbiota, specifically the impact of root hair development (**Figure 2.10**)

Members of these orders have previously been reported as PGPRs (Lugtenberg and Kamilova, 2009, Schlaeppi and Bulgarelli, 2015). Due to the vital role played by root hairs in barley nutrient acquisition it is tempting to hypothesize that if root hair development is impaired this may trigger a plant response to actively recruit members of the microbiota which may be capable of helping to compensate for

the restrictions in acquiring sufficient nutrients.
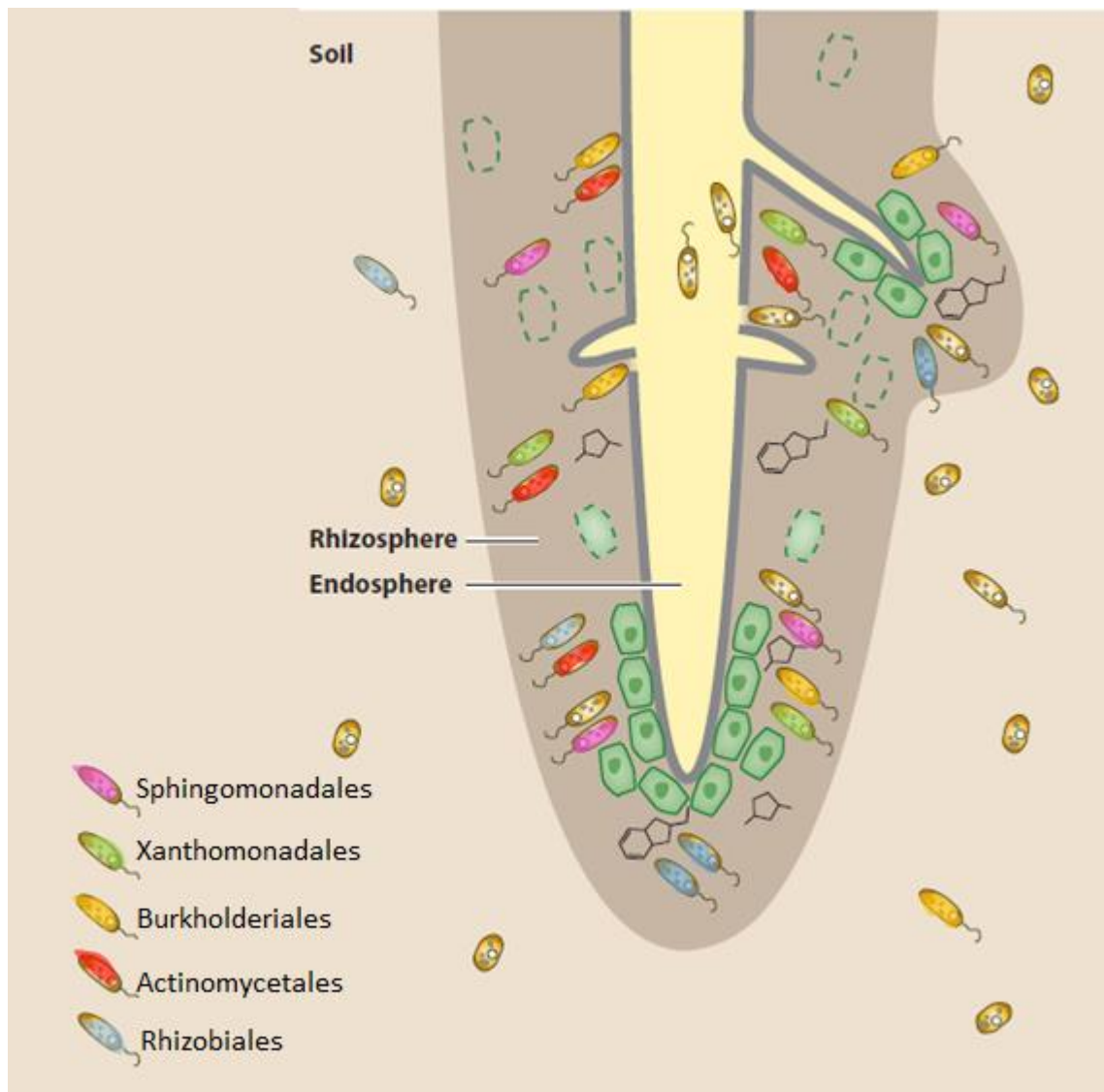
Figure 2.10 The community structure of the barley rhizosphere microbiota. *The bacterial rhizosphere microbiota is, at least in part, modulated by the host genotype influencing root hair phenotype. Barley with root hair mutations show a bias for the orders Sphingomonadales, Xanthomonadales, Burkholderiales, Actinomycetales and Rhizobiales (indicated by colour).*

This proposition would correspond with one proposed for arbuscular mycorrhizal fungi (AMF), in which it was observed to colonise the roots of barley genotypes devoid of root hairs in phosphorus depleted conditions but not observed in that of barley mutants with short root hairs or wild-type plants (Brown et al., 2013). It has been known for decades that AMF form symbioses with phosphate solubilising bacteria (PSB) which allows the uptake of soluble phosphate by the plant via the AMF colonisation that may not normally be in close enough proximity to the roots to be taken up directly (Barea et al., 2005).

Interestingly, the proliferation of rhizosphere-inhabiting *Janthinobacterium* sp.,

the dominant bacteria in *rhp1.b* community, has previously been identified as a distinguishing feature of the bacterial communities associated with the extra radical mycelium of AMF and have been known to protect plant roots from fungal infection by inhibiting growth (Scheublin et al., 2010, Chapelle et al., 2015). Thus, the impact of root hair mutations on the composition of the rhizosphere bacterial microbiota could be, at least partly, the result of the symbiotic interactions between plant roots and soil fungi.

However, it is important to note that although from a different genetic background to those used in this study, when barley plants were grown under soil stress conditions (phosphorus deficiency and water stress), barley with either no or short root hairs produced a lower yield than that of wild type barley (Brown et al., 2012b, George et al., 2014). Therefore, an alternative hypothesis is that the differential recruitment we observed in our experiments is the result of an imbalance (dysbiosis) of the barley microbiota *per se*. It could be argued that a lack of or retardation of root hairs reduces the plants ability to take up sufficient nutrients and fluids which in turn triggers a stress response in the plant. For example, barley plants grown under phosphate-limiting conditions, a stress condition that could be triggered by an impairment of root hair functionality, display a shift in the exudation profile of organic acids (Wang et al., 2015). However, the ability of the root hair mutant plants to take up nutrients has not been explored experimentally.

Organic acids, amino acids and other low molecular weight compounds such as sugars and alcohols usually found in root exudates many can be primary metabolites which are an important nutrient source for rhizosphere microbes and can influence root-microbe interactions shifting the microbiota composition in different soil types (Haichar et al., 2014, Eilers et al., 2010). Consequently, mutant plants, through perturbed nutrient acquisition and in turn a shift in rhizodeposition, may recruit a 'stress-induced microbiota.' In particular, the distinct composition of the root hair mutant microbiota (**Figures 2.6 - 2.9**) might reflect a deficiency in host-microbiota interactions required for optimum plant growth under nutrient stress.

These hypotheses are not necessarily mutually exclusive: a 'stress-induced microbiota' could include an increased proportion of PGPRs but whose metabolic capacities cannot fully compensate for the root hair mutations.

2.5. FURTHER WORK

To further elucidate the results observed in this study and the molecular mechanisms driving the relationships between root hairs, bacteria and other members of the rhizosphere microbiota, a study into the exudation variation between genotypes, the impact of soil density directly on the microbiota in a plant free environment and investigating the ability of the mutant lines to take up sufficient nutrients would be beneficial. This, in partnership with isolation, identification and genetic and proteomic categorisation of rhizospheric bacteria may allow us to ultimately clarify the impact of specific structural and functional configurations of the rhizosphere microbiota on given plant phenotypes and generate a bacterial collection which could be used for colonisation and inoculation investigations in the future.

CHAPTER 3

ROOT HAIR FORMATION AND MUTATIONS SHAPE THE CHEMICAL RHIZOSPHERE ENVIRONMENT

3.1. INTRODUCTION

Plant roots release an array of carbon-based compounds into their surrounding soil through the process of rhizodeposition and exudation (Badri and Vivanco, 2009). The majority of these compounds can be categorised as carbohydrates (sugars), amino acids and organic acids which are not only metabolized by the microbial organisms residing in the soil, but can also elicit a multitude of enzymatic and microbial interactions (Dennis et al., 2010, Somers et al., 2004, Canarini et al., 2019). In addition to stimulatory compounds, a range of secondary metabolites such as coumarins, flavonoids and phenols are also released from roots and act as antimicrobials which can inhibit the proliferation of fungal, eukaryotic and bacterial pathogens (Walker et al., 2003). They can also act as signalling molecules in the establishment of symbiotic relationships with, for example, nitrogen fixing rhizobia (Hassan and Mathesius, 2012). This combination of biological and chemical interactions work together to ensure that root exudates are a key component in shaping the rhizosphere microbiota. Consistently, the application of low molecular weight organic compounds found in root exudates to soil cores were sufficient to shape, at least in part, the soil biota (Hu et al., 2018) .

It is widely accepted that root hairs are the root structures that are the main site of water and nutrient acquisition (Gilroy and Jones, 2000) . However, they are also the main site of exudation of carbon compounds and due to their hollow, delicate structure are a rich source of carbon rich sloughed-off cells (Walker et al., 2003, Prevot and Steward, 1936, Cheng and Gershenson, 2007). As a result, the presence, density and length of root hairs increase exudation and the distribution of the rhizosphere zone, they also enhance plant fitness by allowing more efficient nutrient uptake (Honkanen and Dolan, 2016, White et al., 2013,

Libault et al., 2010, Gilroy and Jones, 2000). It has been demonstrated that in phosphorus limited conditions up to 90% of the total phosphorus obtained by plants was directly via root hairs and that root hairs can account for up to 70% of the root surface area in some plant species (Raghothama and Karthikeyan, 2005).In addition, it has been shown that a lack of root hairs or perturbation of their development can result in the opposite effect, it has been shown that barley "Optic" accessions with no root hairs were much more vulnerable to a combination of water and phosphorus stress being 1.5 - 2.3 fold smaller versus its short, long and wild type root hair homologues that were subjected to the same treatments (Jungk, 2001, Brown et al., 2012b).

This indicates that plants lacking in root hairs are likely to experience nutrient and drought stress compared to their wild type counterparts; a study examining the exudation characteristics of *Andropogon virginicus* grown under conditions with variable phosphorus showed that reduced P bioavailability resulted in the increased exudation of some metabolites, increased amino acid concentration within the roots and decreased organic acid within the roots due to increased exudation (Edayilam et al., 2018). However, plants devoid of root hairs, and in turn perturbed in acquiring nutrients cannot respond in this manner as root hairs are also the primary site of plant exudation. Perturbations to root hair growth and expression may therefore have profound effects on the root exudation profile of the rhizosphere.

It was previously demonstrated (**Chapter 2**) that root hair mutations perturb the barley the rhizosphere microbiota, where it was observed that root hair mutant barley lines *rhl1.a* and *rhp1.b* hosted a reduced complexity bacterial community in the rhizosphere compared to their wild type homologues Karat and Dema, respectively (Robertson-Albertyn et al., 2017). Therefore, it is logical to study the exudate profiles of wild type and root hair mutant barley genotypes. Using a hydroponics-based system and GC-MS analysis, variations in the chemical composition of all 4 genotypes exudates were studied. In addition, Illumina MiSeq technology was used to examine the microbial composition of these samples to identify potential contaminants and seed borne endophytes.

3.2. MATERIALS AND METHODS

3.2.1 Plant genotypes, growth conditions and exudate collection

*Hydroponic protocol adapted from (Giles et al., 2017).

In order to investigate if barley plants with root hair mutations had different exudation profiles to that of wild type barley, the genotypes used in the previous chapter, Dema and Karat alongside their mutant homologues *rhl1.a* and *rhp1.b* were selected to follow-up studies of the rhizosphere microbiome (**Chapter 2**)(Robertson-Albertyn et al., 2017).

The morphology, method of mutation and germination is detailed in **2.2.2**. with the exception that the seeds were washed well with sterile water, but not surface sterilized due to the finding that sterilization negatively effects the germination rate of the mutant genotypes and does not significantly impact the composition of the rhizosphere microbiota (**8.1.1. Appendix 1: Supplementary Database 1**).

Prior to set up, the hydroponics tanks were thoroughly cleaned with dH_2O and then sanitized using 100% EtOH and left to air dry. A total of 315 seedlings per genotype, that showed no sign of infection and had rootlet growth of approximately 1.5 cm, were used for this study and grown in hydroponics solutions for three weeks.

Three aerated 60 L tanks were used per replicate, each housing 140 seedlings of the same genotype secured at the plant collar by foam inserts (**Figure 3.1**). The standard nutrient solution contained 3 mM NH_4Cl, 4 mM $Ca(NO_3)_2$, 4 mM KNO_3, 3 mM $MgSO_4$, 0.1 mM Fe-EDTA, 1 mM KH_2PO_4 with micronutrients (6 µM $MnCl_2$, 23 µM H_3BO_3, 0.6 µM $ZnCl_2$, 1.6 µM $CuSO_4$, 1.0 µM Na_2MoO_4, 1.0 µM $CoCl_2$) and was made up in stocks of each individual compound to be added to the tanks with deionised water .
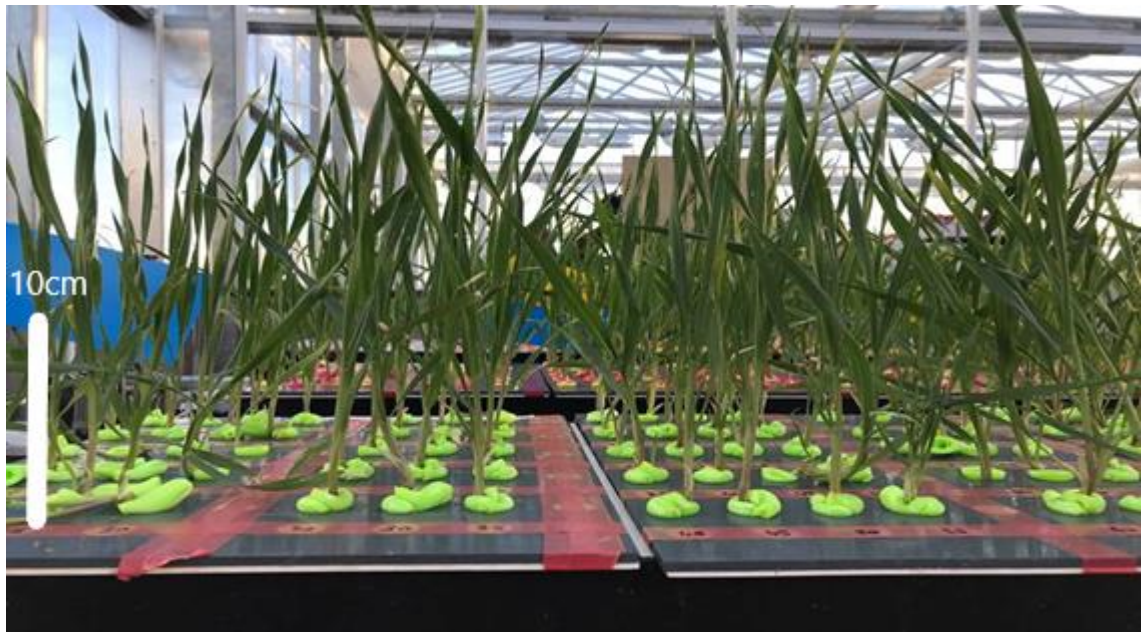
Figure 3.1 Barley hydroponic propagation set up. Barley root exudates were collected using an aerated hydroponic technique adapted from (Giles et al., 2017). Seedlings are held in place at the collar by foam inserts (green) with the root portion submerged below in the hydroponic nutrient solution contained in the black tank.

Nutrient solution was changed every three days beginning with 25% strength, followed by 50% strength and continuing with full strength until harvest. Plants were harvested after 2 weeks of growth under controlled conditions (22 °C day 16 h / 14 °C night) at which point exudates were collected overnight in 150 ml pots containing 75 ml sterile deionised water with 5 plants of the same genotype per pot. Plants were removed from the pots after 24 hours and the remaining liquid in the collection pots was frozen at 20 °C prior to freeze drying.

3.2.2 Exudate freeze drying parameters

(Performed by Ralph Wilson (Cell and Molecular Sciences, James Hutton Institute, Invergowrie, Dundee, UK))
Molecular Sciences, James Hutton Institute, Invergowrie, Dundee, UK))

The exudates were freeze dried using a Christ, Lyo Cabinet 2 - 10 with 2 independent Delta 1 - 24 condenser units over a period of 4+ days until no moisture could be detected in the samples (**Figure 3.2**)

Figure 3.2 Exudates are freeze dried following collection. Samples were freeze dried under controlled conditions over a period of 4 days until moisture sensors registered them as dry.

The general procedure for freeze drying was; the machine was prepared for one hour using the "warmup" setting for two vacuum pumps and two condenser units. Following this samples were loaded, and the main run initiated which consists of both condenser units set to run at -62 °C and the chamber vacuum set for 0.770 mbar, both constant for the duration of the run. Shelf temperature is set to -20 °C. After a day of drying the shelf temperature is set to 0 °C followed a day later by the temperature being increase to 10 °C. Finally, the shelf temperature set to 25 °C and remains at this temperature until samples are confirmed as dry from sample sensors. All data is recorded digitally for quality control and reference purposes.

Due to the yield of exudates per round of 140 plants it was necessary to perform 2 cycles per genotype followed by a third cycle consisting of 35 plants per genotype in a 4 - chamber hydroponics system (one genotype per chamber). Exudates were pooled together by genotype and 4 g of each was sent for GC-MS analysis at Sheffield Hallam University. The total mass of freeze dried exudates obtained from Karat and *rhl1.a* plants exceeded the 4 g required for GC-MS analysis, the excess was retained for DNA extraction in order to identify OTUs that were present in the exudate samples that may be derived from the environment or endophytic bacteria released from the roots. DNA extraction described in **3.3.4.**

3.2.3 Exudate profiling using Agilent GC-MS

*(performed as a service of Sheffield Hallam University by Dr Jamie Young)

3.2.3.1. Amino Acid and Organic Acid Extraction

Amino acid and organic acid extraction was carried out according to (Bobille et al., 2016)

Briefly, 100 mg of sample was transferred into 5 ml of 2 mM formic acid. The suspension was shaken (1 h, 200 rpm, 20 °C). Then, the suspension was centrifuged (10 min, 8000 rpm) and the supernatant was filtered at 0.22 μm to remove particulate matter. The eluate was dried under a gentle stream of nitrogen.

The Amino Acid (AA) extract was derivatised by silylation. A reagent mix was prepared with acetonitrile (AcN), N-methyl-N-tert-butyldimethylsilyl-trifluoroacetamide (MBDSTFA) and triethylamine (TEA) in a 15:15:1 ratio (v/v/v, respectively). 50 μl of reagent mix was added to the dried extract, the microtube was thoroughly vortexed, centrifuged, and incubated in a heated block (95 °C, 30 min).

Measurements were carried out with an Agilent MSD GC-MS. Compounds were separated on a phenyl/ siloxane column; 5% diphenyl/ 95% dimethyl polysiloxane, 30 m × 0.23 mm i.d, 0.25 μm film thickness). The GC was operated in constant pressure mode with helium as carrier gas (1 ml min^{-1}). Injection (1 μl) was performed in splitless mode. The elution profile was: 60 °C for 1 min, linear gradient of 30 °C min^{-1} to 120 °C, linear gradient of 8 °C min^{-1} to 300 °C, 300 °C for 5 min, resulting in a total run time of 39 min. The injector, MS transfer line and source temperatures were respectively 280 °C, 290 °C and 220 °C. Mass spectra were collected using electron impact (EI) ionization at 70 eV.

3.2.3.2. Sugar and Sugar Alcohol Extraction

One hundred mg of sample was transferred into 5 ml of 70% ethanol. The suspension was shaken (1 h, 200 rpm, 20 °C), then centrifuged (10 min, 8000 rpm). Following this, the supernatant was filtered at 0.22 μm to remove particulate matter. The eluate was dried under a gentle stream of nitrogen. The AA extract was derivatised by silylation using 50 μl BSTFA + 1% TMCS added to the dried extract, the microtube was thoroughly vortexed, centrifuged, and incubated in a

heated block (75 °C, 30 min).

Measurements were carried out with an Agilent MSD GC-MS. Compounds were separated on a phenyl/ siloxane column; 5% diphenyl/ 95% dimethyl polysiloxane, 30 m × 0.23 mm and 0.25 µm film thickness). The GC was operated in constant pressure mode with helium as carrier gas (1 ml min^{-1}). Injection (1 µl) was performed in splitless mode. The elution profile was: 60 °C for 1 min, linear gradient of 30 °C min^{-1} to 120 °C, linear gradient of 8 °C min^{-1} to 300 °C, 300 °C for 5 min, resulting in a total run time of 39 min. The injector, MS transfer line and source temperatures were respectively 280 °C, 290 °C and 220 °C. Mass spectra were collected using EI ionization at 70 eV.

Although the methods outlined above will successfully extract amino acids, sugars and organic acids; they are not completely selective for each group of compounds, therefore other classes, such as fatty acids and sterols will also be extracted. These have also been presented in the results.

3.2.3.3. Peak Identification

Peaks were identified using the Agilent MSD software coupled to the NIST 2011 database. Septum and column artefacts were discarded. Peaks with less than an 80% match to the database were manually interrogated using the NIST 2011 database.

3.2.4. DNA extraction of exudates and data analysis

DNA was extracted from ~0.5 g of freeze-dried exudates of Karat and *rhl1.a* genotypes as described in **2.2.3** and the V4 region of the 16S rRNA gene was amplified, purified and subjected to Illumina MiSeq sequencing as per **2.2.4.** Sequencing data was prepared for data analysis as described in **2.2.5** with the exception that the merge command merge_otu_Tables.py was not required.

Statistical analysis was performed in R. Unless otherwise stated, the functions used were retrieved from the default installation of R or the R package Phyloseq (McMurdie and Holmes, 2013). Due to the data consisting of two pooled samples, analysis of the OTUs captured was only taken to taxonomic comparisons. As the method of exudate collection used in this study could not be done axenically it presented the opportunity not only to investigate naturally occurring microbial contaminants from the growth environment, but also to identify endophytic

microorganisms. In addition, it also allowed for the previous root hair mutant rhizosphere microbiota study to be validated further by removing the observed organisms from the exudate study from the rhizosphere microbiota dataset and assessing any changes in the previously observed results. The sequence data from the exudate OTUs was also extracted from the **Chapter 2** OTU dataset and analysis was run again as per **2.2.6** to evaluate the impact of OTUs retrieved from the exudates on our previously published results. Data used for the analysis is available in **8.2.1. Appendix 2: Supplementary Database 2**

3.3 RESULTS

3.3.1. A proportion of root exudates are detected in either wild or root hair mutant barley only.

An average yield of 0.01 - 0.015 g of exudate was obtained per individual plant, there was no significant variation in average yield between genotypes or between the hydroponics tanks used for collection (data not shown), a total of 315 individual plants per genotype were used for this study.

Following GC-MS analysis a total of 68 unique organic and amino acids (**Figure 3.3** and **3.4**) as well as 22 sugars and sugar alcohols (**Figure 3.5** and **3.6**) were detected across all genotypes, these are described in **Tables 3.1** and **3.2**, respectively.
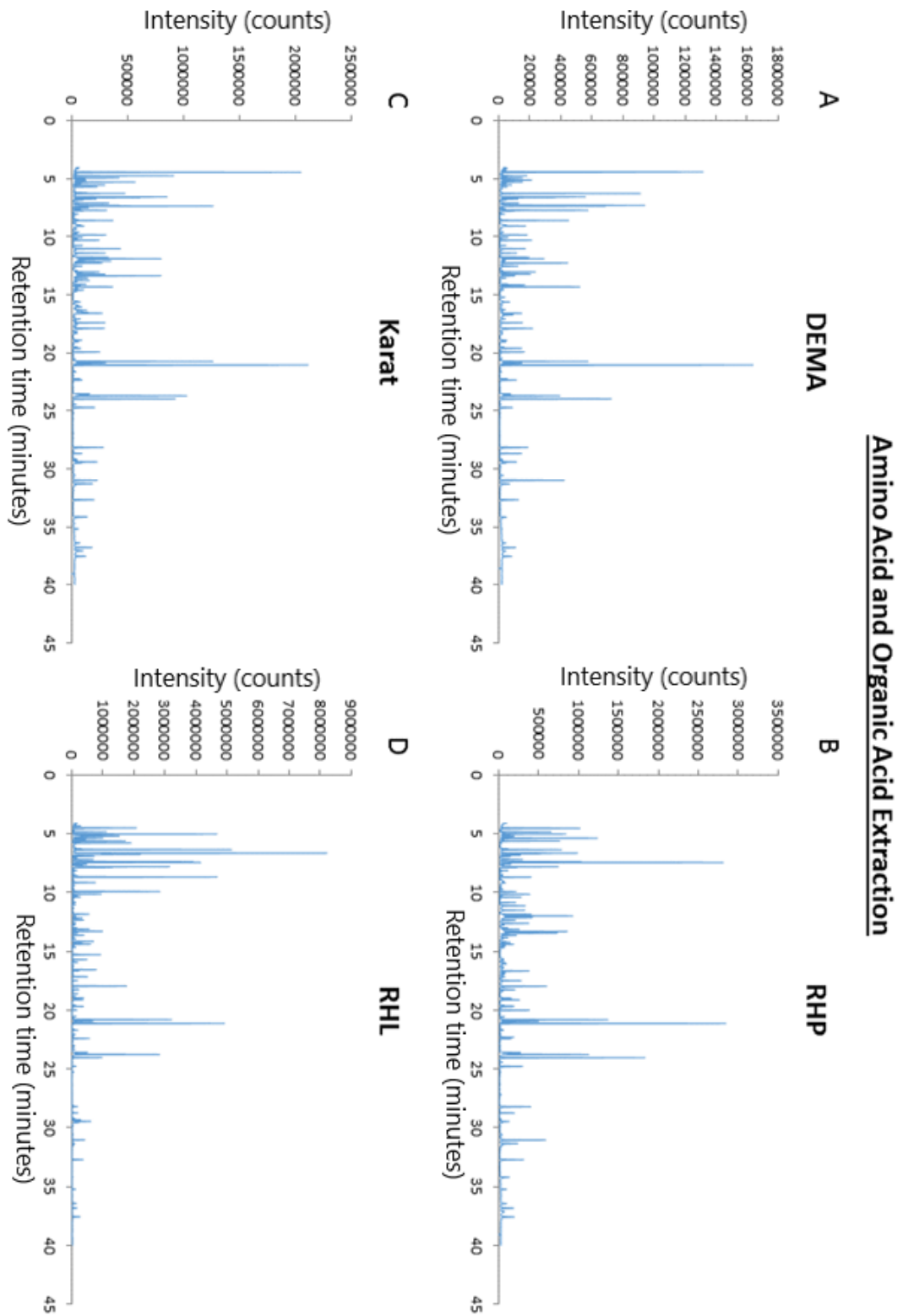
Figure 3.3 Amino acid and organic acid extraction. The total ion chromatograms for the exudates of (A) Dema, (B) rhp1.b, (C) Karat and (D) rhl1.a. Blank extraction can be found in supplementary Figure S2
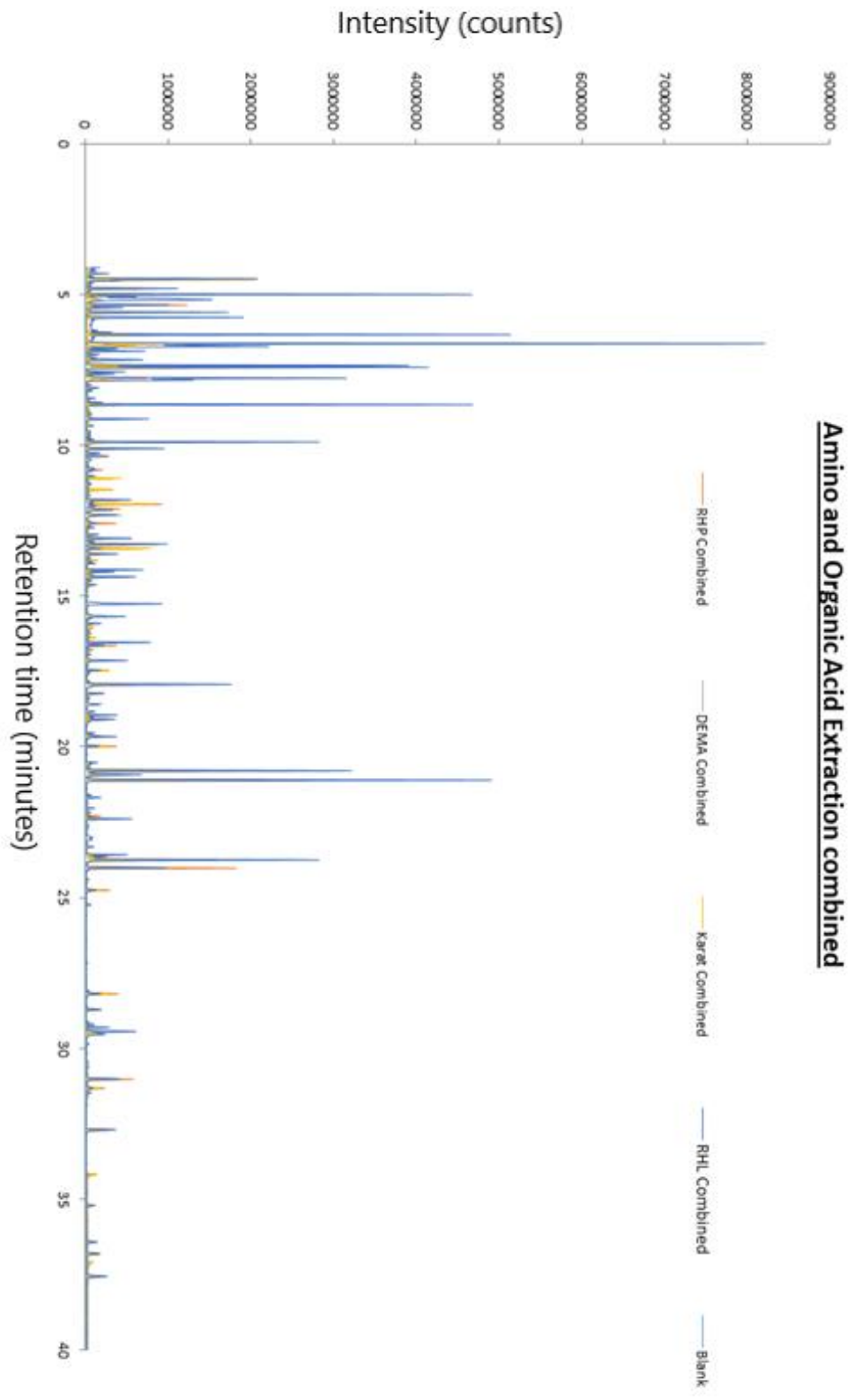
Figure 3.4 Amino and organic acid extraction results combined. *Overlays of the total ion chromatograms for all samples tested plus blank.*

Table 3.1 Organic and amino acids detected using GC-MS in barley exudate samples. *Y/N indicates the presence or absence of each compound in each genotype studied alongside retention time and predicted chemical identifier. Peaks were identified using the Agilent MSD software coupled to the NIST 2011 database. Septum and column artefacts were discarded. Peaks with less than an 80% match to the database were manually interrogated using the NIST 2011 database.*

Retention Time	Chemical Present (Y/N)				Chemical Identifier
	Dema	Karat	*rhl1.a*	*rhp1.b*	
4.47	N	N	Y	N	Alanine
5.0785	N	N	Y	N	3-Hydroxypropanoic acid
5.18	Y	N	N	Y	2-pyrrolidinone
5.3491	Y	Y	Y	Y	3-Hydroxybutyric acid
5.4505	N	Y	Y	N	2-Hydroxyvaleric acid
5.5857	N	Y	Y	N	Unknown
5.5969	N	Y	Y	Y	Urea
6.2506	N	Y	N	N	Glycine
6.3294	Y	Y	Y	Y	Valine
6.6224	Y	Y	Y	Y	4-Hydroxybutyric acid
6.6788	N	Y	Y	Y	Formamide
6.8027	N	N	N	Y	Diethylene glycol
6.8816	N	Y	Y	Y	Benzoic acid
7.107	N	N	N	Y	Octanoic acid
7.1521	N	Y	N	N	4,6-Dioxoheptanoic acid
7.1633	Y	N	Y	Y	Unknown
7.3549	Y	Y	Y	Y	Leucine
7.4112	Y	Y	Y	Y	Glycerol
7.5577	N	Y	Y	N	2-hydroxybutanoic acid
7.569	N	N	N	Y	Alpha-Hydroxyisovaleric acid
7.6366	N	Y	Y	N	Succinic acid
7.7718	Y	Y	Y	Y	L-Isoleucine
8.0986	N	N	N	Y	Succinic acid
8.6508	Y	Y	Y	Y	Thymine
9.8903	N	Y	Y	N	Pyrimidine
9.9016	Y	N	N	Y	Thymine
9.8904	Y	N	N	N	n-methy pyrimidine
10.1043	N	N	Y	N	Dihydrocinnamic acid
10.1157	N	Y	N	Y	Hydrocinnamic acid
10.3636	N	N	N	Y	1-Propionylproline
10.8143	N	Y	N	Y	Decanoic acid
11.8849	N	N	N	Y	1. 4-methoxy-3-methylbenzoic acid methyl ester
12.1102	N	N	N	Y	meso-Erythritol

12.1441	N	Y	N	N	Adenine
12.3243	Y	Y	N	Y	Betaine
12.5835	Y	Y	N	Y	trans-Cinnamic acid,
12.9667	N	N	N	Y	Rhamnose
13.1019	Y	Y	Y	Y	4-Hydroxyphenylethanol
13.4174	Y	Y	N	Y	Gallic acid
13.609	N	Y	N	Y	Fumaric acid
14.1274	N	Y	Y	N	Vanillic Acid
14.2063	Y	Y	N	N	Protocatechuic acid
14.2175	N	N	Y	Y	Phenylalanine
14.3752	Y	Y	Y	Y	p-Hydroxyphenylacetic acid
14.6232	N	Y	N	N	1. Pentan-2-ol
15.254	N	N	Y	N	l-Alanine
15.9303	N	Y	Y	Y	4-Hydroxyhydrocinnamic acid,
16.0881	N	Y	Y	N	2-Hydroxy-3-methylbutyric acid
16.7755	Y	N	Y	N	5,6-Dimethoxy-1-indanone
17.1361	N	Y	N	N	Azelaic acid
17.9361	Y	Y	N	Y	4-Hydroxyhydrocinnamic acid
18.2291	N	N	N	Y	Unknown
18.9503	N	Y	Y	Y	Tetradecanoic acid
19.0968	N	N	Y	Y	n-Pentadecanoic acid
19.6264	Y	N	Y	Y	3-hydroxy-L-tyrosine
20.7983	Y	Y	Y	Y	cis-9-Hexadecenoic acid
21.1026	Y	Y	Y	Y	Palmitic acid,
22.3985	N	Y	N	Y	cis-10-Heptadecenoic acid
23.5705	N	Y	Y	N	9,12-Octadecadienoic acid
23.5817	N	Y	N	Y	9,12-Octadecadienoic acid
23.6493	Y	Y	N	Y	Oleic acid,
23.7507	N	N	Y	Y	trans-13-Octadecenoic acid
24.0212	Y	Y	Y	Y	Octadecanoic acid,
28.7202	Y	Y	Y	Y	1-Monopalmitin
29.4414	Y	Y	Y	Y	Sebacic acid
31.019	Y	Y	N	Y	Stearic acid
36.428	N	N	N	Y	Campesterol
36.8111	Y	Y	N	Y	Stigmasterol
37.5661	N	Y	Y	Y	beta.-Sitosterol
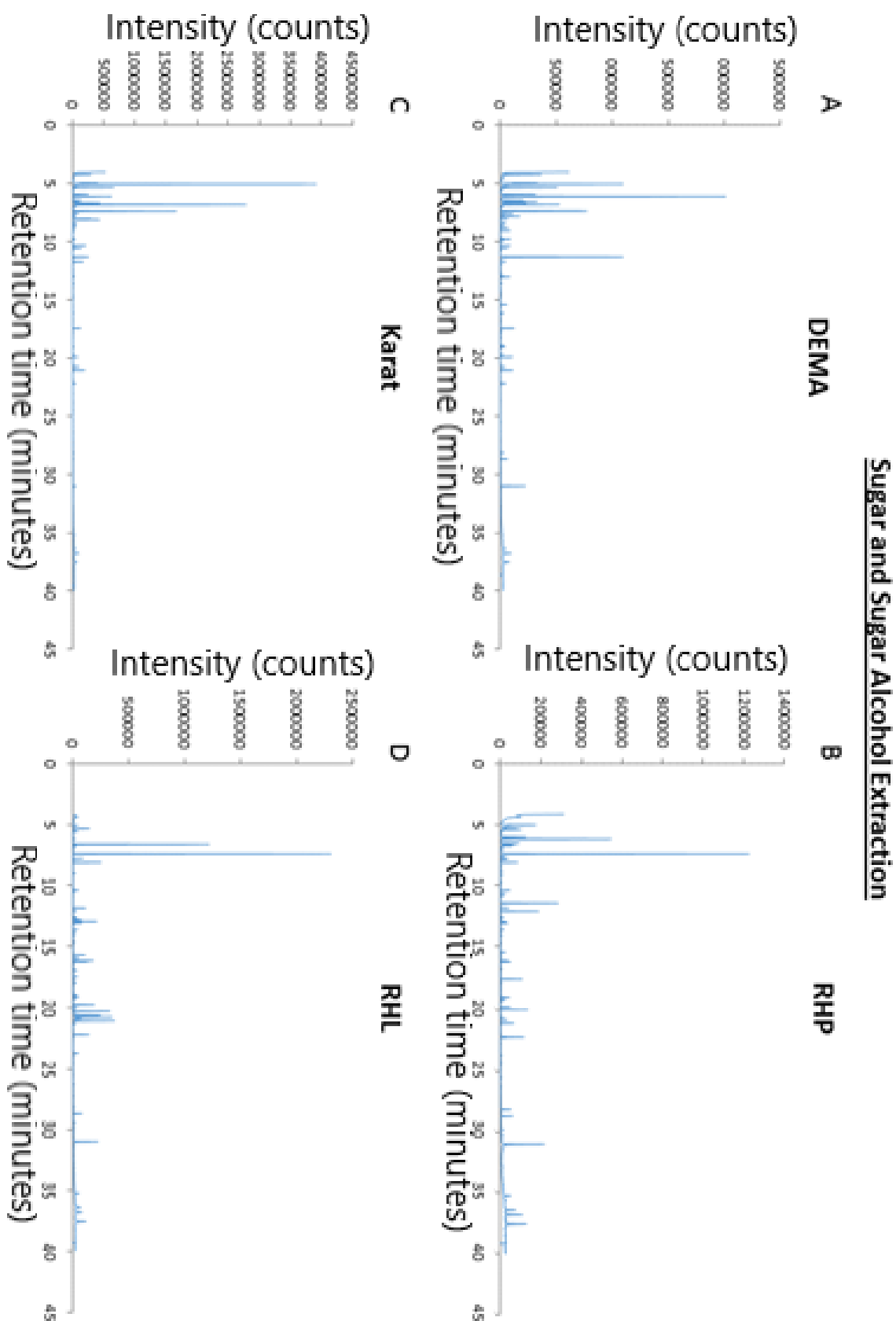
Figure 3.5 Sugar and sugar alcohol extraction. *Total ion chromatograms for the exudates of (A) Dema, (B) rhp1.b, (C) Karat and (D) rhl1.a. Blank extraction can be found in supplementary Figure S3*
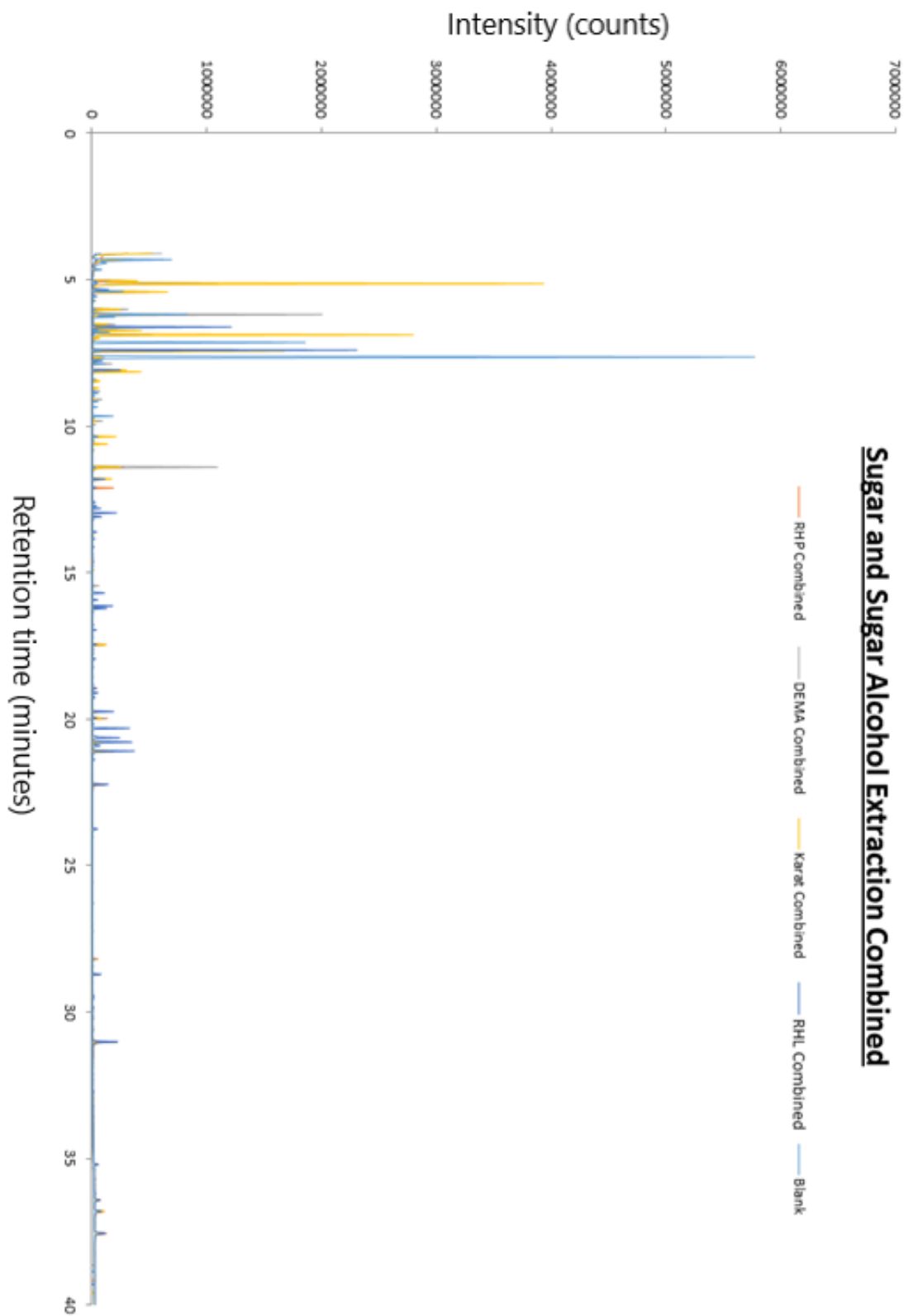
Figure 3.6 Sugar and sugar alcohol extraction results combined. *Overlays of the total ion chromatograms for all samples tested plus blank.*

Table 3.2 Sugar and sugar alcohol detected using GC-MS in barley exudate samples. Y/N indicates the presence or absence of each compound in each genotype studied alongside retention time and predicted chemical identifier. Peaks were identified using the Agilent MSD software coupled to the NIST 2011 database. Septum and column artefacts were discarded. Peaks with less than an 80% match to the database were manually interrogated using the NIST 2011 database.

Retention Time	Chemical Present (Y/N)				Chemical Identifier
	Dema	Karat	*rhl1.a*	*rhp1.b*	
5.3379	Y	N	Y	Y	3-Butanoic acid
5.4055	N	N	N	Y	Hexyl alcohol
6.521	Y	N	N	Y	3-Hydroxyphenylacetic acid
6.6112	Y	N	Y	Y	Hydroxybutyric acid
6.8704	Y	Y	N	N	3-Buten-1-ol
7.4226	Y	Y	N	N	Phosphoric acid
7.7605	N	N	Y	N	2-deoxy Erythro-Pentonic acid
7.862	Y	N	N	N	Diphenyl-Phosphinic acid like molecule
9.7213	Y	N	N	N	Norleucine derivative
12.1103	N	N	N	Y	Erythritol
12.9555	Y	N	Y	N	4-Hydroxyphenylethanol
15.4458	Y	N	Y	N	Fructose
16.2346	Y	N	Y	Y	Xylitol
16.2232	N	N	Y	N	Arabitol
18.9504	Y	N	N	Y	Scyllo-inosose
19.7392	Y	N	Y	Y	D-Mannitol
20.6405	N	N	Y	N	Alpha.-D-Glucopyranuronic acid
20.911	N	N	Y	N	Arabinopyranose
21.1027	Y	Y	Y	Y	Hexadecanoic acid
22.2408	Y	N	Y	Y	Myo-Inositol
35.221	N	N	N	Y	Cholesterol
36.8112	Y	N	N	Y	Stigmasterol
37.5662	Y	N	Y	Y	beta.-Sitosterol

Of the detected exudates 16.7% were present in the samples from all genotypes (Table 3.3) and included compounds such as valine, stearic acid, thymine and palmitic acid which are recognised in the literature as being a feature of barley (and maize) exudation profiles (Naveed et al., 2017).

Table 3.3 Organic and low molecular weight compounds detected in the exudates of all barley genotypes. Y/N depicts if named exudate signal identified or not in a given genotype measured at 70 eV.

Exudate detected in genotype sample (Y/N)				
Amino and Organic Acids	Dema	Karat	*rhl1.a*	*rhp1.b*
Hexadecanoic acid	Y	Y	Y	Y
3-Hydroxybutyric acid	Y	Y	Y	Y
Valine	Y	Y	Y	Y
4-Hydroxybutyric acid	Y	Y	Y	Y
Leucine	Y	Y	Y	Y
Glycerol	Y	Y	Y	Y
L-Isoleucine	Y	Y	Y	Y
Thymine	Y	Y	Y	Y
4-Hydroxyphenylethanol	Y	Y	Y	Y
p-Hydroxyphenylacetic acid	Y	Y	Y	Y
cis-9-Hexadecenoic/palmitoleic acid	Y	Y	Y	Y
Palmitic acid,	Y	Y	Y	Y
Octadecanoic/Stearic acid,	Y	Y	Y	Y
1-Monopalmitin	Y	Y	Y	Y
sebacic acid	Y	Y	Y	Y
Sugars and Sugar Alcohols				
Hexadecanoic acid	Y	Y	Y	Y

A total of 62% of the compounds detected in the study were found in 3 of the 4 genotype exudate profiles in no particular pattern whilst 13% were detected only in the wild type and mutant (i.e., Dema and *rhp1.b*), but not in the corresponding genetic pair (**Table 3.4**).

However, several of these observations may be due to the fractionation process or the analysis itself as structural and steric isomers plus identical compounds with just a variation in retention rate have been detected in all of the genotypes, an example of this is thymine which has been recorded with a retention rate of 9.9 that features only in Dema/*rhp1.b* vs thymine with a retention rate of 8.65 which was detected in all 4 exudation profiles (**Table 3.1**).

Table 3.4 Organic and low molecular weight compounds detected solely in Dema/rhp1.b or Karat/rhl1.a. Y/N depicts if named exudate signal identified or not in a given genotype measured at 70eV

Exudate detected in genotype sample (Y/N)				
Amino and Organic Acids	Dema	Karat	*rhl1.a*	*rhp1.b*
2-pyrrolidinone	Y	N	N	Y
Thymine	Y	N	N	Y
2-Hydroxyvaleric acid	N	Y	Y	N
Unknown	N	Y	Y	N
2-hydroxybutanoic acid	N	Y	Y	N
Succinic acid	N	Y	Y	N
Pyrimidine	N	Y	Y	N
Vanillic Acid	N	Y	Y	N
2-Hydroxy-3-methylbutyric acid	N	Y	Y	N
9,12-Octadecadienoic acid	N	Y	Y	N
Sugars and Sugar Alcohols				
3-Hydroxyphenylacetic acid	Y	N	N	Y
Scyllo-inosose	Y	N	N	Y
Stigmasterol	Y	N	N	Y

Of the 90 compounds identified from the exudates of the 4 barley genotypes 6 were observed to be detected solely in the wild type or solely in the mutant lines. In particular our analysis indicates that phosphoric acid, 3-buten-1-ol and protocatechuic acid could not be detected in the exudates taken from the mutant lines *rhp1.b* or *rhl1.a*. Conversely, phenylalanine, trans-13-octadecanoic acid and n-pentadecanoic acid could not be detected in the wild type exudates (**Table 3.6**).

3.3.2. The bacterial composition of exudate derived microbial communities

This study presented the opportunity to investigate microbial community shifts that may be directly modulated by the composition of the low molecular weight carbon compounds released as exudates by plant roots and to compare these results with previously published findings. It also allowed for the potential identification of endophytic microorganisms that may have been released from plant tissue due to unintentional root damage during the sampling process and the presence of naturally occurring microbial contaminants from the growth environment.

Table 3.5 Organic and low molecular weight compounds detected solely in wild type or mutant genotypes. Y/N depicts if named exudate signal has been identified or not in a given genotype measured at 70 eV

Exudates detected in genotype sample (Y/N)				
Amino and Organic Acids	Dema	Karat	*rhl1.a*	*rhp1.b*
Phenylalanine	N	N	Y	Y
trans-13-Octadecenoic acid	N	N	Y	Y
n-Pentadecanoic acid	N	N	Y	Y
Protocatechuic acid	Y	Y	N	N
Sugars and Sugar Alcohols				
3-Buten-1-ol	Y	Y	N	N
Phosphoric acid	Y	Y	N	N

3.3.2.1 The microbiota obtained from the root exudates of Karat and *rhl1.a* genotypes.

As the DNA analysed was obtained from two pooled samples it was not possible to perform as detailed an analysis as that which was conducted in chapter two on the bacteria isolated from the exudates of a pair of genetically related lines Karat and *rhl1.a.* The average exudate yield per plant was 0.01 - 0.015 g, 0.5 g of exudate was required for DNA extraction meaning it is likely that the exudates analysed per genotype were derived from approximately 62.5 plants combined. A total of 299,772 high quality sequence reads were obtained from the amplicon sequencing of the 16S rRNA gene from the samples which yielded a total of 718 OTUs at 97% identity.

The number of reads per sample was comparable; Karat accounted for 48.03% of the sequence data with 143,985 reads and *rhl1.a* accounted for the remaining 51.59% with 155,787 reads (mean 149,886). Quality filtering was conducted in silico to identify OTUs identified as chloroplast or mitochondria derived but none were detected, confirming that all sequence reads identified were of microbial origin; all original sequence reads and OTUs were retained for further analysis.

3.3.2.2 Bacteroidetes and Proteobacteria account for over 98% of the root exudate microbiota of wild type and mutant barley genotypes.

A total of 15 bacteria and archaeal phyla were observed from the sequencing data. Operational taxonomic unit sequence reads were transformed into relative abundance for each phylum and phyla whose relative abundance was less than 1% were removed from this analysis. This left 3 phyla remaining in the dataset; Actinobacteria, Bacteroidetes and Proteobacteria which accounted for an average of 99% of the total sequence reads represented in this dataset (99.4% retained in Karat and 98.6% in *rhl1.a*). However, it is important to note that Proteobacteria accounted for the largest proportion of the reads classified at phylum level, effectively dominating the dataset. Conversely, Actinobacteria and Bacteroidetes represent a relatively limited proportion of the 3 most represented phyla accounting for an average of 9.4% and 1.6% of the dataset, respectively (**Table 3.6**).

Due to the large representation of Proteobacteria, the taxonomy was interrogated at a higher resolution; i.e, family level. A total of 114 bacterial families were represented in the total sequence data, after trimming of bacterial families whose relative abundance accounted for less than 1% of the sequence data 9 bacterial families were revealed as the most abundant members of the exudate microbiota. The "top 9" most abundant families in the total data set were all members of the Proteobacteria phylum (**Table 3.7**).

Table 3.6 Percentage relative abundance of the top 5 phyla detected in the freeze-dried exudates collected from Karat and rhl1.a barley genotypes. Proteobacteria almost entirely dominate the phyla present in both samples, the majority of 15 phyla detected in samples are present as less than 1% of the entire dataset.

Phylum	Karat Exudates (%)	*rhl1.a* Exudates (%)
Proteobacteria	91.37	84.68
Bacteroidetes	7.25	11.46
Actinobacteria	0.79	2.46
Firmicutes	0.23	1.05
Armatimonadates	0.15	0.18

Table 3.7 Percentage relative abundance of the top 9 families detected in the freeze-dried exudates collected from Karat and rhl1.a barley genotypes. All families belong to the Proteobacteria phylum but represent a broad range of families when families each representing 1% or less of the total data set have been removed.

Family	Karat Exudates (%)	*rhl1.a* Exudates (%)
Enterobacteriaceae	37.58	16.59
Pseudomonadaceae	29.96	22.82
Oxalobacteraceae	9.76	22.64
Xanthomonadacea	5.39	9.4
Flavobacetralaes (f_ weeksellaceae)	4.74	7.89
Alcaligenaceae	4.07	4.94
Sphingobacteriaceae	2.08	3.54
Burkholderiaceae	1.19	3.11
Rhodospirillaceae	0.43	2.79

To visualise the variations in proportions of each high-ranking phyla and family between the exudate samples the relative abundance data was plotted as a simple bar plot (**Figure 3.7**). As can be seen from the data the sequence resolution has almost entirely identified OTUs to the order level specifically with general taxonomy being assigned at the family level.

In order to investigate if the OTUs identified in the exudate data were either the result of environmental contamination from the hydroponic equipment, other glasshouse users, the nutrient solution etc or may have come directly from the roots of the plantlets (i.e., endophytes released into the tanks) the results were compared with a list of previously identified common lab bacterial contaminants obtained by conducting DNA extraction of a sterile distilled water control in the same environment rhizosphere DNA extraction is conducted (Pietrangelo et al., 2018).

Proteobacteria dominate the exudate microbiota at phylum level but fluctuations at family level are not statistically significant
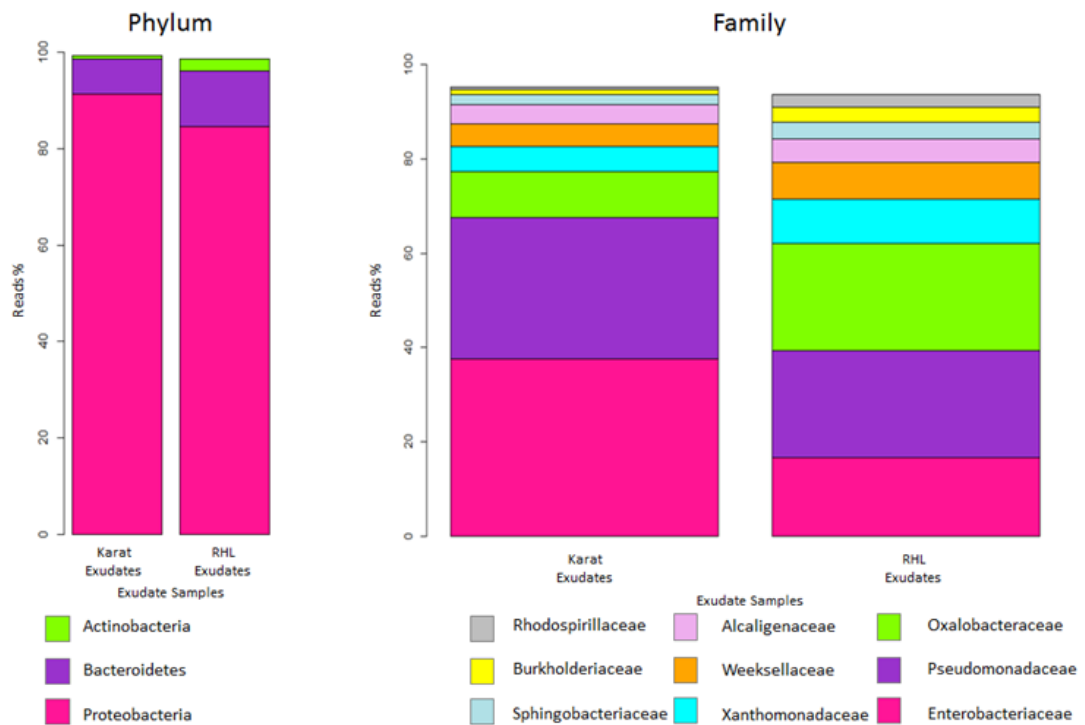
Figure 3.7 Proteobacteria dominate the exudate microbiota at phylum level but fluctuations at family level are not statistically significant. DNA extracted from the exudates of Karat and rhl1.a barley genotypes show a highly dominant proportion of Proteobacteria at the phyla level and feature families from two orders found to be enriched in the rhizosphere of root hair mutants. However, this enrichment is not significant.

3.3.2.3 Known environmental contaminants are not responsible for the majority of microbial reads obtained from barley exudates

Using a list of 56 OTUs identified as contaminants in a previous study 29 OTUs were removed from the exudate data set accounting for just 4% of the OTUs observed in the exudate profiles, all of which were members of the Proteobacteria phylum, i.e., the most represented phylum, in both data sets, particularly the orders Burkholderiales and Pseudomonadales (**Table 3.8**). Reanalysis of the exudate data following the removal of the putative environmental contaminants from the exudate data set resulted in the loss of 12,219 sequence reads, however 95.9% of the sequence data was retained. The "contaminant free" data was again converted into relative abundance and phyla and family level taxonomy distribution was analysed using the same process of removing any which displayed a relative abundance of 1% or less. Following this filtering the mean number of reads retained per samples was 99% at the phyla level and 95.1% at the family level. This data was then compared with the original sequence data

(**Tables 3.9** and **3.10** respectively).

Table 3.8. A small proportion of the exudate microbiota are likely to be environmental contaminants.
The 29 contaminant OTUs listed were removed from the exudate data set. All of these contaminant OTUs
belong to the Proteobacteria phylum.

Number present	Order	OTU ids
12	Burkholderiales	109391, 317424, 331752, 358068, 569952, 576785, 788519, 944197, 1127804, 2360704, 2685602, 4328567
12	Pseudomonadales	142419, 338140, 358042, 513808, 541223, 646549, 728119, 961783, 1109251, 1566691, 2317377, 2534141
2	Rhizobiales	4369105, 4388385
2	Rhodocyclales	4315164, 4418677
1	Sphingomonadales	326054

Table 3.9 Percentage relative abundance of the top 5 phyla detected in the freeze-dried exudates
collected from Karat and rhl1.a barley genotypes compared with data produced following the removal
of OTUs identified as potential contaminants. Proteobacteria almost entirely dominate the phyla present
in both samples, the majority of 15 phyla detected in samples are present as less than 1% of the entire
dataset.

	Original Data (%)		Minus Contaminant (%)	
Phylum	Karat	*rhl1.a*	Karat	*rhl1.a*
Proteobacteria	91.37	84.68	90.7	84.4
Bacteroidetes	7.25	11.46	7.86	11.68
Actinobacteria	0.79	2.46	0.85	2.5
Firmicutes	0.23	1.05	0.23	1.05
Armatimonadetes	0.15	0.18	0.15	0.18

Although removal of the contaminant OTUs made a negligible impact on data at
the phyla level it did impact the family distribution with Pseudomonadaceae,
replacing Enterobacteriaceae as the most abundant, however this change
equally affected both wild type- and mutant-derived sequencing profiles indicating
that mainly contaminant Enterobacteriaceae was removed from the data. The
shift in proportions of all other taxonomic families was negligible and, overall, the
removal of contaminant OTUs did not impact the community structure of the
microbiota gathered from the plant exudates (**Figure 3.8**).

Table 3.10 Percentage relative abundance of the top 9 families detected in the freeze-dried exudates collected from Karat and rhl1.a barley genotypes compared with data produced following removal of "contaminant" OTUs from the exudate dataset. Following the removal of contaminant OTUs all of the most abundant bacterial families remain belonging to the Proteobacteria phylum. Following the removal of contaminant OTUs the Pseudomonadaceae family is now the most abundant. The most abundant families are highlighted in green

	Original data (%)		Minus contaminant (%)	
Family	Karat	*rhl1.a*	Karat	*rhl1.a*
Enterobacteriaceae	37.58	16.59	30.9	10.4
Pseudomonadaceae	29.96	22.82	38.5	27.42
Oxalobacteraceae	9.76	22.64	10.09	22.06
Xanthomonadaceae	5.39	9.4	5.8	9.686
Flavobacteriales (f_ weeksellaceae)	4.74	7.89	4.94	7.77
Alcaligenaceae	4.07	4.94	4.82	5.06
Sphingobacteriaceae	2.08	3.54	1.99	3.25
Burkholderiaceae	1.19	3.11	1.31	3.26
Rhodospirillaceae	0.43	2.79	0.44	2.8

Comparison of Phylum and Family distribution of bacteria extracted from exudate samples before and after contaminant OTU removal
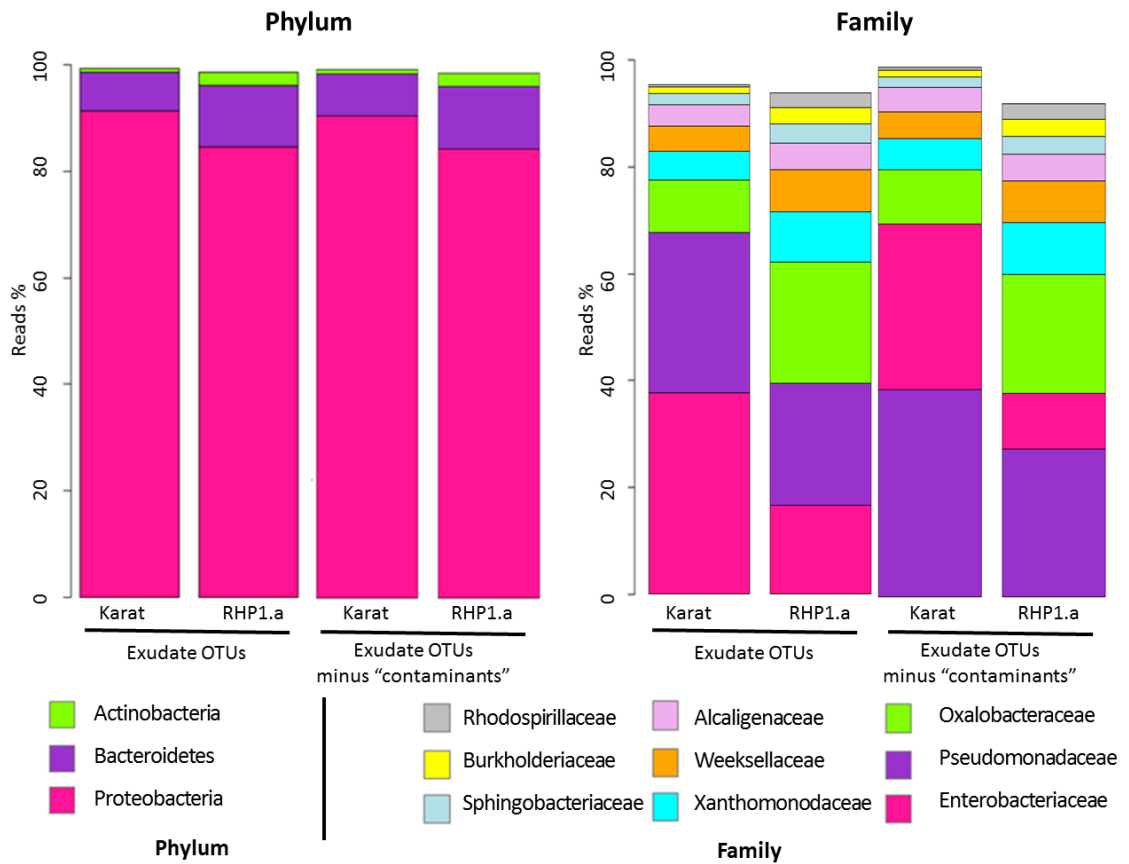
Figure 3.8 Removal of known contaminants makes no significant impact on the taxonomic distribution of the bacteria obtained from barley exudates. *DNA extracted from the exudates of Karat and rhl1.a barley genotypes and analysed at both the phylum and family level. The left-hand proportion of each graph represents the taxonomic composition of the bacteria extracted from the exudates of the genotype described below each bar plot and the right-hand portion of the graph shows the taxonomic distribution of the same organisms minus OTUs identified as potential contaminants.*

3.4 DISCUSSION

This study examined the variation in the exudation of organic and amino acids plus sugars and sugar alcohols between four barley genotypes; Karat and Dema displaying wild type root hair development and *rhp1.b* and *rhl1.a* (the root hair mutants within the wild type backgrounds respectively) and what, if any, the impact root hair mutations had on exudation profiles vs wild type. We identified 6 organic compounds as being detected in the exudates collected from wild type or mutant plants and completely undetected in the other, namely phenylalanine, trans-13-octadecanoic acid and n-pentadecanoic acid detected in wild type exudates only and protocatechuic acid, 3-buten-1-ol and phosphoric acid that were solely detected in the exudes of the mutant plants.

The study went on to investigate the bacterial communities present within the exudates of the wild type Karat and the mutant *rhp1.b* in order to establish if exudates alone would drive community shifts and to examine bacterial OTUs that may be endophytic or the result of environmental contaminants. This study revealed a far simpler microbial community in comparison to soil associated microbial studies with a heavy bias towards members of the Bacteroidetes and Proteobacteria phylum in both exudate datasets. However, at a higher taxonomic resolution it was observed that the orders Enterobacteriaceae and Pseudomonadaceae were present in the wild type exudates in greater proportions than in the mutant derived exudates with Enterobacteriaceae representing over double the relative abundance in wild type exudates versus mutant. However, these were determined not to be statistically significant.

3.4.1. The amino acid phenylalanine was detected only in the exudates of root hair mutant barley

It was interesting to observe that phenylalanine, an aromatic amino acid that is a common component of plant exudates was only detected in the exudation profiles of the root hair mutant plants (Ulehlova B, 1988). Whilst it is highly unlikely that this very common exudate compound was not expressed at all in the wild type plants, it is possible that the observation in this data is the result of a much higher level of secretion from the mutant genotypes.

Phenylalanine is a key component in the phenylpropanoid pathway where it is the skeleton for the production of phenylpropanoids which are a diverse group of compounds that are involved in a variety of crucial activities such as plant defence, structural support, and survival (Dzhavakhiya et al., 2007, Alber and Ehlting, 2012, Kumar et al., 2019, Cabané et al., 2012). It is excreted from plant roots by ABC transporters which, interestingly, have been found as differentially regulated in a comparative proteomic survey of the genetic pairs Karat-*rhl1.a* and Dema-*rhp1.b* (Janiak et al., 2012).

It is tempting to speculate that the higher proportions of phenylalanine in the root hair mutant exudates could be attributed to this differential expression. In addition to this, and more relevant to this study, phenylalanine is also a starting point for the production of compounds such as flavonoids and coumarins (Halpin, 2004) which have been shown to have antimicrobial properties against some common soil bacterial organisms such as *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus subtillis* (Farhadi et al., 2019, Mbaveng et al., 2015) and antifungal properties against *Cryptococcus* sp*, Trichophyton mentagrophytes, Candida parapsilosis, Aspergilluys fumigatus* and *Candida albic*ans (Kurdelas et al., 2010, Silva et al., 2017) ; phenylalanine on its own has also been identified as having antimicrobial properties such as inhibiting the formation of biofilms (Li et al., 2016, Gahane et al., 2018). Interestingly, this putative higher production of phenylalanine is somewhat counter intuitive to the plant as many of the bacterial organisms that it acts on as an antimicrobial against have also been identified as PGPRs, which have the potential to counter-balance the negative impact of root hair mutations in plant's nutrient uptake. For example, *Pseudomonas aeruginosa* has been linked to zinc stress tolerance and *Bacillus subtilis* has been implicated in the solubilisation of phosphate (Saeid et al., 2018, Islam et al., 2014).

However, the observation of a higher detection of phenylalanine in the mutant exudates could be unrelated to the process of exudation. For example, phenylalanine is a precursor for the structural protein lignin, which is a vital component in plant cell wall (Buxton and Russell, 1988, Fukushima, 2001). It cannot be discounted that it is possible that the mutations that result in the hairless phenotype may be responsible for other phenotypic traits such as higher

root cell churn/ sloughed cells or reduced structural robustness which could also contribute to this observation in the root hair mutant exudates.

3.4.2. n-pentadecanoic acid was detected solely in the exudates of root hair mutant barley

The straight chain fatty acid n-pentadecanoic acid could not be detected in the root exudates of wild type plants but was present in the exudates of both mutant genotypes. This was intriguing as the presence of hydrophobic compounds in plant exudates, although not unheard of, is uncommon (Canarini et al., 2019) . Typically, these lipids are bound to proteins which determine their fate, they can serve several functions such as transport to other tissues for storage, modification or degradation, signalling and energy storage in eukaryotic systems (Vogel Hertzel and Bernlohr, 2000, Suh et al., 2015). Straight chain fatty acids have also been implicated in having antimicrobial properties against bacterial species such as methicillin-resistant *Staphylococcus aureus* and *Mycobacteria* and can also serve as a metabolite for some gram positive bacteria that can go on to be used for phospholipid biosynthesis or the production of acyl-CoA (Zheng et al., 2005, Cronan and Thomas, 2009)

Benning and co-workers proposed that these fatty acids may be involved in long distance signalling that may play an important role in both developmental and stress signalling (Benning et al., 2012). In addition, it has also been observed in *Arabidopsis thaliana* that pentadecanoic acid, when added to soil in combination with hexadecanoic acid, palmitoleic acid, octadecanoic acid, and arachidic acid (many of which have been detected in the data from this study) has an inhibitory effect on infection caused by *Pseudomonas syringae,* a highly prevalent plant pathogen (Yuan et al., 2018). Considering the antimicrobial properties of both phenylalanine and n-pentadecanoic acid it is tempting to propose that the reduced complexity microbiota observed in that of the root hair mutant barley plants (described in **Chapter 2**) is, in part, the result of the plants excreting these antimicrobial compounds allowing other members of the microbiota to flourish.

3.4.3. Other compounds are differentially detected in the exudates of wild type and root hair mutant barley

There were exudate compounds that were detected in either wild type or mutant plants only that do not feature in the literature with any relevance to exudate

related activities, either as part of a system or as a primary or secondary metabolite, particularly trans-13-octadecanoic acid, protocatechuic and 3-buten-1-ol. Interestingly though, the chemical structure of 3-buten-1-ol is remarkably similar to that of isoprenol, the only difference being the absence of detection of a methyl group which could have been lost in the fractionation process. Isoprenol (3-methyl-2-buten-1-ol) is common and an intermediate to prenol which is involved in the process of glycosylation, but more relevantly, the assembly of peptidoglycan which is a major component of the bacterial cell wall in bacteria (Vollmer, 2015, Eichler and Imperiali, 2017). This indicates that this could be a bacterially derived compound in the data as neither 3-buten-1-ol or isoprenol are recognised in the literature as being known components of grass exudates.

The final wild or mutant enriched compound was phosphoric acid which was detected in the wild type exudates only. Although this has been reported as a component of plant exudates in the literature at times (but with limited understanding of the function it may have as an exudate). In a quantitative exudate study Naveed et al observed higher than expected levels of phosphoric acid in the exudation profiles of barley which were grown in the same conditions as this study (Naveed et al., 2017). They have postulated that this observation is due to the accumulation of phosphoric acid in the root hair cells during nutrient acquisition and is found in higher levels than predicted as a result of sloughed root hair cells. Further experimentation will be needed to test this scenario.

A 2017 study investigating total carbon released by wild type and root hair mutant barley genotypes (*Hordeum vulgare cv* "Pallas" – wild type and its root-hairless mutant "*brb*") revealed that barley with root hairs released more carbon in to the rhizosphere compared to their cognate wild-type plants (Holz et al., 2018). Although this study did not scrutinise chemical structure of the exudation profiles, and was conducted using a rhizobox instead of hydroponic tanks, it may indirectly complement the findings of this chapter whereby root hair mutants are not impaired in exudation per se but rather these mutants give rise to a distinct exudation profile.

3.4.4. The exudate microbiota: contamination or seed-transmitted endophytes?

The microbial analysis of the exudate profiles ultimately served as confirmation that the method of exudation collection is an open biological system and, in some respects, to extend the database of potential contaminants developed by L Pietrangello (Pietrangelo et al., 2018). This list was created from a DNA extraction kit using sterile water instead of a soil sample, which means it is likely that the contaminants listed are by no means exhaustive. The OTUs that remained in the exudate data following contaminant filtering may be present for a number of reasons including environmental contamination from the plant growth and exudate collection process, but it cannot be ruled out that many may also represent seed-transmitted endophytic microorganisms from the plant roots that are ultimately soil derived. In fact, removing the known contaminants did not alter the bacterial community composition of the pooled exudates from Karat and *rhl1.a* in any significant way at the phylum or family level (**Figure 3.9**). However, it is important to keep in mind that there was a limited range of known contaminants used in this study.

The primary observation from this study was that although the families Enterobacteriaceae and Pseudomonadaceae dominated the communities in both exudate samples their representation was notably greater in the Karat derived exudates, although the orders that these OTUs belong to were not significantly enriched in the pairwise comparisons conducted in **Chapter 2** (**Figure 2.8**). However, the family Oxalobacteraceae, belonging to the order Burkholderiales were far more highly represented in the exudates of *rhp1.b* which indeed, correlates with the observation in **Chapter 2** that the order Burkholderiales is significantly enriched in both *rhp1.b* and *rhl1.a* rhizosphere samples when compared with their wild type homologues. Although it is commonly accepted that rhizodeposition plays an important role in recruitment of the rhizosphere microbiota from the surrounding soil, the composition of bacteria present in plant exudates and derived from the plants remains poorly understood.

Strikingly the dominance of the phyla Bacteroidetes and Proteobacteria in the exudate microbiota is reminiscent of the composition of the rhizosphere of axenically-grown maize seedlings (Johnston-Monje et al., 2016). Likewise, a

recent investigation of soil-grown tomato plants revealed that a significant proportion of the rhizosphere and endosphere microbiota of the given species is indeed seed-derived (Bergna et al., 2018).

3.4.5 Conclusions

It was revealed that in wild type barley antimicrobial compounds such as phenylalanine and N-pentadecanoic acid could not be detected using GC-MS (at the set threshold) but were present in the mutant profiles. Moreover, these antimicrobial compounds have been linked to the inhibition of known PGPR species (Vacheron et al., 2013). Furthermore, the bacterial family Pseudomonadaceae, which is sensitive to both phenylalanine and n-pentadecanoic acid was present in much lower abundance in the exudate samples of the mutant plants compared to the wild types (Askoura et al., 2011).

This observation suggests that shifts in root hair exudation patterns between wild type and root hair mutant plants contribute to the shape, at least in part, of the rhizosphere bacterial microbiota by making the environment chemically less suitable for some PGPR families. Consequently, this would allow other or additional bacterial species to proliferate to an extent which would normally be outcompeted in a wild type rhizosphere environment.

This data supports the multiple propositions that exudates play a role in shaping the microbiota but gives further insight into why root hair mutations are observed to do the same. For instance, it is clear from the exudate data that the compounds phenylalanine and n-pentadecanoic acid which are known *Pseudomonas sp.* antimicrobials, if produced in the exudates of wild type plants are at a level that is below the detection threshold, this corresponds with bacterial sequence data showing a marked increase in the presence of Pseudomonas in the exudate data.

Furthermore, the increased presence of Oxalobacteraceae members, which appear to be less susceptible to these antimicrobials, in both the exudates and rhizosphere of root hair mutant barley adds further support to the suggestion that root hair mutant exudates may selectively interfere with the proliferation of given bacterial species allowing for others to proliferate in a less competitive environment (**Figure 3.9**).
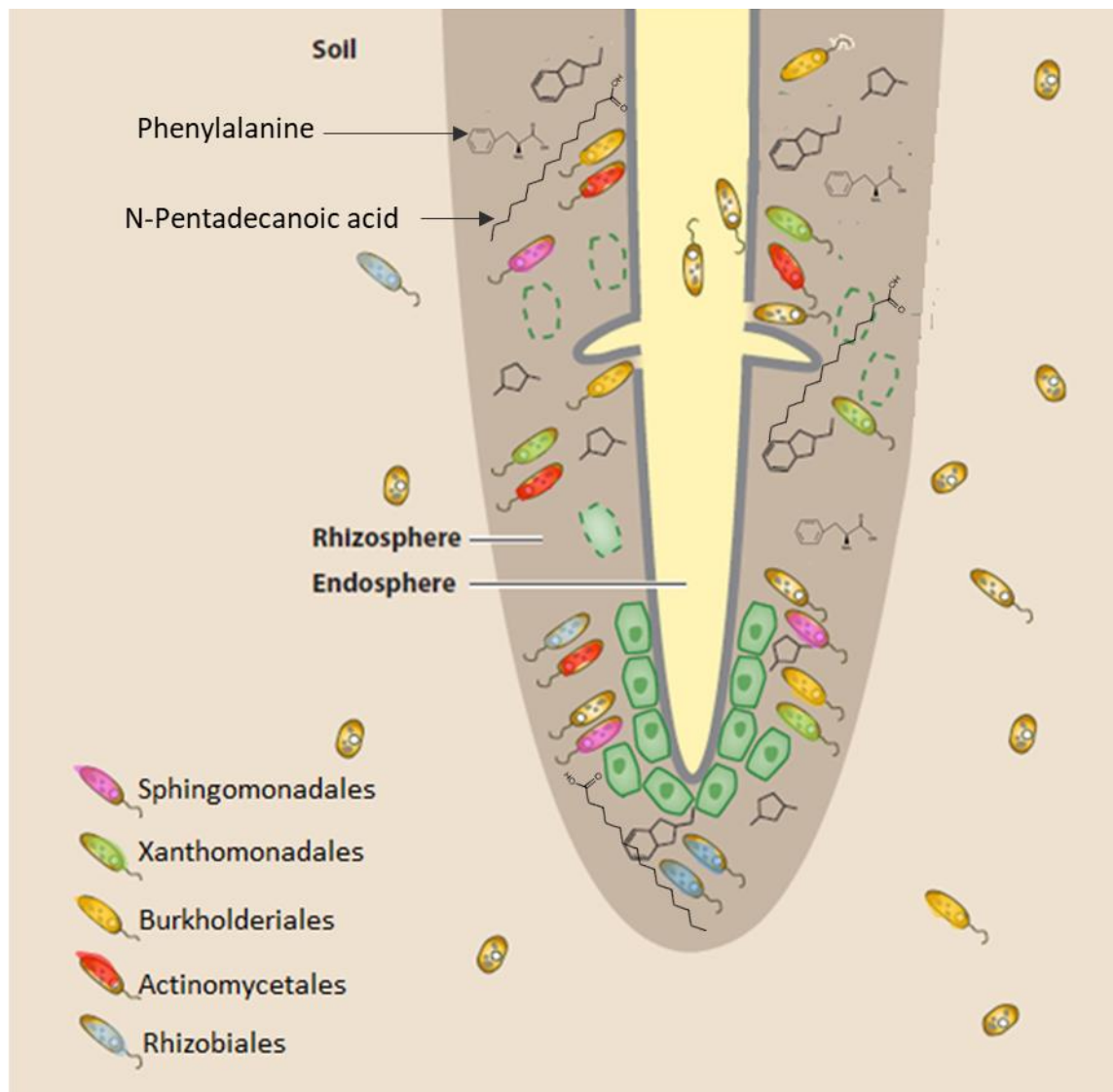
Figure 3.9 The community structure of the root hair mutant barley rhizosphere microbiota. The bacterial rhizosphere microbiota is, at least in part, modulated by the host genotype influencing root hair phenotype and the exudation of small organic compounds excreted by the roots in the form of exudates. Barley with root hair mutations show a bias for the orders Sphingomonadales, Xanthomonadales, Burkholderiales, Actinomycetales and Rhizobiales (indicated by colour). Root hair mutant barley may shape the chemical environment with enhanced production of phenylalanine and straight chain fatty acids

It is possible to test this hypothesis experimentally with the availability of an indexed bacterial collection by direct application of the compound to bacterial cultures.

Interestingly the mutations displayed by the barley plants featured in this study have also been associated with a reduced ability to acquire iron, it has recently been demonstrated in *Arabidopsis thaliana* that the transcription factor MYB72 regulates the biosynthesis of iron-mobilizing fluorescent phenolic compounds which have also been identified as having antimicrobial properties (Stringlis et al., 2018).

Although undetected in this study it would be of interest to examine the exudates of these plants at a higher resolution in order to identify if compounds with a similar mechanism are present in the root hair mutants, offsetting the genetic hurdle associated with Iron acquisition. Furthermore, a recent study examining plant-soil feedback revealed that benzoxazinoids released by maize plants as exudates serve to condition the soil and trigger changes in the structure of the microbiota which then go on to serve the next plant generation; they observed that this conditioning resulted in several desirable observations including increased leaf defences (Hu et al., 2018).

The observations made in this study point towards the need for a more robust understanding of the impact rhizodeposition as a whole has on the microbiota at a molecular level. However, it is of equal importance to understand the microbial factors that serve to condition the soil in which plants will grow thus potentially shaping environment driven exudation patterns. It is also important to consider the necessity of a deeper understanding of the additional environmental factors that may be driving interactions within the rhizosphere that can induce shifts in exudation patterns. Factors such as the chemical and physical properties of the soil are key areas to explore combined with the genetics of the plant itself.

A recent study has identified that barley displaying root hair mutant phenotypes have a far denser rhizosphere as a result of lacking root hairs which serve to assist with creating pores and channels within the soil for the movement of gasses and liquids (Koebernick et al., 2017). Changes to the physical composition of the soil via agricultural compaction or due to root morphology will directly impact the ability of some members of the microbiota to proliferate effectively and may induce stress responses in the plants whereby a shift in exudation may occur. All considered, in order to understand the shifts in exudation patterns which modulate, at least in part, the structure and function of the microbiota it is necessary to understand the interplay between host genetics, plant-soil feedback, the microbial interactions driving plant responses and the physical soil environment.

3.4.6 Limitations of this study

The results in section 3.2 show the variation in the exudation of organic and amino acids plus sugars and sugar alcohols from the roots of four barley

genotypes and what, if any, the impact of the root hair mutations displayed by *rhp1.b* and *rhl1.a* genotypes had on exudation profiles vs barley with wild type root hair phenotypes (Karat and Dema). There are several caveats that need to be considered when interpreting the results of this data including (but not limited to) the fact that a hydroponic environment is not a natural growth environment for barley and may induce a stress response which could influence plant exudation if not set up correctly. Waterlogged barley has been shown to have reduced photosynthetic ability, reduced grain weight and can even result in plant death (Ploschuk et al., 2018). However, this is typically due to a considerable reduction in oxygen exchange at the root surface with waterlogged soil becoming anoxic very quickly (Armstrong, 1980).

It is also important to acknowledge that although known common contaminants were removed from this study this is an open system and as such there is the possibility that microorganisms present in the hydroponics system and surrounding environment may impact the study. For example, microorganisms may enter the root system as endophytes which can elicit changes to the volume and composition of organic carbon released by the plants, it was observed by Van Hecke and co-workers that colonisation of the fungal endophyte *N. coenophialu* in the roots of the grass *Festuca arundinacea* resulted in a significant increase in the release of soluble organic carbon based compounds (p = 0.045) (M. Van Hecke et al., 2005).

Microbes may also be present in the water and exudate collection pots and may metabolise a proportion of the exudates released potentially reducing the presence of certain compounds to an undetectable level (Alegria et al., 2016). However, the collection pots used for this study were heat sterilised and the water used for the collection of exudates was filtered and heat sterilised reducing (but not preventing) microbial impact on results. An additional consideration regarding the interpretation of the results of this study are the results of the GC-MS analysis of the exudates. There are a number of compounds that are the same but have been assigned to different exudate groups due to having a different retention time, for example stigmasterol with a retention time of 36.8112 was detected in the exudates of Dema and *rhp1.b* and assigned as a sugar/ sugar alcohol whist stigmasterol with a retention time of 36.8111 was detected in the exudates of

Dema, *rhp1.b* and Karat and identified as an organic/ amino acid. Stigmasterol is a plant sterol which should be assigned as an alcohol (Kim S, 2019).

Further examples of compounds which are the same but assigned individually to different exudate groups due to retention time are alanine, succinic acid, thymine, hydroxyphenylethanol and 9,12-octadecadienoic acid. These caveats may have been resolved by a quantitative exudate study being conducted as opposed to taking a presence/ absence approach. However, using the method outlined in this study did not allow for the production of a sufficient volume of freeze-dried exudates for this to be within the scope of this investigation.

CHAPTER 4

THE PHYSICAL SOIL ENVIRONMENT INFLUENCES THE MICROBIOTA

4.1 INTRODUCTION

The root hairs of plants are known to increase the surface area that can interact between the plant, the soil and the soil microbiota (Grierson and Schiefelbein, 2002, Danhorn and Fuqua, 2007, Wardle et al., 2004). It has been demonstrated that the structure of roots, including their root hair morphology, can significantly impact the ability of plants to take up nutrients.

Likewise, root hair morphology influences the physical properties of the soil surrounding them by exploring pores and channels, by the release of exudate compounds that can bind soil particles and by shaping the bacterial rhizosphere microbiota (Gilroy and Jones, 2000, Czarnes et al., 2000, Hallett et al., 2009, Moreno-Espíndola et al., 2007, Robertson-Albertyn et al., 2017).

In turn, the rhizosphere microbiota release their own carbon based compounds, mediating both mineral biogeochemical cycling and soil physical formation (Goh, 2004). Based on soil compaction studies it has been widely accepted that due to the influence roots and the soil microbiota have on their surrounding soil, the rhizosphere is likely to have smaller and fewer pores and, consequently, a denser soil environment compared to unplanted, "bulk", (Koebernick et al., 2017). However, the physical structure of the rhizosphere can be dynamic and is modulated by factors such as root phenotype, plant age and wetting and drying cycles (Watt et al., 1994, Haling et al., 2014, Hinsinger et al., 2009, Albalasmeh and Ghezzehei, 2014).

Although it is proposed that the rhizosphere is denser than bulk soil, if the soil environment is too dense or compacted this can be detrimental to plant growth and is widely studied in relation to deforestation and the impact of farm machinery and tilling in agricultural systems (Larson et al., 1994, Nawaz et al., 2013). Soil compaction can result in damage to a multitude of physical, chemical and

biological soil properties which can cause plant stress and yield losses in crops (Kodikara et al., 2018). This is due to factors such as the destruction and reduction in size of soil pores that are essential for gas diffusion, restriction of water and nutrient uptake by the plant, the impedance of root growth for soil exploration and anchorage, plus influencing shifts in the soil microbiota (Beylich et al., 2010).

The impact that is of distinct interest for this study is the lack of oxygenation resulting from compaction and waterlogging which leads to anoxic soil conditions. These conditions ultimately lead to a potentially profound shift in microbial communities and function. Root morphology plays an important role in shaping the soil environment, an important element of this is the formation, frequency, length and distribution of root hairs (Keyes et al., 2017, Brown et al., 2012a). Monitoring root growth and the physical impact that this has on the soil environment has been challenging; typically plants needed to be grown in a lab based environment to observe root growth in real time and responded differently to when growing in a natural soil environment, likewise, monitoring the root influence on soil physical conditions has also faced barriers to studying in situ (Wu et al., 2018).

Nevertheless, due to recent advances in non-invasive synchrotron radiation computed tomography (SRCT) it is now possible to monitor root growth, root hair development and how soil is impacted physically as roots develop (Keyes et al., 2013, Koebernick et al., 2017). Images can be used to quantify water and solute movement and to calculate pore and channel dimensions, which enables changes to soil structure to be linked to root uptake functions and morphology at high resolution (**Figure 4.1)** (Koebernick et al., 2017, Keyes et al., 2013).

A recent SRCT analysis examining the root growth of barley genotypes with either a wild type root hair or root hairless phenotypes revealed that the "rhizosphere" of root hairless plants had fewer and smaller pores than those in the "rhizosphere" of the wild type plants (**Figure 4.2**) (Koebernick et al., 2017). In fact, the root hairless mutant fitted the model produced by Dexter and co-workers that suggests that root growth leads to compaction at the soil root interface, while the wild-type with root hairs caused an increase in porosity in the rhizosphere (Dexter, 1987).

The combination of the observations that soil compaction has been observed to have impact on the structure of the bacterial soil microbiota in long term, large scale studies, and that root hair mutations shape the rhizosphere microbiota (Chapter 2) was the motivation to replicate the Koebernick study conditions and assess the impact of compaction on the rhizosphere microbiota. However, in order to rule out a plant effect on the microbiota, plant-free microcosms were set up with uniformly sieved bulk soil of varying density to assess the impact of shifts in soil density in an acute soil environment representative of the rhizosphere on the microbiota.
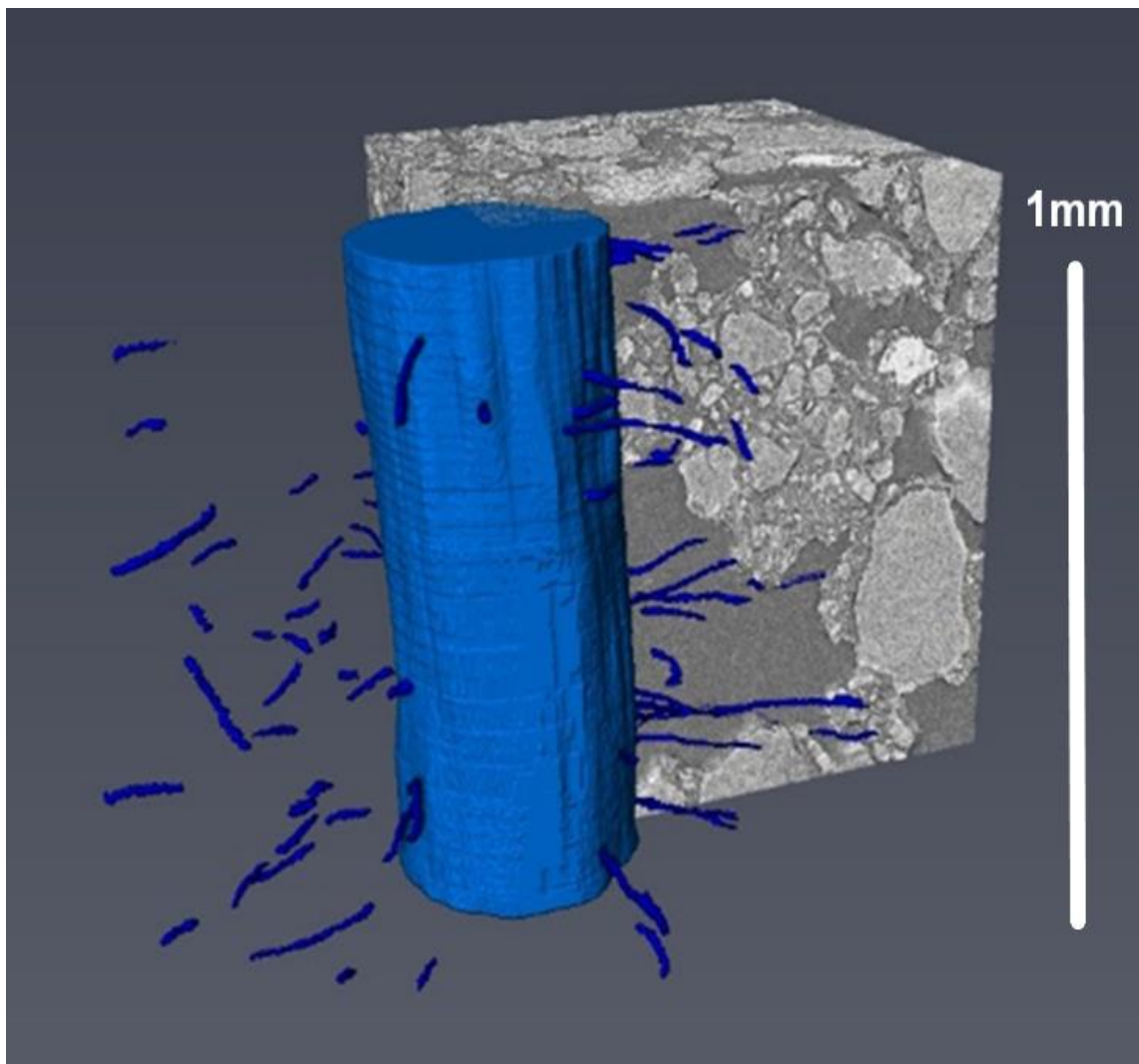
Figure 4.1 High-resolution synchrotron imaging shows that root hairs influence rhizosphere soil structure formation. 3D rendered barley root and hairs showing the surrounding soil. The light blue structure is a section of root and the while dark blue structures are segmented root hairs within a region of 2 × 2 × 1 mm. The vertical length of the root segment is 1 mm. Only root hairs growing in air-filled pores could be visualized which is the reason that root hair structures appear to be fragmented. Used with permission from T.S George. As observed and described by (Koebernick et al., 2017)
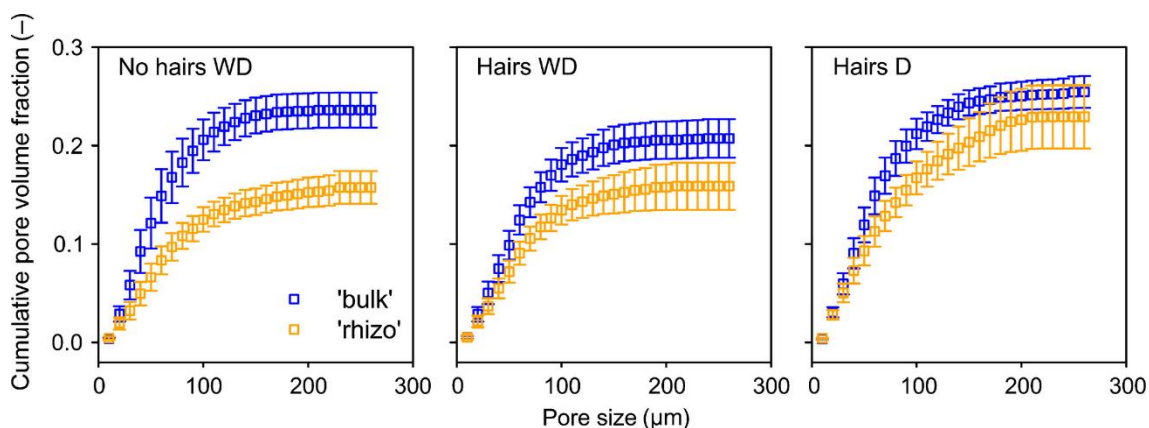
Figure 4.2 Soil density is greater at the root-soil interface when root hairs are absent. *Cumulative pore size distributed across varying distances from the barley root surface. 'Bulk', pore size distribution observed between 500–800 µm from the root surface; 'Rhizo', pore size distribution observed between 0–300 µm from the root surface. Only pores > 5 µm were characterised.* WD*, wet–dry treatment;* D*, dry treatment. As observed and described by (Koebernick et al., 2017)*

4.2. MATERIALS AND METHODS

4.2.1 Set up of soil density study, DNA extraction and data analysis

Soil density studies were adapted based on the set-up used by Koebernick and co-workers (Koebernick et al., 2017). Thirty 1 ml syringe barrels (height = 80 mm, inner diameter = 4.2 mm) were bound together using parafilm in groups of 5. The tip of each syringe was covered with a fine gauze and secured with parafilm around the tip of the syringe with the open tip covered with gauze only. The syringe tips only were then filled with silver sand for drainage. Syringe tips were connected to flexible, 5 mm diameter tubing that had previously been washed through with 70% EtOH and allowed to dry overnight. The 5 pieces of tubing per group were connected to a single main line using aquatic splitters and each main line was connected to an individual flow splitter with the connection points on the splitter that were not occupied by tubing capped off. The splitter was connected to the tap of a 10 L water canister that had previously been cleaned and sterilised with 70% EtOH and firmly secured with a metal clamp band, it was then filled with filtered, distilled water, as was the tubing. The water canister could be raised or lowered, acting as a water table. Each group of 5 syringes was held in place using a clamp stand and positioned at equal heights (**Figure 4.3**).
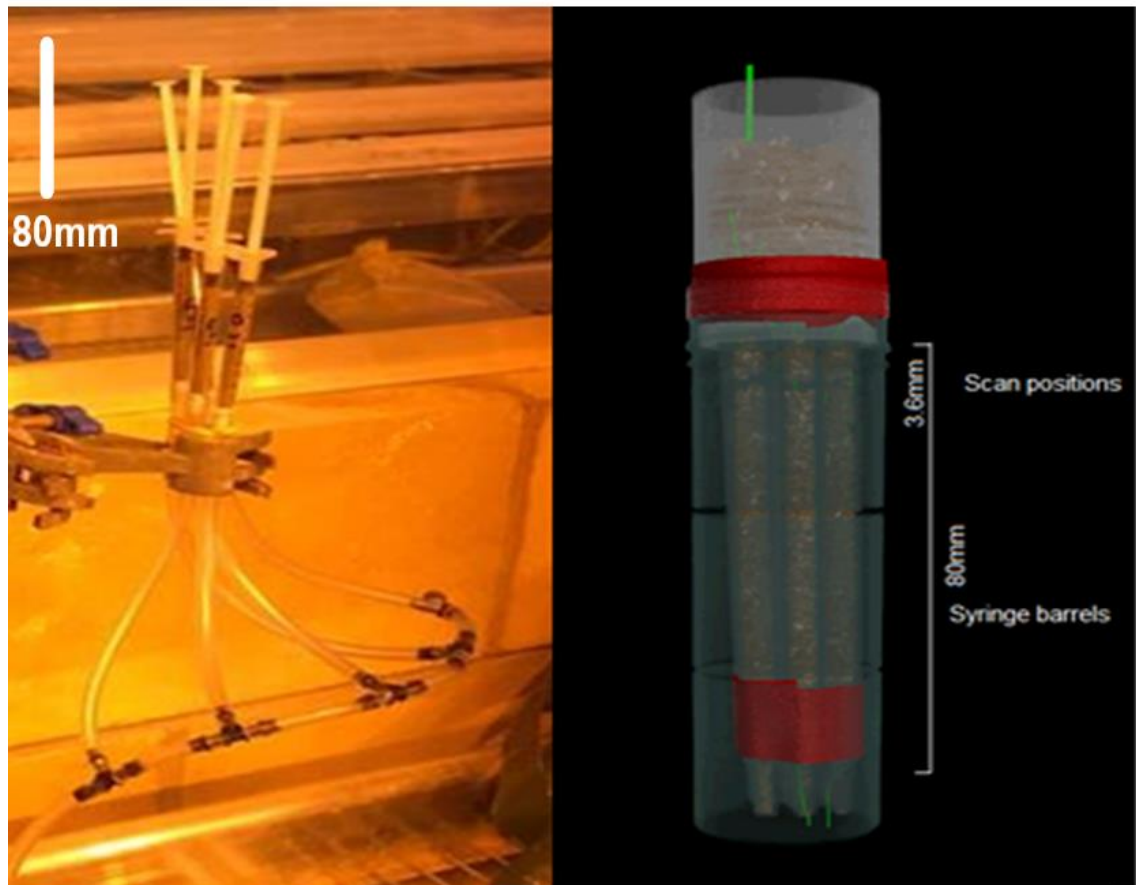
Figure 4.3 Density investigation experimental set up. *Groups of 5 x 1 ml syringes were filled with 1 g, 1.2 g and 1.3 g of soil respectively in order to create 3 bulk soil density environments. Left: set up of one group of 5 samples. (Right) visual representation of the set up for greater clarity of set up - Image adapted with permission from Figure provided by T S George, James Hutton Institute, Invergowrie, UK*

Each syringe group was filled with either 1 g, 1.2 g or 1.3 g of sandy loam textured soil (obtained from the South Bullionfield at the James Hutton Institute, Invergowrie, Dundee, UK) sieved to 1 mm 0.1 g at a time, the top opening of each syringe was then gently plugged with the syringe plunger and soil was compacted until it was level with the 1 ml marker on each syringe resulting in two of the six syringe groups having a soil density of ~1 g cm^3, ~1.2 g cm^3 or ~1.3 g cm^3 to represent bulk, wild type barley rhizosphere and root hair mutant rhizosphere density respectively A wetting/ drying (volumetric water content θ *c.* 0.22 – 0.25 g g^{-1}) treatment (Wet) was applied to one of each density group by lifting the water table to saturation every 2 days, lowering it back down and allowing the samples to drain (**Table 4.1**). The other half of the samples (Dry) were subjected to an initial water saturation as described for the Wet treatment then the tubing was removed from each of their syringes and no further treatment was performed over the course of the study.

Table 4.1 Group set up and treatments for the soil density investigation. *6 sets of 5 syringes were set up with soil density ranging from 1g cm³ to 1.3g cm³. Each density group was subjected to one of two water treatments, Wet, where samples were saturated with water every two days and Dry, where samples were exposed to a single saturation on day one and then left to dry over the observation period.*

Soil Density g cm^3	Treatment
1	Wet
1	Dry
1.2	Wet
1.2	Dry
1.3	Wet
1.3	Dry

Unfortunately, just 3 or 4 syringes per group could withstand the initial wetting phase without soil displacement. However, as there was a minimum of 3 samples per environment it was decided to continue the study using the remaining samples.

Over the period of the 8 day study the experimental set up was kept under controlled glasshouse conditions with a (18/14 °C (day/night)) temperature regime with 16 hour day length supplemented with artificial lighting to maintain a minimum light intensity of 200 µmol quanta m^{-2} s^{-1}) the same environmental conditions under which all other experiments in the thesis were performed. After 8 days the soil within the syringes was excavated by using a sterile scalpel to cut the tip of the syringe off at the 1st measurement mark, past the sand drainage layer and then gently, using the syringe plunger to push the soil out and into 15 ml sterile PBS in a 50 ml non-skirted falcon tube.

In addition to the soil only samples that were generated in this experiment there were an additional 29 syringes provided by Dr Koebernick which were part of a planted experiment with barley genotypes with and without root hairs (differing genotypes to our previous experiments), described in (Koebernick et al., 2017). These were a combination of samples including an individual root and bulk samples with a predicted density of 1.1 g cm^3 (**Table 4.2**). These samples were excavated and prepared in the same way as the density study described above with the exception that the root containing soil was vortexed for 30 s in 15 ml PBS

to remove soil from the root and the root was then removed using EtOH sterilised tweezers. Extraction of DNA was performed as per **2.2.3** with the remaining soil from each sample being stored at -80 °C in the event future analysis was required.

Table 4.2 Group set up and treatments for the density level impact on soil porosity conducted by Koebernick et al 2017. 29 samples consisting of syringes containing either bulk soil, an individual root with no root hairs or an individual wild type root subjected to one of two treatments, Wet, where samples were saturated with water every two days and Dry, where samples were exposed to a single saturation on day one and then left to dry over the observation period.

Sample type	Treatment	Number of samples
No root hairs	Wet	5
No root hairs	Dry	4
Root hairs	Wet	5
Root hairs	Dry	5
No plant	Wet	5
No plant	Dry	5

4.2.2 DNA amplification, sequencing and data analysis.

The V4 region of the 16S rRNA gene was amplified as per **2.2.3** and sequenced using Illumina MiSeq as per **2.2.4**. OTU table construction and data analysis was conducted using QIIME and R as described in **2.2.5** and **2.2.6** but with the modifications summarised below to control for the variations induced by the experimental design (i.e., accounting for samples being gathered one year apart and handled by different individuals during the sampling process).

Statistical analysis was performed in R. The functions used were retrieved from the default installation of R or the R package Phyloseq unless another package is described (McMurdie and Holmes, 2013). Following an initial taxonomic analysis, two observations were made; firstly, several samples showed significant potential contamination which could bias pooled results, secondly, and less surprisingly, the microbial composition of the bulk soil had altered over the year of storage.

As this study was focusing on density alone and the samples from year one were obtained using an adapted protocol, the samples from the previous year were

omitted and only the bulk soil samples from year two were taken forward for this study.

In order to conduct alpha diversity calculations, the OTU table was rarefied to an even sequencing depth of 64,090 sequencing reads per sample which produced 6,908 unique OTUs. Observed OTUs and Shannon diversity index were calculated, visualized and data distribution analysis was conducted as per **2.2.6.**

The differential enrichment of phyla between the varying densities was examined by an analysis of the individual microbiome profiles and was performed using phyla rarefied counts using the package ANCOM (Weiss et al., 2017).

In order to identify beta diversity between individual bacteria differentially enriched between densities analysis was conducted as per **2.2.6** as was the identification of individual bacteria differentially recruited between densities. Owing to the lack of significant interactions between density and treatment (**Figure 4.4**), focus was directed on the effect of density per se and treatment was omitted as a factor in the computation. Pair-wise comparisons between the varying densities were conducted: 1.3 g cm^3 vs 1 g cm^3, 1 g cm^3 vs 1.3 g cm^3, 1.2 g cm^3 v 1 g cm^3. Only an OTU that was identified as significantly enriched in one term vs the other in the pairwise comparisons was considered. I.e., enriched in 1.3 g cm^3 vs 1 g cm^3 and enriched in 1.2 g cm^3 vs 1 g cm^3 with those enriched in 1 g cm^3 being the "middle ground".

In order to determine the number of OTUs assigned to a specific taxonomic order in the comparisons between densities the function count from the plyr package was used and statistical analysis these results was conducted as per **2.2.6**. Venn diagrams were generated to visualise the differentially enriched OTUs using the package VennDiagram and pie charts showing the distribution of top OTUs proportion vs top sequence reads were generated in excel.

Data used for the analysis is available in **8.3.1. Appendix 3: Supplementary Database 3**

4.3 RESULTS

4.3.1. Soil density and water treatment does not affect DNA extraction or sequencing library properties.

To assess the impact of soil density and water treatment on microbial communities the total yield of DNA per sample was examined. Across 22 samples a total of 9,606 OTUs were identified amounting to a total of 2,973,035 high quality sequence reads ranging from 64,728 to 186,331 reads per sample (mean 135,138) were obtained from amplicon sequencing of the 16S rRNA gene. Following the removal of Mitochondrial- and Chloroplast-derived OTUs (20 51 OTUs per organelle, respectively) 9,535 OTUs and 2,958,419 high quality sequence reads were retained for downstream analysis. Despite 71 OTUs being pruned *in silico* from the dataset, the remaining reads termed "no plants" accounted for 99.5% of the original dataset.

A Shapiro-wilk normality test on the DNA concentration showed that data was normally distributed (p=0.4301, w=0.95694) allowing for an ANOVA to be conducted between all samples to investigate DNA concentration as dependent variable and soil vs density as independent variable. This analysis indicated no significant difference in DNA concentration between any samples (p=0.9108), this was confirmed further by conducting multiple comparison of independent samples (Dunns test), in all pairwise comparisons p=0.94-0.99 showing no significant difference in DNA concentration between any sample regardless of density or treatment (**Table 4.3, Figure 4.4**). This was also observed in the percentage of retained reads between samples when plant based OTU reads were removed from the data set.

Again, following a Shapiro-wilk normality test confirming normal distribution of data (p = 0.06664) a two way ANOVA confirmed that neither treatment, genotype or their interaction had a significant impact on high resolution sequence read retention (p value = 0.225, 0.535 and 0.332 respectively) with a one way ANOVA comparing samples only presenting a P value of 0.1312 confirming this further. There was no significant difference between treatments across density samples (**Figure 4.5**).

Table 4.3 Soil density and watering treatment has no significant impact on DNA concentration between samples. Confirmed using pairwise comparisons using Dunns test for multiple comparisons of independent samples.

	1.3 Dry	1.3 Wet	1.2 Dry	1.2 Wet	1 Dry
1.3 Wet	0.94				
1.2 Dry	0.99	0.94			
1.2 Wet	0.94	0.94	0.94		
1 Dry	0.94	0.94	0.94	0.94	
1 Wet	0.94	0.94	0.94	0.94	0.99
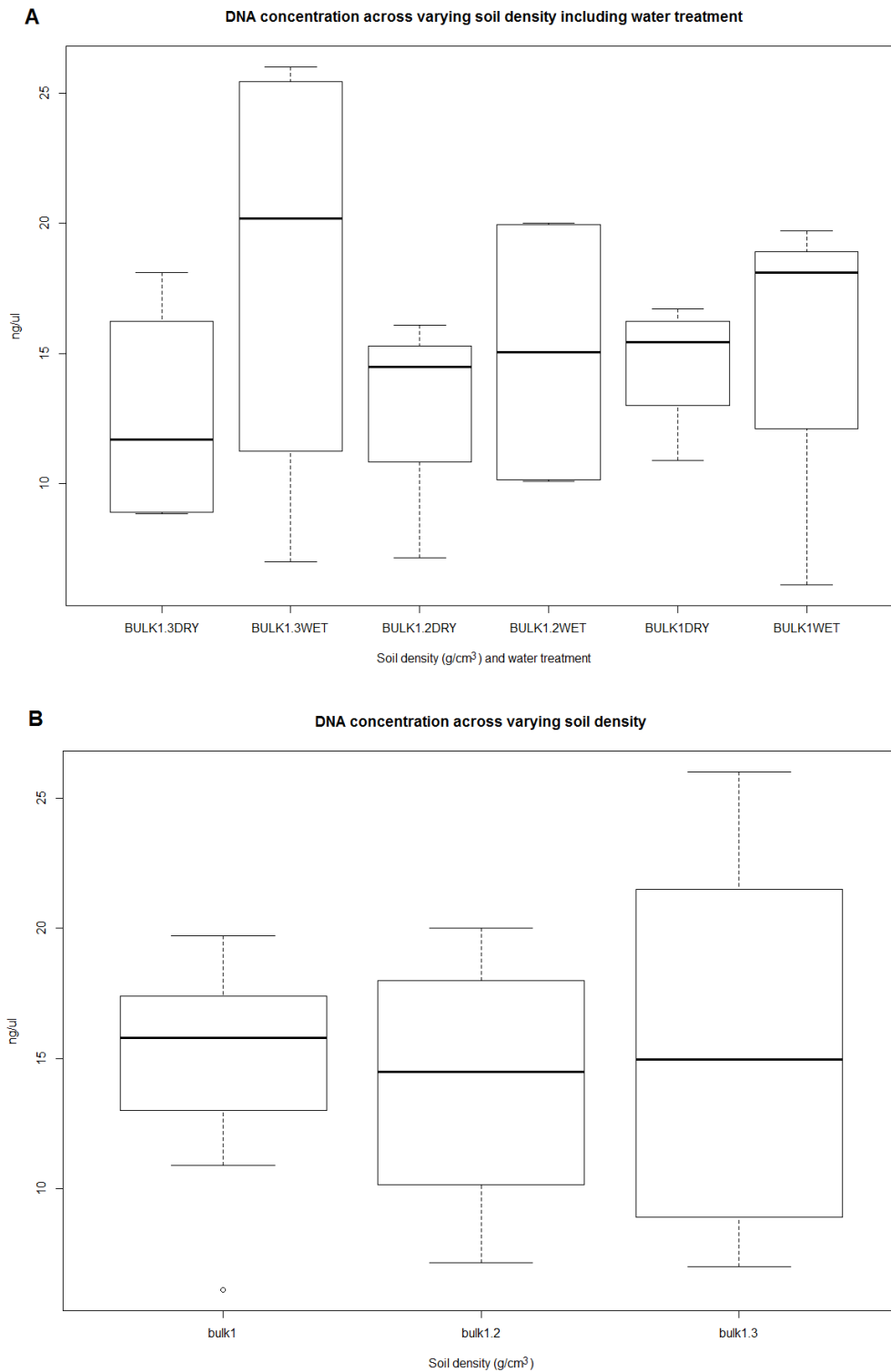
Figure 4.4 Soil density and watering treatment impact on DNA concentration between samples.
Confirmed using Pairwise comparisons using Dunns test for multiple comparisons of independent samples. (A)The DNA concentration for each density sample and treatment and (B) the DNA concentration between samples based on soil density only when treatments are pooled. Higher and lower edges of the box plots represent the higher and lower quartiles, respectively. The bold line within the box denotes the median. Maximum and minimum observed values are represented by the whiskers. The dot denotes an outlier whose value was 3/2 times smaller than the lower quartiles of 6.13 ng/ul from a bulk 1 wet sample.
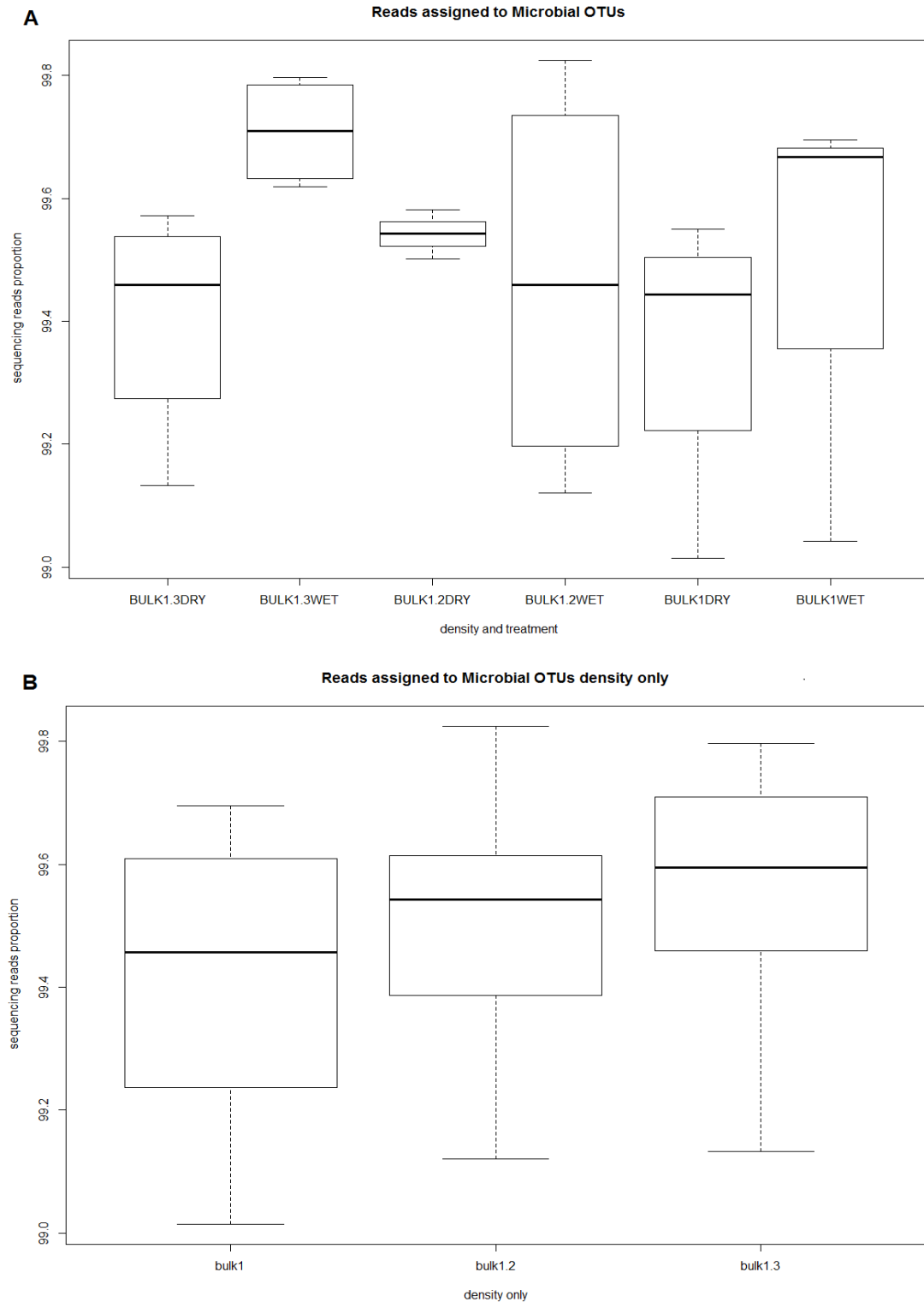
Figure 4.5 Soil density and watering treatment impact on the proportion of non-bacterial OTU high resolution sequence reads between samples. *Confirmed using Pairwise comparisons using Dunns test for multiple comparisons of independent samples. (A) The DNA retained following the removal of mitochondrial and chloroplast reads for each sample type and treatment and (B) The DNA retained following the removal of mitochondrial and chloroplast based on soil density only when treatments are pooled. Higher and lower edges of the box plots represent the higher and lower quartiles, respectively. The bold line within the box denotes the median. Maximum and minimum observed values are represented by the whiskers.*

4.3.2. The soil microbiota is not significantly altered by soil density at the Phyla level

The initial taxonomic analysis at the phylum level revealed a total of 40 individual phyla represented by 9,535 OTUs. This data was converted into percentage relative abundance and after filtering to remove OTUs belonging to phyla that represented less than an average of 1% of the total sequence reads 10 phyla remained representing an average of 98.25% of the high-quality sequence reads in the data set.

There was no significant difference in relative abundance of any phyla as a result of water treatment (**Table 4.4**). Irrespective of the soil density or water treatment, all samples were significantly enriched in members of the phyla Acidobacteria and Proteobacteria whose average relative abundance represented 35.41% and 21.69% of the total community. However, a minor shift in Proteobacteria proportion could be observed between 1.3 g cm^3 samples and 1 g cm^3 with a 9.97% decrease in this phyla as density increases, the same observation was also made with Acidobacteria, relative abundance dropped by 10.78% from 22.63% to 20.19% as density increased from 1 g cm^3 to 1.3 g cm^3.

Conversely, the opposite trend was observed in the third most represented phyla, Bacteroidetes whose community proportions increased from 8.82% to 14.77% as density increased, although this is a small shift as far as the entire bacterial community composition is concerned it represents an increase of 40.3% between sample densities (**Figure 4.6**).

Although there were subtle changes between the other 8 phyla featured in the top phyla data set these were very small and overall showed very little fluctuation with the exception of a marked shift in Firmicutes whose proportions almost tripled as density increased from 1 g cm^3 to 1.3 g cm^{3,} however this represented a small proportion of the overall dataset with the relative abundance increasing from an average of 1% to 2.9% (**Table 4.5**).
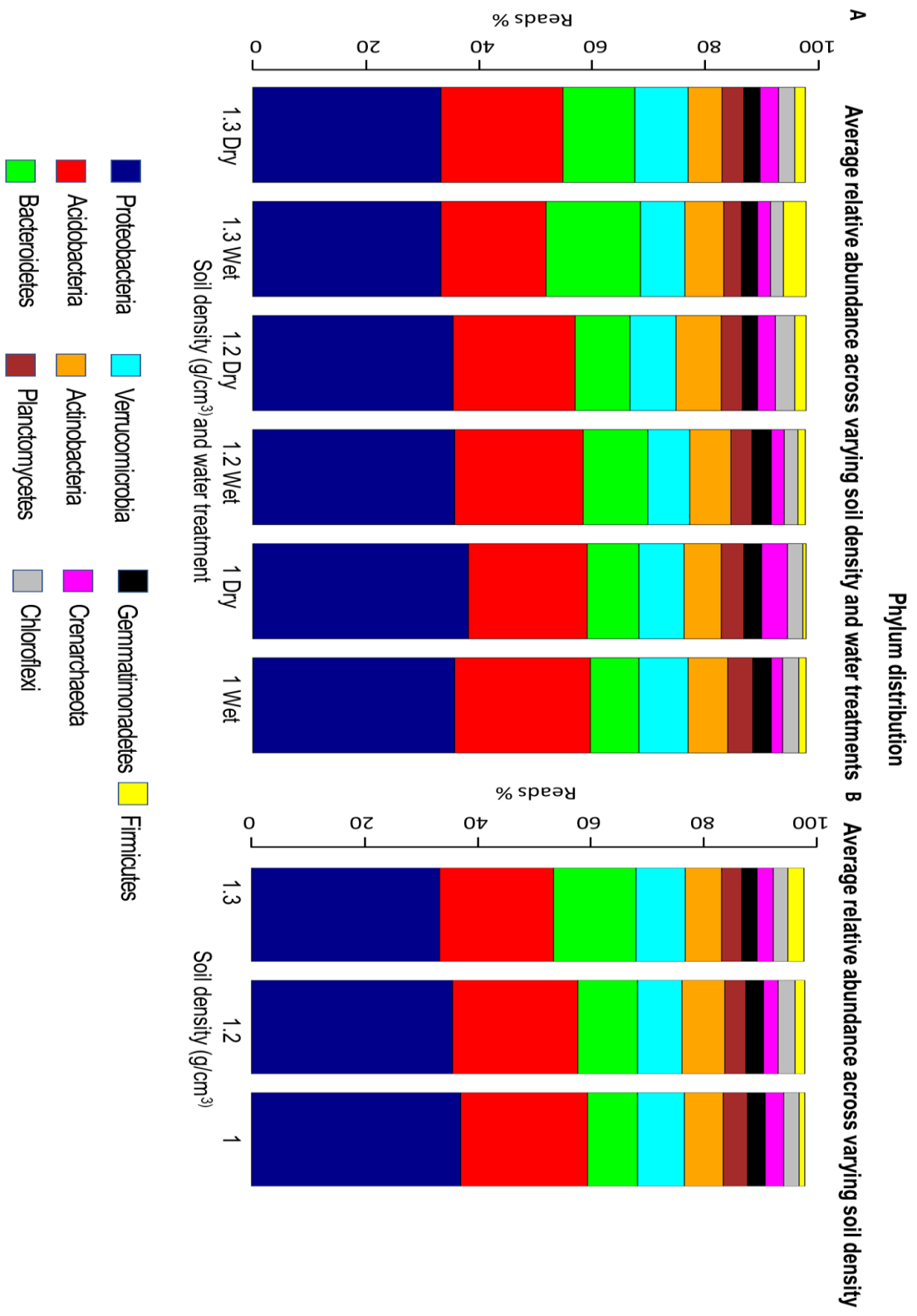
Figure 4.6 The effect of soil density on the microbiota at the phylum level. *Average relative abundance (% of sequencing reads) of the phyla identified in the indicated samples obtained from year 2 Bullionfield soil. Only phyla displaying an average relative abundance > 1% in the whole sequencing dataset have been included but correspond with 97.56% of the total observed reads. (A) Represents the relative abundance across varying soil density and water treatments, (B) represents the average relative abundance across varying soil density only*

Table 4.4 Average relative abundance of bacterial phyla identified in samples studied grouped by density and treatment. Average relative abundance (% of sequencing reads) of the phyla identified in the samples described. Only phyla displaying an average relative abundance > 1% in the whole sequencing dataset have been included but in total correspond to 98.25% of the total observed reads

Phylum	Sample density and treatment relative abundance (%)					
	1.3 Dry	1.3 Wet	1.2 Dry	1.2 Wet	1 Dry	1 Wet
Proteobacteria	33.42	33.46	35.55	35.91	3.83	35.89
Acidobacteria	21.8	18.59	21.66	22.82	21.16	24.1
Bacteroidetes	12.76	16.77	9.76	11.42	9.11	8.52
Verrucomicrobia	9.44	8	8.22	7.52	8.06	8.76
Actinobacteria	6.09	6.81	8.05	7.21	6.57	7.16
Planctomycetes	3.87	3.2	3.75	3.84	4.06	4.43
Gemmatimonadetes	2.78	2.79	2.75	3.45	3.18	3.27
Crenarchaeota	3.39	2.37	3.12	2.19	4.56	2
Chloroflexi	2.78	2.33	3.49	2.51	2.7	2.84
Firmicutes	1.91	3.93	1.9	1.35	0.66	1.28

Table 4.5 Average relative abundance of bacterial phyla identified in samples studied grouped by density only. Average relative abundance (% of sequencing reads) of the phyla identified in the samples described. Only phyla displaying an average relative abundance > 1% in the whole sequencing dataset have been included but in total correspond to 98.25% of the total observed reads

Phylum	Sample density relative abundance (%)		
	1.3 combined	1.2 combined	1 combined
Proteobacteria	33.34	35.73	37.1
Acidobacteria	20.2	22.24	22.63
Bacteroidetes	14.77	10.59	8.82
Verrucomicrobia	8.72	7.87	8.41
Actinobacteria	6.45	7.63	6.86
Planctomycetes	3.53	3.8	4.24
Gemmatimonadetes	2.78	3.1	3.22
Crenarchaeota	2.88	2.66	3.28
Chloroflexi	2.56	3	2.77
Firmicutes	2.92	1.63	0.97

4.3.3. Soil density and water treatment do not alter the distribution of higher taxonomic ranks of the microbiota.

In order to examine these fluctuations at a greater resolution the same analysis as conducted as described above was conducted but at the class and order level. Of the identified 139 bacterial classes in the data set a total of 11 dominant classes whose individual relative abundance exceeded 2.5% and collectively represented over 77% of the sequencing reads were identified. Unlike what was observed at the phylum level, there was no bacterial class markedly dominating the dataset and it emerged that environmental alterations did not have any significant impact on class relative abundance (**Table 4.6, Figure 4.7A**).

Table 4.6 Average relative abundance of bacterial orders identified in samples studied grouped by density and treatment. Average relative abundance (% of sequencing reads) of the orders identified in the samples described. Only phyla displaying an average relative abundance > 2% in the whole sequencing dataset have been included but correspond with 83.8% of the total observed reads

	Sample density and treatment relative abundance (%)					
Class	1.3 Dry	1.3 Wet	1.2 Dry	1.2 Wet	1 Dry	1 Wet
Betaproteobacteria	13.85	13.47	14.54	13.91	14	13.59
Acidobacteria	11.13	9.39	11.45	11.83	10.37	12.53
Alphaproteobacteria	9.14	8.92	10.17	10.69	11.62	11.22
Gammaproteobacteria	7.97	8.66	8.42	8.76	10.34	8.59
Chloracidobacteria	7.87	6.37	7.24	8.06	7.85	8.6
Spartobacteria	8.44	7.023	7.6	6.63	7.18	7.81
Sphingobacteriia	6.4	9.37	5.1	5.17	2.57	2.65
Saprospirae	4.29	5.05	3.15	4.16	3.7	3.74
Thaumarchaeota	3.39	2.37	3.12	2.19	4.56	2
Thermoleophilia	2.47	2.8	3.33	3.03	2.77	3.02
Actinobacteria	2.5	2.76	3.44	3	2.61	2.87
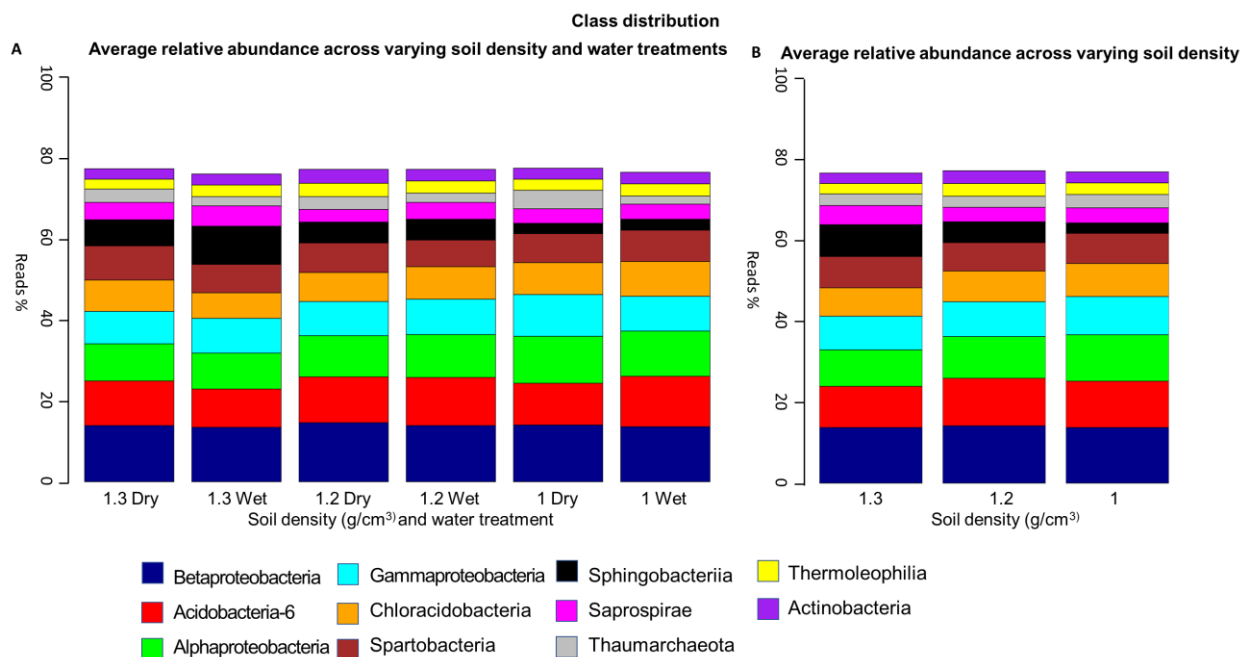
Figure 4.7 Bacterial taxonomic distribution across varying soil densities at the class level. *Average relative abundance (% of sequencing reads) of the bacterial classes identified in the indicated samples obtained from year 2 Bullionfield soil. Only bacterial classes displaying an average relative abundance > 2.5% in the whole sequencing dataset have been included but correspond with 77.08% of the total observed reads. (A) Represents the relative abundance across varying soil density and water treatments, (B) represents the average relative abundance across varying soil density only*

Nevertheless, a shift could be observed in Alphaproteobacteria and Gammaproteobacteria (members of the Proteobacteria phylum); Alphaproteobacteria and Gammaproteobacteria relative abundance dropped by almost 25% and 12% respectively (11.42% to 9% and 9.46% to 8.31%, respectively) mirroring the same observation at the phyla level. Likewise, both Sphingobacteriia and Saprospirae belonging to the Bacteroidetes phylum relative abundance grew with increasing density, particularly Sphinogbacteriia which rose from representing just 2.6% of the total bacterial classes at a density of 1 g cm^3 to 7.9% in the highest density sample set (**Table 4.7, Figure 4.7B**). However, these changes were not statistically significant.

Next, the taxonomic rank of order was investigated to obtain information at an even higher resolution and to elucidate if changes in soil density resulted in more subtle microbial shifts. A total of 281 bacterial orders were identified in the full sample set, again, accounting for 9,535 OTUs. As observed in the phylum data, there was no significant difference in relative abundance as the result of sample treatment type (**Table 4.8 and Figure 4.8A**). The data was then pooled by

environmental density, excluding treatment and converted to relative abundance. Data was initially filtered to remove orders that accounted for less than an average of 1% of the total sequence reads. However, this left 22 orders remaining representing an average of 83.8% of the total high-quality sequence reads which resulted in overcrowding of data. Based on this, filtering stringency was increased to 2% retaining 70.13% of the total data whilst encompassing 12 bacterial orders.

One of the main observations at the order level was that one third of the orders identified were in fact only identified to the Class taxonomic level making it difficult to obtain detailed information. There was also no major dominance from any bacterial order other than "c_Betapoteobacteria ;o_Gallionellales and c_EC1113;o_ representing 11.35% and 10.8% of the total data set respectively and that across all densities no bacterial orders showed any significant change in relative abundance with the exception of the order Rhodospirillales that decreased from 5.36% to 4.32% as density increases from 1 g cm^3 to 1.3 g cm^3 which, is only a very small change based on the total data set, represents a decrease of almost 20% in that Order (**Table 4.9 and Figure 4.8B**).

Table 4.7 Average relative abundance of bacterial orders identified in samples studied grouped by density only. Average relative abundance (% of sequencing reads) of the orders identified in the samples described. Only phyla displaying an average relative abundance > 2.5% in the whole sequencing dataset have been included but correspond to 83.8% of the total observed reads

	Sample density relative abundance (%)		
Class	1.3 g cm^3	1.2 g cm^3	1 g cm^3
Betaproteobacteria	13.66	14.22	13.79
Acidobacteria	10.26	11.64	11.45
Alphaproteobacteria	9.03	10.43	11.42
Gammaproteobacteria	8.31	8.59+	9.46
Chloracidobacteria	7.12	7.65	7.23
Spartobacteria	7.73	6.99	7.5
Sphngobacteriia	7.89	5.13	2.61
Saprospirae	4.67	3.65	3.72
Thaumarchaeota	2.88	2.66	3.28
Thermoleophilia	2.64	2.18	2.89
Actinobacteria	2.63	3.2	2.74

Table 4.8 Average relative abundance of bacterial orders identified in samples studied grouped by density and treatment. Average relative abundance (% of sequencing reads) of the orders identified in the samples described. Only phyla displaying an average relative abundance > 2% in the whole sequencing dataset have been included but correspond to 83.8% of the total observed reads. "O_" indicates order is unknown

	Sample density and treatment Relative abundance (%)					
Class; Order	1.3 Dry	1.3 Wet	1.2 Dry	1.2 Wet	1 Dry	1 Wet
Betaproteobacteria; Gallionellales	11.58	11.07	12.02	11.19	11.0	10.8
EC1113; o_	10.84	9.16	11.05	11.52	10.0	12.2
TA18; PHOS-HD29	6.95	8.25	6.27	7.66	8.17	7.42
Verrucomicrobiae; Verrucomicrobialesz	8.44	7.02	7.36	6.63	7.18	7.81
Acidobacteria-6; iii-15	7.07	5.73	6.48	7.27	7.12	7.75
VC2_1_Bac22; o_	6.4	9.38	5.1	5.17	2.57	2.65
Alphaproteobaceria; Rhodospirillales	4.25	4.39	4.88	5.43	5.26	5.81
Betaproteobacteria; A21b	3.78	3.52	3.84	4.04	4.86	4.08
At12OctB3; o_	4.29	5.05	3.15	4.16	3.7	3.74
Thaumarchaeota; Nitrososphaerales	3.38	2.37	3.12	2.19	4.56	2
Actinobacteria; Actinomycetales	2.5	2.75	3.43	2.94	2.6	2.87
Pla4; o_	2.15	1.73	2.05	2.15	2.36	2.6
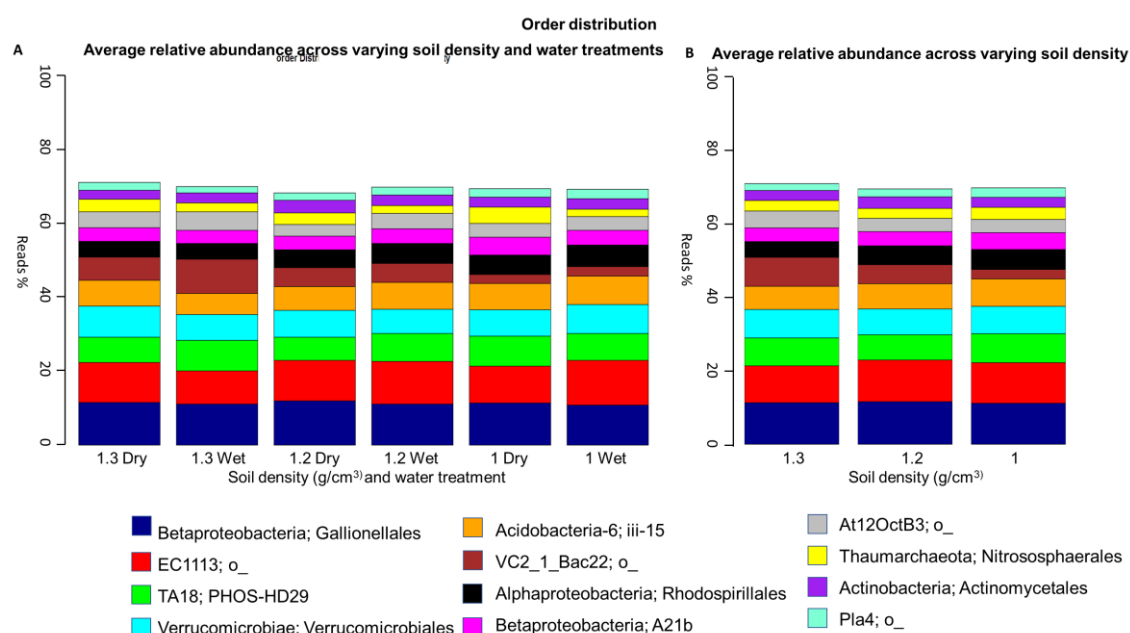
Figure 4.8 Bacterial taxonomic distribution across varying soil densities at the order level. Average relative abundance (% of sequencing reads) of the bacterial orders identified in the indicated samples obtained from year 2 Bullionfield soil. Only bacterial orders displaying an average relative abundance > 2% in the whole sequencing dataset have been included but correspond with 70.13% of the total observed reads. Taxonomy is described as class followed by order (if order taxonomy was available). (A) Represents the relative abundance across varying soil density and water treatments, (B) represents the average relative abundance across samples with varying soil density only

Table 4.9 Average relative abundance of bacterial orders identified in samples studied grouped by density only. Average relative abundance (% of sequencing reads) of the orders identified in the samples described. Only phyla displaying an average relative abundance > 2% in the whole sequencing dataset have been included but correspond to 83.8% of the total observed reads

	Sample density relative abundance (%)		
Class; order	1.3 g cm^3	1.2 g cm^3	1 g cm^3
Betaproteobacteria; Gallionellales	11.33	11.6	11.13
EC1113; o_	10	11.28	11.13
TA18; PHOS-HD29	7.6	7	7.8
Verrucomicrobiae; Verrucomicrobiales	7.73	7	7.5
Acidobacteria-6; iii-15	6.4	6.87	7.44
VC2_1_Bac22; o_	7.89	5.13	2.61
Alphaproteobacteria; Rhodospirillales	4.32	5.16	5.54
Betaproteobacteria; A21b	3.65	3.94	4.47
At12OctB3; o_	4.67	3.65	3.72
Thaumarchaeota; Nitrososphaerales	2.88	2.66	3.28
Actinobacteria; Actinomycetales	2.62	3.19	2.74
Pla4; o_	1.94	2.1	2.48

4.3.4. Soil density and, to a lesser extent water treatment, shape the relationships among individual members of the microbiota.

Finally, the impact of density treatment at the OTU level, representing the building blocks of the community was investigated. Following rarefication, an even sequencing depth of 64,090 reads leaving 6,908 OTUs remaining for downstream analysis (2,627 OTUs were removed from the data set).

At the OTU level, when alpha diversity, was examined there was no significant effect of treatment type on either the number of OTUs observed, the Chao index nor the Shannon diversity index respectively; these analyses are considered to be proxies for bacterial richness and evenness (**Figure 4.9**) (Mann-Whitney-Wilcoxon, *P* > 0.05). Moreover, when examining density only it began to emerge that as density increased the Shannon diversity index reduced significantly indicating that as density increases the number of species and their relative abundance begins to fall (one-way ANOVA p = 0.0165).

Next, Beta diversity was investigated which examines the diversity of species between samples. An abundance threshold was set to remove low-count, poorly-

reproducible OTUs from the analysis so OTUs not seen more than 5 times in 20% of the samples were removed, this resulted in 2,238 of the original 9,535 OTUs passing these parameters. Although this resulted in the loss of 76.5% of the original taxa, 2,873,472 sequence reads were retained accounting for over 97% of the total generated high-quality reads.

This new data set was used to calculate Bray-Curtis (BC) and Weighted UniFrac (WU) distance matrices. Using the BC distance, which is sensitive to OTU relative abundance it was observed that samples were segregating in a gradient like manner as density increased along the X axis. This axis described the largest variation accounting for 29.2% of what was being observed. A similar but less pronounced effect was observed using the WU distance which describes both OTU relative abundance and phylogenetic assignment between samples; apart from a few outliers, samples were orderly partitioned along the Y axis which, although represents the lesser variation still described 21% of the dissimilarity.

The spatial separation observed in the PCoA analyses as soil density changes (**Figure 4.10Ai** and **4.10Aii**) was confirmed statistically following an Adonis test on the density effect on both analyses (**Figure 4.10 Bi** and **Bii**, BC: R^2 = 0.29, P value = 0.0002. WU: R^2 = 0.21, P value = 0.0086). However, although there had been no obvious indication in the PCoA or previous statistical analyses indicating an effect of treatment on the soil microbiota, this approach revealed a limited, but significant, effect of the watering regime on microbiota composition. (**Figure 4.10 Bi** and **4.10 Bii**, BC: R^2 = 0.08, P value = 0.02. WU: R^2 = 0.12, P value = 0.019).
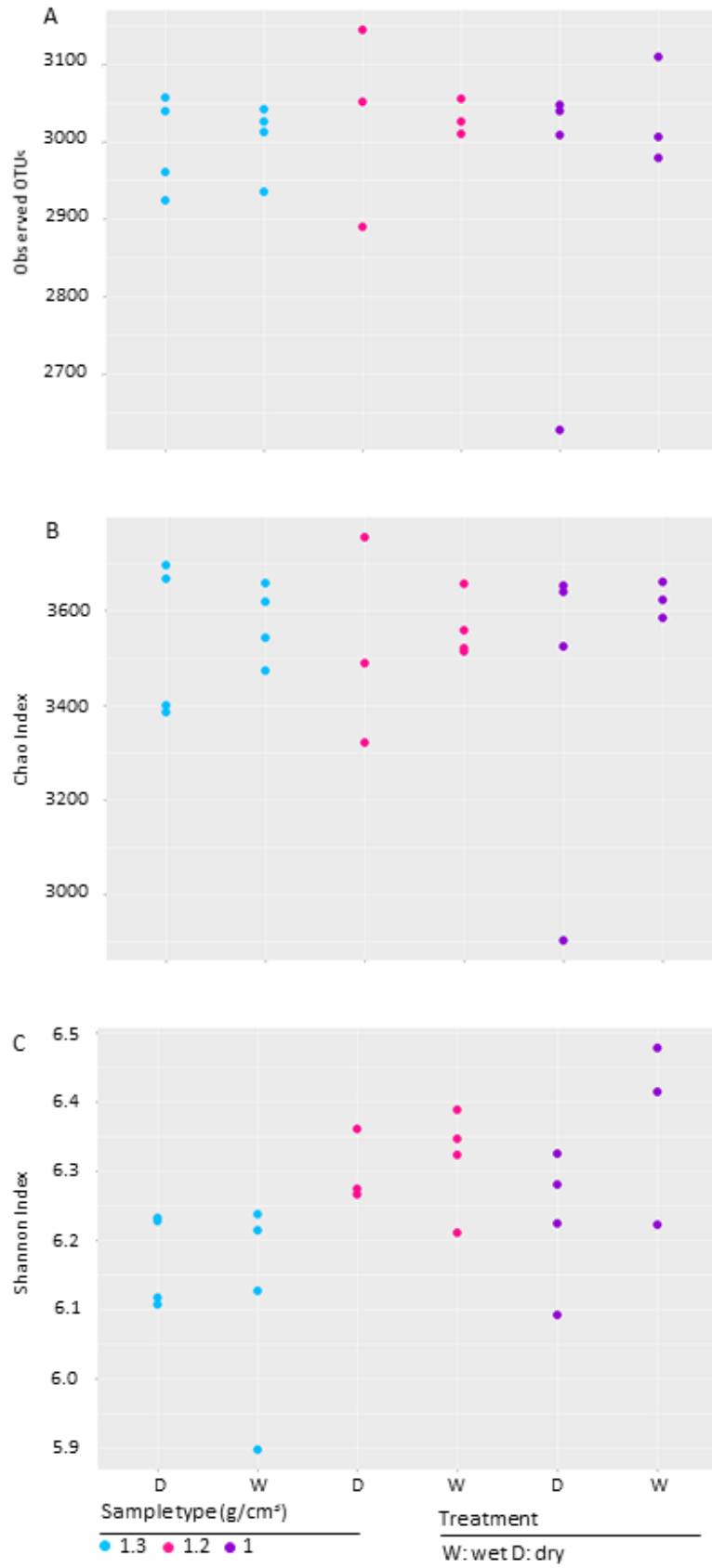
Figure 4.9 Changes to soil density impact the proportionality of the bacterial microbiota, but water treatments do not. *(A) Total number of observed OTUs per sample set. (B) Chao1 Index calculated for varying density samples. (C) Shannon's diversity index calculated for varying density samples.*

Beta Diversity between density samples

Ai — PCoA 16S data, Bray-Curtis distance

Axis 2 (19.7%) — Axis 1 (29.2%)

$$BCij = \frac{1 - 2Cij}{Si + Sj}$$

Treatment: ● Wet ▲ Dry

Aii — PCoA 16S data, Weighted Unifrac distance

Axis 2 (21.4%) — Axis 1 (46.5%)

Density (g cm³): ● 1 ● 1.2 ● 1.3

$$W = \frac{\sum_{i=1}^{N} l_i \left| \frac{Ai}{AT} - \frac{Bi}{BT} \right|}{\sum_{j=1}^{s} L_j}$$

Bi

Bray Curtis	R2	P value
Density	0.29	0.0002
Treatment	0.08	0.0172
Density: Treatment	0.07	0.3676

Bii

Weighted Unifrac	R2	P value
Density	0.21	0.0085
Treatment	0.12	0.0194
Density: Treatment	0.07	0.4523

Figure 4.10 Soil density contributes to soil microbial structure, but water treatments do not. (Ai) PCoA calculated using the Bray-Curtis dissimilarity matrix (sensitive to OTU relative abundances). Different treatment replicates are depicted by shapes whose spatial proximity reflects the degree of dissimilarity of their microbiotas. (Aii) PCoA calculated using the Weighted UniFrac distance matrix (sensitive to both OTU relative abundances and species phylogeny). Different treatments are portrayed by shapes whose spatial proximity to one another reflects the degree of dissimilarity of their microbiotas. (Bi) Permutational analysis of variances calculated using the Bray Curtis dissimilarity matrix for the described effects (Bii) Permutational analysis of variances calculated using the Weighted UniFrac distance matrix for the indicated effects. The R^2 values signify the proportion of variation in distances that can be explained by the described grouping of samples. P-values were calculated for 5,000 permutations.

4.3.5. Individual bacterial OTUs are differentially recruited as soil density changes.

A total of 1,161 OTUs were enriched in 1.3 g cm^3 vs 1 g cm^3 and 1,077 were depleted in the same comparison. The same analysis of OTUs enriched in 1.2 g cm^3 vs 1 g cm^3 yielded 1,102 enriched OTUs and 1,136 depleted. To elucidate which of these were significantly enriched, OTUs with an adjusted P value lower than 0.01 were retained revealing that in 1.3 g cm^3 soil 100 OTUs were significantly enriched and 72 were significantly depleted (Wald test, $P < 0.01$, FDR corrected). The same effect could be seen in soil with 1.2 g cm^3 but to a lesser extent, with 23 OTUs being significantly enriched and 13 being depleted (Wald test, $P < 0.01$, FDR corrected).

Of the OTUs significantly enriched in 1.3 g cm^3 and 1.2 g cm^3 density samples the higher densities shared 19 common significantly enriched OTUs and 11 significantly depleted vs 1 g cm^3 density soil, interestingly, the shared OTUs between 1.2 g cm^3 and 1.3 g cm^3 accounted for the vast majority of the OTUs enriched or depleted in 1.2 g cm^3 indicating that the microbiota is shaped, at least in part, in a density dependent manner, this data also supports the beta diversity data (**Figure 4.11**).
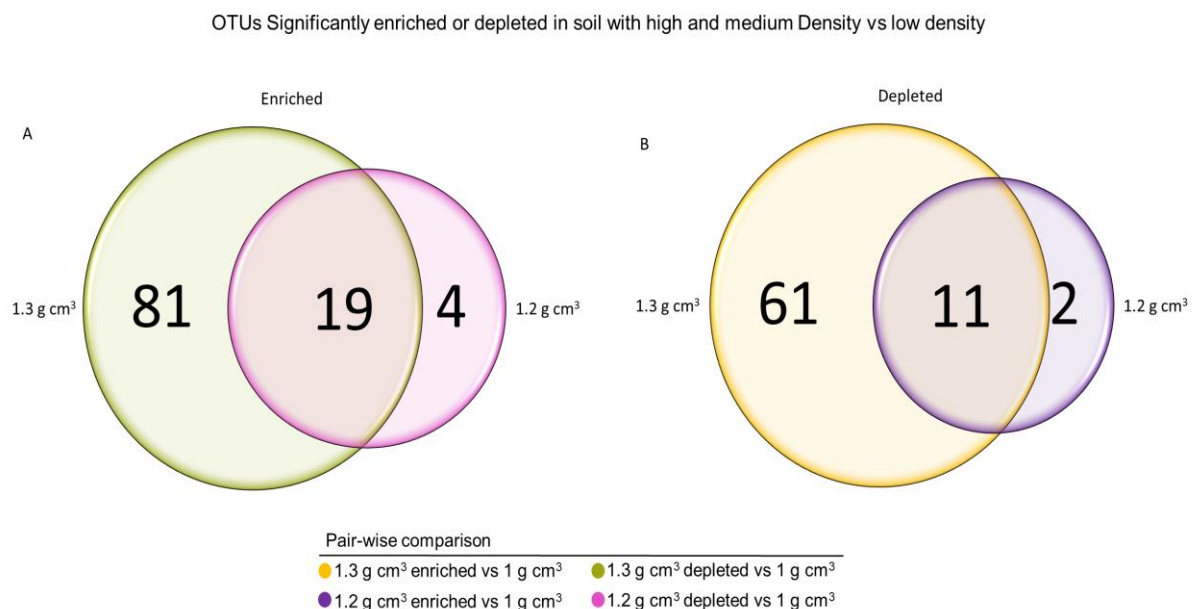
Figure 4.11. Denser soil environments host a multitude of significantly enriched or depleted OTUs vs soil with standard density. The number of OTUs either significantly differentially recruited (A) or (B) significantly reduced or absent in High (1.3 g cm³) or medium (1.2 g cm³) soil vs soil of a standard density (1 g cm³). Colours represent the pair-wise comparisons described. Diagram intersections depict OTUs that are enriched or depleted in both 1.3 g cm³ soil and 1.2 g cm³ vs 1 g cm³.

Following the observation that more unique OTUs were differentially enriched or depleted in high density soil vs standard density soil the taxonomic assignment of these OTUs was examined. Members of the orders Burkholderiales, Bacillales, Rhizobiales, Saprospirales, Sphingomonadales and Xanthomonadales occurred more than 9 times in the data set consisting of OTUs significantly enriched or depleted in higher density soil vs standard density (**Table 4.10** and **4.11**).

Table 4.10 The top taxonomic orders that are significantly enriched or depleted between samples with high/ medium density vs low density. Column 2 represents the total number of unique OTUs assigned to the order described in column 1, Columns 3 and 4 show the number of unique OTUs belonging to these orders that are either significantly enriched or depleted in soil samples that do not have a standard density of 1 g cm^3 (Combined data from differentially enriched OTUs in both 1.3 and 1.2 g cm^3 samples). Table shows orders represented by 5 unique OTUs or over.

Order	Total	Enriched	Depleted
Burkholderiales	34	19	15
Bacillales	14	14	0
Saprospirales	12	11	1
Unassigned	11	7	4
Rhizobiales	11	4	7
Sphingomonodales	11	3	8
Xanthomonodales	9	5	4
Mxyococcales	8	4	4
Sphingobacteriales	7	5	2
Pedosphaerales	6	6	0
Bdellovibrionales	5	0	5
Caulobacteriales	5	0	5

Table 4.11 As soil density increases the number of orders differentially enriched also increases compared to standard density soil. 28 different taxonomic orders are significantly enriched in samples with a density of 1.3 g cm³ vs just 8 in soil samples with a density of 1.2 g cm³, of those 8 orders 7 are also present in the high-density soil samples. Column 2 represents the total number of unique OTUs only present in high density soil samples assigned to the order described in column 1, Column 3 represents the total number of unique OTUs only present in medium density soil samples and column 4 shows the number of unique OTUs belonging to these orders that are present in both high and medium density soil samples. Table shows the top 15 Orders enriched in the data set.

Order	1.2 only	1.3 only	shared	Total
Burkholderiales	1	12	6	19
[Saprospirales]		10	1	11
Bacillales		8	6	14
Xanthomonadales		5		5
Unassigned	1	5	1	7
[Pedosphaerales]		6		6
Solirubrobacterales		3		3
Sphingomonadales		3		3
Myxococcales		3	1	4
[Chthoniobacterales]		2		2
Gemmatales		2		2
Pirellulales		2		2
RB41		2		2
WD2101		2		2
Rhizobiales	1	2	1	4
Sphingobacteriales		2	3	5

None of these featured in the top 12 orders in terms of assigned OTUs meaning that these orders are present, at a maximum of less than 2% of the total high-quality sequence read data. Therefore, the enrichment analysis performed is likely to have captured "intimate" intermicrobial relationships rather than a "passive" flow-through from existing data. Remarkably, 4 of the 6 orders identified as being enriched or depleted in this data set (Burkholderiales, Rhizobiales, Sphingomonadales and Xanthomonadales) are among the orders identified as being differentially enriched in the root hair mutant study (**Chapter 2**). In order to visualise and examine the structure of the enriched and depleted orders in soil samples with density higher than standard (1 g cm³) an analysis of the number

of unique OTUs representing the orders and the proportion of sequence reads assigned to these OTUs was conducted.

Firstly, as the unique enriched and depleted orders in soil density samples of 1.2 g cm^3 only accounted for 6 of the 172 observed OTUs, high and medium density data was combined and will be referred to as "higher density" for the remainder of these results. However, a subset was created that consisted of the unique enriched or depleted OTUs that were common between both datasets to be analysed alongside the pooled data. All data was analysed using both the number of unique OTUs and the number of high-quality sequencing reads assigned to orders in order to get a detailed picture of the significantly differentially represented community structures.

Subsequently, the unique OTUs assigned to the orders found to be enriched in higher density soil were analysed (**Figure 4.12 A**), it was observed that Bacilliales, Burkholderiales, and Saprospirales dominated the significantly enriched community, accounting for over 50% of the most highly represented OTUs (**Figure 4.12 B**). However, when the same data was analysed using the number of assigned sequence reads instead of the number of OTUs a significant shift in the proportionality of orders was observed, notably that despite only accounting for 6.1% of the unique OTU data Sphingobacteriales now represented over 60% of the most highly abundant sequence reads (**Figure 4.12 C**).

Likewise, the combination of Baciliales, Burkholderiales and Saprospirales now only accounted for just over 25% of the data set. The different analytical approaches also added or excluded orders. In the top unique OTU data Myxococcales, Pedospaerales and Rhizobiales accounted for around 17% of the data but based on the number of assigned reads for these orders they did not feature in the most abundantly represented orders in the sequence data. Likewise, when using assigned reads to define the most highly represented orders although their proportions are small the orders Cythoniobacteriales, Sphingomonodales and WD2101 were now featured (**Figure 4.12 D**).
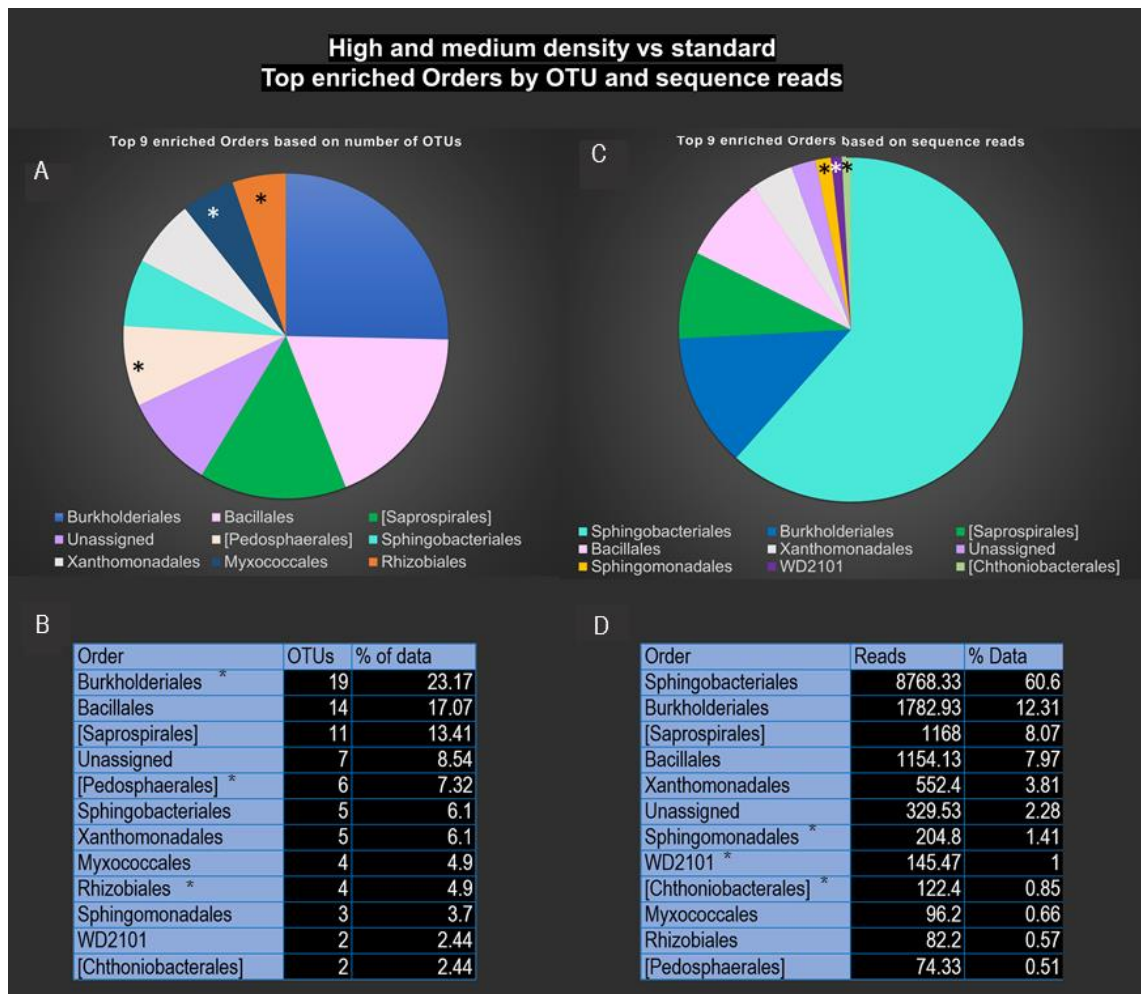
Figure 4.12 Higher density soil is dominated by Burkholderiales based on unique OTUs, however this is not a true reflection of the true dominant order which, based on sequence reads is Sphingobacteriales. (A) shows the proportion of orders represented by the number of unique OTUs. Asterisks denote orders that appear in the top represented orders based on OTU number that do not appear in the most highly represented orders when based on sequence reads. (C) shows the percentage of orders represented by unique OTUs plus the proportion that would have been represented by orders that feature as most represented based on sequence reads that were not represented by enough unique OTUs to feature in the top orders based on OTU number. (B) depicts the proportion of orders represented based on sequence reads (the sum of the mean reads per OTU per sample). Asterisks identify the orders which were not present in the most commonly represented orders based on unique OTU numbers but now featured when based on sequence reads. (D) shows the number of sequence reads assigned to each order (sum of the mean per OTU per sample) and the percentage of the data it represents plus the percentage of the data that would have been represented when incorporating the three orders that were present in the OTU data but excluded from the sequence based analysis due to low abundance.

As the sequence-based data shows a quantitative view of the microbiota community structure, it was decided to focus on this data for the downstream analysis. The orders that were significantly enriched in both the high and low density soil (higher conserved enriched orders) were then examined (**Figure 4.13**), despite being represented by only 1 unique OTU, Saprospiralles (a sub group of Sphingobacteriales) dominates the common orders accounting for over

53% of the total common sequence reads, followed by Sphingobacteriales which represents over 35% and 3 OTUs of the remaining data (**Figure 4.13A** and **4.13B**). Intriguingly, when using the number of unique OTUs to define the microbial community structure Saprospirales only represents around 5% of the total dataset with Bacillales and Burkholderiales appearing to be the most abundant, accounting for over 60% of the dataset combined (**Figure 4.13 C**).

Enriched Orders shared between medium and high density based on sequence reads

A

Legend:
- [Saprospirales]
- Sphingobacteriales
- Burkholderiales
- Bacillales
- Unassigned
- Rhizobiales
- Myxococcales

B

Order	Reads	% Data
[Saprospirales]	12832	53.89
Sphingobacteriales	8735.53	36.69
Burkholderiales	1143.73	4.80
Bacillales	967.2	4.06
Unassigned	80.4	0.34
Rhizobiales	37.93	0.16
Myxococcales	13.93	0.06

C

Order	OTUs	% Data
Bacillales	6	31.58
Burkholderiales	6	31.58
Sphingobacteriales	3	15.79
Unassigned	1	5.26
[Saprospirales]	1	5.26
Myxococcales	1	5.2
Rhizobiales	1	5.26

Figure 4.13 A single OTU of Saprospirales dominates both high and medium density soil. *(A) depicts the proportion of orders represented based on sequence reads (the sum of the mean reads per OTU per sample). (B) shows the number of sequence reads assigned to each order (sum of the mean per OTU per sample) and the percentage of the data it. (C) depicts the proportion of orders represented based on the number of unique OTUs.*

Interestingly, Saprospirales is only present in the common shared OTUs due to 1 unique OTU, a further 10 unique OTUs are present in the 1.3 g cm^3 soil density data indicating that the presence of this order may correlate in a gradient type manner as soil density increases (**Table 4.11**). A moderate proportion of depleted orders are represented by Rhizobiales and Xanthamonadales which, combined, form around 20% of the dataset encompassing the 8 most depleted orders (**Figure 4.14 A and B**).

Top 8 Depleted Orders based on sequence reads

A

- Burkholderiales
- Rhizobiales
- Xanthomonadales
- Sphingomonadales
- Caulobacterales
- Unassigned
- N1423WL
- WD2101

B

Order	Reads	% Data
Burkholderiales	5512.43	58.79
Rhizobiales	1049.71	11.2
Xanthomonadales	812	8.66
Sphingomonadales	477	5.09
Caulobacterales	292.29	3.12
Unassigned	277.71	2.96
N1423WL	264.29	2.82
WD2101	253.14	2.7
Myxococcales	244.57	2.61
Bdellovibrionales	193.43	2.06

C

Order	OTUs	% Data
Burkholderiales	15	27.27
Sphingomonadales	8	14.55
Rhizobiales	7	12.73
Bdellovibrionales	5	9.09
Caulobacterales	5	9.09
Unassigned	4	7.27
Myxococcales	4	7.27
Xanthomonadales	4	7.27
N1423WL *	1	1.82
WD2101 *	2	3.64

Figure 4.14 Members of the order Burkholderiales are significantly depleted in soil with 1.2 g cm^3 and 1.3 g cm^3 density. (A) shows the proportion of orders represented based on sequence reads. Asterisks identify the orders which were not present in the most commonly represented orders based on unique OTU numbers but now featured when based on sequence reads. (B) shows the number of sequence reads assigned to each order and the percentage of the data it represents plus the percentage of the data that would have been represented when incorporating the two orders that were present in the OTU data but excluded from the sequence based analysis due to low abundance. (C) shows the percentage of orders represented by unique OTUs plus the proportion that would have been represented by orders that feature as most represented based on sequence reads that were not represented by enough unique OTUs to feature in the top orders based on OTU number.

As observed with **Figure 4.11**, analysing the data using unique OTU abundance impacted the picture we see of the bacterial orders, although Burkholderiales remains to be the most depleted order it represents just 27% of the data, the orders N1423WL and WD2101 that were represented in relatively low proportions in the sequence-based grouping do not feature based on the number of representative OTUs (**Figure 4.14 C**). The orders Bdellovibrionales and Myxococcales did not have enough sequence reads to feature in the sequence-based group despite being composed of 4 and 5 unique OTUs respectively.

Finally, the orders that were depleted in both high and medium density soil were investigated (**Figure 4.15**).

Depleted Orders in common based on sequence reads

A

- Burkholderiales
- Xanthomonadales
- Nitrospirales
- Unassigned
- Bdellovibrionales
- Sphingobacteriales
- Sphingomonadales
- [Saprospirales]
- Flavobacteriales

B

Order	Reads	% Data
Burkholderiales	875.29	44.27
Xanthomonadales	693.86	35.09
Nitrospirales	125.43	6.34
Unassigned	121.43	6.14
Bdellovibrionales	57	2.88
Sphingobacteriales	34.43	1.74
Sphingomonadales	31	1.57
[Saprospirales]	20.43	1.03
Flavobacteriales	18.43	0.93

C

Order	OTUs	% Data
Xanthomonadales	2	18.18
Bdellovibrionales	2	18.18
Burkholderiales	1	9.09
Sphingomonadales	1	9.09
[Saprospirales]	1	9.09
Flavobacteriales	1	9.09
Sphingobacteriales	1	9.09
Unassigned	1	9.09
Nitrospirales	1	9.09

Figure 4.15 A single strain each of Burkholderiales and Xanthomonadales represent almost 80% of the depleted orders conserved between 1.2 g cm³ and 1.3 g cm³ density soil samples. (A) depicts the proportion of orders represented based on sequence reads. (B) shows the number of sequence reads assigned to each order (sum of the mean per OTU per sample) and the percentage of the data it accounts for. (C) depicts the proportion of orders represented based on the number of unique OTUs.

Of these conserved orders only 1 or 2 unique OTUs represented each but the sum of the mean sequence reads per sample revealed that, despite each being represented by just one unique OTU Burkholderiales and Xanthomonadales represented almost 80% of the depleted orders present in both high and medium density soil. Interestingly, as with Saprospirales although only 1 Burkholderiales OTU is depleted in both higher soil densities a total of 15 unique Burkholderiales OTUs are depleted over all, 14 of which are only depleted in the high (1.3 g cm^3) soil indicating that depletion of Burkholderiales orders increases with increasing soil density and may represent a gradient-like correlation.

4.4 DISCUSSION

*high density soil: 1.3 g cm^3

**medium density soil: 1.2 g cm^3

***standard density soil: 1 g cm^3

****higher density soil: total unique OTUs enriched in 1.3 g cm^3 and 1.2 g cm^3 soil density combined

*****higher shared soil: unique OTUs enriched in both 1.2 g cm^3 and 1.3 g cm^3

4.4.1. Soil density and treatment does not affect the ability for bacterial microbiota to proliferate in soil.

This data shows that shifts in soil density impact the community composition of the soil bacterial communities: while the higher ranks of the soil microbiota appears sufficiently resilient to withstand the pressure imposed by these physical drivers, evidence suggests that the abundance of individual members of the community (i.e., OTU level) do respond in a density-dependent manner. For example, the concentration of DNA and number of unique OTUs observed between samples showed no significant differences (**Table 4.3, Figure 4.5, Figure 4.9A**), instead it was the proportion of the unique OTUs assigned to the soil environments that shifted, particularly the proportion of sequence reads assigned to individual OTUs that were impacted by these changes (**Figure 4.12**

and Figure 4.14). It is important to note that watering treatment showed no significant effect on the bacterial community profiles until analysed at the OTU level. However, this effect was far less pronounced and was independent of soil density suggesting, and complimenting some studies, that a multitude of factors contribute to shaping the soil microbiota (Deng et al., 2018, Hacquard, 2016). Although this study focusses on the impact of soil density on the bacterial soil microbiota it cannot be ruled out that watering treatment may have played a small role in the observations of this investigation. For example, it can be observed that samples that were subjected to the "Wet" treatment consistently showed an increase in the relative abundance of the classes Saprospirae and Sphingobacteria compared to the "Dry" treated samples of the same density (**Table 4.6**). However, in all but one of these comparisons these changes accounted for less than 1% of the total data and did not change the pronounced effect of soil density on the overall community structure (**Figure 4.7**).

At the phylum, class and order level subtle shifts in the relative abundance of the most highly represented microbial communities were observed, but these were not statistically significant (**Figures 4.6, 4.7 and 4.8**). This indicates that the rhizosphere effect has the greatest influence on the soil microbiota and that the bacterial communities in the soil remain somewhat stable at these taxonomic ranks in the absence of plant roots under controlled conditions (**Chapter 2; Figure 2.5**)(Berg et al., 2006, Gregory, 2006, Whipps, 2001). However, this picture changes when data is examined at the OTU level, revealing the extent of the impact that soil density has on the microbiota; specifically the observation that as soil density increases, the presence of some OTUs become disproportionately increased or decreased in the community of individual samples compared to soil of standard density (**Figure 4.9B**).

Ultimately this was further confirmed when beta diversity was examined, showing a distinct shift in the composition of the bacterial communities in a density-dependent manner. This suggests that these community profile shifts are likely to be the result of bacterial responses to the physical properties of a higher density environment, with it either promoting or inhibiting the proliferation of specific species. For the remainder of this discussion the specific changes at the OTU level in response to soil density increases and the potential consequences of this will be examined.

4.4.2. Enriched and depleted OTUs in high density soil vs standard density

When investigating the selective enrichment or perturbation of OTUs between higher and standard density samples it was clear that there was a positive and negative gradient like effect as soil density increased. Of the number of unique OTUs that were significantly differentially enriched in higher density soil a clear trend was observed with 4 unique OTUs enriched in medium density soil only, increasing to 19 unique OTUs enriched in higher shared soil then increasing again to 81 unique OTUs in the high density substrate only.

The same trend was observed in unique OTUs that were significantly differentially depleted as soil density increased; In soil with medium density just 2 OTUs were significantly diminished, this increased to 11 unique OTUs that were commonly depleted in higher density soils (i.e., combined dataset 1.2 and 1.3) and increased notably again to 61 unique OTUs significantly depleted in high density soil only (**Figure 4.11**).

This indicates that the impact of density on the microbiota increases as soil compaction becomes more pronounced. This observation is described visually in **Figure 4.16**. The influence of soil compaction on the soil microbiota has been explored in previous studies; mainly in long term forestry studies or in relation to tilling practices, for example, the impact of logging associated compaction on the soil microbiota showed that compaction significantly reduced microbial abundance, increased diversity and consistently changed the shape of the soil microbiota (Hartmann et al., 2013). Likewise, Miransari and co-workers demonstrated that the introduction of Arbuscular mycorrhizal fungi helped to reduce compaction stress and promote root growth in wheat (Miransari et al., 2008). However, studies looking at delicate and localized changes in soil density in relation to root architecture and the impact this has on the plant associated microbiota have yet to be conducted in great depth.
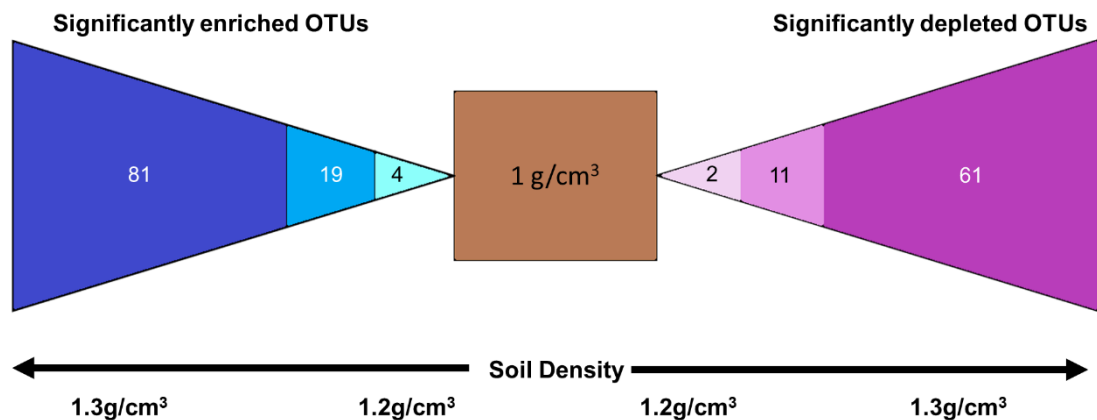
Figure 4.16 OTU enrichment or depletion occurs in a gradient like manner as soil density increases. Numbers indicate the number of unique OTUs either significantly enriched or depleted as soil density increases from 1.2 g cm³ to 1.3 g cm³ versus standard density soil. The middle term in each triangle indicates unique OTUs enriched or depleted in both 1.2 g cm³ and 1.3 g cm³ soil respectively.

4.4.3 Enriched OTUs in high density soil

As soil compaction is widely reported to have a negative effect on a multitude of factors affecting plant fitness including germination (Skinner et al., 2009) and root hair morphology (Alessa and Earnhart, 2000) it was interesting to discover that more unique OTUs were significantly enriched in high density soil than depleted. This observation mimics our previous observation that root hair mutants, whose rhizosphere soil density is higher compared to wild type plants, host a reduced-complexity community compared to plants with fully developed root hairs (Robertson-Albertyn et al., 2017).

When the taxonomy of the enriched OTUs in this study was compared to the enriched OTUs in the rhizosphere study in **Chapter 2** there were some marked differences in the Orders identified, as well as some correlations with the OTUs identified as having high relative abundance in the exudates of the root hair mutants (**Chapter 3**). These are described below.

4.4.3.1. Sphingobacteriales dominate the OTUs enriched in high density soil

The taxonomic order Sphingobacteriales significantly dominated the OTUs enriched in high density soil when based on assigned high quality sequence reads (**Figure 4.12**). Although this order was not identified as significantly enriched in the rhizosphere of root hair mutants it was observed to be 60% more

abundant in the exudates of the root hair mutant *rhl1.a* (**Figure 2.8, Table 3.11, Chapter 3**) which could indicate that this particular bacterial order not only can proliferate well in a compacted soil environment, but may be nurtured further by the exudation patterns and rhizodeposition of the root hair mutant barley lines investigated. This could be tested experimentally by repeating this study but including exudate inoculant to test this theory.

Many members of the Sphingobacteriales order use carbohydrates as their sole source of nutrition, they have also been identified as playing roles in Carbon cycling and the decomposition of organic matter which would support this proposition (Tian et al., 2015, Lambiase, 2014, Taylor et al., 2012, Steyn et al., 1998). However, despite their significant presence in the denser soils and increased presence in the root hair mutant exudates this does not correspond with the data studying the enrichment of orders in the root hair mutant rhizosphere (**Chapter 2**).

This indicates that other edaphic or plant factors are at play during the recruitment of Sphingobacteriales in the barley rhizosphere. For example, Sphingobacteriales have also been shown to be vulnerable to long-term organic changes in soil and nitrogen availability, both of which can be influenced by the presence of plants and the chemical composition of the substrate (Hester et al., 2018, Campbell et al., 2010).

4.4.3.2. The order Burkholderiales is enriched in higher density soil

The second most abundant order enriched in higher density soil overall was Burkholderiales, although it was represented by 19 unique OTUs the total assigned sequence reads accounted for 12.3% of the data set. Not only was this order found to be significantly enriched in the rhizosphere of the root hair mutants (**Chapter 2, Figure 2.8**) it also had double the relative abundance in the exudates of *rhl1.a* compared to the wild type (31.6% and 15.7% respectively) (**8.3.1. Appendix 3: Supplementary database 3**).

Many members of the Burkholderiales order have been identified as having plant growth promoting abilities, some members of the order have been implicated in the solubilisation of phosphate in maize plants and can outcompete some fungi, they have also been observed to promote plant growth by producing the enzyme 1-aminocyclopropane-1- carboxylate (ACC) deaminase which reduces the plant

ethylene hormone (Xiao et al., 2017, Zhao et al., 2014, Poupin et al., 2013). In addition to this Burkholderiales have been observed to be significantly enriched in a compacted soil environment as the result of tillage versus a zero-tillage environment (Ceja-Navarro et al., 2010).

Compellingly, the majority of OTUs in the higher density dataset belonging to the Burkholderiales order were assigned to the Oxalobacteraceae family, which display a broad range of phenotypic and ecological properties. All species of Oxalobacteraceae (with the exception of the anaerobe Oxalobacter) are aerobic but some strains can act as facultative anaerobes which would potentially be of benefit in a high density soil environment (Baldani et al., 2014). This family also includes the genus Janthinobacter, which was observed to be significantly enriched in the rhizosphere of root hair mutant barley and seen to be dominant in the root hair mutant *rhp1.b* (**Chapter 2**).

Although, the taxonomic resolution of this data does not confirm the presence of Janthinobacter it still strongly compliments both the rhizosphere and exudate study, indicating that members of the order Burkholderiales are particularly suited to inhabiting the dense rhizosphere of barley root hair mutants and may be responsible for aiding the acquisition of phosphate and other essential nutrients.

However, although the high diversity of this order allows some members to proliferate well in a high density soil or lower root hair environment, equally it means that many cannot survive these conditions. This is described in the depleted OTUs dataset **4.4.4.1**

4.4.3.3. Saprospirales represent the third most enriched order in higher density soil

Saprospirales was the third most abundant order based on both the number of unique OTUs and assigned sequence reads. Interestingly the order was represented by 11 unique OTUs representing approximately 8% of the total higher density data set (**Figure 4.12**). However, 10 of these OTUs did not feature in the medium density soil data, the remaining, single OTU was enriched in the higher density shared data set. Despite this order being represented by just one OTU, it dominated the shared data set accounting for almost 54% of the total sequence reads assigned to it.

This compliments the potential "gradient effect" that was described in **Figure 4.16**. In addition to the gradient like behaviour observed in response to soil density, as the name suggests, many members of the order Saprospirales have been identified as being saprophytic.

Specifically, having the ability to degrade chitin and other complex organic matter (Glavina Del Rio et al., 2010, King et al., 2010). This is particularly interesting as chitin is a major structural component of the fungal cell wall and it has been demonstrated that fungi are far less resilient in high density soil environments and are adversely affected by compaction (Lord and Vyas, 2019, Mercado-Blanco et al., 2018).

Nevertheless, some species of arbuscular mycorrhizal fungi have been shown to associate with root hair mutant barley roots to increase surface area for nutrient acquisition, particularly with regard to phosphorus uptake (Brown et al., 2013, Li et al., 2014). Thus, it is tempting to speculate that members of the order Saprospirales are consuming some fungal species that are unable to proliferate as efficiently as density increases resulting in an increase in this order and subsequent decrease in the presence of some fungal species.

It is also prurient that in the higher density data the proportion of assigned sequence reads to this order is only 8% in comparison to over 50% in the shared data. This could indicate that in high density soil some fungi cannot proliferate at all reducing the source of nutrition for Saprospirales, resulting in an overall decline in the number of individual bacteria compared to medium density soil. A hypothesis that could be tested as a result of this data is that the order Saprospirales declines in high density soil as a result of fungal decline in response to a high-density soil environment.

4.4.4. Depleted orders in higher density soil

Due to the widely reported observation that soil compaction perturbs the microbiota it was of no surprise that many orders were depleted in higher density soil compared to soil of a standard density.

It has been demonstrated that the rhizosphere microbiota of root hair mutant barley plants has a different bacterial composition to that of wild type plants which have a standard rhizospheric soil density (Robertson-Albertyn et al., 2017,

Koebernick et al., 2017).

Significantly, fewer orders were identified as having OTU members appreciably depleted in the higher density soil compared to those that were enriched. Furthermore, the Order Burkholderiales was amongst the most represented in both the enriched and depleted datasets which is a clear indication of the sheer diversity of members of this bacterial order.

Due to the fact that only 2 unique OTUs (belonging to the orders Myxococcales and Sphingobacteriales) were depleted only in medium density soil the discussion around the most significantly depleted orders will focus on those significantly depleted in the combined high and medium density datasets termed "higher density". The percentage enrichment in standard soil versus 1.2 g cm^3 and 1.3 g cm^3 density soil respectively has been used as a proxy to infer OTU depletion in the 1.2 g cm^3 and 1.3 g cm^3 density samples.

4.4.4.1. The order Burkholderiales has the greatest depletion of unique OTUs in higher density soil

The order Burkholderiales accounted for 58.8% of the sequence reads assigned to depleted OTUs in higher density soil (**Figure 4.14**). These reads encompassed 15 unique OTUs, only one of which was depleted in both 1.2 g cm^3 and 1.3 g cm^3 density samples, the rest being present in a 1.2 g cm^3 density environment and only being perturbed at 1.3 g cm^3 density (**Figure 4.15**). This observation further supports the "gradient" theory proposed by this study (**Figure 4.16**).

Unlike the OTUs that were enriched in 1.3 g cm^3 density soil, members of the Oxalobacteraceae family did not dominate the members of the Burkholderiales order, instead, of the OTUs that could be assigned at family level, it was represented by a 50/50 mixture of members of Comamonadaceae and Oxalobacteraceae. The greater presence of Oxalobacteraceae is somewhat expected due to the diversity between species which has already been discussed. Moreover, it was interesting to observe that no Comamonadaceae were enriched in the higher density soil, only depleted. This indicates that Comamonadaceae may be more sensitive to changes in environmental density than their taxonomic relatives. However, it is of note that the family Comamonadaceae is also highly diverse, encompassing over 100 species belonging to 29 genera with a large phenotypic diversity including aerobic

organotrophs, anaerobic denitrifiers and photoautotrophs (Willems, 2014). It is of further note that OTUs assigned to this family were not detected in the bacteria extracted from either set of exudate samples described in **Chapter 3**.

The majority of Comamonadaceae are free living saprophytes which suggests they can assist with the cycling of nutrients in soil such as Fe III (*Comamonas guangdongensis)* and Sulphur *(Comamonas sp.* has the ability to mobilize sulphate esters)(*Willems, 2013, Hummerjohann et al., 2000)*. However, as is a common feature of members of the Burkholderiales order, they do inhabit a diverse range of environments and have a broad range of phenotypes; a number of species within this family are known plant pathogens such as *Acidovorax sp.* and *Xylophilus sp.* (Willems, 2013).

The data suggests that as soil density increases the tolerance of the higher density environment is lost by some members of the Burkholderiales Order, particularly Comamonadaceae; as a large proportion of this family are beneficial saprophytes it could be concluded that plants being grown in poorly managed, compacted soil or plants with root hair mutations may not receive the full benefit of a symbiotic relationship with organisms from this bacterial family.

4.4.4.2. Rhizobiales

Although a very small proportion of the order Rhizobiales were observed to be significantly enriched in higher density soil (**Figure 4.12**) this was overshadowed by the much larger number of unique OTUs and sequence reads identified as being depleted. This order is well documented as featuring beneficial bacteria, as members engage in mutualistic symbiosis with plants, specifically the fixation of nitrogen by forming nodules in legumes (Lagunas et al., 2015). Although its members also form a core part of the rhizosphere microbiota they are typically considered to be transient commensal species in non-legumes (Garrido-Oter et al., 2018, Jiao et al., 2019).

Inversely, in a study investigating potential PGPR qualities of rhizobia in the model species *Arabidopsis thaliana* it was shown that commensal Rhizobiales spp have the ability to positively manipulate root development (Garrido-Oter et al., 2018). This discovery suggests that Rhizobiales spp may perform even more additional beneficial functions for plants, but this remains to be fully elucidated (Hacquard et al., 2015, Yeoh et al., 2017).

Fascinatingly, although this order was not represented in the bacterial sequence reads obtained from the exudates of wild type and mutant root hair barley (**Chapter 3**), Rhizobiales was significantly enriched in the rhizosphere of root hair mutant barley (**Figure 2.8**). These observations, at first glance, conflict with the data obtained from this study and indicate that soil density per se might not be the sole determinant of Rhizobiales recruitment in the rhizosphere; conversely other or additional edaphic and plant factors likely mediate the assemblages of these bacteria at the root-soil interface.

4.4.4.3. Xanthomonadales

Similar to the observed distribution of enriched Rhizobiales, members of the order Xanthomonadales featured in both the enriched and depleted datasets, but with a far more pronounced presence in the depleted data. Remarkably, although they were significantly depleted in the higher density dataset they were identified as being enriched in the rhizosphere of root hair mutant barley which had a greater soil density in the rhizospheric region compared to wild type barley. (**Chapter 2, Figure 2.8**). However, all but two of the OTUs responsible for these observations were conserved between the two studies showing that at a higher taxonomic resolution the distribution of OTUs would be less likely to be conflicting between datasets.

The depleted members of Xanthomonodales appeared to be particularly sensitive to soil density with 85% of the total observed depletion occurring at medium soil density, again, this data supports the "gradient theory" described earlier (**Figure 4.14, 4.15 and 4.16**). Many members of the Xanthomonodales order are well documented phytopathogens and are responsible for several devastating plant diseases, notably bacterial blight in barley caused by *Xanthomonas camperstris*.

Although *Xanthomonas camperstris* infects the leaves and stem of barley, it has been shown to be present on and within seeds allowing the introduction of this pathogen to the soil which then allows transmission through water channels which introduce this pathogen to the soil and allow it to infect via the water in the soil (Swings et al., 1990, Aung et al., 2018, Maharshi.R.P, 2006). Due the transmission method of some members of the Xanthomonadales order being through soil water channels this data could indicate that although compacted soil is, on the whole, negatively correlated with plant fitness and microbial richness it

could reduce the rate of infection in some crop systems and may also help to protect plants with root hair mutations from some diseases caused by pathogenic Xanthomonadales. However, it is important to consider that although Xanthomonadales comprises one of the largest phytopathogenic bacterial orders not all members are pathogenic.

4.4.5. Conclusions

The motivation for this study was based on the observation that plants with root hair mutations had higher density soil at the root soil interface which affected pore size, water channels and potentially gas diffusion (Koebernick et al., 2017, Stepniewski et al., 1994, Schjønning et al., 2015). These density dependent soil features ultimately result in a reduction in oxygen levels in the soil which can impact bacterial respiration pathways which is fatal for obligate aerobes and may change the chemical soil environment due to the production of lactic acid or ethanol by facultative anaerobes. This led to the hypothesis that shifts in the bacterial rhizosphere microbiota in root hair mutant barley compared to their wild type counterparts is, at least in part, the result of changes to the soil environment caused by soil compaction.

Although the effects of soil compaction on a multitude of factors including the microbiota have been studied, these studies have been over the course of years in forestry or tilling projects, not a short time period of 10 days and then linked back directly to both the plant microbiota and exudation profiles of wild type and mutant root hair plants.

Examining this data individually and collectively across the previously detailed studies allow us to paint a more detailed picture of the interplay between a multitude of factors that contribute to shaping the plant microbiota and influencing plant fitness.

Whilst it is clear that soil density has a significant effect on the presence (or absence) of multiple bacterial orders, particularly Burkholderiales, Rhizobiales, Saprospirales, Sphingomonadales and Xanthomonadales this data also confirms that, ultimately it is a combination of many physical, chemical and biological factors which shape the soil microbiota, particularly that of the rhizosphere. For example, the aeration of the soil as a result of compaction or the chemical composition as a result of facultative anaerobic respiration. This was especially

apparent in the observation that although in higher density soil a marked depletion of the orders Rhizobiales and Xanthomonadales occurred this was in direct contrast to the data obtained on enriched orders in the rhizosphere of root hair mutants which, in principle, also have a more dense soil environment.

Furthermore, these orders were not detected at all in the DNA extracted from the exudates of wild type or mutant plants that were grown hydroponically making it unlikely that the exudation of the plants studied explained the enrichment of these orders observed in the rhizosphere. Nonetheless, with there being such diversity within bacterial orders in-depth analysis at a higher taxonomic resolution may give further insights into these potentially conflicting observations. This was supported during the analysis of the order Burkholderiales, an order commonly associated with many PGPR functions (Xiao et al., 2017) .

Notably, members of the order Burkholderiales were observed to be a) primarily enriched in the rhizosphere of root hair mutants, b) present in higher abundance in the exudates of root hair mutants and c) significantly enriched in higher density.

Put together, these observations suggest a plant-mediated mechanism whereby seed transmitted, putative PGPRs, are released in the rhizosphere of root hair mutants. However, the order Burkholderiales is paradigmatic to exemplify the metabolic diversity of the microbiota as members of this order were also observed to be significantly depleted in higher density soil. Although this does not rule out the concept that these observations may be plant mediated, it indicates that a greater understanding of plant-microbe-soil interactions at the species and even molecular level is required to fully describe and utilize these relationships for practical agricultural applications.

Paramount towards the implementation of these applications will be the characterisation of the genetic potential of individual members of the microbiota in the form of plant growth promoting functional genes, a task that will be described in the following **Chapter 5**.

4.4.6. Limitations of the study

This provisional data points towards soil density playing an important role in contributing to shaping the soil microbiota. However, due to the number of samples that withstood the initial treatments it would be important to repeat this

study using a much larger sample set to validate the data. An analysis of the fungal microbiota would also serve to improve understanding of the shifts observed in some bacterial orders in response to soil density in this study. It is also important to consider that this study was conducted using DNA sequencing, not RNA meaning that it cannot be determined if the sequence reads were obtained solely from living bacteria.

CHAPTER 5

THE ISOLATION AND CATEGORISATION OF INDIVIDUAL BACTERIAL MEMBERS OF THE RHIZOSPHERE MICROBIOTA

5.1. INTRODUCTION

Identifying whether the specific structural and functional composition of rhizosphere microbiota communities are specifically related to a particular plant genotype or environment is both challenging and complex. Although the use of 16S rRNA sequencing can provide detailed insight into the taxonomic diversity and abundance of bacterial species at the OTU level this investigation alone does not allow us to fully understand the potential functional applications of the bacteria enriched in the rhizosphere or the physical, chemical and biological interactions driving the composition of these communities.

By isolating members of the barley bacterial rhizosphere microbiota, an indexed bacterial library can be generated to allow for the opportunity to determine which bacterial taxa can successfully proliferate in the rhizosphere of barley displaying different phenotypic traits and in a variety of environments. The collection can also be analysed at the genetic level with a high resolution for potential functionality to be identified and then used to determine bacterial isolates which can be used in PGPR studies in barley and other agriculturally important plant species. The aim of this study was to isolate, identify and categorize the functionality of individual members of the barley rhizosphere microbiota to create a bacterial collection that can be utilized for future inoculation studies and collaborations.

To achieve the aforementioned tasks, using the barley genotypes Dema and Karat and their respective root hairless mutant homologs *rhl1.a* and *rhp1.b* grown in the Scottish agricultural soil termed Quarryfield alongside bulk soil samples, an indexed library of 53 bacterial isolates was generated using traditional microbiology techniques. This library was subjected to whole genome sequencing in order to identify PGPR functionality and to elucidate the accuracy

of using 16S rRNA sequencing as a taxonomic identifier vs higher resolution analysis of the whole genome.

The bacterial library generated encompassed 16 bacterial species representing 11 bacterial genera. Provisional genomic analysis of the isolate library revealed the presence of at least 5 PGPR gene groups based on KEGG orthology including, *pho, ding, pqq, nif* and *hcn* genes which have been identified as performing roles in the acquisition of phosphorus and nitrogen and enhancing the efficacy of biological fertilizers to enhance plant nutrient uptake.

5.2. MATERIALS AND METHODS

5.2.1. Barley germination and growth environment

Seeds of the barley varieties Karat and its root hair mutant homologue *rhl1.a* alongside Dema and its root hair mutant isogenic line *rhp1.b* were reproduced from plants grown from seeds provided by Agnieska Janiak that were grown to full maturity in controlled glasshouse conditions as described in **Chapter 2.2.2** (Janiak and Szarejko, 2007, Xiao et al., 2017). Spikes were manually excised from the plants and gathered using a mechanical thresher ensuring that seeds from each plant genotype were processed separately to avoid incorrect genotype allocation to the new seed generation. Seeds were germinated and grown in Quarryfield soil (**Table 2.1**) as described in **Chapter 2.2.2**.

5.2.2. Bacterial extraction from the rhizosphere

Plants and bulk soil samples were excavated from the soil and the "rhizosphere compartment" obtained as described in **Chapter 2.2.3**. Roots were removed using sterile tweezers and discarded. In order to obtain bacterial "fractions" the rhizosphere samples were placed in 20 ml PBS and shaken on a roller bed for 15 minutes to fully mix. Then, using a 1 ml pipette, 750 µl aliquots of the suspension were transferred to individual 2 ml Eppendorf tubes containing 750 µl of sterile 70% glycerol, the rhizosphere samples were gently resuspended as required. Aliquots were then vortexed to ensure thorough mixing with the stabilizing agent. Aliquots of the PBS and glycerol used in this process were also retained for contamination checks. Glycerol samples were stored at - 20 ˚C for future

processing and rhizosphere stocks were stored at 5 °C.

5.2.3. Bacterial dilution optimisation

In order to determine the most suitable dilution factor that would allow sufficient bacterial growth to observe diversity without overcrowding and to estimate the number of CFUs in the rhizosphere samples a series of serial dilutions were conducted. Serial dilutions were prepared from rhizosphere stocks that had been stored at 5 °C for 24 - 72 hours by diluting to $10^1 - 10^6$, respectively using sterile PBS in a category 2 laminar flow cabinet.

To simultaneously investigate the variation of CFUs that could be obtained, 500 µl of each dilution was spread out on 3 different nutrient media selected for their varying bacterial growth properties (**Table 5.1**). Four replicates, plus a PBS control, were generated per media type per bacterial sample and two plates each were incubated for 48 - 72 hours at either 27 °C or on the bench at room temperature (approximately 20 °C) (**Table 5.2**). A total of 120 "mother plates" were generated per dilution. For each media type, a new batch of buffer or package of sterile spreader two controls were taken and incubated at 20 °C and 27 °C, respectively. Following incubation, the number and variety of CFUs per plate was recorded and images taken to record observed morphology. Plates were stored at 5 °C for colony picking, isolation and stock preparation.

Table 5.1 Nutrient media used to isolate a variety of bacterial species from rhizosphere soil. Bacterial growth properties as described in the literature (Sandle, 2019, Biosciences, Reasoner and Geldreich, 1985) and nutrient media composition as described by DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) (Zellkulturen, 2012, Zellkulturen, 2007a, Zellkulturen, 2007b)

Media	Bacterial growth properties	Composition
Nutrient	A general, complex, non-specific media	Peptone, Meat extract, Agar, distilled water. (Ph7)
PP	Meets the nutritional requirements of some bacterial, fungal and eukaryotic cells where a single source of casein or meat peptones has not been unsatisfactory.	Polypeptone, yeast extract, $MgSO_4$, agar, distilled water (pH7)
R2A (Reasoners 2 Agar)	Used to study slow-growing bacterial species that would normally be suppressed quickly by faster growing species if there is limited volume or resources	Yeast extract, proteose peptone (Difco no.3), casein, glucose, soluble starch, Na-pyruvate, K_2HPO_4, $MgSO_4$, Agar, distilled water. (Ph 7.2)

Table 5.2 Rhizosphere bacterial dilution and nutrient optimisation replicates and controls. Sample replicates were conducted per dilution ($10^1 - 10^6$) as described below and controls were conducted per media, buffer or spreader batch as described..

Hypothetical sample	Nutrient media	Number of plates	Incubation temperature
Each sample per dilution			
Sample "x"	Nutrient	2	27 ˚C
Sample "x"	Nutrient	2	20 ˚C
Sample "x"	PP	2	27 ˚C
Sample "x"	PP	2	20 ˚C
Sample "x"	R2A	2	27 ˚C
Sample "x"	R2A	2	20 ˚C
Controls per media			
Media control	Nutrient, PP, R2A	1	20 ˚C
Media control	Nutrient, PP, R2A	1	27 ˚C
Spreader control (conducted with each new batch of spreaders)	Nutrient, PP, R2A	1	20 ˚C
Spreader control (conducted with each new batch of spreaders)	Nutrient, PP, R2A	1	27 ˚C
PBS control	Nutrient, PP, R2A	1	20 ˚C
PBS control	Nutrient, PP, R2A	1	27 ˚C

5.2.4. Colony picking

It was determined that a dilution factor of e^{-3} was the most suitable for this project as samples from this dilution range resulted in 30 to 300 CFUs per "mother plate" to be taken forward for isolation. This range is considered to be statistically significant and is a common baseline for bacterial isolation studies originally proposed over a century ago (Breed and Dotterrer, 1916). A plate containing less than 30 CFUs can mean that small discrepancies in dilutions or the presence of a contaminant can have a non-proportionate effect on the final count.

Likewise, plates containing more than 300 CFUs are considered to be too dense, resulting in poor isolation due to CFUs likely to be growing together or in such close proximity to one another that picking an individual colony is very difficult. Individual CFUs were selected for isolation from the 120 "mother plates" that were retained based on their visual morphology; size, shape, edge, texture, proximity

to other CFUs, colour and opacity with a focus on obtaining a broad range of CFUs from each rhizospheric environment whilst ensuring a manageable selection **(Figure 5.1)**. Therefore, dilution, spreading and picking of isolates was conducted on several occasions over the course of the study using the rhizosphere glycerol stocks in place of the fresh soil suspension.

For small CFUs the entire colony was lifted from the plate and streaked out on corresponding media, for large CFUs the same process was conducted but by picking up half of the CFU. Plates were incubated as per the rhizosphere "mother plate" until sufficient growth could be observed **(Figure 5.2A)**.
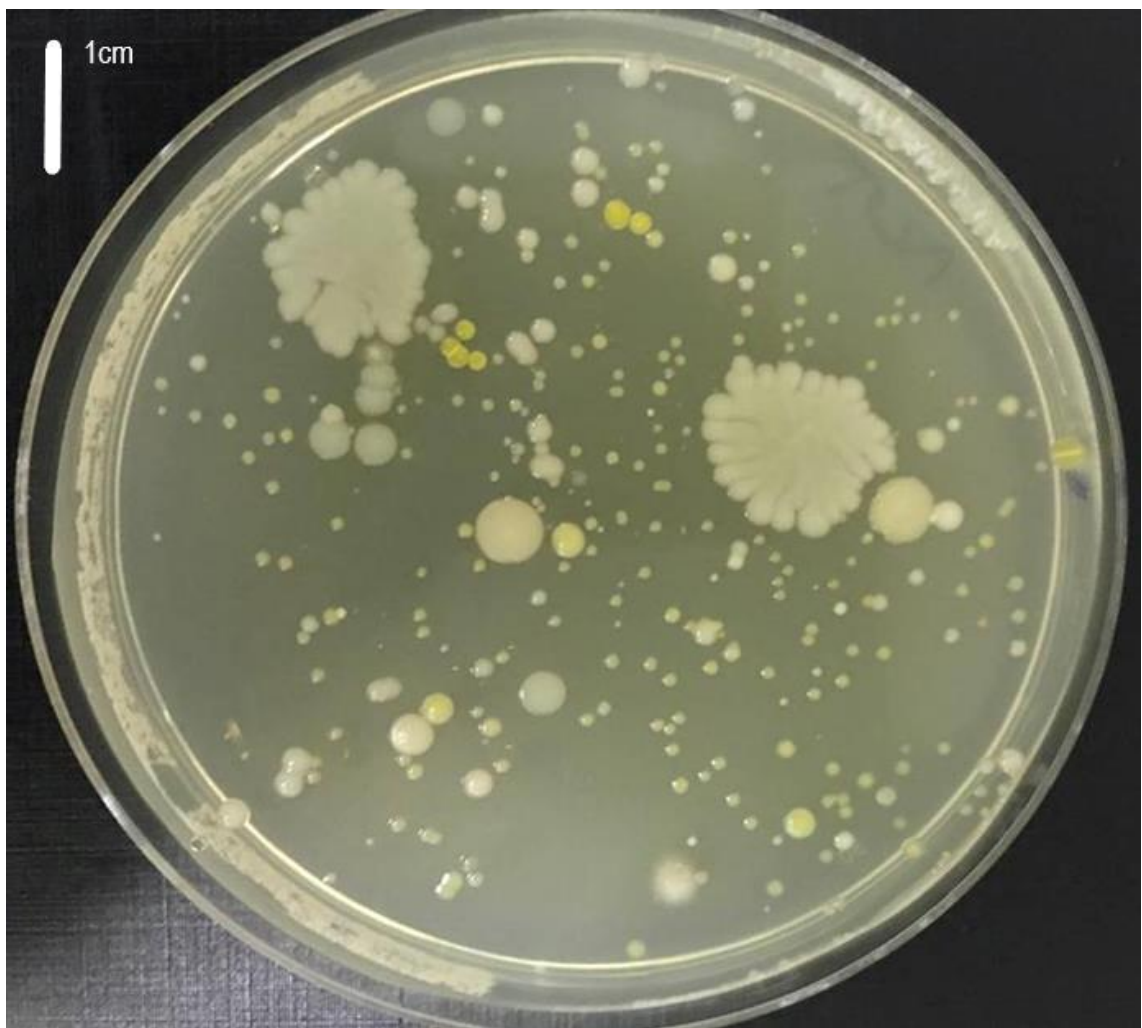
Figure 5.1 Representative example of a rhizosphere bacteria "mother plate". The technique used to isolate bacteria from rhizospheric soil provided a broad morphological range of CFUs. This sample was derived from an e-³ rhp.1b soil sample and grown on nutrient agar at 20 ˚C
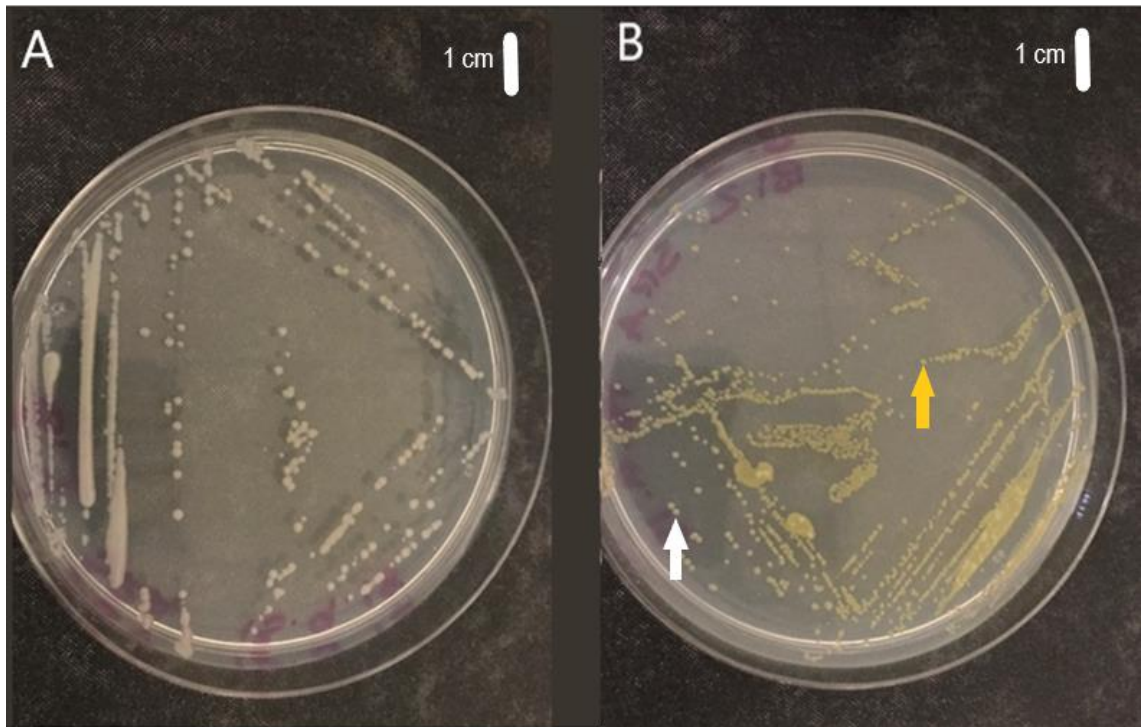
Figure 5.2 Fully and partially isolated rhizosphere bacteria. *Plates show examples of the results of a round of colony picking from a rhizosphere mother plate. Figure 5.2A has been isolated from the rhizosphere of rhl1.a on nutrient agar and shows uniform colonies that were later identified as Agrobacterium HJ13-3 with over 90% taxonomic confidence. Figure 5.2B shows a plate that was also from a rhl1.a nutrient agar plate. However, it required further colony picking due to contamination (small white colonies indicated by the white arrow). The yellow colonies (indicated by the yellow arrow) were later identified as Microbacteriaceae family (closest neighbour testaceum) with over 90% taxonomic confidence.*

Plates with uniform CFUs corresponding to the morphology of the CFUs picked from the "mother plate" were selected to generate liquid broth stocks. A single small colony or half of a single large colony was dropped into a 15ml falcon tube containing 5 ml of liquid media corresponding to the "mother plate" from which it was isolated. The CFU was agitated to allow dispersal throughout the media, then incubated at 27 ˚C with 150 rpm agitation to generate bacterial broth solutions. Two broths per CFU were generated plus controls for each media type and for each new batch of loops opened. Plates that displayed non-uniform CFUs were subjected to the colony picking process again, selecting CFUs corresponding to the morphology of the original colony selected until uniformity was achieved (**Figure 5.2B**).

After 48-hour incubation, 750 μl of broth was added to 750 μl sterile 70% glycerol in a 2 ml Eppendorf tube, vortexed to ensure samples were well mixed then stored at -80 ˚C for future use (this was performed to generate a minimum of 4 replicates per isolate).

5.2.5. Extraction and amplification of DNA from individual bacterial isolates

The FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, USA) was used to extract DNA from individual samples as per manufacturer's instructions for soil using 500 ul of defrosted isolate glycerol stock as described in **Chapter 2.2.3**. Amplicons were generated using selective PCR amplification of the hypervariable V4 region of the 16S rRNA gene using the PCR primers:

515F (5'-GTGCCAGCMGCCGCGGTAA-3')

806R (5'-GGACTACHVGGGTWTCTAAT-3')

as described in **Chapter 2.2.4.**

Of the 194 isolates selected for amplification a total of 84 were successfully amplified and purified using Agencourt beads as per **Chapter 2.2.4** then subjected to Sanger sequencing (Sanger et al., 1977) at the genomic facilities of the James Hutton Institute using the 806R primer as a template on an ABi3730 DNA analyser which is a 48 capilliary array (36 cm) machine.

5.2.6. Preliminary taxonomic classification and Phylogenetic tree assembly

In order to provisionally identify the identity of the isolated bacterial species the FASTA files obtained from Sanger sequencing (**Chapter 5.2.5**) were subjected to a BLAST analysis (Altschul et al., 1990). They were also subjected to OTU clustering using the closed reference approach in QIIME using the Greengenes database (**Chapter 2.2.5**). Individual identities of the OTUs matching the database were compared with the list of OTUs differentially enriched in the root hair mutant investigation to identify OTU members in the bacterial collection. Generation of a phylogenetic tree was conducted by concatenating and submitting the FASTA files of isolates to the SILVA 132 Alignment, Classification and Tree Service (Pruesse et al., 2012). The resulting phylogenetic tree was annotated with iTOL (Letunic and Bork, 2007).

5.2.7. Next generation sequencing

*Next generation sequencing was conducted as a service using the "standard service" by Microbes NG (*http://www.microbesng.uk*) which is supported by the BBSRC (*grant number BB/L024209/1*). This is briefly described below*

Three beads were washed with extraction buffer containing lysozyme (or

lysostaphin for *Staphylococcus sp.*) and RNase A, incubated for 25 min at 37 ˚C. Proteinase K and RNase A were added and incubated for 5 min at 65 ˚C. Genomic DNA was purified using an equal volume of SPRI beads and resuspended in EB buffer. DNA was quantified in triplicate with the Quantit dsDNA HS assay in an Ependorff AF2200 plate reader. Genomic DNA libraries were prepared using Nextera XT Library Prep Kit (Illumina, San Diego, USA) following the manufacturer's protocol with the following modifications: two nanograms of DNA instead of one were used as input, and PCR elongation time was increased to 1 min from 30 seconds.

DNA quantification and library preparation were carried out on a Hamilton Microlab STAR automated liquid handling system. Pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for Illumina on a Roche light cycler 96 qPCR machine. Libraries were sequenced on the Illumina HiSeq using a 250bp paired end protocol. Reads were adapter trimmed using Trimmomatic 0.30 with a sliding window quality cut off of Q15 (Bolger et al., 2014). De novo assembly was performed on samples using SPAdes version 3.7 (Bankevich et al., 2012), and contigs were annotated using Prokka 1.11(Seemann, 2014).

5.2.8. Taxonomy, gene and protein prediction

Although provisional taxonomic and gene identification was conducted by MicrobesNG (**Chapter 5.2.7**) this data was analysed in further detail. The taxonomy of isolates was confirmed using Amphora (http://pitgroup.org/amphoranet/) (Kerepesi et al., 2015) and based on this 53 isolates were taken forward for genetic analysis based on taxonomic confidence. Genetic heat maps and genomic class subsetting was conducted using RAST (Rapid Annotation using Subsystem Technology) (Aziz et al., 2008, Overbeek et al., 2014, Brettin et al., 2015).

Using an established pipeline for genomic analysis developed by Jamie Orr (James Hutton Institute, Invergowrie, Dundee, UK) genes were predicted from each isolate using Prokka (v1.13) (Seemann, 2014). To compare only components of characterised metabolic pathways, predicted genes from all isolates were concatenated and annotated with eggNOG-mapper (v1.0.3) (Huerta-Cepas et al., 2018, Huerta-Cepas et al., 2017). The resultant annotation

file was parsed in python to generate a table of taxonomic ID to Kyoto Encyclopaedia of Genes and Genomes (KEGG) ortholog (KO) identifier. From this table a presence-absence matrix of all KOs predicted at least once in each isolate was generated in R. This matrix was used to compute a principal component analysis (PCA) of KOs.

To account for variation in KOs which resulted from isolates using different KOs for the same metabolic reaction, a similar matrix of reaction identifiers was generated from eggNOG annotations in python and R as above and plotted in a PCA.To compare all predicted proteins between isolates, predicted proteomes were clustered using OrthoFinder (v2.2.1) (Emms and Kelly, 2015), and functionally annotated using InterProScan (v5.29-68.0) (Jones et al., 2014). Clusters and annotations were aggregated using KinFin (v1.0) (Laetsch and Blaxter, 2017). Cluster and KO intersections were plotted using UpSetR (v1.3.3) (Conway et al., 2017).

5.3. RESULTS

5.3.1. A taxonomically-diverse bacterial collection of the barley microbiota.

Analysis of Sanger sequence data using Greengenes (**Chapter 5.2.6.**) resulted in broad taxonomic classification and allocation of OTU identities to all but 10 of the isolates sequenced **Table 5.3.** This allowed comparisons to be made between the bacterial isolate assigned OTU identities and the root hair mutant rhizosphere (**Chapter 2**), exudate (**Chapter 3**) and density (**Chapter 4**) investigations.

Of the isolates successfully assigned to an OTU, 63 appeared in at least one of the studies conducted and 28 were present in all 3. Remarkably 22 isolates could not be linked to any of the previous studies despite successful OTU allocation to exactly half of them and the fact that they had been isolated from a replicate environment of the root hair mutant study. For example, although isolate bi120 was isolated from the rhizosphere of Dema barley in Quarryfield soil, its assigned OTU did not feature in any rhizosphere or bulk sample from Quarryfield soil in the root hair mutant study.

Table 5.3 Provisional taxonomic assignment of isolates based on OTU identities generated from Sanger sequencing reads. *Taxonomy indicates the identification of each isolate at the highest taxonomic resolution available based on OTU assignment and the presence or absence of that OTU in previous studies. "RHM" refers to the root hair mutant rhizosphere (chapter 2), "EXU" refers to the exudate study (chapter 3) and "DEN" refers to the density study (chapter 4). Isolates highlighted in green depict OTUs enriched in the indicated study. Colourless indicates present in the dataset indicated but not significantly enriched or depleted. OTUs identified in the exudate study are marked as present only as no enrichment comparisons could be made (chapter 3) (m) indicates enrichment in mutant samples, (wt) indicates enrichment in wild type samples (Chapter 2), (d) indicates depleted in 1.3 g cm3 soil (chapter 4).*

Sample ID	Assigned OTU	Sanger Taxonomy	Isolation environment		OTU present		
			Soil	media	RHM	EXU	DEN
bi43	4318815	Arthrobacter	Rhl	nut	Yes (m)	yes	Yes
bi97	4401816	Arthrobacter	Bulk	nut	Yes (m)	yes	Yes
bi120	204714	Arthrobacter	Dema	r2a			
bi41	4456886	Bacillus	Rhl	nut		yes	Yes
bi58	4456886	Bacillus	Bulk	r2a		yes	Yes
bi63	4456886	Bacillus	Rhl	r2a		yes	Yes
bi80	4456886	Bacillus	Bulk	r2a		yes	Yes
bi84	4456886	Bacillus	Bulk	nut		yes	Yes
bi86	4365717	Bacillus	Rhl	nut	yes		Yes
bi96	4456886	Bacillus	Bulk	nut		yes	Yes
bi132	4483006	Bacillus	Bulk	r2a			
bi133	4456886	Bacillus	BulK	r2a		yes	Yes
bi134	4456886	Bacillus	Bulk	r2a		yes	Yes
bi82	4365717	Bacillus	Bulk	nut	yes		Yes
bi02	806092	Calvibacter	Rhl	nut			
bi04	4329518	Chryseobacterium	Dema	nut	Yes (m)	yes	
bi12	4329518	Chryseobacterium	Dema	nut	Yes (m)	yes	
bi104	4329518	Chryseobacterium	Dema	nut	Yes (m)	yes	
bi124	575419	Devosia	Karat	r2a	yes		
bi13	4449851	Enterobacter	Dema	nut	yes	yes	Yes
bi45	4449851	Enterobacter	Rhp	nut	yes	yes	Yes
bi49	238208	Lysinibacillus	Bulk	nut			
bi33		Lysobacter	Karat	r2a			
bi30	100208	Massilia	Bulk	r2a			
bi118	992035	Massilia	Bulk	r2a	Yes (wt)	yes	Yes (d)
bi44	2791042	Microbacterium	Karat	nut		yes	
bi61	634117	Microbacterium	Rhl	r2a			
bi68	2791042	Microbacterium	Rhp	r2a		yes	
bi69	3251963	Microbacterium	Dema	r2a	yes	yes	Yes
bi74	2791042	Microbacterium	Rhp	r2a		yes	
bi87	2791042	Microbacterium	Karat	nut		yes	
bi93	3251963	Microbacterium	Dema	nut	yes	yes	Yes
bi98	3251963	Microbacterium	Rhl	nut	yes	yes	Yes
bi105	3251963	Microbacterium	Dema	r2a	yes	yes	Yes
bi115	2791042	Microbacterium	Rhl	r2a		yes	
bi121	634117	Microbacterium	Dema	r2a			
bi127	3251963	Microbacterium	Rhl	r2a	yes	yes	Yes

Sample ID	Assigned OTU	Sanger Taxonomy	Isolation environment		OTU present		
			Soil	media	RHM	EXU	DEN
bi07	635391	Pedobacter	Rhl	nut	yes		Yes
bi27	1126297	Pedobacter	Rhl	r2a	yes	yes	Yes
bi36	1126297	Pedobacter	Rhl	r2a	yes	yes	Yes
bi78	1126297	Pedobacter	Rhl	r2a	yes	yes	Yes
bi126	1126297	Pedobacter	Rhl	r2a	yes	yes	Yes
bi26	4401816	Pseudarthrobacter	Dema	r2a	Yes (m)	yes	Yes
bi71	4401816	Pseudarthrobacter	Dema	r2a	Yes (m)	yes	Yes
bi06	1109251	Pseudomonas	Dema	nut	Yes (m)	yes	Yes
bi10	4435982	Pseudomonas	Rhl	nut	Yes	yes	
bi11	4435982	Pseudomonas	Dema	nut	Yes	yes	
bi28	4435982	Pseudomonas	Karat	r2a	Yes	yes	
bi38	4435982	Pseudomonas	Rhl	r2a	Yes	yes	
bi39	238388	Pseudomonas	Rhl	r2a			
bi47	4327501	Pseudomonas	Rhp	nut		yes	
bi50	4435982	Pseudomonas	Rhl	nut	Yes	yes	
bi55	1109251	Pseudomonas	Rhp	nut	Yes (m)	yes	Yes
bi64	276867	Pseudomonas	Karat	r2a	Yes (m)	yes	Yes
bi70	4419276	Pseudomonas	Dema	r2a		yes	
bi75	238388	Pseudomonas	Rhp	r2a			
bi89	821562	Pseudomonas	Karat	nut	yes	yes	
bi110	4478861	Pseudomonas	Karat	r2a		yes	
bi111	4478861	Pseudomonas	Karat	r2a		yes	
bi117	4435982	Pseudomonas	Bulk	r2a	Yes	yes	
bi123	1109251	Pseudomonas	Rhp	r2a	Yes (m)	yes	Yes
bi130	1109251	Pseudomonas	Rhl	r2a	Yes (m)	yes	Yes
bi131	4478861	Pseudomonas	Rhl	r2a		yes	
bi05	645742	Rhizobium	Bulk	nut	yes	yes	
bi46	241317	Rhizobium	Rhp	nut			
bi01	645742	Rhizobium	Rhl	nut	yes	yes	
bi08	645742	Rhizobium	Karat	nut	yes	yes	
bi91	4460853	Rhodococcus	Rhp	nut	yes	yes	Yes
bi35	3330580	Stenotrophomonas	Dema	r2a	Yes (m)	yes	Yes
bi72	3330580	Stenotrophomonas	Dema	r2a	Yes (m)	yes	Yes
bi81	3330580	Stenotrophomonas	Rhl	pp	Yes (m)	yes	Yes
bi102	3330580	Stenotrophomonas	Rhp	nut	Yes (m)	yes	Yes
bi122	3330580	Stenotrophomonas	Rhp	r2a	Yes (m)	yes	Yes
bi66	3330580	Stenotrophomonas	Karat	r2a	Yes (m)	yes	Yes
bi106	748483	Yersinia	Dema	r2a			
bi03		Unassigned	Karat	nut			
bi09		Unassigned	Bulk	nut			
bi73		Unassigned	Dema	r2a			
bi76		Unassigned	Rhp	r2a			
bi79		Unassigned	Rhl	r2a			
bi83		Unassigned	Bulk	nut			
bi85		Unassigned	Rhl	nut			

However, 19 of the isolates were identified in the RHM study as being significantly

enriched in the root hair mutant rhizosphere samples versus their wild type counterparts, accounting for just under half of the total OTUs found in the RHM dataset. Conversely, although 37 isolates were present in the density study, only one (992055, *Massilia sp.*) was significantly depleted in high density soil with all other isolates forming part of the insignificantly influenced microbiota observed.

Enrichment could not be identified in the exudate study dataset due to the nature of the sample set in the study. Of the provisionally identified bacteria that were isolated from an environmental replicate of the root hair mutant study, just 59% corresponded with the root hair mutant microbiota study, the others being unassigned or featuring in a soil free (exudates) or different soil (density) environment. However, all isolates assigned as Flavobacteriales and Sphingomonadales, and all but one assigned as Xanthomonadales were present in the root hair mutant study.

In order to provisionally investigate how related the isolates that had not been identified in previous studies were to those that had been identified, a phylogenetic tree was constructed (**Figure 5.3**). There were several striking observations; the first being that of the 19 isolates belonging to the order Actinomycetales only 4 were enriched in the RHM data and were all assigned to the same clade (*Arthrobacter sp.* / *Pseudoarthrobacter sp.*) with the remaining 14 isolates being assigned almost exclusively to the Microbacterium genus across 3 OTUs, 1 of which (3251963) was detected in all 3 previous datasets and another (2791042) which was present only in the exudate study.

Corroborating the relationships, the isolates assigned to each of these OTUs (3251963 and 2791042) appear in two separate phylogenetic clades together. However, the phylogenetic tree shows that these isolates are unlikely to be the same species despite the taxonomy assigned based on their OTU id, instead it is more likely that they are closely related. Interestingly, isolates belonging to the third OTU belonging to the Microbacterium genus that was not present in any of the previous studies was only separated from the other two Microbacterium OTUs by 2 common ancestors.
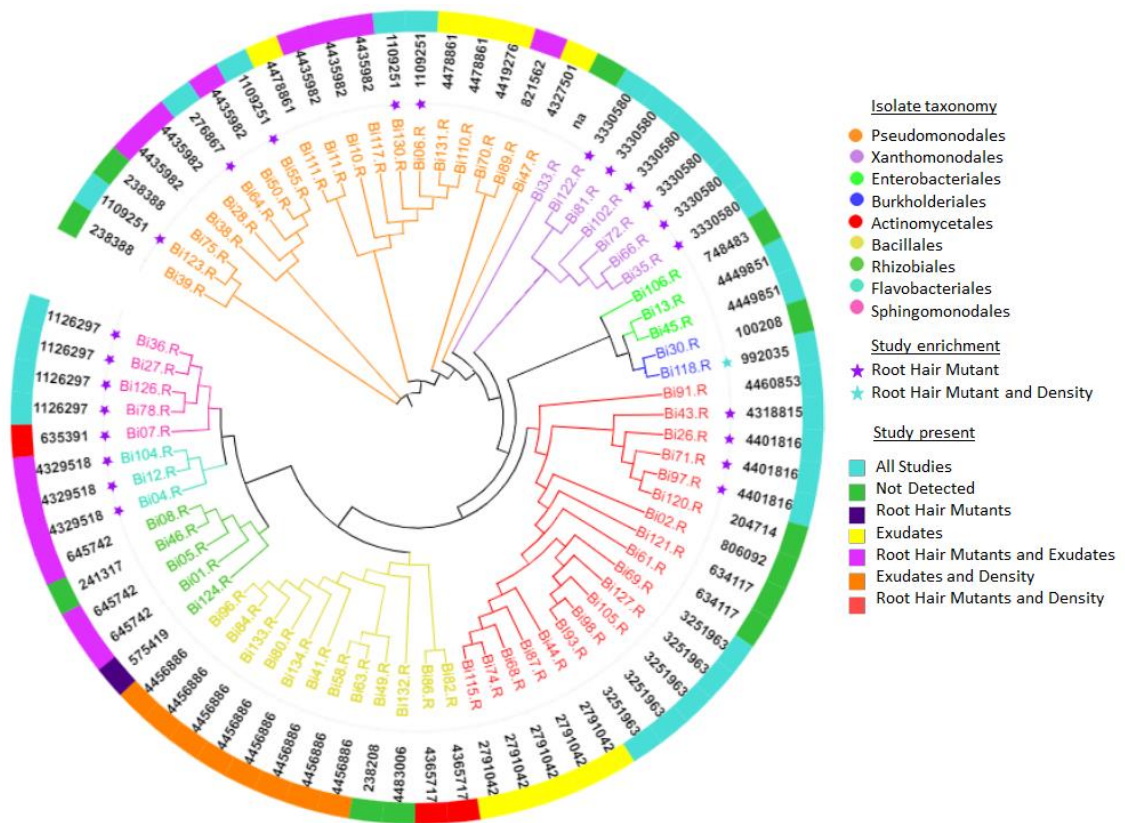
Figure 5.3 Phylogenetic tree constructed based on Sanger sequence reads of 75 bacterial isolates.
Colours on the outer ring depict the study in which the indicated OTU (second ring) was present. Stars represent the study in which the indicated OTU was significantly enriched or depleted. Clades are coloured based on the taxonomic order to which the indicated isolate was assigned based on its OTU allocation.

Conversely, 19 isolates assigned across 8 OTU identities were identified as belonging to the Pseudomonas genus where taxonomic assignment, OTUs and clades did not follow such a clear pattern. Notably, the bacterial isolate bi123, one of four assigned to OTU 1109251 and observed to be present in all previous studies is in a clade shared only by isolates assigned to OTU 238388, which was not detected in any of the same studies.

Several other discrepancies with clade placement in relation to OTU assignment and presence in previous studies were observed. For example, isolates bi11 and bi111 are assigned to different OTUs, 4435982 and 4478861, respectively. Isolates assigned to OTU 4435982 were present in both the RHM and exudate study whilst isolates assigned to OTU 4478861 were not detected in any of the RHM data. These two isolates have been identified as being almost phylogenetically identical. A further discrepancy is the marked scattering of clade

assignment to isolates assigned to the OTU 1109251 which has been identified in all previous datasets. Isolates were distributed between 3 distinct, distantly related clades.

5.3.2. Sequencing taxonomic assignment

Next generation sequencing data provided a much higher taxonomic resolution of the isolated bacterial species and identified 30 isolates that had likely contained contamination or were likely to consist of two or more species. Potentially contaminated or mixed samples were provisionally identified due to abnormally high sequence size, high contig number, low N50 score or mixed GC content peaks in relation to other isolates (**Supplementary Table S1, Figure 5.4**).

The GC content itself was not a factor in determining sequence quality as this can vary from 20-75% depending on the bacterial species to which it belongs; the majority of the clean isolates had a GC content of either 30-40% or 60-70% (**8.4.1. Appendix 4: Supplementary Database 4**) (Sueoka, 1962, Nakabachi et al., 2006).

It is important to consider all of these factors when determining genome annotation quality as one factor on its own may not be sufficient to indicate quality. By comparing genome assemblies with other isolates, samples that could be mistaken as being of sufficient quality for future processing can stand out as abnormal compared to the other isolates (**Figure 5.5**). Although the assembly data offers a strong indicator of sequence quality and purity, taxonomy was investigated to ensure no samples were erroneously removed from the study.

Of the 30 isolates identified as unsuitable to be taken forward for further analysis based on the N50 and GC score of their genome assemblies (**Table 5.1**) all but two were revealed to be assigned to more than one bacterial species. A clear example of this is bi105 which, even at phylum level, shows clearly that the genome of more than one bacterial species has been assembled (**Figure 5.6**). The two isolates that appeared to be clean and belonging to a single species with over 90% certainty were bi86 and bi132, which were assigned to *Bacillus megatarium* and *Bacillus* sp. respectively. However, these were later re-excluded from the data due their assemblies being rejected by RAST due to both having a very high contig number (over 3000) combined with a very low N50 score (under 1000) (**Table 5.3**).
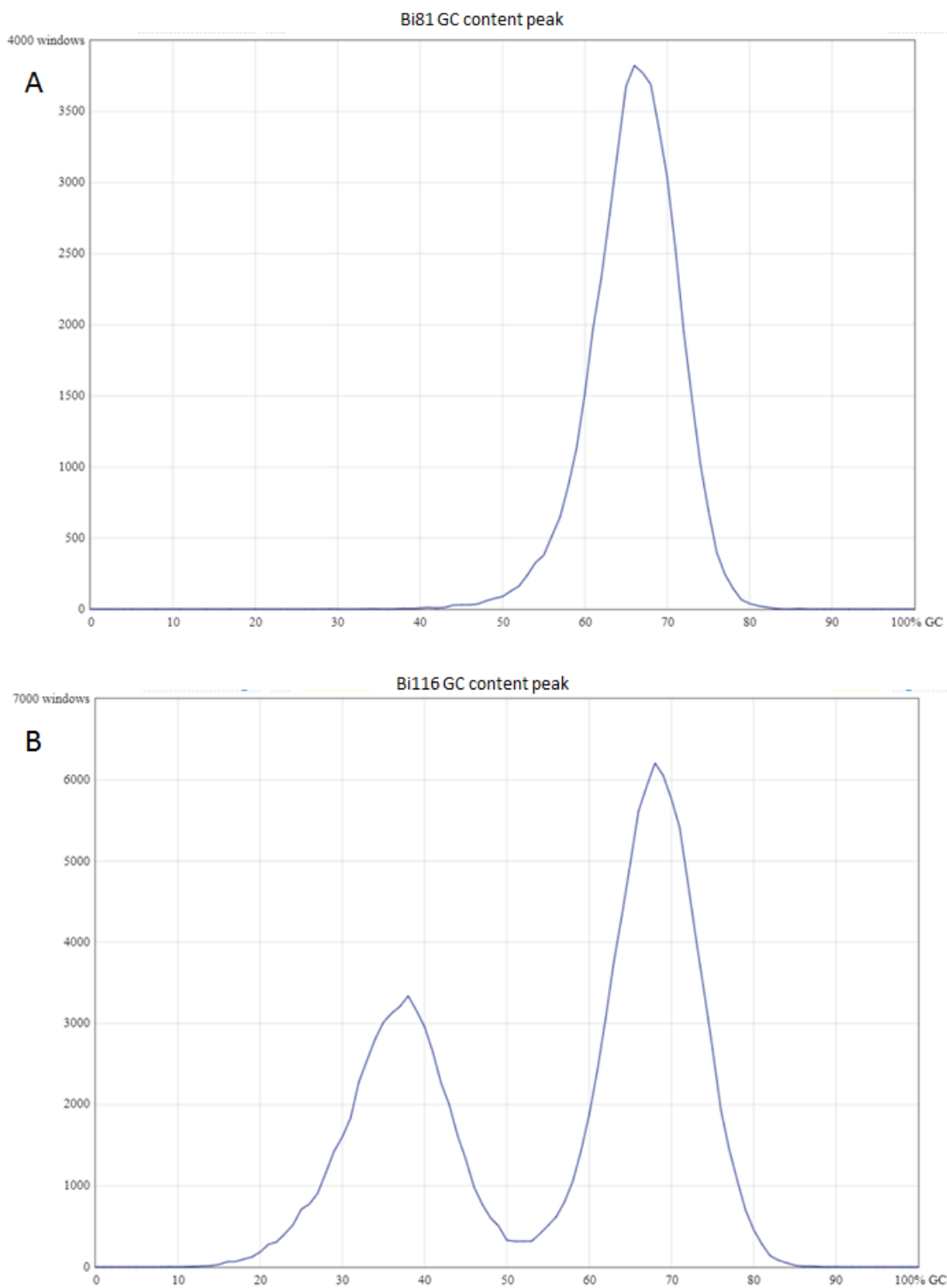
Figure 5.4 Representative GC plots used in the process of quality checking. *(A) is an example of a good quality GC plot which was obtained from isolate bi81. (B) is an example of a GC plot that indicates an impure sample. The two clearly separate peaks indicate that isolate bi116 is composed of two bacterial species, with each peak likely to be the GC content of each individual.*
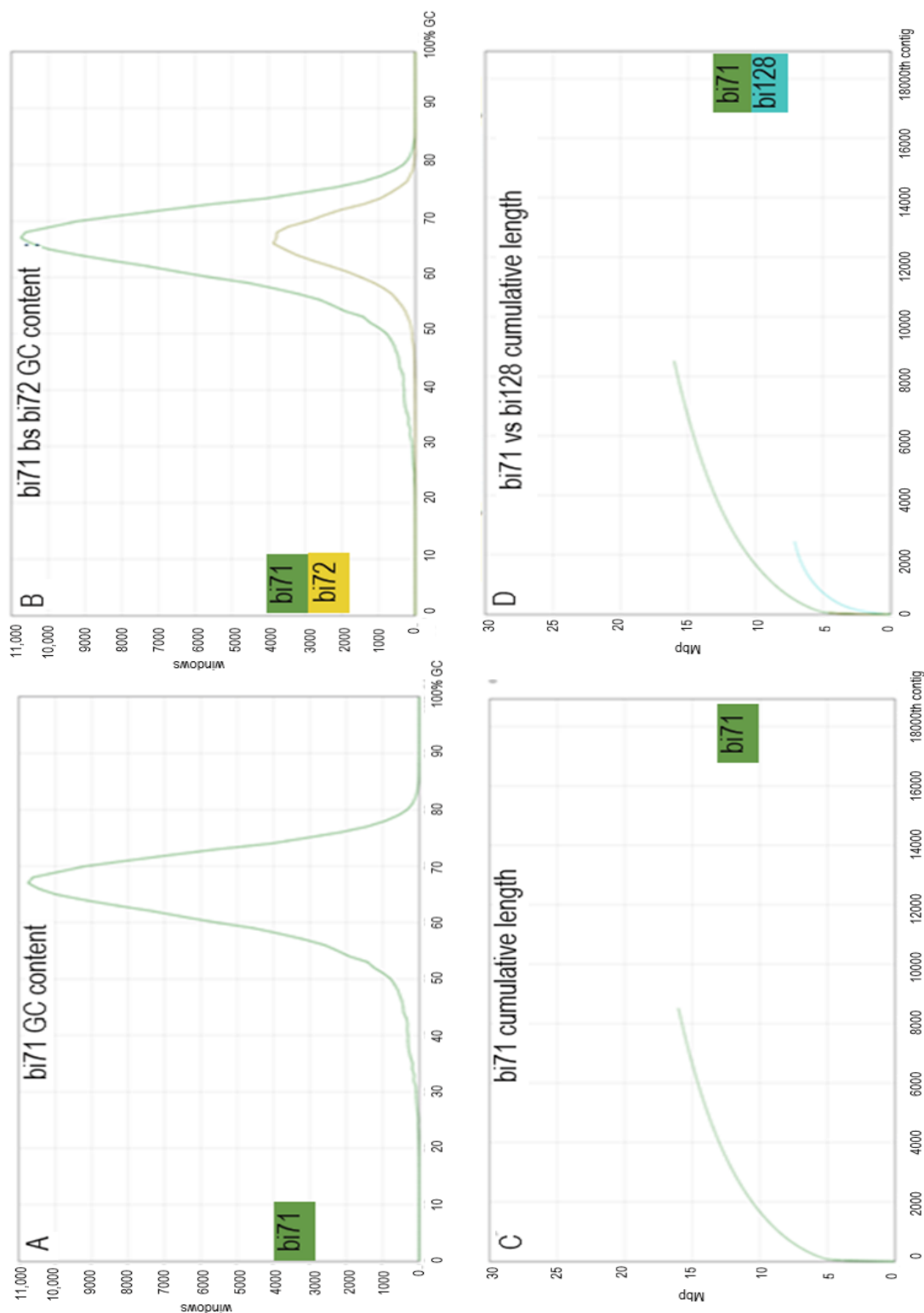
Figure 5.5 Next generation sequence quality filtering by comparative data analysis. *When investigating the quality of genome assemblies, it is important to compare individual genomes. Outputs can appear to be of sufficient quality e.g.(A) shows a single high-quality GC peak, however when compared with bi72 (B) it is clear that a significantly higher number of base pairs are assigned to bi71 than bi72. The same can be observed when comparing cumulative contig length (C), when comparing with another isolate (D) it is clear that the genome assembly of bi71 is abnormally large, indicating that this sample is composed of more than one bacterial species.*

Bi105 Phylum assignment
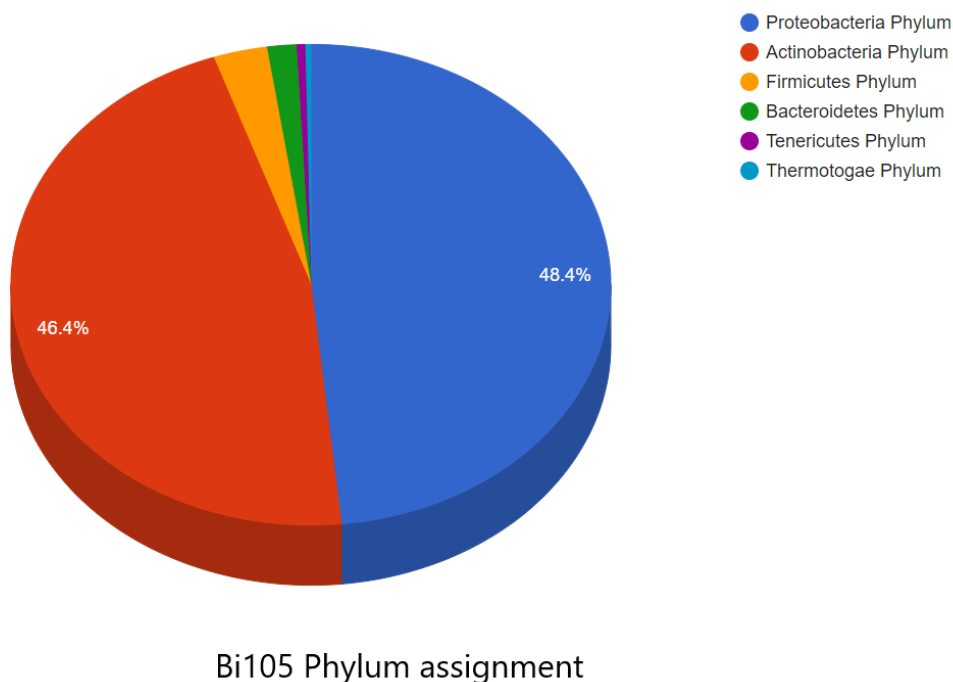
Figure 5.6 Amphora taxonomic assignment based on genome assembly data for isolate bi105*. Genomic assembly indicates that the DNA provided for isolate bi105 was composed of at least two different bacterial species belonging to the proteobacteria and actinobacteria phylum.*

After assembly quality filtering, 53 isolates remained to be taken forward for detailed genomic analysis. Interestingly, when comparing the high-resolution taxonomy assigned following a detailed Amphora analysis with the original, lower resolution, OTU based taxonomic assignment, the majority of the isolates that had been identified to genus level using Greengenes OTU assignment corresponded with taxonomic data gathered from Amphora. (**Table 5.4**)

Nevertheless, there were some clear discrepancies. For example, OTU 645742 was assigned to 3 isolates, predicting that they were members of the rhizobium genus. At a higher taxonomic resolution two of these isolates were identified as Agrobacterium H13-3, the terms Agrobacterium and Rhizobium are often used interchangeably so this corresponds with the lower resolution data.

One isolate, bi08, was predicted to be *Pseudomonas fluorescens PfO-1*, a completely different organism. A second OTU, 241317 initially assigned to Rhizobium was also assigned to *Agrobacterium h13-3*. Likewise, isolate bi118 was assigned as Massilia using OTU data, but was identified at higher resolution as *Janthinobacter marseille*. Although both belong to the Oxalobacteria family they are separate genera and as such may feature differing properties of interest.

Table 5.4 Higher resolution taxonomic assignment of isolates selected for further study. *OTU taxonomic assignment is based on the OTU ID allocated to the 16S rRNA sequence data assigned to the indicated bacterial isolate using the Greengenes data base. Amphora taxonomic assignment is based on the full genomic alignment of the indicated isolates.*

Sample ID	OTU ID	OTU Taxonomic assignment	Amphora taxonomic assignment
bi01	645742	Rhizobium	*Agrobacterium H13-3* A
bi02	806092	Clavibacter	*Microbacteriaceae* sp
bi03			*Microbacterium testaceum B*
bi04	4329518	Chryseobacterium	*Flavobacteriaceae bacterium 3519-10*
bi05	645742	Rhizobium	*Agrobacterium H13-3* A
bi06	1109251	Pseudomonas	*Pseudomonas brassicacearum* A
bi08	645742	Rhizobium	cn *Pseudomonas fluorescens PfO-1* A
bi10	4435982	Pseudomonas	cn *Pseudomonas fluorescens PfO-1* B
bi11	4435982	Pseudomonas	*Pseudomonas brassicacearum* B
bi12	4329518	Chryseobacterium	*Flavobacteriaceae bacterium 3519-10*
bi13	4449851	Enterobacter	*Enterobacter* sp
bi26	4401816	Pseudoarthrobacter	*Arthrobacter sp. FB24*
bi27	1126297	Pedobacter	*Pedobacter heparinus*
bi28	4435982	Pseudomonas	cn *Pseudomonas fluorescens PfO-1* B
bi35	3330580	Stenotrophomonas	*Stenotrophomonas maltophilia*
bi36	1126297	Pedobacter	*Pedobacter heparinus*
bi38	4435982	Pseudomonas	cn *Pseudomonas fluorescens PfO-1* B
bi44	2791042	Microbacterium	*Microbacterium testaceum* C
bi45	4449851	Enterobacter	*Enterobacter sp*
bi46	241317	Rhizobium	*Agrobacterium H13-3* B
bi55	1109251	Pseudomonas	*Pseudomonas brassicacearum* A
bi64	276867	Pseudomonas	cn *Pseudomonas fluorescens PfO-1* C
bi66	3330580	Stenotrophomonas	*Stenotrophomonas maltophilia*
bi70	4419276	Pseudomonas	*Pseudomonas brassicacearum* C
bi72	3330580	Stenotrophomonas	*Stenotrophomonas maltophilia*
bi75	238388	Pseudomonas	*Pseudomonas brassicacearum* D
bi76			*Microbacterium testaceum* D
bi80	4456886	Bacillus	*Bacillus* sp
bi81	3330580	Stenotrophomonas	*Stenotrophomonas maltophilia*
bi82	4365717	Bacillus	*Bacillus megaterium*
bi83			*Arthrobacter* sp
bi84	4456886	Bacillus	*Bacillus* sp
bi85			*Microbacterium testaceum* A
bi89	821562	Pseudomonas	cn *Pseudomonas fluorescens PfO-1* D

Sample ID	OTU ID	OTU Taxonomic assignment	Amphora taxonomic assignment
bi91	4460853	Rhodococcus	cn *Rhodococcus jostii RHA1*
bi96	4456886	Bacillus	*Bacillus megaterium* B
bi98	3251963	Microbacterium	*Microbacterium testaceum* E
bi102	3330580	Stenotrophomonas	*Stenotrophomonas maltophilia*
bi106	748483	Yersinia	*Rahnella sp. Y9602*
bi110	4478861	Pseudomonas	cn *Pseudomonas aeruginosa* B
bi111	4478861	Pseudomonas	cn *Pseudomonas aeruginosa* B
bi112			cn *Pseudomonas aeruginosa* A
bi115	2791042	Microbacterium	*Microbacterium testaceum* C
bi118	992035	Massilia	cn *Janthinobacterium marseille*
bi121	634117	Microbacterium	*Microbacterium* sp
bi122	3330580	Stenotrophomonas	*Stenotrophomonas maltophilia*
bi123	1109251	Pseudomonas	*Pseudomonas brassicacearum* A
bi126	1126297	Pedobacter	*Pedobacter heparinus*
bi128			cn *Marine actinobacterium PHSC20C1*
bi130	1109251	Pseudomonas	*Pseudomonas brassicacearum* A
bi131	4478861	Pseudomonas	cn *Pseudomonas aeruginosa* B
bi133	4456886	Bacillus	*Bacillus* sp
bi134	4456886	Bacillus	*Bacillus* sp

Another discrepancy was due to the use of dated vs current taxonomic nomenclature for example, Chryseobacterium is described as Flavobacteria (Booth, 2014). It is likely that this is the result of minor sequencing discrepancies or that the highly variable 16S region of these species is quite similar but using the full genomic sequence data allowed more accurate taxonomic assignment. Interestingly, many isolates assigned to the same higher taxonomic level had been assigned different OTU identities.

For example, the 7 *Pseudomonas brassicacearum* isolates were assigned to 4 different OTUs; 4 to 1109251, then one each to 4435982, 4419276 and 238388, respectively. The same was observed with *Microbacterium testaceum*: 6 isolates were assigned to 2 OTUs plus 3 had no assigned OTU during the provisional taxonomic assignment. This was particularly remarkable as the different OTUs assigned to these individual isolate species were not all present at a detectable level in the same studies. For instance, the *Pseudomonas brassicacearum* species assigned to OTU 4435982 was detected in both the root hair mutant study and the exudate study, but was not present in the density study, conversely the *Pseudomonas brassicacearum* isolate assigned to OTU 238388 was not detected in any of the previous studies. With this in mind, it was necessary to

investigate the phylogeny of these species to unravel why this phenomenon was being observed and if there was a genetic functional basis for it.

5.3.3 Comparative genetic analysis of Bacterial isolates

Using the table of taxonomic ID to Kyoto Encyclopaedia of Genes and Genomes (KEGG) ortholog (KO) identifier, generated as described in **5.2.8** 6,429 unique KOs were predicted across all isolates. A PCA was generated based on the presence or absence of each KO in each isolate, the isolate taxonomy and the environment(s) in which the originally assigned OTU had been detected. The overarching observation was a clear species-based segregation accounting for both the primary and secondary differentiation values between isolates (28.86% and 12.55%, respectively) and the environmental effect, if any, could not be ascertained (**Figure 5.7**).

When examining the KOs that overlapped between isolates it was observed that there was a group of 514 KOs that were conserved between all isolates (**Figure 5.8**). In the case of Microbacterium and Stenotrophomonas groups, all isolates assigned to each group shared the same KOs based on this analysis. However, this same observation did not extend to isolates assigned to the Pseudomonas and Bacillus groups: Bacillus samples had 240 gene intersections shared only between themselves. In addition in Pseudomonas, where 4 isolates share 24 KOs that did not featured in any other sample (within or outwith Pseudomonas), which were assigned to 3 different *P. brassicacearum* OTUs (bi75, bi06, bi11, bi55). A further *P. brassicacearum* isolate, bi70, assigned to yet another OTU, had its own set of 24 unique KEGG orthologs versus the other isolates.
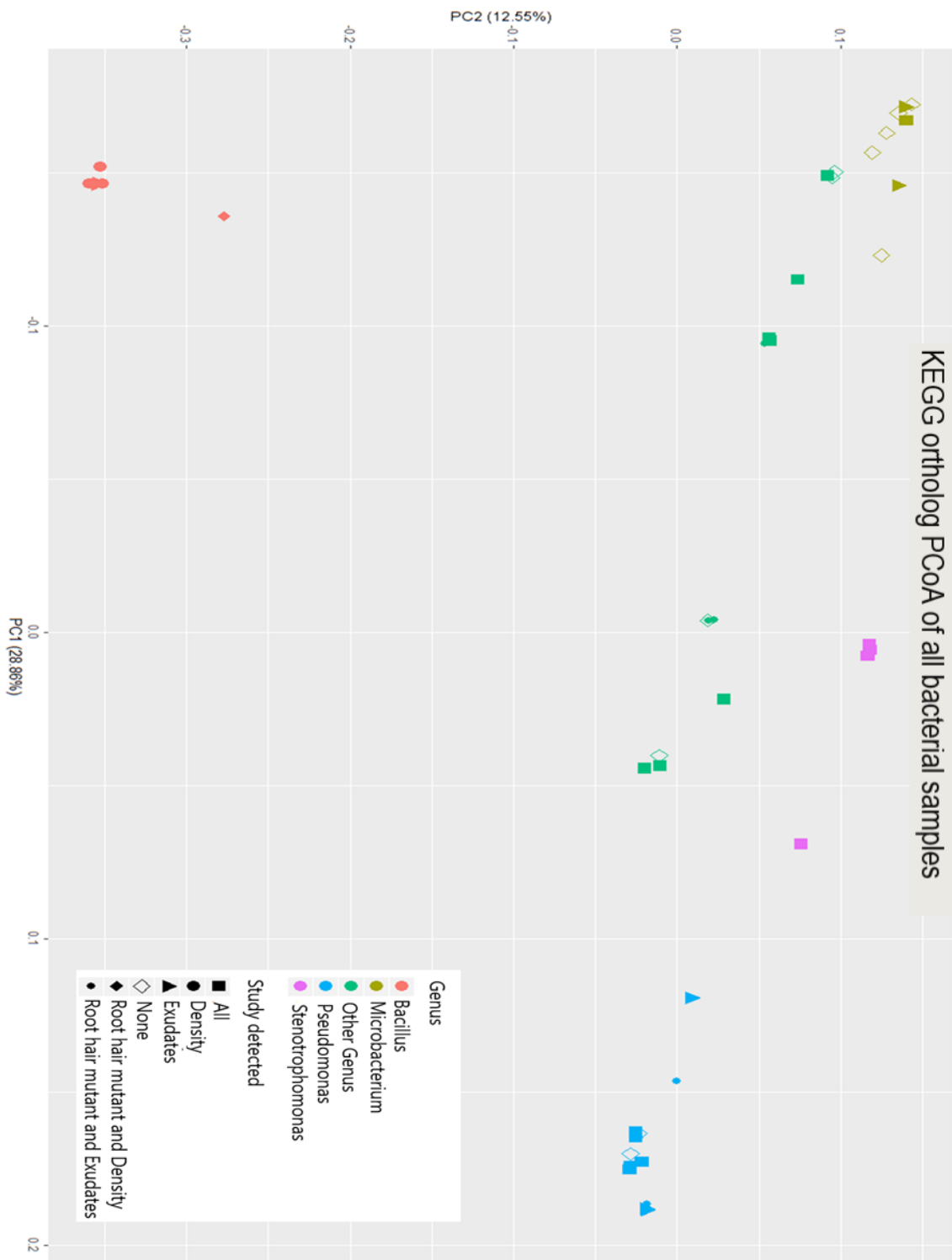
Figure 5.7 PCoA of KEGG orthologs assigned to each bacterial isolate. *Each shape denotes the study each isolate was identified in based on OTU assignment from 16S sequence data. Colours represent the genus assigned to each isolate based on Amphora analysis of full genome sequence data. The proximity of each individual isolate to another reflects the relatedness of each isolate based on the presence or absence of KEGG orthologs.*
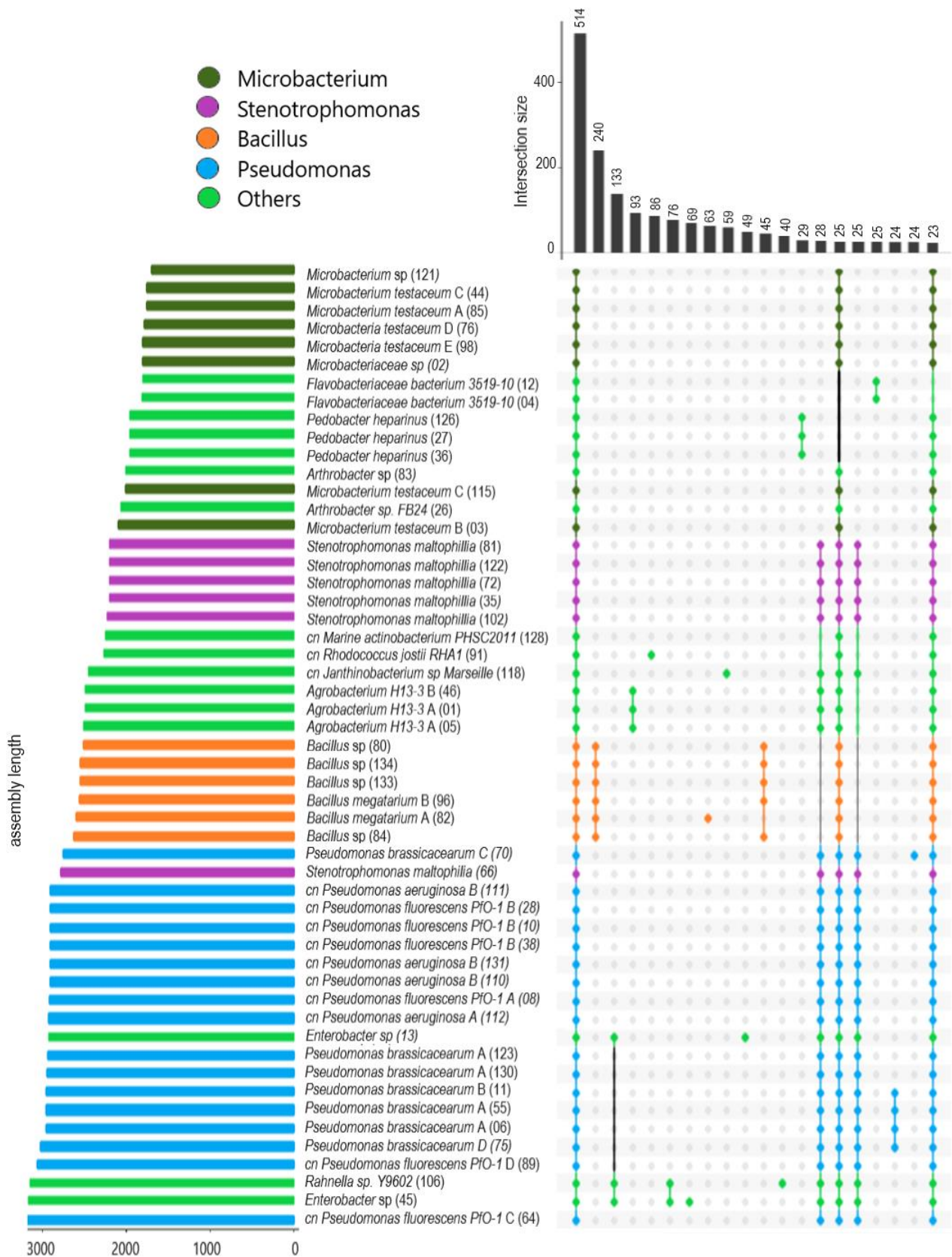
Figure 5.8 KEGG ortholog intersection map of all isolates. *Colours depict the assigned genus of each isolate based on Amphora taxonomic assignment from full genome sequence reads. Intersection size denotes the number of KEGG orthologs shared by the isolates indicated by the corresponding circle below the intersection. The number in brackets next to the isolate description denotes the isolate ID and the length of the coloured bar next to each isolate represents the length of the sequence assembly of the individual sample.*

5.3.4. Gene ortholog variability between and within Pseudomonas spp.

With the purpose of investigation into the genetic drivers of differentiation between and within species and to attempt to discover any environmental effect on this, the isolates were split into 5 analytical groups based on taxonomic assignment; Bacillus, Microbacteria, Pseudomonas, Stenotrophomonas and "Others" which consisted of the remaining isolates that did not correspond with the aforementioned groups.

Principal components analysis and KEGG ortholog overlaps were recalculated for each group and a repeat of the PCA and KO overlap analysis was conducted. Although this was performed for all groups, only the data for Pseudomonas is presented going forward due to the degree of gene ortholog differences observed within the species. Analysis of Bacillus, Microbacteria, Stenotrophomonas and "Others" is detailed in **8.4.1.Appendix 4: Dataset 4**, **8.4.2, Appendix 5 Supplementary Database 5** and **8.4.3, Appendix 6: Figures S2 – S12.**

Principle coordinates analysis between Pseudomonas isolates revealed three levels of diversity between *Pseudomonas brassicacearum* species that could not be explained by OTU assignment alone (**Figure 5.9**). Specifically, 4 *P.brassicacearum* assigned to a single OTU "A" were clearly segregating from each other based on the relatedness of the presence or absence of KEGG orthologs on both the primary and secondary terms of comparison, with one pair being grouped at the highest point of both terms of comparison (bi06, bi55) and the second pair being grouped at -0.2 of both terms. Likewise, two further *P. brassicacearum* assigned to unique OTUs "B" and "D" were closely related to the *P. brassicacearum* OTU "A" pair grouped at the extreme terms of comparison indicating far more relatedness based on functional gene assignment (bi130, bi123) than the other two "A" isolates. Furthermore *P. brassicacearum* assigned to OTU "C" was highly divergent from all other isolates featuring at -0.75 in the secondary term of comparison with no related isolates. A similar but less extreme distribution was observed between isolates assigned to *Pseudomonas flourescens* and *Pseudomonas aurgenosa* with noteable segregation within species along the primary term of comparison. However, although a clear deviation was observed, this corresponded with OTU assignment, where those assigned to a particular OTU group being grouped together.
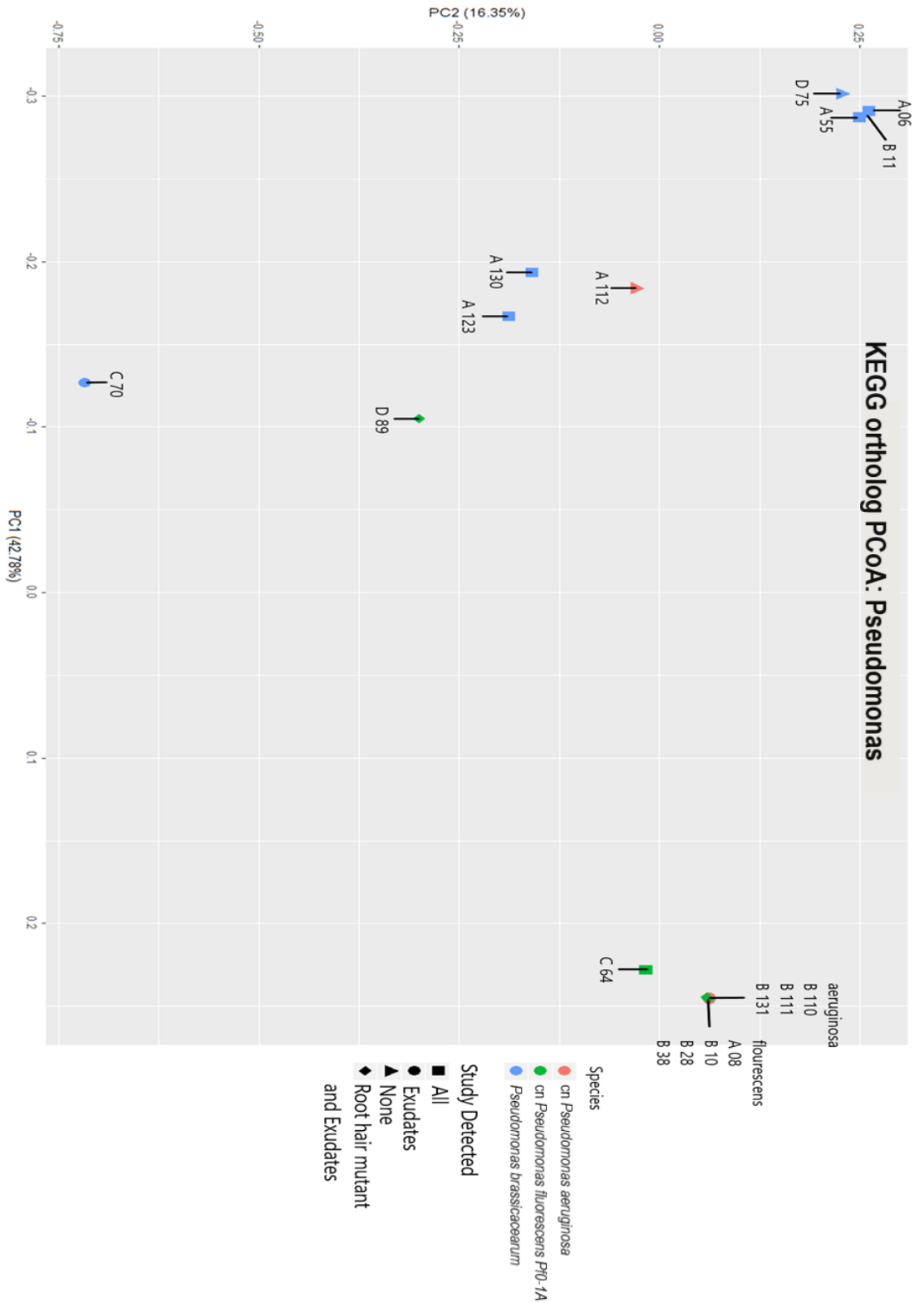
Figure 5.9 PCoA of KEGG orthologs assigned to each Pseudomonas isolate. *Each shape denotes the study each isolate was identified in based on OTU assignment from 16S sequence data. Colours represent the species assigned to each isolate based on Amphora analysis of full genome sequence data. The proximity of each individual isolate to another reflects the relatedness of each isolate based on the presence/absence and similarity of KEGG orthologs.*

In order to elucidate the genetic basis of within species segregation in the Pseudomonas group, the KEGG ortholog intersections were investigated (**Figure 5.10**). The basis for the segregation observed between *P. brassicacearum* "A" was clear; bi130 and bi123 shared 21 KOs that were not detected in bi06 and bi55, likewise the latter pair shared 84 KOs undetected in the isolates initally identified as being the same based on OTU assignment. Interestingly, the *P. brassicacearum* assigned to OTU groups "B" and "D" were identical to the OTU group "A" isolates that they were grouped with during pairwise comparison. An interesting observation was that among the gene orthologs identifed as being present only in *P. brassicacearum* isolates bi06, bi55 (groupA), bi11(groupB) and bi75 (groupD) compared to bi123 and bi130 (groupA) were a selection of genes encoding for phosphate binding DING proteins (**8.4.2. Appendix 5: Supplementary Database 5**).

Notably, over 170 KEGG orthologs were unique to *P. brassicacearum* "C" compared to the other *P. brassicacearum* assigned isolates. However, this degree of difference could be more indicative of assembly quality as opposed to such a strong species divergence. The same proposition could be made regarding P. brassicacearum "D" being the only sample not to share an intersection of 19 orthologs that were assigned to all other Pseudomonas samples.

The ortholog groups identified as being uniquely present in *P. brassicacearum* "C" were primarily assigned to sulphur metabolism and iron acquisition which were represented by a different KO with a highly similar functional assignment in the other *P. brassicacearum* isolates (**8.4.1 Appendix 4: Supplementary Database 4**). However, KEGG orthologs identified as being involved in alkanesulfonate assimilation and pyruvate metabolism in *P. brassicacearum* "C" were observed in all but two of the *Pseudomonas fluorescens PfO-1* isolates (**8.4.1 Appendix 4 Supplementary Database 4**) despite being absent in all other *P. brassicacearum* species. When examining the diversity within isolates assigned to *Pseudomonas fluorescens* those that had been assigned to OTU group "A" and "B" were completely conserved with regards to KO assignment and the isolate belonging to group "C" (bi64) was highly similar with the exception of 142 KEGG orthologs, which were mainly assigned to polyamine metabolism genes (**8.4.1 Appendix 4 Supplementary Database 4**). Remarkably, 30 of these

KOs which were only assigned to bi64 in the *P. fluorescens* species grouping were conserved in the *P. brassicacearum* isolate bi70 (*P. brassicacearum* group "C") that was most divergent from the other isolates assigned to that species.
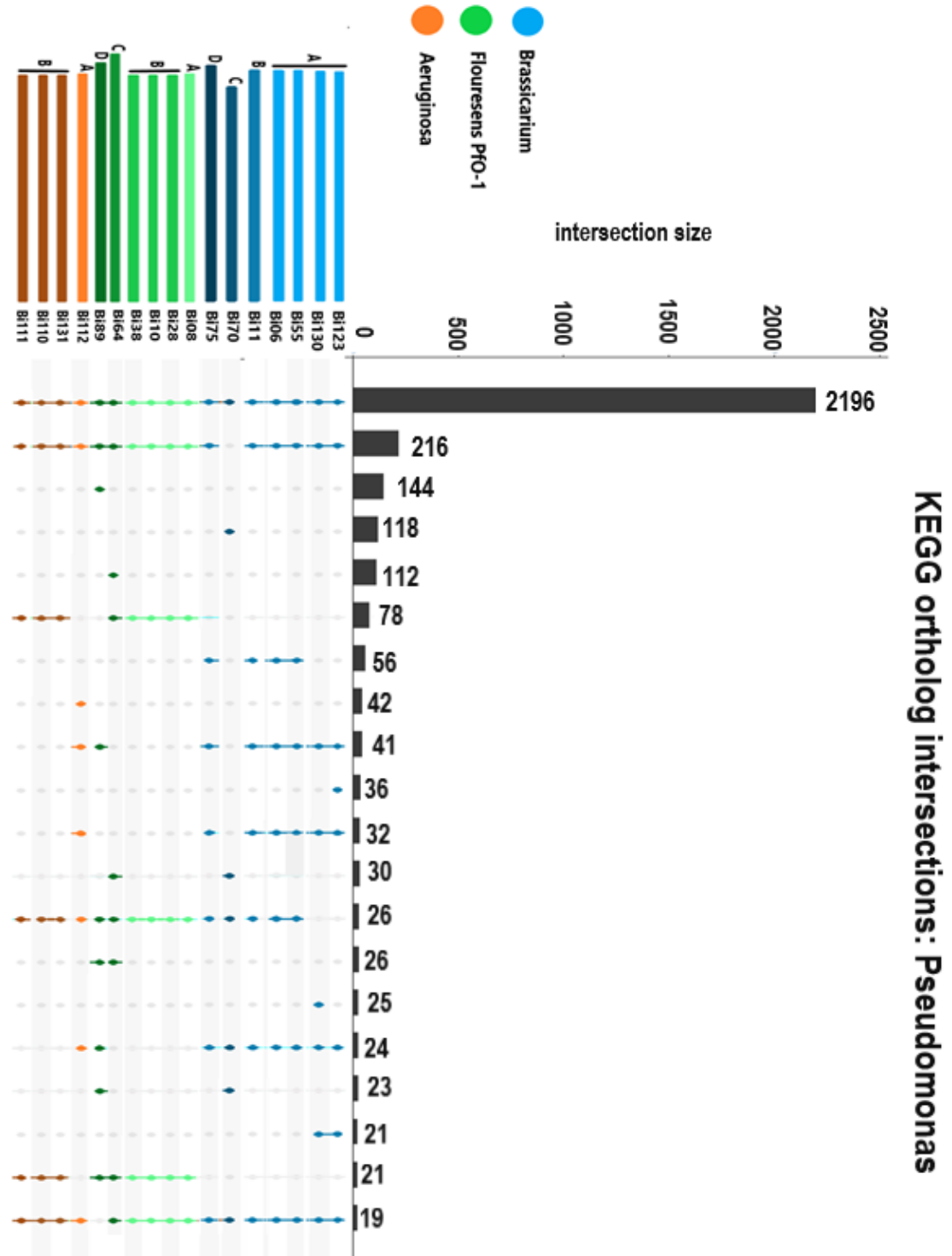
Figure 5.10 KEGG ortholog intersection map of Pseudomonas isolates. *Colours depict the assigned species of each isolate based on Amphora taxonomic assignment from full genome sequence reads. Intersection size denotes the number of KEGG orthologs shared by the isolates indicated by the corresponding circle below the intersection. The length of the coloured bar next to each isolate represents the length of the sequence assembly.*

These 30 conserved genes were mainly assigned to lactose utilization and zinc resistance (**8.4.1 Appendix 4 Supplementary Database 4**). When examining the genetic variance that separated *P. fluorescens* isolate bi89, assigned to OTU group "D", from the other 5 *P. fluorescens* isolates, over 200 KOs were identified as unique when they were compared. Interestingly several were identified as being genes involved in the formation of the type IV pillus, flagellum formation and phosphate binding DING proteins.

Similar, to the *P. fluorescens* isolate assigned to OTU group "C", over 60 of these KEGG orthologous were conserved in isolates assigned to the *P. brassicacearum* species and were mainly assigned to type IV pillus and flagellum formation (**Table 5.5**). Finally, the functional diversity between isolates assigned to *Pseudomonas aeruginosa* was examined. There were 145 KEGG orthologs that were differentially assigned between isolates assigned to OTU group "A" and "B", respectively. Specifically, the *P. aeruginosa* isolates bi110, bi111 and bi132 assigned to group B based on OTU identity had genes identified as orthologous to coenzyme b12 biosynthesis, alkanesulfonate assimilation and inorganic Sulphur assimilation, none of which were detected in *P. aeruginosa* isolate bi112 which was assigned to OTU group A. Notably the gene orthologs assigned to coenzyme B12 biosynthesis were also undetected in the *P. brassicacearum* isolates bi123 and bi130 (identified as diverging from isolates assigned to the same species and OTU group (A)) plus *P. brassicacearum* isolate bi70 (OTU group C). Importantly, 2,196 KOs were conserved across all *Pseudomonas* species which were assigned to a range of functions/ proteins including, but not limited to amino acids and derivatives, carbohydrate metabolism, the TCA cycle, DNA metabolism and repair, respiration, phosphate metabolism and membrane transport. It is likely that these are primarily *Pseudomonas* housekeeping genes. A further 235 orthologs were conserved between all but one *Pseudomonas* isolate (bi70); however, it is likely that these are also essential housekeeping genes and may not have been detected in isolate bi70 due to a sequencing or assembly anomaly. Despite examining KO based inter and intra species relatedness at a higher resolution (genus groups) there were no obvious, distinct relationships between the study environments in which the isolates had been detected (based on OTUs assigned to the isolates based on 16S sequence data) and the presence or absence of gene orthologs.

Table 5.5 Genes detected in Pseudomonas fluorescens PfO-1 (OTU group D) vs P.fluorescens and P.brassicacearum isolates. "Subsystem" denotes the subsystem belonging KEGG orthologs. Numbers represent the number of orthologous genes identified per species described. Colours increase with intensity as the number of copies of an ortholog increases vs its presence in other species.

Genes present in Pseudomonas fluorescens PfO-1 (OTU Grp D) vs flourescens and brassicarium isolates

Subsystem	cn Pseudomonas fluorescens PfO-1 A (0E	cn Pseudomonas fluorescens PfO-1 B (1C	cn Pseudomonas fluorescens PfO-1 B (2E	cn Pseudomonas fluorescens PfO-1 B (3E	cn Pseudomonas fluorescens PfO-1 C (64	cn Pseudomonas fluorescens PfO-1 D (8S	Pseudomonas brassicacearum A (06)	Pseudomonas brassicacearum A (55)	Pseudomonas brassicacearum A (123)	Pseudomonas brassicacearum A (130)	Pseudomonas brassicacearum B (11)	Pseudomonas brassicacearum C (70)	Pseudomonas brassicacearum D (75)
A TRAP transporter and a hypothetical	0	0	0	0	0	3	3	3	0	0	3	3	3
Adenylylsulfate reductase	0	0	0	0	0	2	0	0	0	0	0	0	0
Exopolysaccharide Biosynthesis	0	0	0	0	0	5	0	0	5	5	0	4	0
Flagellum	0	0	0	0	0	24	24	23	24	23	24	5	24
Flagellum in Campylobacter	0	0	0	0	0	5	5	5	5	5	5	5	5
General Secretion Pathway	0	0	0	0	0	11	0	0	0	0	0	0	0
Na(+)-translocating NADH-quinone oxidoreductase and rnf-like group of electron transport complexes	0	0	0	0	0	5	5	5	6	6	5	0	4
Phd-Doc, YdcE-YdcD toxin-antitoxin (programmed cell death) systems	0	0	0	0	0	3	0	0	0	0	0	0	0
Phosphate-binding DING proteins	0	0	0	0	0	8	8	8	0	0	8	0	8
Siderophore Achromobactin	0	0	0	0	0	1	1	1	1	0	1	5	3
Sulfate assimilation related cluster	0	0	0	0	0	8	0	0	0	0	0	0	0
Trans-envelope signaling system VreARI in Pseudomonas	0	0	0	0	0	7	7	7	0	0	7	0	7
tRNA processing	0	0	0	0	0	6	0	0	0	0	0	0	0
tRNA splicing	0	0	0	0	0	3	0	0	0	0	0	3	0
Type IV pilus	0	0	0	0	0	20	21	20	20	20	21	18	20

A cursory analysis was performed to investigate if there were any specific orthologs present or absent in the isolated *Pseudomonas* group in a plant or non-plant dependent basis (present or absent in the root hair mutant rhizosphere study and/ or exudate study versus the soil density study only). This investigation yielded one KO that fell into this category; KO: k21929 was present only in plant populating *Pseudomonas* and undetected in all *Pseudomonas* isolates that had been detected in a non-plant environment based on OTU assignment. However, this is a gene of unknown function and is possibly a sequencing artefact. This observation was not pursued and, instead, analysis of significant presence or absence of KOs, the presence of PGPR genes that may be harnessed for future studies and proteomics of the isolates was examined for all isolates taken forward.

5.3.5. Determination of PGPR functional genes

In order to identify which genes or gene orthologs may be of interest for future research applications, a list of a selection of known PGPR genes was constructed based on the literature (Bruto et al., 2014, Rosier et al., 2018) and the focus on OTU assignment and environmental enrichment was replaced with a focus on genes implicated in phosphate and nitrogen acquisition assistance followed by genes identified in the literature as enhancing nutrient uptake from biological fertilizers. This approach was taken in order to identify bacterial isolates that may have downstream applications e.g. in inoculant trials.

5.3.5.1 Phosphate solubilising genes

Sequences within the bacterial isolates identified as orthologous to genes aiding phosphate acquisition processes in plants were determined using RAST analysis and using KEGG ortholog assignment. RAST analysis revealed the presence of DING proteins in several Pseudomonas isolates (bi06, bi08, bi11, bi55, bi89) assigned to the species *P. fluorescens* and *P. brassicacearum*. These proteins did not appear in isolates assigned to any other genus. In addition to this a further Pseudomonas specific enrichment was revealed, although all Pseudomonas isolates had regions identified as "High affinity phosphate transporter and control of PHO regulon" genes, the isolates bi08, bi10, bi38, bi06 and bi11 had double the number of coding regions compared to the other Pseudomonas isolates.

Remarkably, these observations were not specific to the species of Pseudomonas and did not appear to have any correlation with original OTU assignment as the number of DING orthologs varied between samples assigned to the same OTU (**Table 5.6**). Using KEGG ortholog assignment the presence of pyrroloquinoline quinone (PQQ) genes was investigated. The PQQ operon system plays an important role in phosphate solubilisation; PQQ produced by the PQQ operon in bacteria is required by glucose dehydrogenase as a cofactor in the process of direct oxidation of glucose to produce the organic acid gluconic acid which goes on to solubilize insoluble phosphate compounds (Suleman et al., 2018).

Table 5.6 Enrichment of phosphate binding DING proteins and PHO regulon control genes in Pseudomonas sp. "Subsystem" denotes the subsystem belonging to detected KEGG orthologs. Numbers represent the number of orthologous genes identified per species described. Colours increase with intensity as the number of copies of an ortholog increases vs the number of copies detected in other bacterial samples.

Bacterial Isolate	Subsystem	
	High affinity phosphate transporter and control of PHO regulon	Phosphate-binding DING proteins
cn *Pseudomonas aeruginosa* A (112)	4	0
cn *Pseudomonas aeruginosa* B (110)	4	0
cn *Pseudomonas aeruginosa* B (111)	4	0
cn *Pseudomonas aeruginosa* B (131)	4	0
cn *Pseudomonas fluorescens* PfO-1 A (08)	8	0
cn *Pseudomonas fluorescens* PfO-1 B (10)	8	0
cn *Pseudomonas fluorescens* PfO-1 B (28)	4	0
cn *Pseudomonas fluorescens* PfO-1 B (38)	8	0
cn *Pseudomonas fluorescens* PfO-1 C (64)	4	0
cn *Pseudomonas fluorescens* PfO-1 D (89)	4	8
Pseudomonas brassicacearum A (06)	8	8
Pseudomonas brassicacearum A (55)	4	8
Pseudomonas brassicacearum A (123)	4	0
Pseudomonas brassicacearum A (130)	4	0
Pseudomonas brassicacearum B (11)	8	8
Pseudomonas brassicacearum C (70)	4	0
Pseudomonas brassicacearum D (75)	4	8

Further interrogation of the results showed that a variety of pqq genes belonging to the PQQ operon were present in a number of bacterial isolates with functions ranging from performing roles in the oxidation of 3a-(2- amino-2-carboxyethyl)-4,5-dioxo-4,5,6,7,8,9-hexahydroquinoline- 7,9-dicarboxylic-acid to form PQQ, PQQ transport and PQQ biosynthesis protein coding regions among other functions (**Table 5.7, 8.4.1 Appendix 4 Supplementary Database 4**). Notably, *ppqB, C, D, E, F* and (in 7 isolates) *H* were almost exclusively found together in *Pseudomonas* derived isolates, apart from the bi13, *Enterobacteria* genus, isolate in which *ppqB, C, D, E* and *F* were also detected. Although the majority of isolates did not carry any ppq gene orthologs, in addition to these there were 4 isolates that carried a single *pqq* gene ortholog (*Arthrobacter FB24* (bi26); *pqqF*, *Stenotrophomonas maltophilia* (bi66); *pqqF*, *Pseudomonas aeruginosa* (112); *pqqB* and *Janthinobacterium* (bi118); *pqqL*) and one (*Microbacterium testaceum*

(bi03)) that carried a pair of pqq gene orthologs, *pqqC* and *pqqD*. Interestingly, *pqqE* was not detected in this isolate when it has been demonstrated that *pqq* genes are present in a conserved order and that, occasionally, *pqqCDE* can be fused with *pqqD* being fused to the C-terminus of *pqqC* and/ or the N-terminus of *pqqE* (Barr et al., 2016, Shen et al., 2012).

Table 5.7 Pqq gene orthologs detected in bacterial isolates. *The letter Y indicates that the named pqq gene ortholog has been identified in the corresponding bacterial isolate. Blank cells indicate no pqq gene detected in the data.*

Isolate ID	Species	PQQ gene ortholog						
		pqqB	*pqqC*	*pqqD*	*pqqE*	*pqqF*	*pqqH*	*pqqL*
bi03	*Microbacterium testaceum* B (03)		y	y				
bi06	*Pseudomonas brassicacearum* A (06)	y	y	y	Y	y	y	
bi08	cn *Pseudomonas fluorescens PfO-1* A (08)	y	y	y	Y	y		
bi10	cn *Pseudomonas fluorescens PfO-1* B (10)	y	y	y	Y	y		
bi11	*Pseudomonas brassicacearum* B (11)	y	y	y	Y	y	y	
bi13	*Enterobacter* sp (13)	y	y	y	Y	y		
bi26	*Arthrobacter* sp. *FB24* (26)					y		
bi28	cn *Pseudomonas fluorescens PfO-1* B (28)	y	y	y	y	y		
bi38	cn *Pseudomonas fluorescens PfO-1* B (38)	y	y	y	y	y		
bi55	*Pseudomonas brassicacearum* A (55)	y	y	y	y	y	y	
bi64	cn *Pseudomonas fluorescens PfO-1* C (64)	y	y	y	y	y		
bi66	*Stenotrophomonas maltophilia* (66)					y		
bi70	*Pseudomonas brassicacearum* C (70)	y	y	y	y	y	y	
bi112	cn *Pseudomonas aeruginosa* A (112)	y						
bi118	cn *Janthinobacterium sp. Marseille* (118)							y
bi123	*Pseudomonas brassicacearum* A (123)	y	y	y	y	y	y	
bi130	*Pseudomonas brassicacearum* A (130)	y	y	y	y	y	y	
bi131	cn *Pseudomonas aeruginosa* B (131)	y	y	y	y	y		

	pqq gene proposed function
	Insulinase (Peptidase family M16)
	Prolyl oligopeptidase
	coenzyme PQQ biosynthesis protein PqqF (TIGRFAM)
	Elongator protein 3, MiaB family, Radical SAM
	With pqqC converts a biosynthetic intermediate to pyrroloquinoline quinone
	Ring cyclization and eight-electron oxidation of 3a-(2-amino-2-carboxyethyl)-4,5-dioxo-4,5,6,7,8,9-hexahydroquinoline- 7,9-dicarboxylic-acid to PQQ
	May be involved in the transport of PQQ or its precursor to the periplasm

5.3.5.2 Bacterial isolate samples observed to carry PGPR gene orthologs related to nitrogen acquisition

It is well documented that *nif* genes are associated with the fixation of nitrogen, *nif* genes are of particular interest when present in rhizobia due to the symbiotic relationship they have been documented as forming with nodulating plants (Lagunas et al., 2019). Although barley does not nodulate, the presence of *nif* gene orthologs in the bacterial isolates was examined using KEGG orthologs with a view to identifying potential PGPR properties in isolates that may be useful in other agricultural plant species (**Table 5.8**).

Unsurprisingly, bi46 *Agrobacterium H13-3* was identified as carrying the most nif gene orthologs, *nifALSU*; A*grobacterium* is well described in the literature as having nitrogen fixing capabilities, carrying *nif* genes and inducing nitrogen fixing nodules in legumes (Saha et al., 2017).However, 20 isolates across a variety of genera including *Janthinobacter, Pedobacter* and *Pseudomonas* were observed to have *nifA* orthologs which are commonly considered to indicate that a particular bacterial species is responsive to nitrogen within the soil.

Notably, only one of the bacterial isolates were observed to have a gene ortholog that corresponded to the *nifH* gene which is the biomarker most widely used to study the ecology and evolution of nitrogen-fixing bacteria. There were also no orthologs for *nifD* or *nifK* which encode the two components required to form the nitrogenase enzyme. Combined this indicated that none of the bacterial samples

appear to carry the full gene compliment for the *nif* operon. However, this could be the result of sequence assembly errors so it would be logical to explore the presence of the *nif* operon using a pcr with PolF or PolR primers (Ayyaz et al., 2016)

Table 5.8 Nif gene orthologs detected in bacterial isolates. *Y indicates the presence of the gene identified in the corresponding bacterial isolate*

isolate ID	Species	nif gene ortholog					
		nifA	*nifJ*	*nifL*	*nifS*	*nifU*	*nifH*
bi01	*Agrobacterium H13-3* A (01)	Y			Y	Y	
bi04	*Flavobacteriaceae bacterium 3519-10* (04)	Y				Y	
bi05	*Agrobacterium H13-3* A (05)	Y			Y	Y	
bi06	*Pseudomonas brassicacearum* A (06)	Y					
bi08	cn *Pseudomonas fluorescens PfO-1* A (08)	Y					
bi10	cn *Pseudomonas fluorescens PfO-1* B (10)	Y					
bi11	*Pseudomonas brassicacearum* B (11)	Y					
bi12	*Flavobacteriaceae bacterium 3519-10* (12)	Y				Y	
bi26	*Arthrobacter sp. FB24* (26)						Y
bi27	*Pedobacter heparinus* (27)	Y				Y	
bi28	cn *Pseudomonas fluorescens PfO-1* B (28)	Y					
bi36	*Pedobacter heparinus* (36)	Y				Y	
bi38	cn *Pseudomonas fluorescens PfO-1* B (38)	Y					
bi45	*Enterobacter* sp (45)		Y				
bi46	*Agrobacterium H13-3* B (46)	Y		Y	Y	Y	
bi55	*Pseudomonas brassicacearum* A (55)	Y					
bi64	cn *Pseudomonas fluorescens PfO-1* C (64)	Y				Y	
bi75	*Pseudomonas brassicacearum* D (75)	Y					
bi115	*Microbacterium testaceum* C (115)	Y					
bi126	*Pedobacter heparinus* (126)	Y		Y		Y	
bi130	*Pseudomonas brassicacearum* A (130)	Y					
bi131	cn *Pseudomonas aeruginosa* B (131)	Y					
bi133	*Bacillus* sp (133)				Y	Y	
bi134	*Bacillus* sp (134)				Y	Y	

nif gene proposed function					
	Activates transcription of nitrogen fixation promoters	Oxidoreductase	Nitrogen fixation regulatory protein	Cytosine desulpherase	Nitrogen fixation protein

5.3.5.3 PGPR genes related to enhancing the efficacy of biological fertilizers to enhance plant nutrient uptake.

Hydrogen Cyanide (HCN) channels, encoded by the three genes *hcnABC*, have been associated with having PGPR capabilities as biocontrol agents and more recently as being involved in biogeochemical processes in the soil such as metal chelation, indirectly increasing the availability of inorganic phosphate and enhancing nutrient uptake by plants (Rijavec and Lapanje, 2016, Ahemad and Kibret, 2014, Voisard et al., 1989). With the clear potential of isolates that can produce HCN to contribute to increasing the availability of inorganic phosphate the presence of *hcnABC* gene orthologs was investigated using KO analysis.

Analysis revealed that 7 of the bacterial isolates in our collection carried the full complement of *hcn* genes required for HCN synthesis; interestingly all of the isolates identified as having the potential to synthesise HCN belonged to the Pseudomonas genus, specifically, all *P.brassicacearum* isolates with the exception of bi70 (which has been identified as being notably different from the other *P.brassicacearum* isolates based on OTU assignment and common gene orthologs) and a single *Pseudomonas aeruginosa* isolate (bi112)

5.4 DISCUSSION

5.4.1 Traditional microbiology isolation techniques can be used to identify a diverse selection of culturable members of the barley rhizosphere microbiota

Despite initially obtaining a physiologically diverse range of bacterial isolates from the barley rhizosphere and bulk soil samples potentially representing hundreds of bacterial species, the process of meticulous isolation, amplification and extraction of clean, high concentration DNA and latterly next generation sequence assembly quality exclusion resulted in the final bacterial library consisting of 53 bacterial isolates considered to be of sufficient quality to take forward.

However, this represented 17 bacterial species across 11 genera; many of which have been identified as carrying genes orthologs to known PGPR genes. These bacterial isolates also represented all of the enriched orders described in the studies described in chapters 2, 3 and 4. This demonstrates that although the process of developing a robust bacterial library can result in discarding a high proportion of the original CFUs observed that it does not necessarily negatively impact the range of bacterial species that can be isolated. It is commonly reported in the literature that just 1% of the bacterial microbiota can be successfully cultured from soil but can reach up to 10% in the rhizosphere environment due to the enrichment of particular bacterial species and the nutrient rich environment (Ling et al., 2015, Gurusinghe et al., 2019).

Notwithstanding, this has been contested by the Schulz-Lefert group in collaboration with the Vorholt group, who have provided evidence that these figures are likely to be underestimated. Using *Arabidopsis thaliana* as a model they demonstrated that over 50% of the dominant members of the microbiota could be cultured *in vivo* (Bai et al., 2015). Although, based on the number of unique OTUs identified in the root hair mutant study described in **chapter 2** this initial collection accounts for just a limited fraction of the barley rhizosphere microbiota it does demonstrate the diversity that can be obtained by using traditional microbiology techniques and a small, provisional sample set.

5.4.2 Whole genome sequence quality filtering and isolate selection

In order to identify which bacterial isolates were the best candidates for future analysis and applications it was necessary to apply filters based on several parameters relating to sequence quality. By using GC content analysis as an initial step, it was possible to identify sequence assemblies which are clearly derived from more than one species (**Figure 5.4**). Following this, the more intuitive steps of considering sequence length, N50 and the number of contigs is typically conducted. This filtering process often relies heavily on the overall quality of the entire dataset and the organisms being studied in order to assign parameters as opposed to following set filtering parameter protocols (Ekblom and Wolf, 2014).

An example of this is when considering sequence length. Although bacteria can have a whole genome ranging in length from 130 kbp to around 14 Mbp, it is advisable to calculate the average read length based on the total data set (McCutcheon and von Dohlen, 2011, Han et al., 2013). By performing this small check it allows the identification of assemblies that markedly differ from the average, although this does not necessarily identify an assembly issue it allows the taxonomy assigned to be checked and compared with draft genome data to ensure sequence length correlates with previous studies on that organism. Often, a particularly large sequence length can indicate a contaminated sample that contains the sequence data of more than one bacterial species.

One of the main identifiers of whole genome sequence quality is the N50 score. This score describes the quality of assembled genomes that are composed of contigs of varying lengths, it is commonly described as the minimum contig length needed to cover half of the entire genome; put simply it means that half of the genome sequence is composed of contigs equal or greater in size to the N50 score (Mäkinen et al., 2012). The N50 based quality filtering comes hand in hand with the number of contigs in an assembly; ultimately a high N50 score in combination with a low contig number indicates a more reliable genome assembly which is composed of a lower number of long, continuous reads as opposed to many short reads which may result in assembly misalignment.

Following this filtering process, a further rigour was applied in examining the assigned taxonomy using amphora to ensure accurate and high-quality

taxonomic assignment as described in **Chapter 5.3.3**. Considering this in totality, the isolates taken forward for this study were based on the highest quality genome assemblies in the dataset, however it is important to recognise that there are several studies that have been conducted that are based on more robust sequence data.

Tools such as Quast (QUality ASsesment Tool) are available to thoroughly evaluate genome assemblies computationally. It analyses N50 (the minimum contig length needed to cover 50% of the genome), L50 (the number of contigs whose length is greater or equal to the N50 score), NG50 (where the length of the reference genome is being covered), NA50 and NGA50 (not applicable to this study as it is based on sequence blocks as opposed to contigs), the number of N's per 100 kbp (the number of uncalled bases per 100kbp) and GC%, misassemblies and the number of genes and operons covered. Unfortunately, the duration of this study did not allow this additional step to be conducted but it is undoubtedly a tool which would be beneficial for larger, future studies and would provide a higher level of confidence during quality filtering.

5.4.3 Taxonomic assignment of bacterial isolates

When comparing the high-resolution taxonomy with the OTU based taxonomic assignment, most of the isolates that had been identified to genus level using Greengenes OTU assignment corresponded with taxonomic data gathered from Amphora (**Table 5.4**). Although there were some discrepancies (described in **Chapter 5.3.4**) 46 of the 53 isolates taken forward following NGS quality filtering had been assigned an OTU based on 97% sequence identity on the Greengenes database, of these 44 were accurately taxonomically assigned at the genus level accounting for over 95% of the dataset.

Conspicuously, it was observed that more than one OTU identity was assigned to the same bacterial species on several occasions, but this is likely due to small variations in both the V4 sequences generated in this study and featuring in the Greengenes database (**Table 5.4**) there were also isolates assigned to either the Agrobacterium or Rhizobium genus interchangeably. However, the assignment of Agrobacterium to the rhizobium genus has been a source of debate (Farrand et al., 2003, Young et al., 2003).

This indicates that the use of 16S Illumina MiSeq sequencing for the taxonomic

classification of future bacterial isolate libraries is sufficient for identifying isolates of interest to take forward for further analysis based on the total taxonomic diversity of new and current isolates. This information can be used to select isolates that have not yet been cultured in our collection and to identify candidates for whole genome sequencing in order to streamline the bacterial library construction process.

Nevertheless, it is important to note that several isolates were assigned dated taxonomic descriptions when using the Greengenes database (as described in **Chapter 5.3.4**), therefore it would be prudent to use the SILVA database going forward not only to ensure up to date assignment of OTU IDs but also to ensure continuity and replicability in line with current publications.

5.4.4 Correlations between functional annotation and OTU taxonomic assignment

When examining the relatedness of bacterial isolates based on the presence or absence of KEGG orthologs using PCoA (**Figure 5.7**) it initially appeared that isolates assigned to the same genus were, for the most part clustered in relatively close proximity to one another, indicating that isolates belonging to the same genus were closely related functionally. However, on closer examination it was clear that analysing the total isolates in one output was masking functional diversity within and between species and was likely a by-product of a high proportion of housekeeping genes conserved within genera.

When examining the KO intersects between all isolates it was clear that there were notable variations in intersects within and between species, indicating a potential variation in the presence of PGPR genes and other genes of potential interest (**Figure 5.8**). However, the majority of intersects did correlate between and within species, indicating an area of the data that can be investigated at a later time to examine the genetic conservation and to identify key housekeeping genes within and between species and how they may vary.

The greatest variation in KO intersect groups was observed in isolates assigned to *Pseudomonas* sp and by focussing on this genus it was apparent that although Amphora taxonomic assignment correlated with OTU assignment and distribution

(although more than one OTU identity was assigned to isolates identified as belonging to the same species **Table 5.4**, **Chapter 5.4.3**) this was not mirrored when examining the relatedness of the Pseudomonas isolates based on the presence or absence of KOs.

A clear example of this lack of correlation is the distribution of the *Pseudomonas brassicacearum* isolates assigned to OTU group "A", two of these isolates have been clustered based on KEGG ortholog presence or absence similarity with the *P. brassicacearum* isolates assigned to two different OTUs, this in itself is not likely to be of significance. Comparatively, the other two *P. brassicacearum* isolates also assigned to OTU group A were clustered completely independently of their corresponding OTU isolates, instead being more closely functionally related to an individual strain (and OTU group assigned) of both *P. fluorescens* and *P. aeruginosa*, respectively.

An additional observation using EC numbers from RAST annotation revealed that *Pseudomonas fluorescens* isolate bi89 assigned to its own unique OTU group described as "D" showed far more similarity to isolates taxonomically assigned to *Pseudomonas brassicacearum* species than other bacterial isolates identified as *P. fluorescens* (**Table 5.6**). Moreover, when examining the presence or absence of subsystems in comparison between bi89 and all other *P. fluorescens* and *P.brassicacearum* isolates, respectively, it was apparent that many of the genes absent in *P. fluorescens* versus bi89 and *P.brassicacearum* are likely to be the result of an annotation or assembly issue.

For example the physical absence of "flagellum" gene orthologs is highly unlikely; *Pseudomonas spp.* including *P. fluorescens* have been heavily documented as flagellate organisms with *Pseudomonas fluorescens* even being implicated in flagella driven chemotaxis towards exudate compounds in tomato root colonization with their flagella playing a role in competitive root colonization (de Weert et al., 2002, Capdevila et al., 2004, Wisplinghoff, 2017).

A further illustration is the highly unlikely probability that there are no ECs assigned at all to tRNA processing or splicing, given its crucial role in the accurate translation of messenger RNA codons during protein synthesis and, ultimately, cellular gene expression. A far more likely scenario is that the SEED does not yet have a sufficient array of *Pseudomonas fluorescens* gene orthologs in particular

subsystems documented to recognize orthologous genes or conserved domains that may differ slightly from genetic information inherited by the database thus far.

Although these observations could be the result of sequencing or whole genome assembly issues it also indicates that although V4 16S data can be used to infer taxonomy for screening purposes relatively accurately it cannot be confidently used as an indicator of functionality for individual bacteria even based on the functional data sets that have been constructed from the isolates from this study.

There are tools available that can be used to predict the functional composition of a metagenome using marker gene data and a database of reference genomes from 16S Illumina MiSeq data. PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) uses an "extended ancestral-state reconstruction algorithm" to predict which gene families are present in 16S metagenomic datasets, specifically those created from heavily bacterially populated environments such as the rhizosphere, it then combines gene families to estimate the composite metagenome (Langille et al., 2013).

Although PICRUSt can be used as an excellent resource for examining potential functional diversity in the rhizosphere of different barley genotypes grown in varying environments, it cannot be used accurately in isolation studies and when higher resolution analysis is required.

5.4.5 Correlation between KEGG ortholog assignment and environmental enrichment

During the process of analysing the taxonomic assignment of the bacterial isolates taken forward during this study it was surprising to observe that there was no clear correlation between the environment in which isolates that had been identified as being enriched based on OTU assignment and their functional assignment based on KEGG orthologs or RAST analysis (present or absent in the root hair mutant rhizosphere study and/ or exudate study versus the soil density study only). There was one exception to this; in the Pseudomonas isolates in a plant vs non-plant comparison it was revealed that just one KO (KO: k21929) was present only in plant-populating Pseudomonas and undetected in all Pseudomonas isolates that had been detected in a non-plant environment based on OTU assignment.

When examining the functionality of this gene ortholog it was revealed that this corresponds with a gene ortholog of unknown function. Although it could be tempting to propose that a currently uncategorized gene may play a role in driving bacterial proliferation in a plant based environment it is far more realistic to propose that the conserved presence of just one gene ortholog from a set of over 6,400 in a particular environment is unlikely to have any significant relevance. It is arguably more interesting that from a data set consisting of so many gene orthologs that only one was detected in a plant associated only environment.

5.4.6. The identification of plant-growth promoting rhizobacteria genes

Plant growth promoting rhizobacteria are a highly diverse group of bacterial species that colonize the rhizosphere and roots of plants. PGPRs have the capability of promoting plant growth in a number of indirect and direct ways. Indirectly PGPRs act as biocontrol agents by local antagonism of pathogens or induction of systemic acquired resistance against pathogens. Directly they can act by synthesizing and then providing the plant with organic compounds such as phytohormones or by facilitating the uptake of nutrients not readily available to plants in the soil.

For example, the production of low molecular weight organic acids that solubilize organic phosphate is a key function of some PGPRs. There is extensive literature on the modes of action of PGPRs, but the categorization of the genetic drivers of these actions are yet to be fully elucidated, primarily because it is highly likely that not all PGPR traits and their corresponding genes have been identified and because the identification of PGPR genes is often limited to a small sample of bacterial clades (Bruto et al., 2014). Using lists of confirmed PGPR genes and operons collated by (Bruto et al., 2014) and (Rosier et al., 2018), the metagenomic data generated from this study was mined for the presence of several well documented PGPR genes with a view to identifying candidates from the bacterial library to use in future studies.

5.4.6.1 Phosphate-solubilising gene ortholog presence in this study

There is an abundance of genes that have been identified in different bacterial organisms that have been implicated in the solubilisation of organic phosphate (Po). The most commonly studied are phytase genes (*phy*) which produce phosphatases that specifically trigger the release of P from phytic acid, *mps*

genes which produce gluconic dehydrogenase which interacts with pyrroloquinoline quinone (pqq) (produced by the pqq operon) to produce gluconic acid which solubilizes organic phosphate, *gapY* genes which produce gluconic acid in a pqq independent manner to name just a few.

Due to the time permitted for analysis this study focused on three gene ortholog groups implicated in the process of the solubilisation of organic phosphate; Pho, DING and pqq

5.4.6.1.1. Pho genes

This study identified the presence of "High affinity phosphate transporter and control of PHO regulon" homologous genes in all Pseudomonas isolates. It was interesting to observe that the *P. fluorescens* isolates bi08, 10 and the *P. brassicacearum* isolates bi06 and 11 carried double the number of orthologs (8) compared to the other taxonomically complimentary isolates.

Arguably, in order to fully understand the applications of this observation it is necessary to investigate the individual gene orthologs detected in each isolate and if they correlate with the full Pho operon using a multiple annotation tool approach. This allows a more detailed breakdown of the individual orthologs and may identify if they are involved in the production of extracellular enzymes or involved in the transportation and storage of acquired P, specifically homologues of alkaline phosphatases (*phoA*), phospholipases (*phoD*), glycerophosphodiester phosphodiesterases (*glpQ* and *ugpQ*), phytases (*phyC*) and 5′-nucleotidases (*ushA*) (Wanner and Chang, 1987).

The PHO operon is associated with the production of the alkaline phosphatase pho which is controlled by a two component system (consisting of an inner-membrane histidine kinase sensor protein and a cytoplasmic transcriptional response regulator) that is considered to be a highly efficient regulatory mechanism in bacteria that is believed to have evolved in response to surviving in a low Pi environment (Santos-Beneit, 2015).

Riccio and co-workers used an indicator medium (TPMG) composed of Tryptose-Phosphate agar supplemented with phosphatase substrate phenolphthalein diphosphate and methyl green stain in order to screen bacterial phosphatase producing clones (Riccio et al., 1997). It could be possible to use this technique

to test the PHO operon activity in the Pseudomonas isolates described above to complement further computational analysis.

Despite the fact that the Pho operon has evolved in order to aid bacterial survival, as it involves the release of extracellular enzymes it has been proposed to act as a passive PGPR by mineralising Po into organic forms within the rhizospheric region making it also freely available to its plant host making these *Pseudomonas sp.* isolates potential candidates for PGPR studies in barley inoculation trials (Chhabra et al., 2013, Lidbury et al., 2019).

5.4.6.1.2. DING proteins

DING proteins (named due to their N-terminal sequence) are phosphate binding proteins which are expressed in low phosphate environments in *Pseudomonas spp.* (Berna et al., 2008). It has been proposed that due to their role in phosphate binding they may play a role in biomineralization, but curiously DING proteins have also been isolated in eukaryotic sources and can bind to ligands other than phosphate (Berna et al., 2002, Riah et al., 2000, Scott and Wu, 2005).

It has been proposed that, in fact, DING proteins may actually be related to signal transduction in Eukaryotes (Berna et al., 2008). However, sequencing of Pseudomonas has identified that numerous species carry genes which are up to 80% conserved with eukaryotic DING genes leading to the proposition that all DING like proteins originated from the Pseudomonas genus (Lewis and Crowther, 2005).

Although the potential of DING protein expression in relation to PGPR studies has not been examined to any great extent in the literature, it is of interest that gene orthologs were detected in one *P. fluorescens* isolate (bi89, which was identified as sharing more unique orthologs with *P.brassicacearum* than the other *P. fluorescens* isolates) and 4 *P. brassicacearum* isolates (bi06, 55, 11 and 75).

It would be prudent to consider that it is likely that, as these are surface proteins, it is highly likely that any phosphate acquisition activity will solely benefit the organism but greater turn-over of the rhizosphere microbiota and, in turn the release of P will enhance plant acquisition of phosphate indirectly. Unfortunately, the isolates in which this protein has been detected all also carry Pho regulon genes which would mean that it would be problematic to establish which genes

were driving phosphate solubilisation using traditional assays.

5.4.6.1.3. The PQQ Operon

Pyrroloquinoline quinone (PQQ) is thought to be produced by hundreds of bacterial species where it functions as a redox cofactor in substrate oxidation using a non-glycolytic pathway for the production of cellular ATP (Shen et al., 2012). Specifically, it acts as a cofactor with gluconic dehydrogenase to oxidize glucose producing gluconic acid which solubilizes organic phosphate. In order to make P fully available for uptake by an organism solubilisation is the first step that must then be followed by mineralisation (which is conducted by Pho genes). Meaning that the Pho and pqq gene complements working together have a large impact on organic P availability.

As a result of this interaction, PQQ is considered to have plant growth promoting functionality (Barr et al., 2016, Bruto et al., 2014). PQQ also works as a cofactor with methanol dehydrogenase in the production of bacterial ATP, but this does not result in phosphate solubilisation (Puehringer et al., 2008).

PQQ production is regulated by the pqq operon which consists of a number of *pqq* genes, the number of genes involved is yet to be agreed based on conflicting proposals in the literature and may vary depending on the bacterial species. Nevertheless, there is agreement that the combination of 5 to 8 of these genes within the operon facilitate the synthesis, regulation, transport and release of PQQ.

Puehringer and co-workers proposed that in *Klebsiella pneumoniae pqqABCDEF* are required to form a complete operon, suggesting that *pqqA* codes for the PQQ precursors glutamate and tyrosine which are recognised by *pqqE* then accepted by *pqqF* before being catalysed by *pqqC* (Puehringer et al., 2008). Gliese and co-workers suggest a similar operon which they have identified in *Pseudomonas aeruginosa*, *pqqABCDEH* where *pqqH* is proposed to be the gene PA1990 which is co-transcribed with *pqqABCDE* and responsible for the excretion of PQQ (Gliese et al., 2010).

A more simplified model is proposed by Shen and co-workers that a core operon consisting of just four genes, *pqqBCDE* is all that is required for the synthesis of PQQ, furthermore they suggest the *pqqF* should be considered a non-essential

gene in the synthesis of PQQ (Shen et al., 2012). This study identified 13 bacterial isolates that carry the proposed "core" pqq operon (pqqBCDE), 6 *Pseudomonas brassicacearum* isolates, 5 *Pseudomonas fluorescens* isolates, and 1 *Pseudomonas aeruginosa* and *Enterobacter sp*. isolate respectively. Each of these isolates were also identified as carrying a pqqF gene ortholog, in addition to this, all of the *P. brassicacearum* isolates carried a pqqH ortholog.

Interestingly, *pqqA* orthologs were not detected in any of the bacterial isolates which suggests an incomplete operon based on the research of Gliese and Puehringer (Gliese et al., 2010). However, this could be due to missing data in the KEGG ortholog annotation database, an assemblage or sequencing error is less likely based on the number of isolates affected. Presence of a functional pqq operon could be validated quite simply by culturing isolates in glucose based liquid media with the addition of gluconic dehydrogenase from *Pseudomonas sp*. and bromothymol blue pH indicator.

5.4.6.2 Nitrogen fixing (*nif*) genes

It has been substantively documented that *nif* genes are associated with the fixation of atmospheric nitrogen by bacteria, termed diazotrophs, into a form that can be utilized by living organisms by the production of nitrogenase and by encoding regulatory genes for the process of nitrogen fixation (Patel et al., 2019). The relationship between rhizobia and nodulating plants has been researched in great depth. Although barley does not nodulate, it has been proposed to benefit from associative fixation in which the level of nitrogen fixation is determined by a number of factors including a low oxygen pressure rhizosphere environment, exudate availability and bacterial competitive success (Vlassak, 1979, Roley et al., 2018).

Nevertheless, the efficacy of associative nitrogen fixation has been queried, some have suggested that the levels of nitrogen-fixation attained by free living diazotrophs in cereals are not high enough to support the plant's needs and cannot compete with those obtained with chemical fertilizers (Rosenblueth et al., 2018).

It has also been suggested that as N fixation is a carbon expensive process that the presence of free living diazotrophs in the rhizosphere is likely a consequence of the carbon rich compounds produced through the process of rhizodeposition

(Smercina et al., 2019).

Although barley is the model plant for the studies described in this work it was of interest to explore the presence of *nif* genes to identify potential isolates that could not only be used to compliment future SynCom (bacterial synthetic communities) studies but could also be used as a collaborative resource in nitrogen fixation studies in legumes and other nodulating plant species.

The *nif* regulon varies between bacterial species, but the most commonly described is that of *Klebsiella pneumoniae* which is composed of up to 8 operons consisting of 20 *nif* genes. However, at least 20 *nif* genes have been identified across other bacterial species (Brill, 1980, Merrick and Edwards, 1995). Typically the nitrogen fixation activator gene *nifA* is used as a marker to indicate that an organism may have the ability to fix nitrogen (An et al., 2007).

This study identified *nifA* gene homologues in 20 bacterial isolates across multiple genera. In addition to this *nifJLSU* were also identified sporadically across others (**Table 5.6**). Although the detection of 5 *nif* genes does not infer nitrogen fixation potential there may still be potential in these isolates and that additional gene orthologs that would result in a full gene compliment have simply not been detected computationally. As proposed regarding *pqq* genes, the failure to detect a gene ortholog does not necessarily indicate its absence and this may instead be the result of missing data from annotation databases. In order to investigate this further it would be prudent to begin with a multi-assembly analysis to optimise gene ortholog detection and use laboratory-based investigations such as PCR.

5.4.6.3 Hydrogen Cyanide channel (HCN) genes

Abundant studies articulate how bacterial species that express hydrogen cyanide (HCN), encoded by the three genes *hcnABC*, have been associated with having plant growth promoting capabilities. Hydrogen cyanide has been primarily attributed to being a biocontrol agent based on its toxicity against plant pathogens and it has been recently observed that the inoculation of tomato plants with a bacteria that can secrete a combination of DAPG (diacetylphloroglucinol) and HCN, *Pseudomonas brassicacearum* could significantly reduce the bacterial

canker symptoms induced by *Clavibacter michiganenis* (Voisard et al., 1989, Paulin et al., 2017).

More recently this theory has been challenged and it has been proposed that HCN does not actually work as a true biocontrol agent exhibiting an increase in HCN does not increase pathogen destruction; instead it has been attributed to being involved in biogeochemical processes in the soil such as metal chelation, indirectly increasing the availability of inorganic phosphate and enhancing nutrient uptake by plants (Rijavec and Lapanje, 2016, Ahemad and Kibret, 2014).

Heydari and co-workers isolated a strain of *Pseudomonas fluorescens* that was identified as carrying an HCN gene homolog, they reported that inoculation with this strain resulted in the enhancement of length of the stem and roots plus the germination rate of rye, wild barley, and wheat (Heydari.S, 2008). Based on these findings the potential of the cyanotic bacterial isolates is exciting and of particular relevance to the barley genotypes with varying root hair phenotypes described in **Chapter 2** and **3**, respectively.

If these isolates can be used to increase P availability in soil and enhance the area in which roots can explore there is the potential to offset the reduced ability to uptake P due to root hair mutation and improve plant fitness using PGPR inoculant treatments on root hair mutant plants.

Considering the potential of the HCN operon ortholog carrying isolates in this study to perform multiple roles in plant growth promotion it would be advantageous to initially verify HCN production, this can be performed by streaking the isolates of interest on nutrient agar and placing Cyantesmo paper on the inner lid of the plate (Buntić et al., 2019). A colour change in the paper from white to grey would confirm the production of HCN.

5.4.7. Conclusions and future perspectives

Not only did this study demonstrate the broad taxonomic variety of bacterial genera that can be isolated from the rhizosphere of barley using traditional microbiology techniques, it also identified that 30% of these isolates are potentially PGPR candidates. It has been predicted that just 1-2% of the rhizosphere microbiota have PGPR functions, indicating that although this may be the case in the total rhizosphere microbial community, the ability to extract

potential PGPR candidates from the rhizosphere is significantly higher than this (Beneduzi et al., 2012).

Excitingly, three of the isolated bacteria carry gene orthologs for all five of the plant growth promoting functions that were investigated (*P.brassicacearum* 06, 11 and 55), the prospect of isolates with multiple beneficial functions provides a wealth of opportunities for future applications in a variety of agriculturally important crops.

There are a plethora of ways in which this study can be explored further, starting with the use of a multi-tool annotation analysis of the whole genome sequence data in order to validate the gene orthologs identified and to potentially increase the recognition of the five plant growth promoting functions used in additional isolates. By data mining for additional PGP properties such as the presence of *phl, bud, ipd* gene orthologs or homologs of genes implicated in essential processes like systemic acquired resistance (SAR) such as salicylic acid and N-acyl homoserine lactone; increases in root growth such as the nod factors lipo-chitooligomers (LCOs), indoles and cyclopeptides; induced systemic resistance (ISR) such as acetoin and dimethyl disulphide (DMDS) to name just a few. Data mining can also be expanded to explore proteomics data (not presented, **8.4.2 Appendix 6: Supplementary Figures S13 – 23**), bacterial secretion systems, pathogenicity and antibiotic potential.

The variety of bacterial species that can be isolated from the rhizosphere can also be significantly expanded by taking a more specific and directed approach to isolation via media selection. This study primarily used nutrient agar and R2A which are both commonly used with success in bacterial isolation from the rhizosphere, but by moving to more specialized media this is likely to repress the growth of many common soil borne organisms allowing the proliferation of species that may often be outcompeted in a standard isolation environment. Media such as Czapek Dox which contains sucrose as the C source and nitrate as the sole source of nitrogen, Vegitone modified MRS broth which is recommended for the isolation of Lactobacillus (antimicrobial PGP properties), Yeast mannitol agar for the cultivation of Rhizobium and Actinomycetes isolation media for Actinomycetes (production of indole acetic acid) are all good candidates for this.

By using the methods proposed throughout the discussion of this study the PGP functions provisionally identified can be validated relatively simply in a laboratory environment. Completing these validations is essential in order to make evidence-based decisions on the most suitable bacterial isolates to be taken forward for future studies.

An obvious next step following validation is to take the most promising bacterial candidates forward for inoculation trials, for example the isolates bi06, bi55 and bi11 which were identified as carrying orthologs for 5 PGPR functional genes (**Figure 5.11**). By combining select isolates based on complimentary functionality a selection of SynComs can be generated and applied directly to the roots of barley seedlings prior to potting in "sterile" substrate. Not only can the efficacy of each SynCom be monitored by plant success, the rhizosphere can also be extracted, and the composition of the microbiota examined to assess which isolate strains persist.

3 bacterial isolate samples carry orthologs for 5 PGPR functional genes

- PHO regulon control
- Phosphate-binding DING proteins
- Nif
- HCN Operon
- PQQ Operon

Sample ID	Species	Isolation location
Bi06	Pseudomonas brassicacearum A	rhl rhizosphere
Bi55	Pseudomonas brassicacearum A	rhp rhizosphere
Bi11	Pseudomonas brassicacearum B	karat rhizosphere
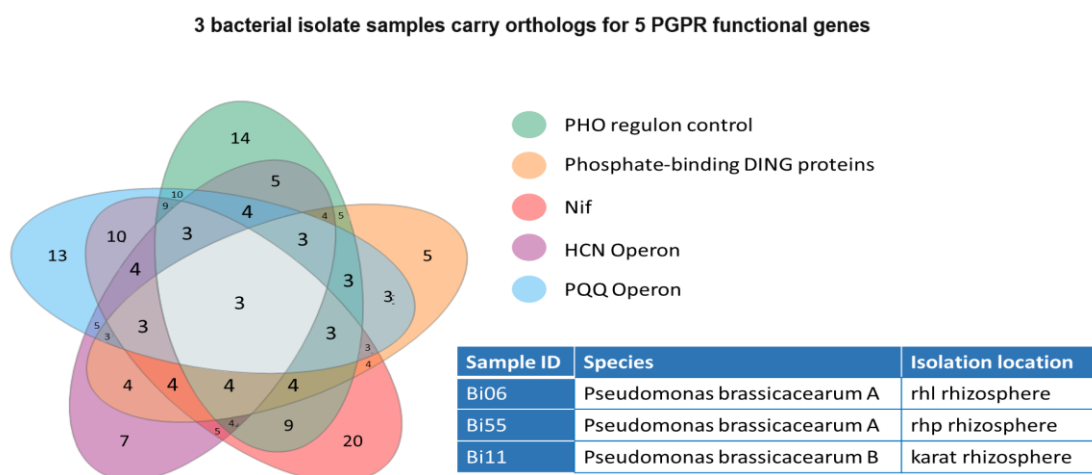
Figure 5.11 Three bacterial isolate samples carry orthologs for 5 PGPR functional genes. bi06, bi55 and bi11 carry all 5 PGPR functions investigated. Venn diagram describes the number of bacterial isolates identified as carrying the indicated gene orthologs and overlapping regions indicate the number of samples identified as carrying the indicated combination of orthologs.

Ultimately, the overarching goal is to develop a SynCom community that can thrive and enhance plant fitness in a natural soil environment without the addition of agrochemicals. However, although this collection has great potential for isolate studies it is also an excellent resource for collaborative studies with other crops of agricultural importance and the identification of functions of relevance to the wider scientific community.

CHAPTER 6

DISCUSSION

At the outset of this study in 2015, the science of the rhizosphere microbiota was and remains to be a rapidly expanding hot topic. This was particularly true for model plants such as *Arabidopsis thaliana* but for crop plants such as barley the benefit of the application of next generation sequencing technologies were not keeping pace with the advances gathered from model plants (Bulgarelli et al., 2012, Bai et al., 2015). The scientific community were aware that an in-depth understanding of the molecular mechanisms of the rhizosphere microbiota were indeed a tool that had the potential to be harnessed to contribute to the improvement of crop yields and to reduce the environmental and economic impact of agrochemicals (Busby et al., 2017). However, for many cultivated plants, in depth understanding of the underlying drivers that shape the rhizosphere microbiota, and in turn the specific microbial interactions occurring at the root soil interface, had only just started to gain momentum.

This discussion will address the specific knowledge that the scientific community had about the importance of rhizospheric microbial communities at the inception of the study and how these have advanced in the intervening years, it will go on to discuss and interpret the results and context of this study and the impact this has had on contributing to this knowledge, it will conclude with proposals for future studies based on this research and the potential these results have to the wider scientific community and for agriculture.

6.1 The state-of-the-art at the inception of the project

Prior to the implementation of this study a major hot topic in microbiology was the gut microbiota and how particular bacterial communities inhabiting the gut could impact human health, how dysbiosis of the gut microbiota could be responsible, at least in part, for several human illnesses ranging from inflammatory bowel disease to obesity and depression and how particular diets or medical treatments could strongly influence the composition of the gut microbiota (Nicholson et al., 2012, Walters et al., 2014, Foster and McVey

Neufeld, 2013, Le Bastard et al., 2018, Carding et al., 2015, Turnbaugh et al., 2009). Gut microbiome studies boomed, resulting in global collaborations such as the human genome project which gathered microbiome data (mainly generated by the use of 16S rRNA gene profiling) from research groups around the world to build a more detailed picture of the factors driving the shape of the gut microbiota and the impact this could have on human health and wellbeing. There were also large scale studies exploring the benefits of inoculation therapies to resolve gut dysbiosis (Lander et al., 2001). One such development was that of probiotic dairy products mainly containing *Bifidobacterium spp.* which had been implicated in aiding the digestion of dietary fibre, passively aiding the human immune system and helping to facilitate the breakdown of complex vitamins and minerals in order for them to be available for uptake in the gut (Gomes and Malcata, 1999, Gibson, 2004, Kailasapathy and Chin, 2000). Another development was a full microbial transplant where a subject with severe dysbiosis would receive a full complement of the gut microbiota of a healthy host which was proposed to be efficacious based on a highly cited study involving transgenic mice. This study took mice with a genetic propensity toward obesity (ob) and mice which had a propensity for a lean build (lb) (this was demonstrated by feeding the mice an identical diet and the observation that despite the same calorific intake the ob mice would consistently have significantly increased body biomass vs their lb counterparts) and subjecting them to a full gut microbiota transplant. Fascinatingly, the transplanted microbiome overrode the genetic blueprint regulating body weight, suggesting that the restructuring of the gut microbiota alone can have a profound impact on the host (Ridaura et al., 2013) .

Why is this relevant to a plant rhizosphere microbiota study?

In some respects, plants wear their gut on the outside in the form of roots and metaphorically mirror the mammalian gut: 'Roots are but as guts inverted' - Jetho Tull (1762) (Tull, 1762). Roots are the site of water and nutrient acquisition, have a large surface area due to root hairs in a similar manner in which villi increase gut surface area, they are also the most heavily microbially populated region of a plant (Ramírez-Puebla et al., 2013). Roots are also the site in which the microbes that reside in synergy with the plant also benefit from rhizodeposition as a food source (Bulgarelli et al., 2013). It was understood that root morphology

(in particular root hair development) had a direct impact on the formation of the rhizosphere and that the exudation of low molecular weight compounds by the plant were likely to play a significant role in microbial recruitment (George, 2019). However, understanding of the complex interplay between an undefined number of physical, biological and environmental factors including those outlined above, in shaping the rhizosphere microbiota, plus the molecular mechanisms and interactions occurring at the root soil interface were yet to be fully elucidated.

6.2 Root hairs as a determinant of the rhizosphere bacterial microbiota.

Root structure has been of scientific interest for decades, particularly the way in which the architecture, transport systems and ability to explore substrates allow plants to maintain optimal nutrient levels in changing soil environments (Hodge et al., 2009, George, 2019). In particular, it was well understood that plant root system traits such as length, biomass, density and surface area influence many aspects of plant health (Comas et al., 2013). One research area surrounding root hair morphology was the development of root hairs in flowering plants, it was observed that plants devoid of root hairs or with perturbations in development had difficulty thriving in nutrient limited conditions, had a significantly reduced surface area, reduced exudation and rhizodeposition and a markedly smaller rhizosphere (Brown et al., 2012b, Grierson and Schiefelbein, 2002, George, 2019). With the emerging importance of the rhizosphere microbiota and the clear impact root hairs have on factors highly likely to impact this community it was surprising that the impact of root hairs on the microbiota had not yet been investigated. This offered an opportunity to explore the impact that root hairs have on the rhizosphere microbiota.

To fill this knowledge gap, in **chapter 2** it was revealed that, although soil is a clear driver of the composition of the rhizosphere microbiota at a high taxonomic resolution, that root hairs play a fundamental role in shaping the microbiota. A bias for members of the orders Actinomycetales, Burkholderiales, Rhizobiales, Sphingomonadales, and Xanthomonadales was identified among the bacteria differentially recruited between barley plants displaying wild-type and root hair mutations. These orders also represented the dominant members of the community, many of which have been identified as being PGPRs or having PGP

potential from published literature. This study suggested that root hairs do indeed shape the rhizosphere microbiota, but the mechanism driving this was unclear, as was the function of the microbial communities differentially recruited. There were several possible explanations for these observations; the first was that the barley genotypes displaying perturbed, or absent, root hairs were recruiting a microbial community in order to compensate for the challenges the underdeveloped root hairs present to the plants, such as difficulty thriving in nutrient depleted conditions. Another possibility was that retardation of root hair formation elicited a stress response in the plant by triggering a shift in exudation patterns, resulting in the chemo-attraction of a different microbial community. Likewise, the other side to this argument would be that the lack of root hairs resulted in a marked reduction in rhizodeposition on the whole, not only changing the chemical signals within the rhizosphere, but reducing the availability of plant-based carbon as a nutrient source for the microbiota ultimately indicating a loss of control from the mutants to shape their associated microbial community.

The information generated in this study is not only beneficial from a plant-microbe interaction perspective, but also from a breeding perspective. Cultivars with poorly formed root hairs are seldom selected directly by breeding programs due to the well documented hurdles that are faced, particularly the acquisition of P, but the optimal conditions used for breeding could lead to the unintended loss of root hair length due to breeding progress. By taking this study further and exploring the functionality of the rhizosphere microbiota represented in both wild type and root hair mutant barley it would be possible to ascertain if the shifts in the microbiota impact over all functionality either positively, compensating for the impairment of root hairs; negatively, that the biophysical impact of root hair mutations perturb the recruitment of PGPRs or if this taxonomic shift does not actually change the overall functionality of the microbiota with the same roles being performed in both environments, but by different bacterial organisms. Unravelling this could present an opportunity, not only to expand the scope of breeding programs by confirming that root hair mutations do not affect microbial community functionality in the rhizosphere, but also by identifying key members of the microbiota that allow the host plant to compensate for the root hair mutation. Of course, if a beneficial or compensatory role is identified in the rhizosphere microbiota of root hair mutant plants then we are faced with the

challenge of isolating and successfully inoculating these key microorganisms. The ultimate benefit of this would be to allow breeders more flexibility when considering crosses to consider using plants with root hair mutations in breeding programs where many of the other traits displayed are desirable, with the knowledge that root hair mutant phenotypes can be compensated for microbially. In addition to this, if we can identify key bacterial species that allow root hair mutant plants to obtain sufficient nutrients to proliferate normally we can potentially harness these organisms to enhance the nutrient uptake of wild type plants reducing the amount of agrochemicals required to maintain or enhance yields.

This level of investigation was not possible during this study, instead it was decided to investigate possible causes of the root hair effect on the rhizosphere by examining the exudation profiles between genotypes and conducting a plant independent study on the impact of soil density in immediate proximity to the soil microbiota.

6.3. Root hair mutants display an altered exudation profile

Another area of investigation which has been of interest for decades and remains to be of high scientific interest today is the impact of the genetic and environmental factors on plant exudation profiles and how the changes triggered by these factors shape the microorganisms residing at the root soil interface. It was known that temperature, light intensity, soil moisture content, soil composition and agrochemical inputs could all effect plant physiological processes and, in turn, the low molecular weight compounds released by the plant (Cheng et al., 2010, Graham et al., 1982, Raynaud, 2010, Neumann and Römheld, 2007). Furthermore, it was demonstrated that a higher abundance of carbon in the rhizosphere provoked changes in the structure, abundance and diversity of the microbiota with a study conducted in the late 1980s proposing that root exudates stimulated the growth of arbuscular mycorrhizae which, alongside rhizobia have been long understood to infer beneficial roles in the rhizosphere (Grayston et al., 1997, Berg, 2009, Bécard and Piché, 1989). Using *Arabidopsis thaliana* as a model it was presented that 8 accessions each displaying variations in their exudation patterns were host to microbial communities that varied in composition and relative abundance (Micallef et al.,

2009). What was lacking in the literature was a detailed study into correlations in the community structure of the microbiota in relation to the exudation profile of crop hosts.

Although such a detailed correlative analysis is yet to be presented in crop plants, as a first step towards a comprehensive understanding of the link between exudation profiles and microbiota recruitment, in Chapter 3 an analysis of the organic and amino acids alongside the sugar alcohols exuded by the four barley genotypes used throughout the study was carried out. Six organic compounds were detected as present or absent in either wild type or mutant barley. Specifically, phenylalanine, trans-13-octadecanoic acid and n-pentadecanoic acid detected in wild type exudates only and protocatechuic acid, 3-buten-1-ol and phosphoric acid that were solely detected in the exudates of the mutant plants. The presence of both phenylalanine and N-pentadecanoic acid at a detectable level in the exudation profiles of the mutants only was particularly interesting. Both compounds have been identified as having antimicrobial properties, particularly against *Pseudomonas spp.* which could indicate that the excretion of these compounds is playing a role in the reduced complexity of microbiota that was observed in the rhizosphere study detailed in chapter two. Furthermore, by potentially contributing to this simplified microbiota it could pave the way for the proliferation of other microorganisms which may normally be outcompeted in wild type conditions.

This data supports the assertion that exudates play a role in shaping the microbiota but gives further insight into why root hair mutations are observed to do the same. For instance, it is clear from the exudate data that the compounds phenylalanine and N-pentadecanoic acid, if produced in the exudates of wild type plants, are at a level that is below the detection threshold, this corresponds with bacterial sequence data showing a marked increase in the presence of *Pseudomonas* in the exudates of these plants. Furthermore, the increased presence of Oxalobacteraceae members, which appear to be less susceptible to these antimicrobials, in both the exudates and rhizosphere of root hair mutant barley adds further support to the suggestion that root hair mutant exudates may selectively interfere with the proliferation of given bacterial species allowing for others to proliferate in a less competitive environment (**Figure 6.1**).
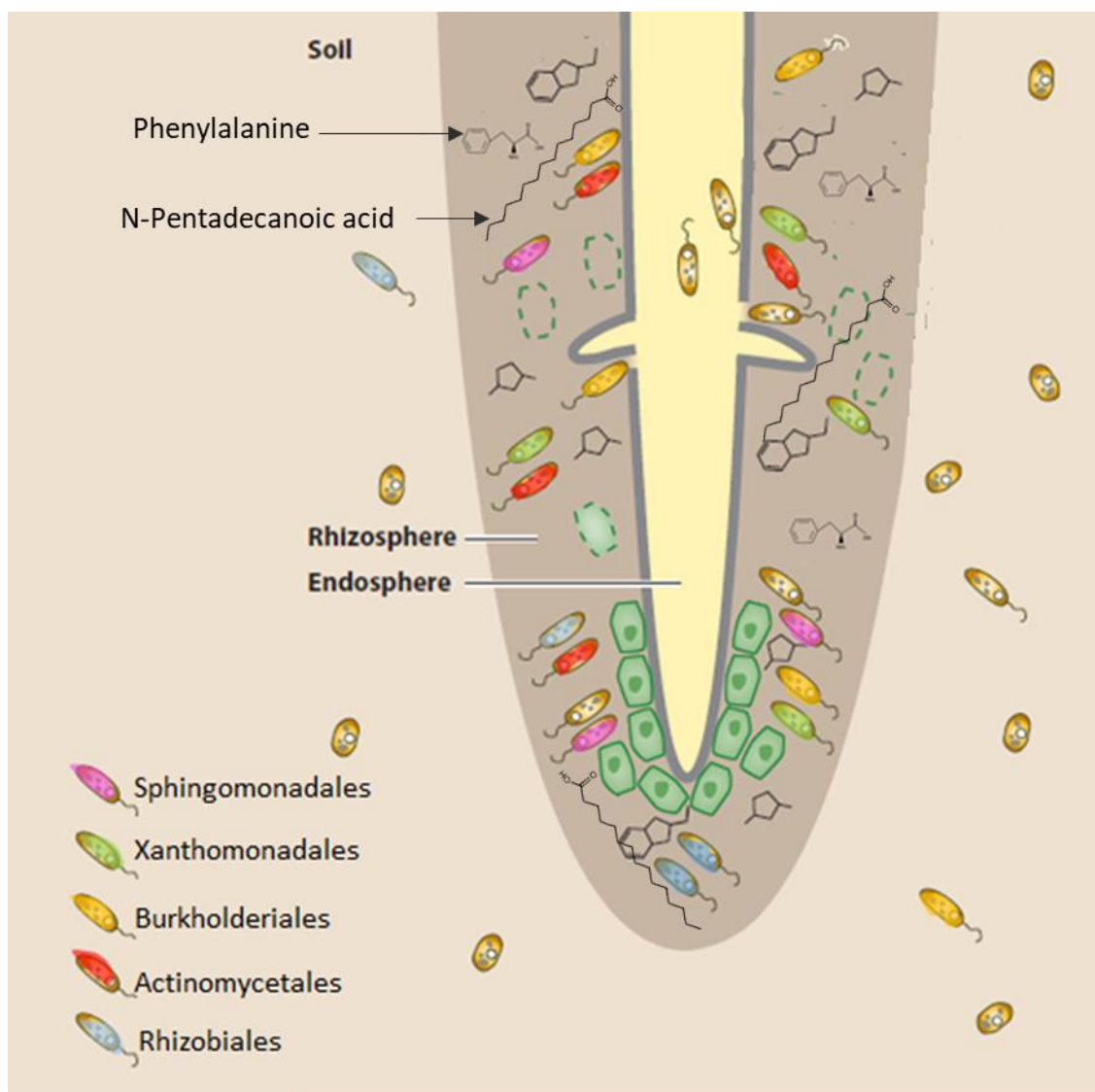
Figure 6.1 Exudates play a role in shaping the community structure of the root hair mutant barley rhizosphere microbiota. *The bacterial rhizosphere microbiota is, at least in part, modulated by the host genotype influencing root hair phenotype and the exudation of small organic compounds excreted by the roots in the form of exudates. Barley with root hair mutations show a bias for the orders Sphingomonadales, Xanthomonadales, Burkholderiales, Actinomycetales and Rhizobiales (indicated by colour). Root hair mutant barley may shape the chemical environment with enhanced production of phenylalanine and straight chain fatty acids*

6.4 Soil density as a selective filter for the rhizosphere microbiota

Soil compaction is a common and serious issue that is of high importance to ecological scientists and farmers (Soane and van Ouwerkerk, 1994). It is typically the result of the use of heavy machinery and inappropriate tilling practices resulting in increased soil density, which in turn affects the ability for plant roots to explore soil efficiently to gain nutrients and by shifting gas and water transit and diffusion within the soil due to changes in pore and channel

sizes (Soane and van Ouwerkerk, 1994, Unger and Kaspar, 1994). Prior to the implementation of my experimental research the importance of the physical effects of soil density were also being investigated in relation to the soil microbiome in addition to crop fitness (Hartmann et al., 2013, Hartmann et al., 2015, Hamza and Anderson, 2005, D.Janušauskaite, 2013). These studies were mainly conducted in forest or field based studies, where it was observed that there was a direct correlation between soil compaction and the microbiota and in turn the observation of significant changes in soil processes (Hartmann et al., 2013, D.Janušauskaite, 2013). These observations suggested that not only does soil compaction impact plants in a physical manner, but also biologically by changing the environment in which the microbiota can proliferate and, ultimately, shifting the composition of the hosts rhizosphere microbiota impeding its success further. At this time studies conducted in to the impact of soil compaction on the rhizosphere microbiota of crops was sparse, but it was revealed that the cropping techniques of maize impacted the rhizosphere microbiota (Hargreaves et al., 2015). Clearly the impact of soil compaction on the microbiota and, in turn, the beneficial processes inferred on their plant host required expansion. Furthermore, as it has been widely presented that the process of root growth results in compaction of soil at the root soil interface, it was also surprising that the impact of root hairs on soil density and the effect this had within the rhizosphere on the microbial communities inhabiting this region had not been investigated in great depth (Dexter, 1987, Young, 2006, Young, 1998, Vollsnes et al., 2010). Crucially, my investigation revealed that soil density has a selective impact on the microbiota owing to the fact that shifts in community composition could be clearly observed to be occurring in response to soil density in a gradient like manner. This was particularly prominent in the abundance of the orders Burkholderiales, Rhizobiales, Saprospirales, Sphingomonadales and Xanthomonadales. Notably, members of the order Burkholderiales, who are commonly associated with having PGP capabilities, were observed to be significantly enriched in higher density soil in addition to also being enriched in both the exudates and rhizosphere of root hair mutant barley. This makes further investigation into the applications of this order appealing, particularly for studies exploring enhancing plant success in suboptimal environmental conditions.

Not all results were congruent to previous studies; this study also revealed a somewhat contradictory story in that the orders Rhizobiales and Xanthomonadales were significantly depleted in high density soil, but had been observed to be enriched in the rhizosphere of the root hair mutant barley genotypes which, in principle, have a denser rhizosphere than the wild type plants (Koebernick et al. 2017). This observation adds weight to the argument that there is no single factor that shapes the microbiota, instead it is clear that there is a complex interplay between the chemical, physical and biological environment, creating an ecosystem that can arguably be described as being in a state of constant flux. However, by utilizing resources such as metagenomics and proteomics combined with the continued advances in soil and plant genomic science, we are slowly unravelling the mechanisms at play in this complex system in order to apply this knowledge to agricultural practices and advances.

6.5 Towards an indexed bacterial collection of the barley microbiota

The availability of collections of individual members of the microbiota allows scientists to be in the exciting position of discerning between correlation and causal relationships. By exploring the causation of the bacterial colonization of plants and increasing our understanding of the processes dictating them, it also opened up further research avenues that had already been successfully explored in mammals (Ridaura et al., 2013). Namely, the isolation, categorisation and transplantation/inoculation of members of the bacterial rhizosphere microbiota to boost plant fitness by enhancing nutrient acquisition and pathogen defence and to reduce the requirement of agrochemicals.

The significance of arbuscular mycorrhiza (AM) and rhizobia have been well documented. Arbuscular mycorrhizal fungi cannot complete their life cycles without forming a symbiotic relationship with the roots of plants, and are considered to be an integral part of the natural ecosystem. At the time of this study it was commonly accepted that AM benefit from the uptake of plant derived carbon in exchange for the facilitation of the cycling of nutrients, particularly phosphate and nitrogen. Arbuscular mycorrhiza had also been implicated in tolerance to water stress by regulating water absorption, transpiration and photosynthesis (Marschner and Dell, 1994, Augé, 2004).

Similarly, rhizobia live in symbiosis with their plant host, specifically nodule forming legumes. Rhizobia have been of such interest due to their ability to fix atmospheric nitrogen, and convert it into a state which can be utilized by the plant host; this is of significant ecological relevance as nitrogen is the most common element that is deficient in agricultural soils. Rhizobia are also of interest in this study due to the mechanism of nodule formation in legumes which occurs by Rhizobia inducing curling in root hairs to form pockets in which they can enter to form symbiosis. Although barley does not nodulate, given that a significant proportion of this study examined root hairs and their associated impact on the rhizosphere the observations regarding shifts in rhizobial abundance may be of relevance to those who study nodulating crops.

However, evidence had been building over decades that, in addition to the clear importance of AM and rhizobia, a highly diverse array of other bacterial organisms could elicit beneficial interactions within the rhizosphere in the form of pathogen control and nutrient cycling, termed plant growth promoting rhizobacteria. In particular the genra *Pseudomonas* and *Bacillus* had been well documented for their ability to trigger induced systemic resistance in their plant hosts and inoculation trials using known PGPRs such as *Pseudomonas flourescens*, *Chryseobacterium balustinum* and *Serratia fonticola* on crop plants such as soya bean and maize were performed with mixed results ranging from having a deleterious effect on the plant host or failure to thrive in the competitive soil environment to increasing the host yield significantly (Lucas Garcia, 2004, Planchamp et al., 2015, Egamberdieva et al., 2015*).* Furthermore, a number of studies were being conducted to isolate members of the rhizosphere microbiota from an array of environments and hosts and testing their potential as PGPRs based on their ability to antagonise known pathogens or promote the cycling of nutrients (Chung et al., 2005, Majeed et al., 2015). Furthermore, with the increasing accessibility of whole genome sequencing and targeted amplicon metagenomics in combination with the improved quality and availability of metabolic databases functional genomics of both individual bacterial organisms and entire communities was rapidly expanding. This was demonstrated with the establishment of the earth microbiome project in 2010 (Thompson et al., 2017). With the availability of such excellent resources and techniques it was clear that by combining areas in current research that would benefit from expansion and

the tools available that this study could make a significant contribution to current scientific research. Although projects such as this can be referred to as "stamp collecting" they play a vital role in providing a deeper understanding of genetic drivers of microbial community composition and identifying key patterns in the function of these communities and their individual members to allow us to harness these for agricultural, medical and industrial purposes, to name a few.

A key area of scientific interest is the development of several indexed bacterial collections for several crop plants. Following the parallels that can be drawn from the advances in medical science in relation to the human microbiome it is clear that the development of personalised microbial therapies in agriculture is an exciting and promising avenue to pursue. In Chapter 5 it was demonstrated that the barley rhizosphere is indeed culturable. By using traditional microbiological isolation techniques, it was possible to isolate a broad taxonomic range of bacterial genra. Excitingly, 30% of these isolates were identified as potential PGPR candidates after metagenomic analysis with the opportunity to mine this data further using a multi tool analytical approach to identify additional genes of interest and candidate isolates. Given that it has been predicted that just 1 – 2% of the rhizosphere microbiota have PGPR capabilities this result is encouraging and indicative that, although this may be the case, PGPRs appear to be able to proliferate in isolation conditions with great success. Clearly the development of this bacterial library opens up many research opportunities ranging from harnessing the current collection for SynCom inocula studies in a range of crops to further data mining and validation studies to explore the applications of members of this collection in the wider scientific community in fields such as bioremediation and antimicrobial research to name just two. In addition to this it was exciting to observe that 3 *P.brassicacearum* isolates carried orthologs for all 5 of the PGP functions that were investigated in this study. Although this is not a unique observation, recently *Bacillus sp.* isolates were identified to have the ability to perform 20 – 80% of 5 tested PGP functions including nitrogen fixation, phosphate mobilization and IAA production, it does define an excellent opportunity to take a more simplified approach to SynCom studies. By using isolates with multiple capabilities, it is likely that inter specific competition will be much reduced and will simplify rhizosphere establishment and persistence studies.

6.6 What's next?

It is becoming clear that optimum plant growth is the result of the tri-partite interplay between soil biophysical characteristics, the microbiota and the host plant. Investigating the use of a microbiota which can promote increased nutrient availability and enhance the plants natural defence systems is a very attractive research avenue to explore for enhancing sustainable crop production. From a translational biology perspective this tri-partite interaction supports taking a tri-partite approach should be taken when it comes to improving agricultural sustainability, focussing on the interaction between the soil, the microbiota and the host plant (**Figure 6.2**)
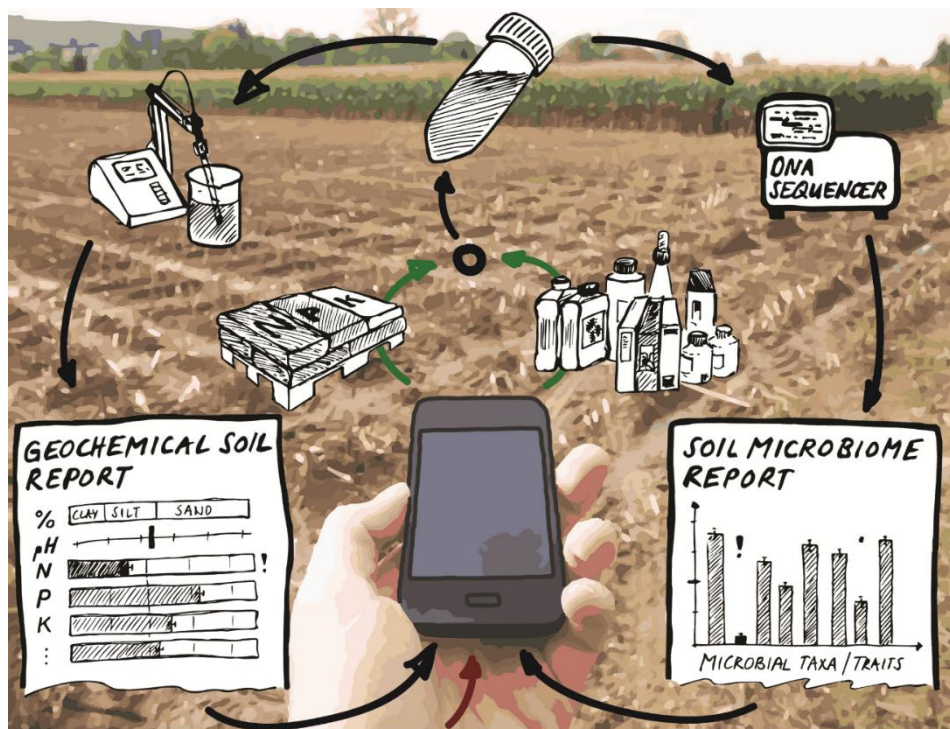
Figure 6.2 The microbiota as a form of agricultural management. The rhizosphere microbiome can be harnessed to improve crop fitness and to assist with soil management (Schlaeppi and Bulgarelli, 2014)

Agricultural practices such as different tillage regimes appear to modify the physical and chemical properties of the soil, in turn shifting the structure and function of the soil microbiota which, in turn, impacts the availability of nutrients for plant growth (Degrune et al., 2017). In addition to physical agricultural practices, the effects of soil fertilization treatments have also been shown to influence the microbiota (Kalia and Gosal, 2011). On this basis it is clear that an increased awareness of the impact of agricultural practices on the functions of the microbiota associated with enhanced crop nutrition may help to steer agricultural management in the future. This could be promoted by the existence

of translational sequencing efforts, such as the Earth Microbiome Project, which so far, has gathered information for more than 200,000 microbiota samples across the globe (Thomson 2017).

Another approach to enhancing crop growth and nutrition is to focus on the structure and function of microbial communities and directly harnessing the microbiota through microbial inoculants. As previously described, there is clear evidence that some members of the microbiota have PGP capabilities such as aiding nutrient acquisition and indirect pathogen protection. These observations have formed the basis for the isolation and categorization of individual members of the microbiota, and lead to commercialisation for their application as microbial inoculants in agriculture (Islam MT, 2012, Owen et al., 2014). Unfortunately, commercial probiotics have had limited success in both crop enhancement and agricultural interest (Benintende, 2010, Bashan, 2013). Under controlled conditions the success of these inoculants is often very inconsistent which serves to reflect the complexity of the interactions occurring at the root soil interface (Bashan, 2013). On this basis a deeper and more robust understanding of the complexity of these interactions is absolutely key if reliable microbe-based crop therapies are to be developed and taken on by agricultural communities. Specifically, a detailed understanding of the dynamics and the stability of the microbial communities proliferating at the root-soil interface, particularly the ability to persist of the microbial inoculum upon application and the "fate" of members i.e. over proliferation, microbe-microbe interactions (positive and negative), horizontal gene transfer, competition etc. Although DNA based analysis of microbiomes gives insight in to taxonomic and functional diversity it cannot investigate the impact of interactions between the plant host and its microbiota. However, DNA studies can contribute to the SynCom approach, where individual members of the plant microbiota are isolated on microbiological media and used to colonise germ-free hosts. Sequencing technology can identify isolated microorganisms of interest based on the presence of PGPR genes to go forward as members of a SynCom and could provide novel insights into the relationships among members of these communities and their impact on plant traits. This approach, conducted under soil conditions, aimed to decipher the impact of plant-microbiota interactions on specific host traits will be key in developing novel and/or more efficacious inoculants for agriculture.

The final and arguably most traditional approach to improving crop sustainability is targeted plant breeding. By expanding the scope to also consider the "health" of the microbiota that is recruited by a plant it may be possible to breed plants to naturally recruit a microbiota that confers more beneficial traits. There have been a multitude of investigations which invariably identified a limited, but significant, effect of the host genotype on the recruitment and maintenance of the microbiota thriving at the root-soil interface, which ultimately sparked the concept of 'breeding for the plant microbiota'. However, for this to come to fruition it is first necessary to identify the genetic factors that modulate plant microbiota recruitment. Studies performed using Arabidopsis under controlled conditions highlighted this, suggesting that the signalling of phytohormones, such as salicylic acid and ethylene, as well as host physical morphology were important features in the recruitment and maintenance of both the above and below ground microbiota (Bodenhausen et al., 2014, Lundberg et al., 2012). This is of key relevance as these findings were echoed by a genome-wide association mapping study of 196 field-grown Arabidopsis accessions revealing that genes involved in defence responses, as well as host cell-wall features, contribute to the establishment of the leaf microbiota (Horton et al., 2014).

All considered these results point towards the importance of host genetic components, such as immune response and root structure that could serve as breeding programme targets to enhance the microbiota and in turn, positive host-microbe interactions that may serve to enhance plant fitness and resilience in adverse growth environments. Ultimately, by identifying plant genetic components of the microbiota combined with the approaches outlined above will lay the foundation for the application of this combined knowledge as a 'holobiome' in an agricultural setting to enhance crop sustainability. The development of these approaches signifies the beginning of a very exciting time in agricultural and environmental science, converting the soil microbiota from an observation to a true tool that can be utilized to help overcome the challenges facing agriculture in times to come by "naturally" increasing the nutrients in the soil available for plant acquisition and stimulating plant immune responses. Using this approach may facilitate the reduction of common agrochemicals which are both environmentally and economically expensive and may serve to allow the utilisation of land which is currently not suitable for food production. These

potential applications will not only reduce the impact agriculture has on climate change but will also aid farmers in developing countries who do not have sufficient access to the agrochemicals required to meet food production requirements and help to offset reductions in soil fertility commonly experienced in developing countries as a result of climate and soil exhaustion due to over-cultivation.

In the immediate future it will be prudent to proceed in two ways; firstly, by mining the current bacterial isolate data for additional functional identification, removing contaminates from samples that were identified as impure, verifying functionality identified in silico using in vitro techniques, performing competition assays between isolates identified as having PGPR function and expanding the bacterial isolate collection. Secondly, by performing inoculation studies investigating the impact of different SynComs on plant fitness and viability in varying nutritional environments and studying shifts in SynCom communities post transplantation to have a better understanding of the host-microbe and microbe-microbe interactions occurring.

If it is determined that a specific SynCom can be used to enhance plant fitness and remain robust whilst doing so then field trials will present a new challenge. The richness and diversity of soil will undoubtedly present significant competition with regard to SynCom proliferation, establishment and persistence so devising an efficient and agriculturally viable mode of bacterial transplantation will be key. Exploring seed coating, dried SynCom incorporation into agrochemical treatments or the inoculation of plant reproductive organs to internally "pre-package" seeds with SynComs.

6.7 Conclusion

Overall this research has not only given new insights into the impact root hair formation has on the bacterial rhizosphere microbiota it has also added to existing knowledge regarding rhizodeposition at the root-soil interface and has generated knowledge regarding the immediate impact of proximal density on the rhizosphere microbiota. Finally, the bacterial library created as part of this study has not only shown the array of PGPR genes that can be obtained by using traditional techniques, but also now provides a foundation for the production of SynComs for future studies, collaborations and wider scientific

applications such as medicine, antimicrobials and industrial phytoremediation applications. Collectively, the scientific outputs of this body of work will be deployed to devise novel strategies aimed at enhancing sustainable crop production in the UK and globally.

CHAPTER 7

SCIENTIFIC DISSEMINATION AND INSPIRATION

Public engagement is an umbrella term for an almost limitless variety of ways in which any theme can be translated and delivered to an equally unlimited variety of recipients. The term "public engagement" is interpreted in many ways by different people. However, in this complementary chapter to my thesis I will describe my perception of the relevance, benefits and importance of science public engagement followed by a brief overview of the events and initiatives I have been involved with and the awards that I received as a result of these activities.

7.1 What's the big deal?

When you ask scientists to describe what public engagement means to them you are likely to get a different reply each time (**Figure 7.1**). This is because it truly is different for everyone and the motivation for being involved differs just as much.

"What does public engagement mean to you?"

"It is key for the future of scientific research."

"By nurturing the knowledge of the public we nurture the freedom of the society we live in."

"Without being able to communicate your research in an accessible and understandable manner, do you really understand it yourself?"

"Public engagement helps research become a dialogue between the many people who benefit from it, rather than the sphere of only a few."

Senga Robertson-Albertyn
@sengarella1

I'm compiling an outreach chapter for my thesis and I need your help!

I need one line describing why you think public engagement is important.

"When done well, it is the way universities share their knowledge, listen to others, and change the world"

"Public engagement is important because we are the public, in so many arenas outside our tiny little sphere"

"Because the next Einstein may be stuck in a city where the only job is to cut up chickens. "

"Engagement enables, enriches, energises and empowers science"

"Without public engagement, we would not know what problems to address, or what science we need to produce."

"Accountability of funding for the recipient and funding body, and so the results of research are used to their full potential and not mis-used."

"It takes science from seeming elitist to some to being accessible to all."

Figure 7.1 "What does public engagement mean to you?": *A selection of social media answers in response to asking what people felt was important about public engagement.*

Some of the most common motivations and benefits of sharing scientific research with the greater community include greater research impact, increased accountability and transparency of research, increased responsiveness to societal needs, building trust between the scientific community and the public, developing new transferable skills, funding requirements, inspiring people and getting fresh insights in to research triggered by public responses (**Figure 7.2**).
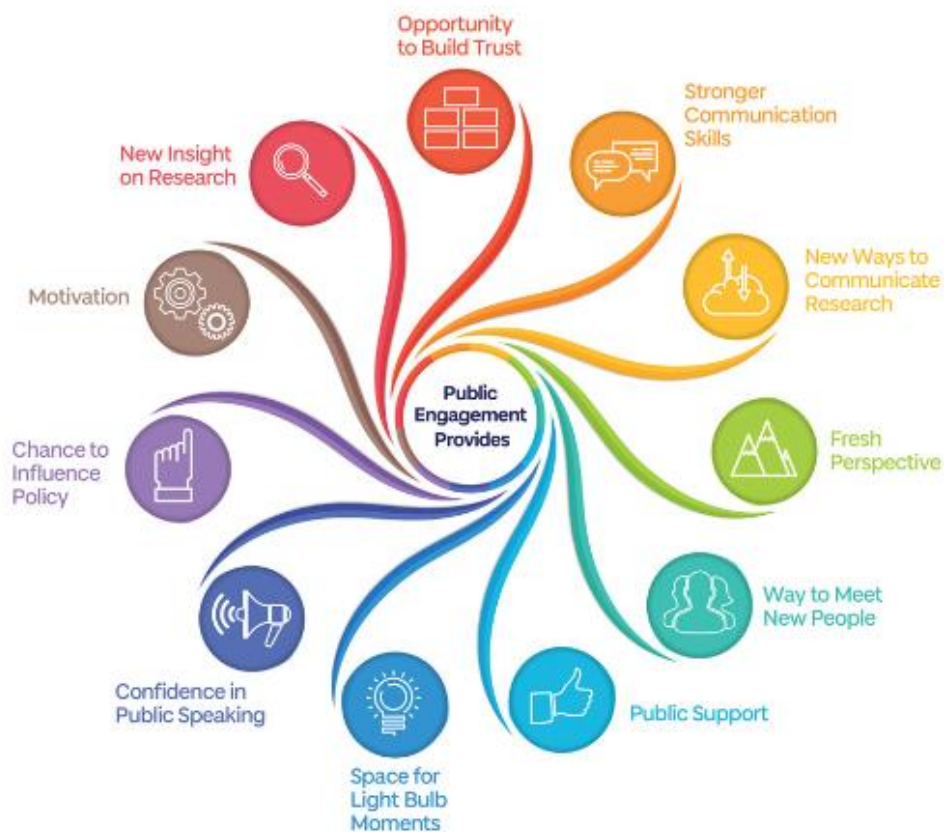
Figure 7.2. University of Dundee "Why engage" motivations. Feedback collated from a survey issued to academic staff (Lambert and Stanley-Wall 2019)

My passion for public engagement is driven by the desire to inspire people from underprivileged backgrounds to believe in their potential and abilities. One of the many benefits I receive from outreach work is when asked "but why?". If this question cannot be answered simply and quickly it is a trigger to re-evaluate both my understanding of the scientific research goals I have set and how relevant they are to the "big picture".

Over the past decade the importance of scientific outreach has been increasingly recognised for the reasons detailed above and has become a common requirement in grant applications. Arguably the recognition public engagement is

now receiving by funding bodies has rapidly improved and expanded the variety, reach and format of the ways in which scientific research is communicated and the mutual benefits to participants and creators.

7.2. (Out)reach for the stars: The only limit is your imagination

Gone are the days where outreach options would revolve around school, science centre or museum activities. Now, the diversity in the ways we can engage and the audiences we can engage with is so vast that it could be the subject matter of an entire thesis; and with the implementation of outreach Honours and Masters courses coupled with undergraduate and postgraduate modules and courses by academic institutions such as the University of Dundee (Gold Engage Watermark from the National Co-Ordinating Centre for Public Engagement) this is likely to be a reality in the near future.

Whilst traditional outreach approaches are still very popular and impactful the world is now our outreach oyster. From describing the humorous aspects of scientific life to stand-up comedy events such as Bright Club and informal evening sessions such as Café Science and a Pint of Science aimed at a general adult audience to creative arts activities such as drama workshops, music festivals and animation projects. However, public engagement does not rely on physically interacting with the public; social media platforms such as Twitter are an excellent resource to not only announce recent publications but to simply and briefly discuss current scientific trends and opinions. There are also a wide range of online tools, videos, blogs and even online competitions such as I'm a Scientist, get me out of here where Scientists compete against each other and avoid elimination from the competition by answering an array of questions posed by upper primary school aged children in a combination of offline questions and fast paced online chats. These approaches represent a fraction of the ways in which we can engage with and learn from each other (after all, scientists are members of the public too) and demonstrate that there is an outreach avenue out there for everyone to help towards making science accessible to all.

7.3 Personal contributions to outreach

Over the duration of this study I have had the privilege of being part of a University and research group that not only share my passion and enthusiasm for all forms of public engagement but also recognise the two way benefits it presents. In this

section I will describe a selection of the events and initiatives that I have been involved in with a view to showcasing the knowledge exchange opportunities that outreach work presents, starting with the project that sparked my interest.

7.3.1. The beginning: "Microbe Motels : How to make a healthy poo" (2015).

Microbe motels is a simple, fun activity using cheap, easily attainable resources to translate the importance and composition of the gut microbiome, the roles played by particular phyla and to translate the importance of dysbiosis (when the proportions of members of a microbial community become imbalanced). It also gently reinforces the message that not all bacteria are "bad" and the importance of health and wellbeing regarding diet and hygiene.

After being introduced to the human gut microbiome, participants learn about the known roles of bacterial phyla in the gut, they are then presented with a mock gut, food and coloured liquid bacteria and invited to add the food to the gut followed by the proportions of bacteria they deem to be important based on their roles. Colour is assigned to the phyla Firmicutes, Actinobacteria, Proteobacteria and Bacteroidetes based on their average composition in a healthy western gut, ultimately creating brown if added in the correct proportions. This is mixed with the food in the mock gut which opens up the opportunity to discuss digestion and diet. Once mixed, the contents of the gut are expelled in to a small toilet or other suitable receptacle and the results are discussed. If the participant has created a realistic "poo" sample, we celebrate with them for having a balanced gut microbiome. However, quite often the sample will be an unusual colour or texture which opens up the dialogue around dysbiosis where associated medical conditions such as irritable bowel syndrome, eczema, depression etc can be discussed.

This activity was very popular and since its development has now become a University of Dundee core outreach activity, has been taken on by the Dundee science centre and has been delivered across dozens of large-scale outreach events with each event having footfalls of around 600 participants. It has also been delivered to multiple schools across the UK (and a few international events) and resulted in a well-received manuscript in the Journal of Microbiology and Biology Education (JMBE) (detailed in **Chapter 10**). Following publication this activity was selected to feature in the White House Office of Science and

Technology Policy Active Learning Day (25 October 2016) and was also featured as a spotlight article in JMBEs education blog. Needless to say, the reach that this activity has had has been significant and truly cemented my enthusiasm for outreach.

7.3.2. Outreach highlights (2015 – present)

Over the past 4 years I have been involved in a wide variety of outreach events delivered to a broad range of audiences. This section will briefly describe a selection of these.

7.3.2.1. Stand-up comedy: Bright Club and the Dundee festival of the future

Bright club is a UK wide initiative has been running for over ten years, it describes itself as a platform that transforms researchers into standup comedians. Bright club events are held in bars and comedy clubs in close proximity to participating universities and feature researchers at all career stages taking a lighthearted approach to the funny side of academia. I have performed at several bright club events discussing everything from using public outreach to overcome fear of public speaking to the challenges that can be faced while on a research exchange overseas. Taking part in these events gave me the opportunity to perform during Dundee's Festival of the Future in 2018 in a similar style event. Standup comedy outreach not only helps to demonstrate that academics are "normal" people that are just as dysfunctional as everyone else it is also an excellent opportunity to gain skills and mechanisms to overcome the very common fear of public speaking due to the excellent workshops and follow ups that are available for performers by the organisers.

7.3.2.2 Scientific open days

Over this research period I have participated in an average of 3 public outreach days per year in events such as "Plant Power Day", "Meet the expert", "Magnificent Microbes" and Open farm days, all of which have been organized by The James Hutton Institute or Life Sciences, University of Dundee. Events such as these allow you to interact with a broad range of people and, importantly, are free to attend which allows them to be accessible to all. Public open days are often inter-divisional so not only can you benefit from inspiration from those attending you also have the opportunity to learn about the work of others, deliver

activities outside of your normal research area and establish potential future research collaborations. The most recent venture undertaken by my research group was working with open day attendees to create an animated video describing the rhizosphere and the host-microbe microbe-microbe interactions that take place within it. Not only did this activity appeal to multiple learning styles, particularly tactile and visual it also provided a final product that all participants could enjoy and celebrate being part of. The video can be viewed here:

https://youtu.be/JdbDRyfiad4

7.3.2.3 I'm a scientist, get me out of here!

In addition to open days I also took part in careers events, Gatsby plant science sessions and debate sessions aimed at young adults plus visited several Scottish schools to deliver outreach activities. This section will now go on to discuss my participation in the online competition "I'm a scientist, get me out of here!"

I'm a Scientist, get me out of here is an online event where upper primary school students connect with real scientists about real science. It's a competition between scientists, where students are the judges and no question is out of bounds. Students challenge the scientists over a seven-day period with a combination of fast-paced daily online text-based live chats featuring up to 30 students and unlimited offline questions **(Figure 7.2**).

Participants can ask the scientists anything they want, and vote for their favourite scientist to win a prize of £500 to communicate their work to the public. This highly rewarding event supported by the Wellcome Trust is not only fun but gives the opportunity to identify "top topics" alongside gaps in knowledge in particular age groups.

I was very fortunate to win my zone in 2017 and to receive £500 to spend on an outreach event of my choice.
(https://plutoniumn17.imascientist.org.uk/profile/sengarobertsonalbertyn/)
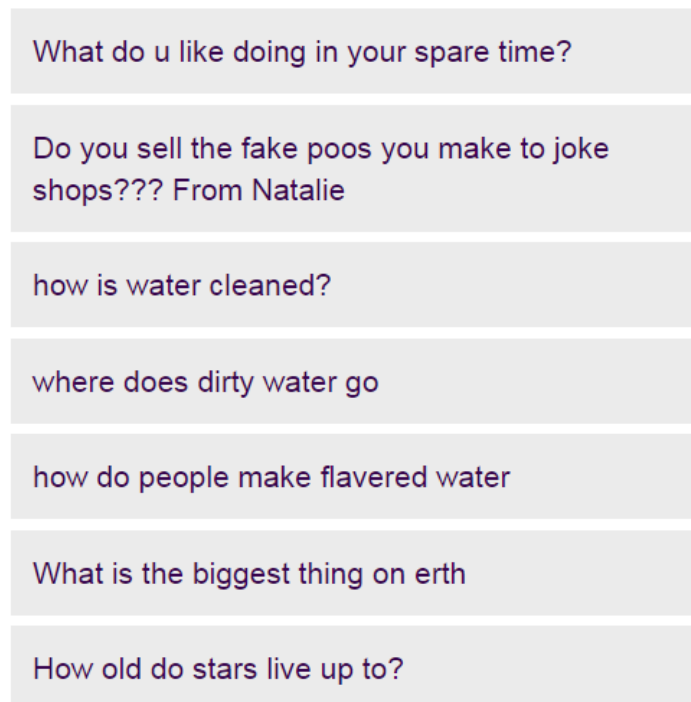
Figure 7.3 Sample set of offline questions submitted during "I'm a scientist, get me out of here". A selection of offline questions posed during the competition.

 I also built relationships with several schools which developed in to visiting the students of two Scottish schools involved in the competition and delivering several hands-on activities with them. In addition to this I arranged to send electronic Microbe Motel activity packs to every school group that was involved in the competition that requested one.

Winning this event combined with generous funding from the University of Dundee's public outreach fund allowed me to organise my own medium scale outreach event; Cell-ebration of Science which I will describe briefly in section **7.3.2.4.**

7.3.2.4. Cell-ebration of Science (2018)

Cell-ebration of science was the first medium scale outreach event that I had the responsibility of arranging facilitated by significant support from the School of Life Science public outreach team. I used publicly available information to identify and contact local schools that fell in to the "under privileged" category, this was based

on the average income of the catchment area and the percentage of students that qualified for free school meals in addition to several other factors. Of the respondents 30 upper school students from each of two schools were invited to participate in the event.

The event held over a full day split in to a morning and afternoon session, featured activities from a range of divisions across the whole University of Dundee School of Life Sciences including Forensic Anthropology, Plant Sciences and the Protein Phosphorylation Unit to name just a few. Activities ranged from bioinformatics and making "genes jump" to making DNA jewellery and "blasting biofilms". In addition to hands on activities there were also two drama workshops run per session exploring themes such as "What does a Scientist do?" and role playing as scientists in "end of the world scenarios".

In addition to a wide selection of activities and topics to choose from the decision was made to cover all transport costs and to cater for all attendees. Although this did cause some additional considerations and contingencies in relation to health and safety it was a very important part of the event, ensuring that all participants could attend without any financial requirement from their families.

This event was very successful and the feedback, both verbal and written was incredibly positive. Not only did it offer a fun, educational day out for 60 young people it also brought together multiple life science divisions and established an external collaboration with the drama expert that gave their time on the day. On a professional level I benefited from gaining a number of transferrable skills pertaining to event and people management that I would otherwise not have had the opportunity to obtain.

7.3.2.5 Behind the scenes

In addition to the activities such as those described above I have also had the privilege of being involved "behind the scenes" by sitting on the University of Dundee science education and public outreach funding panel (September 2016-2019) and the Dundee School of life sciences Brian Cox Public Engagement award panel (2017, 2019). In addition to this I was also a contributor to the successful application of the Gold Engage Watermark from the National Co-

Ordinating Centre for Public Engagement and a reviewer of Reviewer of School of life Science, University of Dundee public engagement strategy (May 2017). Not only do appointments such as these help to appreciate and understand the funding process, they also give the opportunity to witness the sheer variety of outreach themes, topics, approaches and audiences being explored and to understand what turns good outreach into great outreach.

7.4 Public engagement awards

As the result of my involvement in the outreach initiatives described earlier and my supporting role behind the scenes, I have had the great fortune to have been recognised for my contributions in the form of 3 awards. Namely:

The Brian Cox award for engaged researcher of the year (2018)
The Microbiology Society Award for Outreach (2018)
The Royal Society of Edinburgh's medal for Public engagement (2019)
The Steven Fry award for engaged early career scientist of the year (2020)

In combination with my scientific achievements these awards have been true highlights of my PhD research career and have given me the opportunity to deliver numerous presentations about the benefits of outreach across the UK including delivering the Microbiological Society keynote presentation at the Early Career Scientists forum in April 2019.

7.5 Concluding remarks

This complementary section of my thesis has been presented not only to acknowledge the relevance and increasing importance being placed on scientific outreach work but to convey that successes and achievements throughout the course of a PhD study (and beyond) can come in many forms that are, currently, not traditionally recognised in a thesis. However, science and how its impact is measured is an ever-evolving process which I believe is rapidly moving towards recognition of outreach "as a part of" research instead of outreach "in addition to" research.

CHAPTER 8

SUPPLEMENTARY INFORMATION

8.1 Chapter 2 Supplementary

Root hair mutations displace the barley rhizosphere microbiota

8.1.1 Appendix 1 (Chapter 2/ Supplementary data sheet 1.xlsx)

Appendix information can be obtained by contacting the author or Dr Davide Bulgarelli directly due to University guidelines not allowing the use of external storage such as a USB

Supplementary Database 1 contains the following information:

Worksheet ws1: Map_JH02_JH03_RHM_phyloseq.txt. This is the design file of the experiment;

Worksheet ws2: JH02_JH03_RHM_otu_table_nc2.txt. This is the OTU count table generated in QIIME;

Worksheet ws3: JH02_JH03_RHM_noPlant_OTUs_id.txt. The OTU ids after the in silico depletion of OTUs classified as either chloroplast or mitochondria;

Worksheet ws4: JH02_JH03_RHM_L2_visualisation.txt. This is the rarefied Phylum-level count matrix generated in QIIME where counts are collapsed for sample type;

Worksheet ws5: JH02_JH03_RHM_otu_table_nc2_noPlants_L2_rare_sample.txt. This is the rarefied phylum-level count matrix generated in QIIME used for statistical analysis in R;

Worksheet ws6: OTU_table_transposed.txt. This is the rarefied phylum-level count matrix generated in QIIME and converted in 'wide format';

Worksheet ws7: This is the result of the ANCOM analysis;

Worksheet ws8: JH02_JH03_RHM_dat_tax_noPlants_ordered.txt. This is file contains the taxonomy information where individual ranks are organised column-wise of the OTUs identified in the survey after the in silico depletion of OTUs classified as either chloroplast or mitochondria;

Worksheet ws9: JH02_JH03_RHM_data_phyloseq_rare_table_counts_2.txt. This is the OTU count table rarefied at even sequencing depth for alphadiversity calculation;

Worksheet ws10-13: Statistical and taxonomic information of the OTUs significantly enriched in and discriminating between rhizosphere and unplanted soil samples in the genotypes Karat, *rhl1.a*, Dema and *rhp1.b*, respectively (Quarryfield soil data);

Worksheets ws14-17: Statistical and taxonomic information of the OTUs differentially recruited in the pair-wise comparisons: Karat enriched vs. *rhl1.a; rhl.1a* enriched vs. Karat; Dema enriched vs. *rhp1.b; rhp1.b* enriched vs. Dema, respectively (Quarryfield soil data);

Worksheet ws18-21: Statistical and taxonomic information of the OTUs significantly enriched in and discriminating between rhizosphere and unplanted soil samples in the genotypes Karat, *rhl1.a*, Dema and *rhp1.b*, respectively (Tayport soil data);

Worksheets ws22-25: Statistical and taxonomic information of the OTUs differentially recruited in the pair-wise comparisons: Karat enriched vs. *rhl1.a; rhl1.a* enriched vs. Karat; Dema enriched vs. *rhp1.b; rhp1.b* enriched vs. Dema, respectively (Tayport soil data);

Worksheets ws26-27: Statistical and taxonomic information of the OTUs differentially recruited in the pair-wise comparisons: Karat enriched vs. Dema; Dema enriched vs. Karat respectively (Tayport soil data);

Worksheet ws28: Cumulative hypergeometric probabilities computed for Actinomycetales, Burkholderiales, Rhizobiales, Sphingomonadales and Xanthomonadales;

8.1.2. Supplementary Figure S1

Figure S1 Root hairs fine-tune the composition of the rhizosphere microbiota in a soil dependent manner
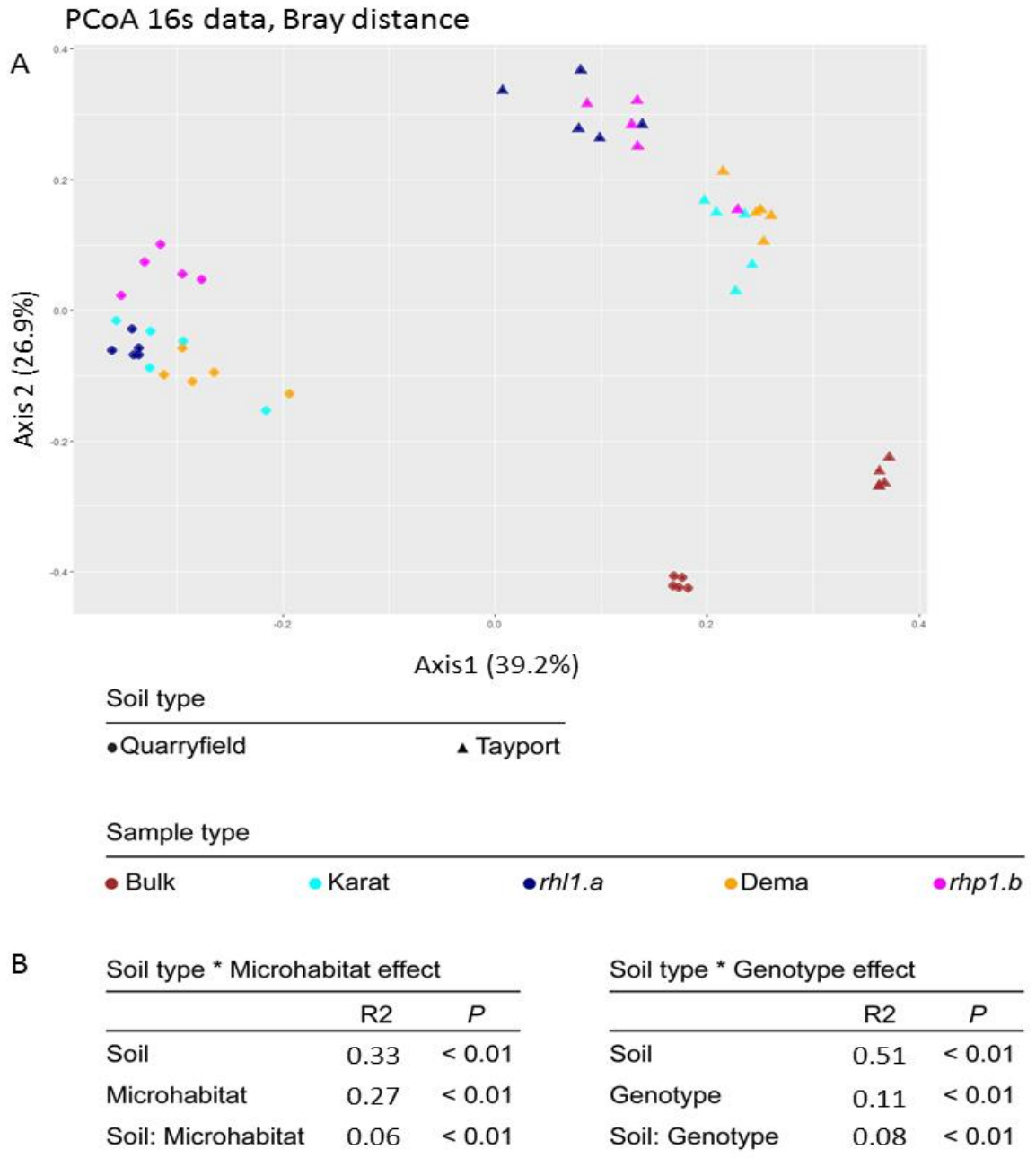
Figure S1 Root hairs fine-tune the composition of the rhizosphere microbiota in a soil dependent manner. *(A) PCoA computed using the Bray-Curtis distance (sensitive to OTU relative abundances). Replicates of bulk and rhizosphere samples are depicted by shapes whose spatial proximity reflects the degree of similarity of their microbiotas. (B) Permutational analysis of variances calculated using the Bray-Curtis distance matrix for the indicated effects. The R-squared values depict the proportion of variation in distances explained by the specified grouping of samples. Note that for the calculation of the Soil type * Genotype effect, bulk soil samples were omitted from the analysis. P values calculated for 5,000 permutations.*

8.2 Chapter 3 Supplementary

ROOT HAIR FORMATION AND MUTATIONS SHAPE THE CHEMICAL RHIZOSPHERE ENVIRONMENT

8.2.1 Appendix 2 Supplementary Database 2 (USB: Chapter 3/ Supplementary data sheet 2.xlsx)

Supplementary Database 2 contains the following information:

Worksheet ws1: Map_JH08_EXU.txt. This is the design file of the experiment;

Worksheet ws2: JH08_otu_table_nc2.txt. This is the OTU count table generated in QIIME;

Worksheet ws3: JH08_EXU_noPlant_OTUs_id.txt. The OTU ids after the in silico depletion of OTUs classified as either chloroplast or mitochondria;

Worksheet ws4: JH08_otu_table_nc2_noPlants_groupedL2.txt. This is the rarefied Phylum-level count matrix generated in QIIME used for statistical analysis in R;

Worksheet ws5: JH08_EXU_dat_tax_noPlants_ordered.txt. This is file contains the taxonomy information where individual ranks are organised column-wise of the OTUs identified in the survey after the *in silico* depletion of OTUs classified as either Chloroplast or mitochondria;

Worksheet ws6: JH08_EXU_data_phyloseq_rare_table_counts_2.txt. This is the OTU count table rarefied at even sequencing depth for alphadiversity calculation;

8.3 Chapter 4 Supplementary

THE PHYSICAL SOIL ENVIRONMENT INFLUENCES THE MICROBIOTA

8.3.1 Appendix 3 Supplementary Database 3 (USB: Chapter 4/ Supplementary data sheet 4.xlsx)

Supplementary Database 3 contains the following information:

Worksheet ws1: Map_JH08_phyloseq.txt. This is the design file of the experiment;

Worksheet ws2: JH02_JH08_DEN_otu_table_nc2.txt. This is the OTU count table generated in QIIME;

Worksheet ws3: JH08_DEN_noPlant_OTUs_id.txt. The OTU ids after the in silico depletion of OTUs classified as either chloroplast or mitochondria;

Worksheet ws4: JH08_DEN_otu_table_nc2_noPlants_L2_rare_sample.txt. This is the rarefied phylum-level count matrix generated in QIIME used for statistical analysis in R;

Worksheet ws5: JH08_DEN_dat_tax_noPlants_ordered.txt. This is file contains the taxonomy information where individual ranks are organised column-wise of the OTUs identified in the survey after the *in silico* depletion of OTUs classified as either chloroplast or mitochondria;

Worksheet ws6: JH08_DEN_data_phyloseq_rare_table_counts_2.txt. This is the OTU count table rarefied at even sequencing depth for alphadiversity calculation;

Worksheet ws7-10: Statistical and taxonomic information of the OTUs significantly enriched in and discriminating between 1.3, 1.2 and 1.1 g cm^3 datasets;

8.4 Chapter 5 Supplementary

THE ISOLATION AND CATEGORISATION OF INDIVIDUAL BACTERIAL MEMBERS OF THE RHIZOSPHERE MICROBIOTA

8.4.1 Appendix 4: Supplementary Database 4 (USB: Chapter 5/ Supplementary data sheet 5.xlsx)

Worksheet ws1: Bacterial isolates logfile: This details the assigned taxonomy based on Sanger and Amphora sequencing, Isolation conditions and NGS quality filtering data;

Worksheet ws2: seq_wide_ENV.csv: This details the KEGG ortholog assignment of all isolates including environmental factors;

Worksheet ws3: seq_wide_ENVP.csv: This details the KEGG ortholog assignment of Pseudomonas isolates including environmental factors;

Worksheet ws4: seq_wide_ENVM.csv: This details the KEGG ortholog assignment of Microbacterium isolates including environmental factors;

Worksheet ws5: seq_wide_ENVS.csv: This details the KEGG ortholog assignment of Stenotrophomonas isolates including environmental factors;

Worksheet ws6: seq_wide_ENVB.csv: This details the KEGG ortholog assignment of Bacillus isolates including environmental factors;

Worksheet ws7: seq_wide_ENVO.csv: This details the KEGG ortholog assignment of "Others" isolates including environmental factors;

Worksheet ws8: eggnog.csv: Combined KO, GO and EC ortholog assignment for all isolates;

8.4.2 Appendix 5: Supplementary Database 5 (USB: Chapter 5/ Supplementary data sheet 6.xlsx)

This database shows all isolates sorted by gene category based on the assignment of GO terms from RAST

Worksheet ws1: Amino acids and derivatives

Worksheet ws2: Carbohydrates

Worksheet ws3: Cell division and cell cycle

Worksheet ws4: Cell wall and capsule

Worksheet ws5: Cofactors, vitamins, prosthetic groups, pigments

Worksheet ws6: DNA metabolism

Worksheet ws7: Dormancy and sporulation

Worksheet ws8: Fatty acids, lipids, and isoprenoids

Worksheet ws9: Iron acquisition and metabolism

Worksheet ws10: Membrane transport

Worksheet ws11: Metabolism of aromatic compounds

Worksheet ws12: Miscellaneous

Worksheet ws13: Motility and chemotaxis

Worksheet ws14: Nitrogen metabolism

Worksheet ws15: Nucleosides and nucleotides

Worksheet ws16: Phages, prophages, transposable elements, plasmids

Worksheet ws17: Phosphorus metabolism

Worksheet ws18: Potassium metabolism

Worksheet ws19: Protein metabolism

Worksheet ws20: Regulation and cell signalling

Worksheet ws21: Respiration

Worksheet ws22: RNA metabolism

Worksheet ws23: Secondary metabolism

Worksheet ws24: Stress response

Worksheet ws25: Sulfur metabolism

Worksheet ws26: Virulence, disease and defence

8.4.3 Supplementary Figures

Figure S2: KEGG ortholog intersection map of Microbacterium. *Intersection size denotes the number of KEGG orthologs shared by the isolates indicated by the corresponding circle below the intersection. The length of the coloured bar next to each isolate represents the length of the sequence assembly.*

Figure S3: PCoA of KEGG orthologs assigned to each Microbacterium isolate based on taxonomy. *Each colour denotes the study each isolate was identified in based on OTU assignment from 16S sequence data. Shapes represent the species assigned to each isolate based on Amphora analysis of full genome sequence data. The proximity of each individual isolate to another reflects the relatedness of each isolate based on the presence or absence of KEGG orthologs.*

Figure S4: PCoA of KEGG orthologs assigned to each *Microbacterium* isolate based on environment. *Each colour denotes the study each isolate was identified in based on OTU assignment from 16S sequence data. Shapes represent the species assigned to each isolate based on Amphora analysis of full genome sequence data. The proximity of each individual isolate to another reflects the relatedness of each isolate based on the presence or absence of KEGG orthologs.*

Figure S5: KEGG ortholog intersection map of Bacillus. *Intersection size denotes the number of KEGG orthologs shared by the isolates indicated by the corresponding circle below the intersection. The length of the coloured bar next to each isolate represents the length of the sequence assembly.*

Figure S6: PCoA of KEGG orthologs assigned to each Bacillus isolate based on taxonomy. *Each shape denotes the study each isolate was identified in based on OTU assignment from 16S sequence data. Colours represent the species assigned to each isolate based on Amphora analysis of full genome sequence data. The proximity of each individual isolate to another reflects the relatedness of each isolate based on the presence or absence of KEGG orthologs.*
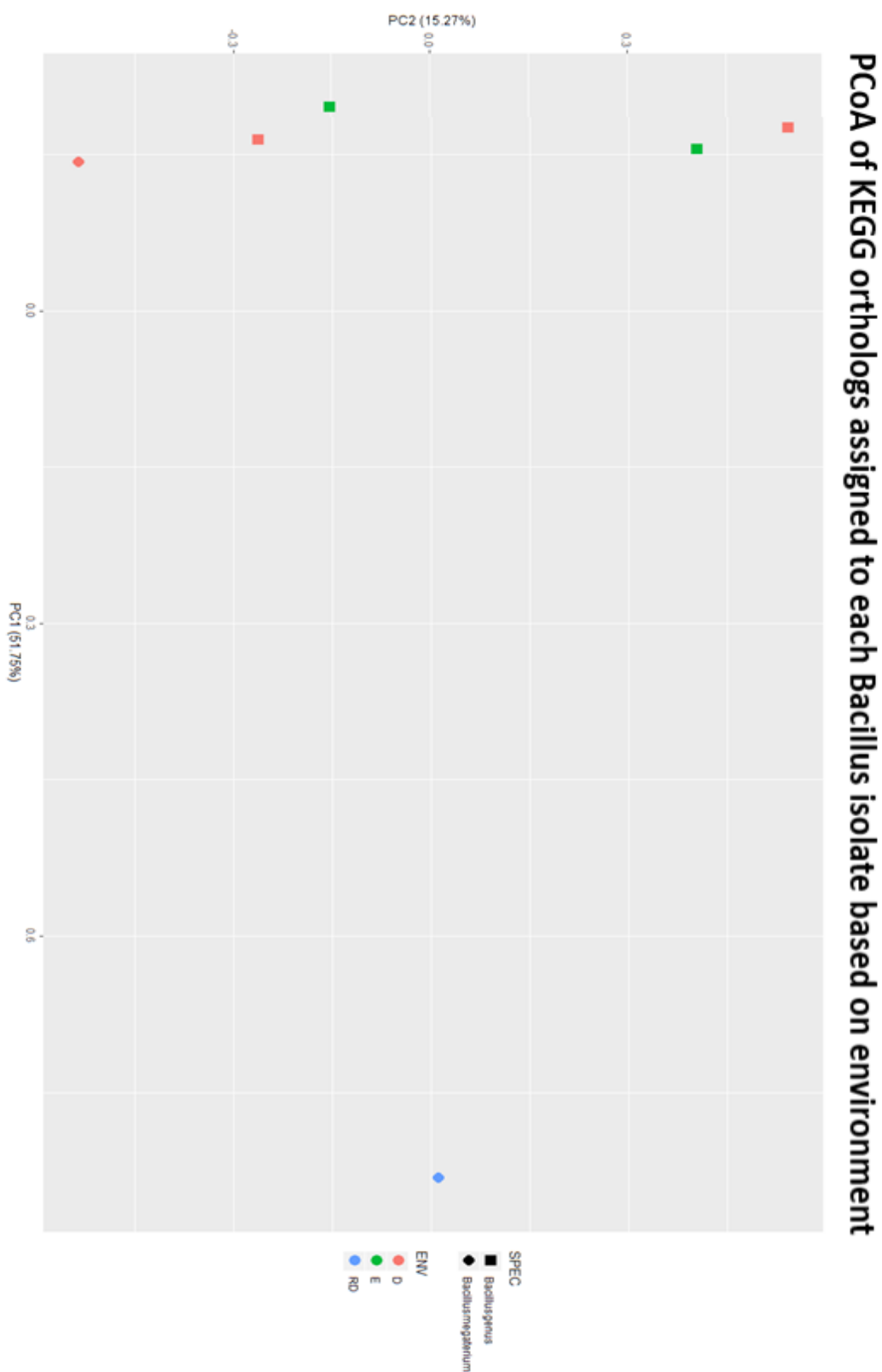
Figure S7: PCoA of KEGG orthologs assigned to each Bacillus isolate based on environment. *Each colour denotes the study each isolate was identified in based on OTU assignment from 16S sequence data. Shapes represent the species assigned to each isolate based on Amphora analysis of full genome sequence data. The proximity of each individual isolate to another reflects the relatedness of each isolate based on the presence or absence of KEGG orthologs.*
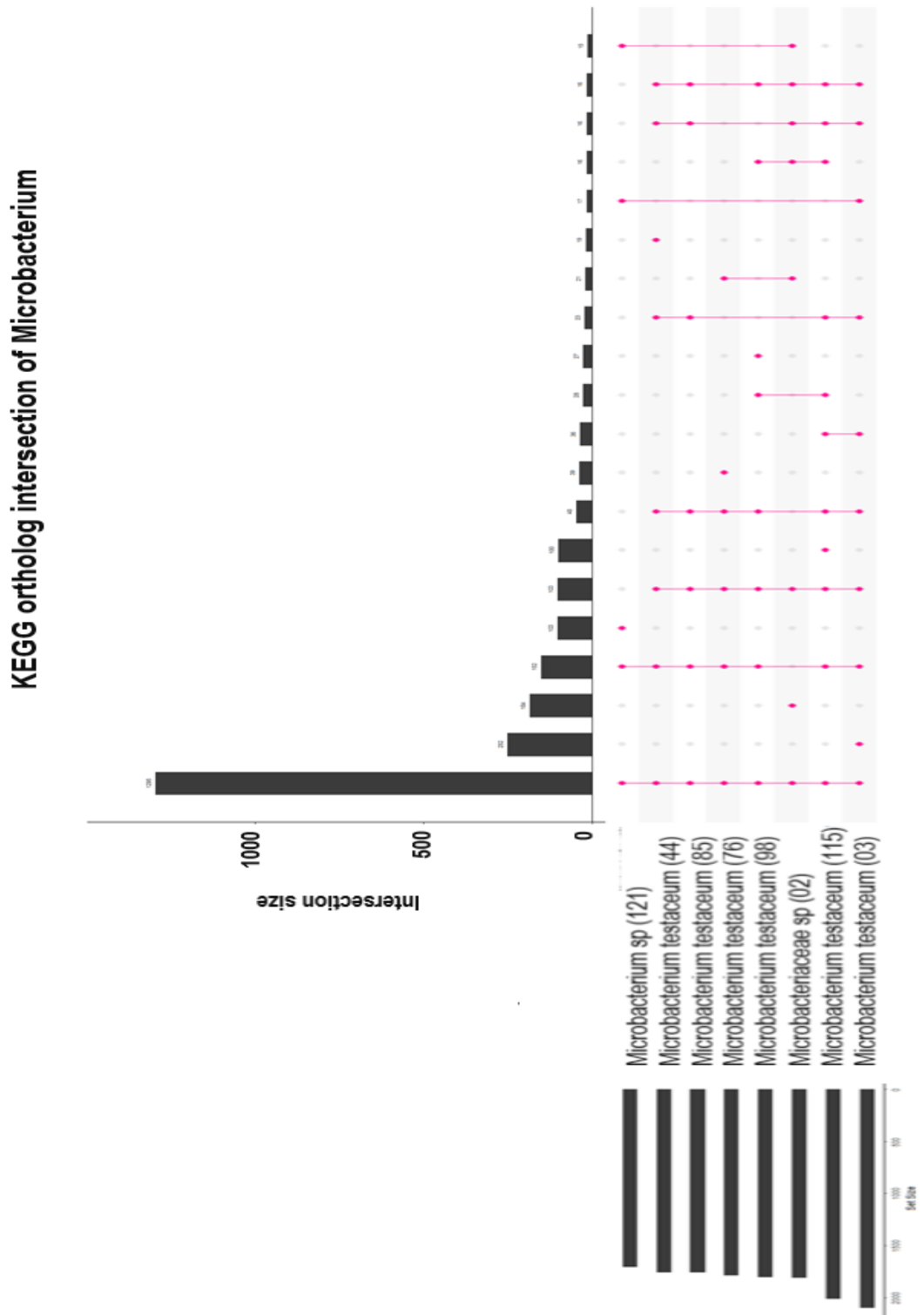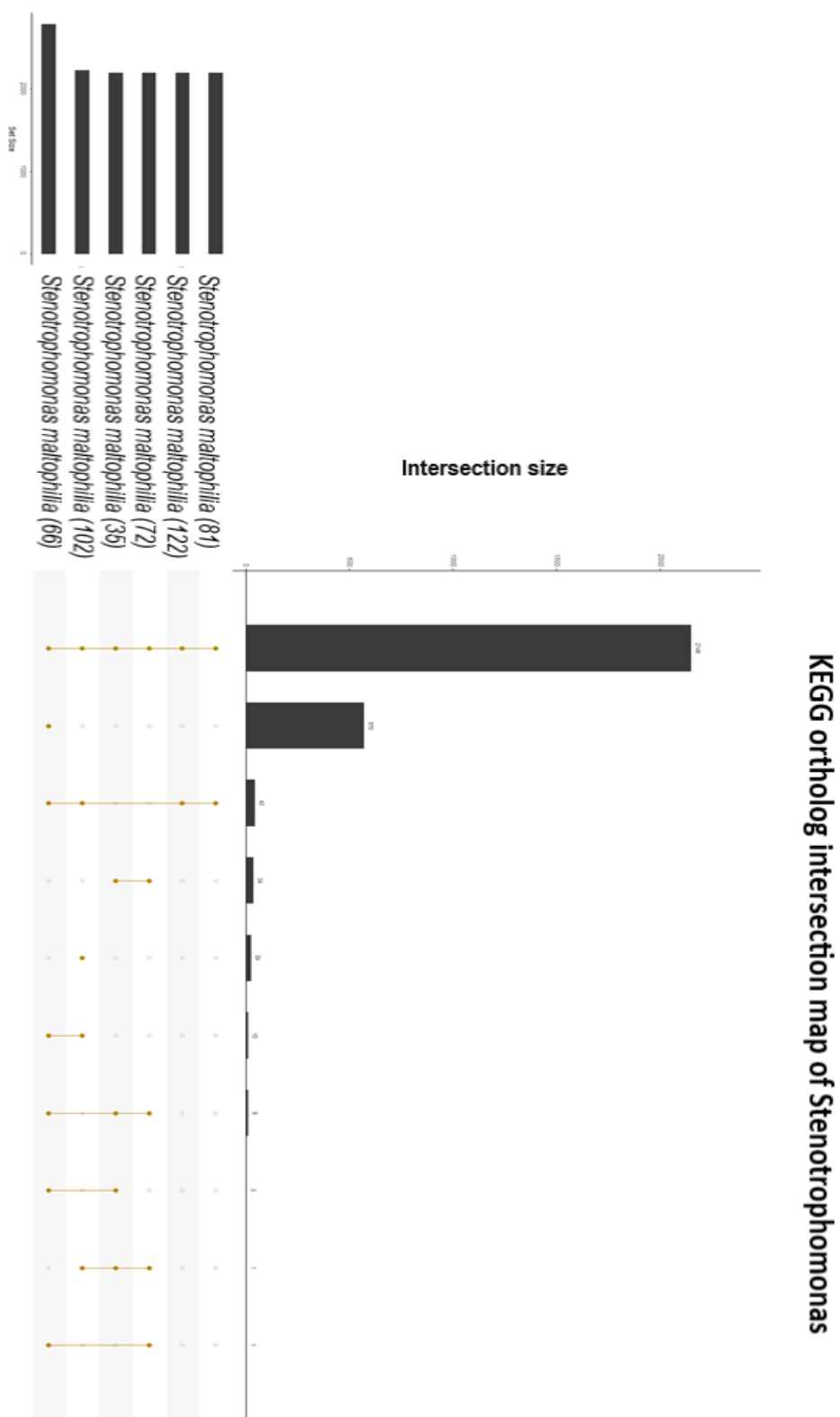
Figure S8 KEGG ortholog intersection map of Stenotrophomonas. *Intersection size denotes the number of KEGG orthologs shared by the isolates indicated by the corresponding circle below the intersection. The length of the coloured bar next to each isolate represents the length of the sequence assembly.*
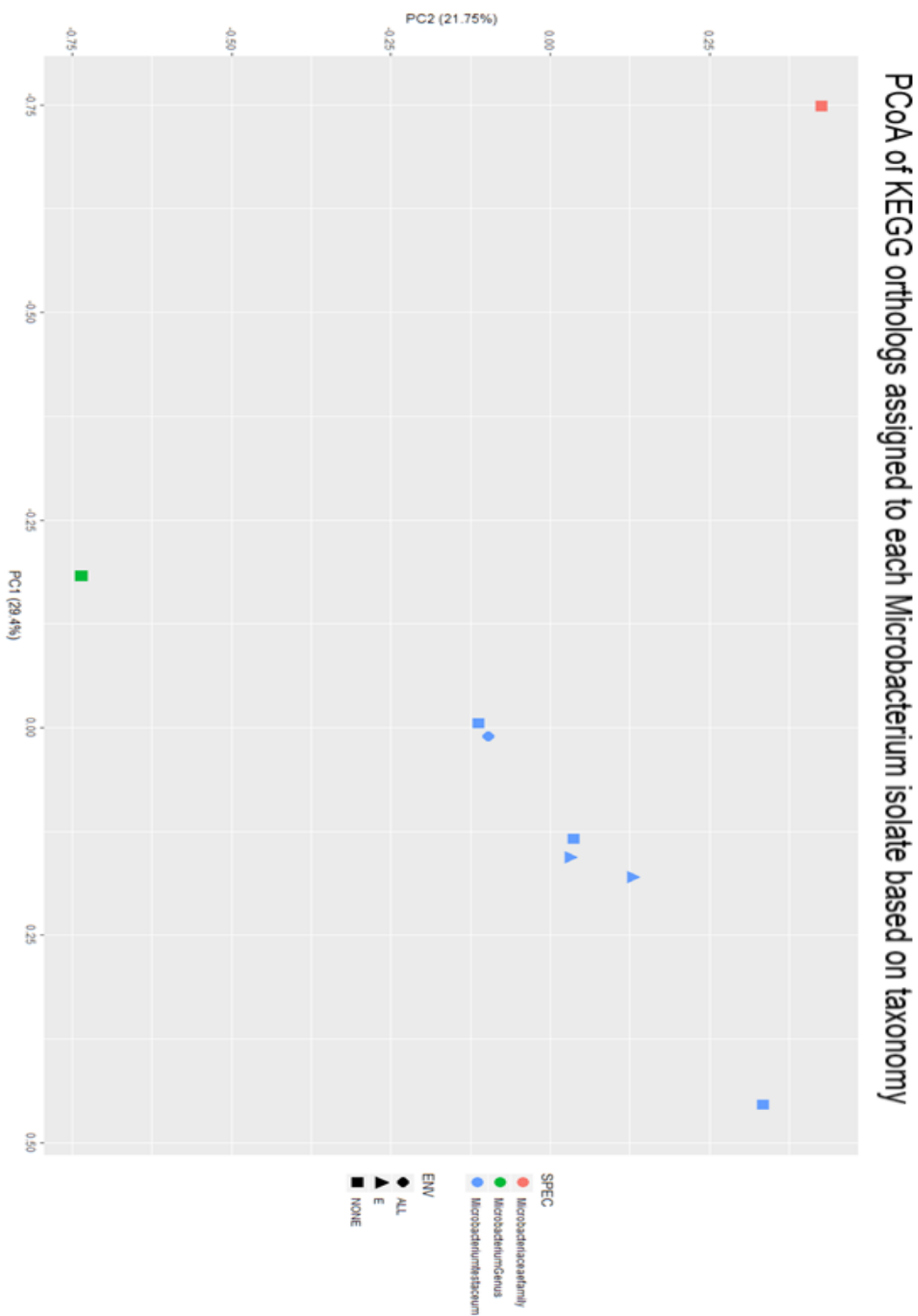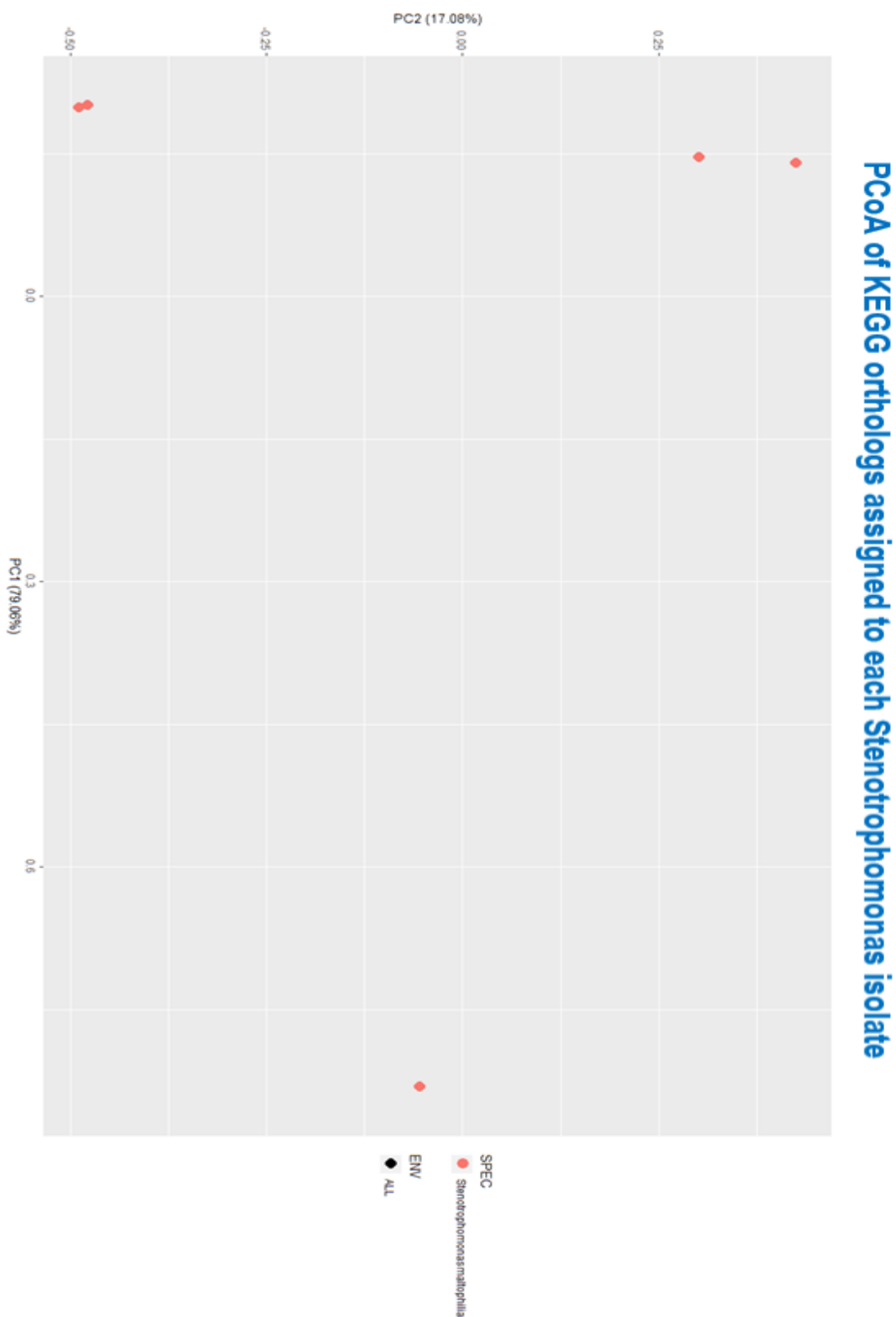
Figure S9: PCoA of KEGG orthologs assigned to each Stenotrophomonas isolate. *Each shape denotes the study each isolate was identified in based on OTU assignment from 16S sequence data. Colours represent the species assigned to each isolate based on Amphora analysis of full genome sequence data. The proximity of each individual isolate to another reflects the relatedness of each isolate based on the presence or absence of KEGG orthologs.*
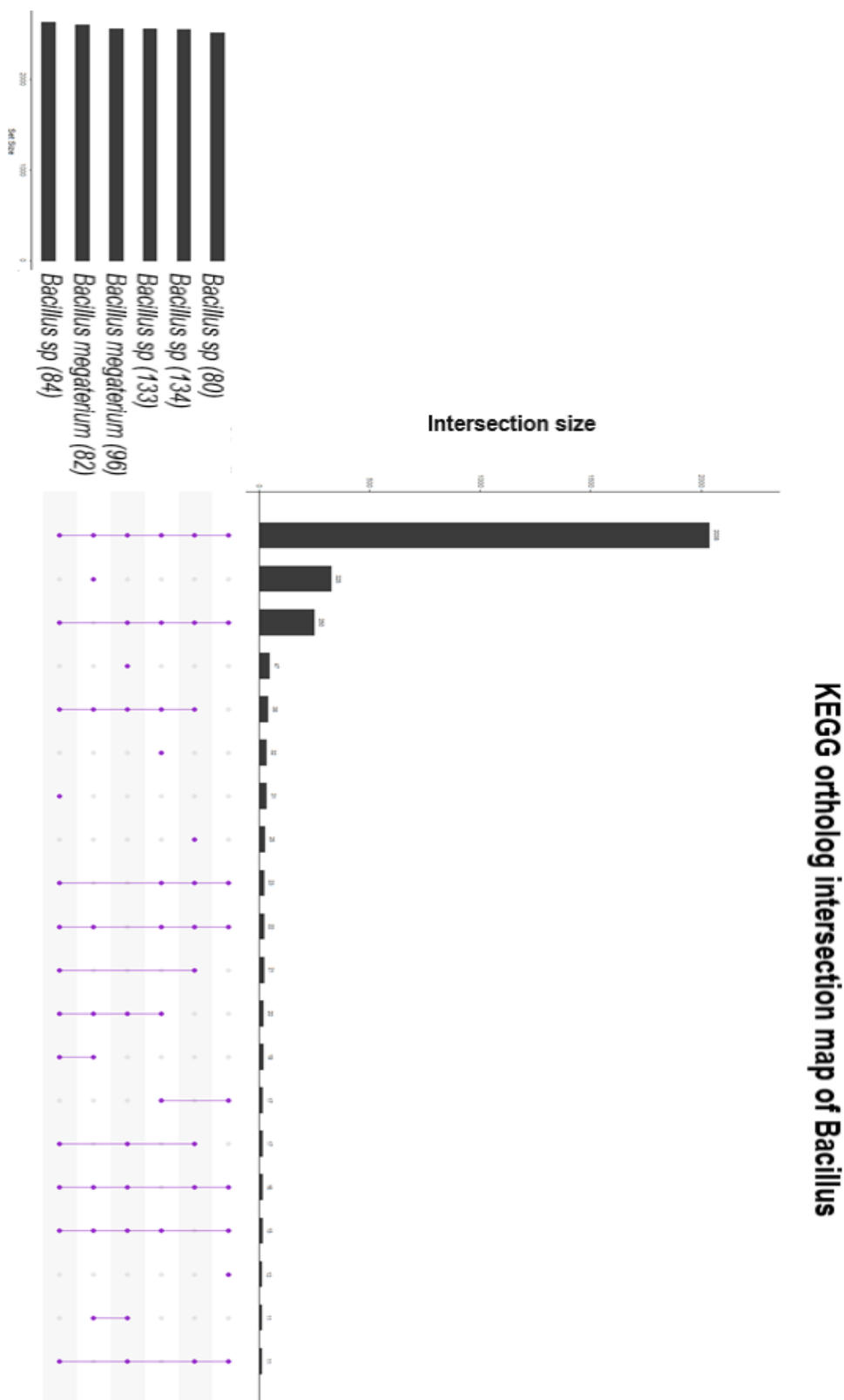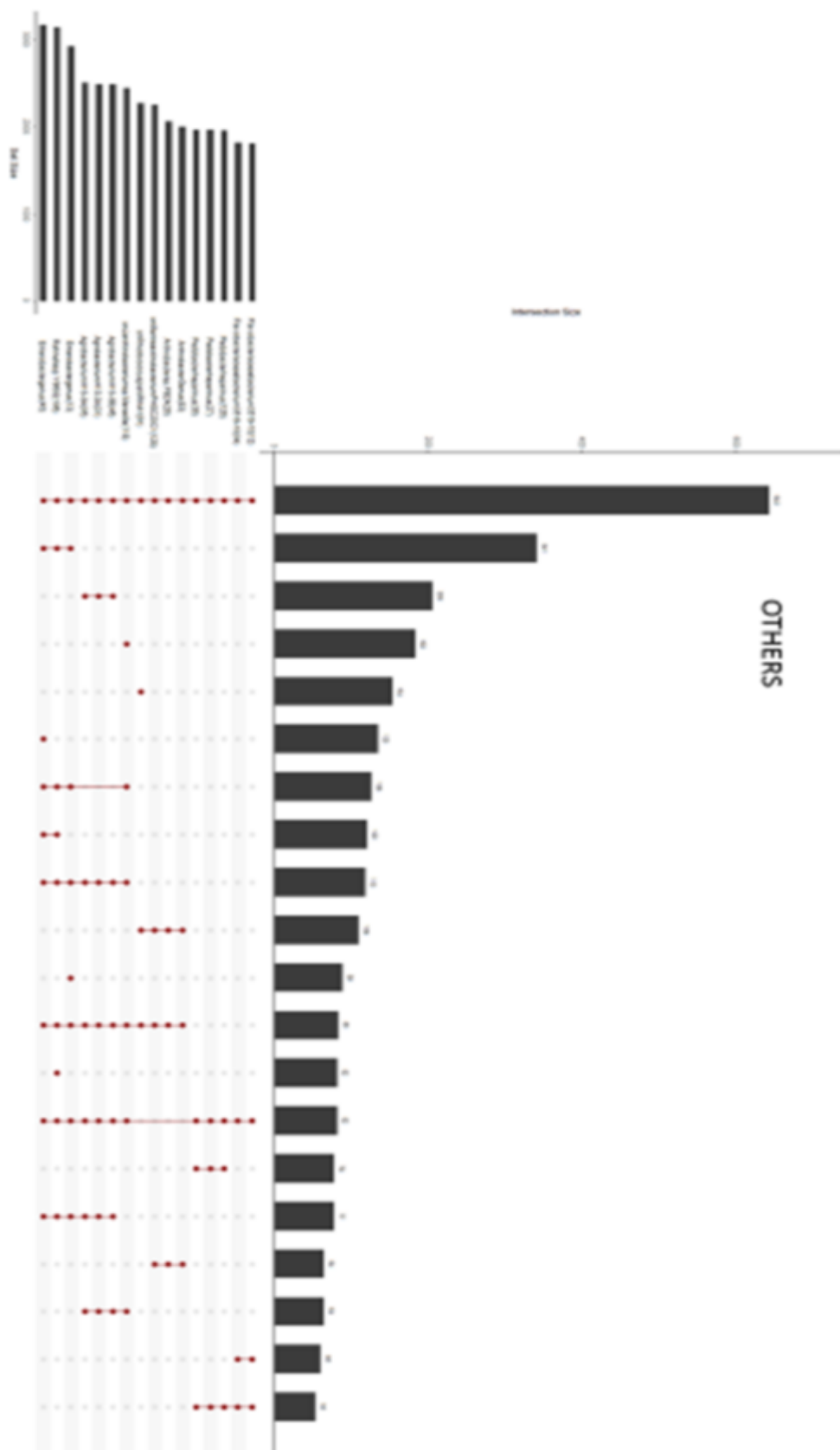
Figure S10 KEGG ortholog intersection map of "Other" isolates*. Intersection size denotes the number of KEGG orthologs shared by the isolates indicated by the corresponding circle below the intersection. The length of the coloured bar next to each isolate represents the length of the sequence assembly.*
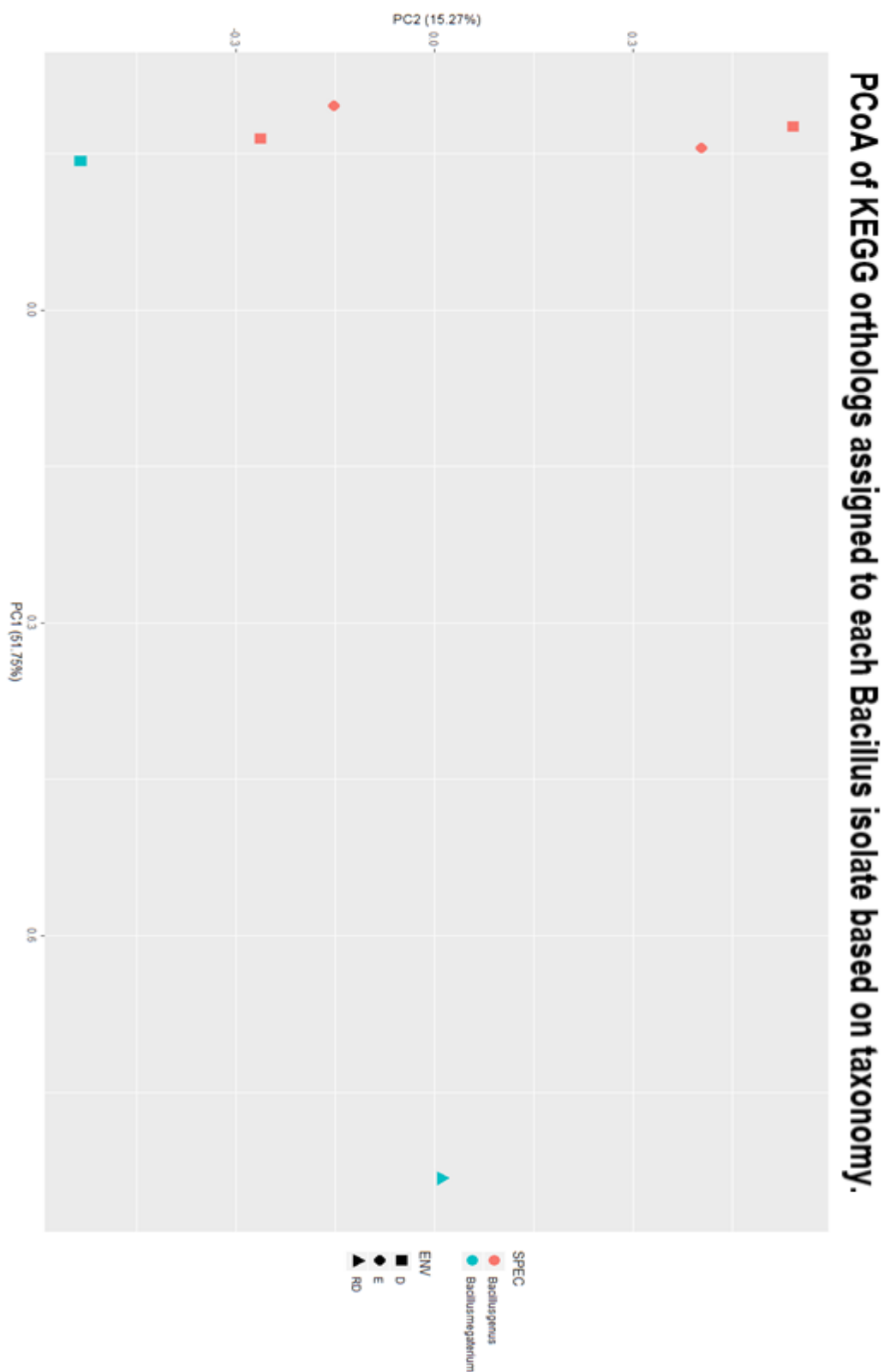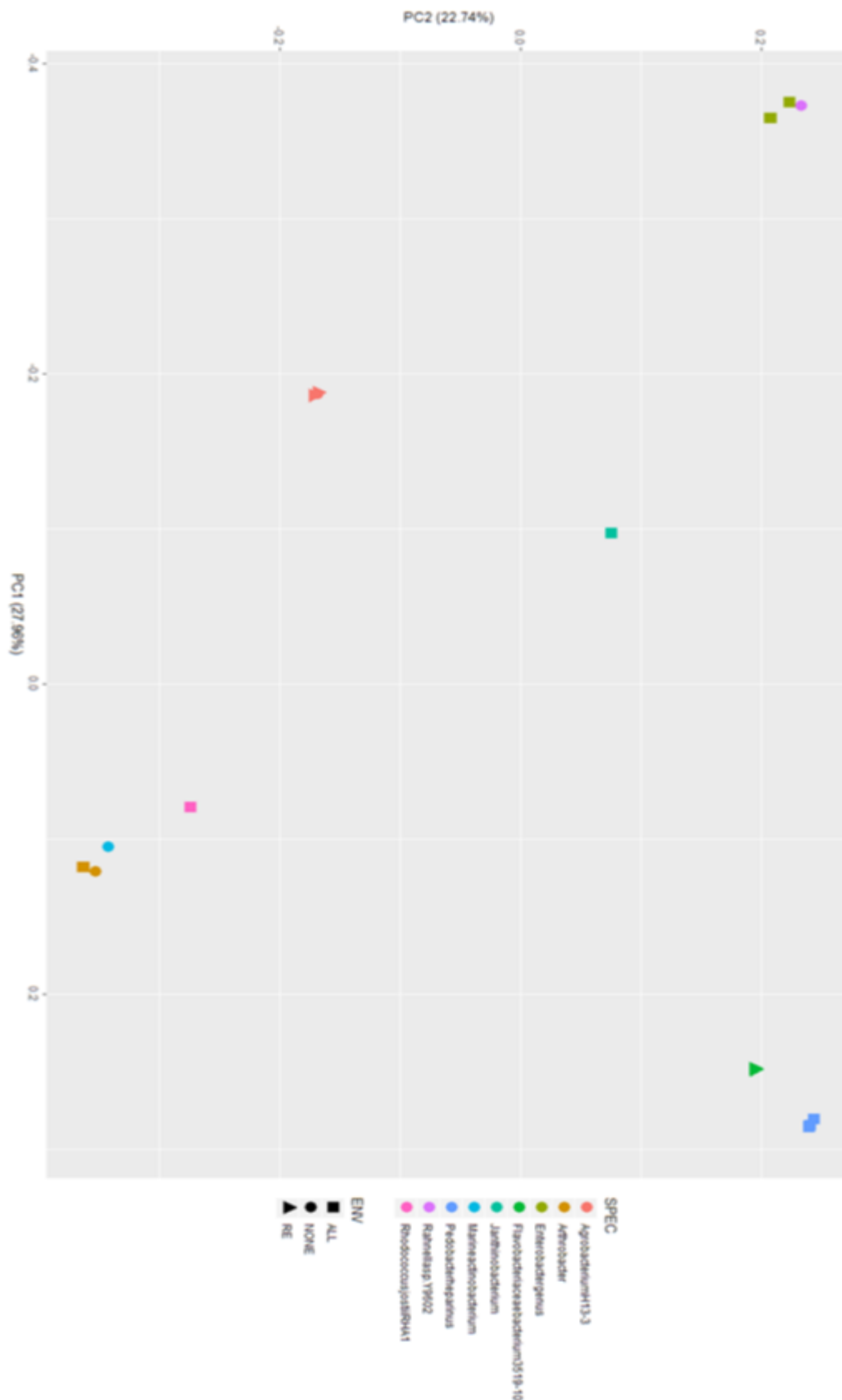
Figure S11 PCoA of KEGG orthologs assigned to each "Others" isolate. Each shape denotes the study each isolate was identified in based on OTU assignment from 16S sequence data. Colours represent the species assigned to each isolate based on Amphora analysis of full genome sequence data. The proximity of each individual isolate to another reflects the relatedness of each isolate based on the presence or absence of KEGG orthologs.

Figure S12 PCoA of KEGG orthologs assigned to each "Others" isolate based on environment. Each colour denotes the study each isolate was identified in based on OTU assignment from 16S sequence data. Shapes represent the species assigned to each isolate based on Amphora analysis of full genome sequence data. The proximity of each individual isolate to another reflects the relatedness of each isolate based on the presence or absence of KEGG orthologs.

Figure S13 Single, shared and singleton proteomics of all bacterial isolates. *Colours depict Single, shared or singleton proportions*
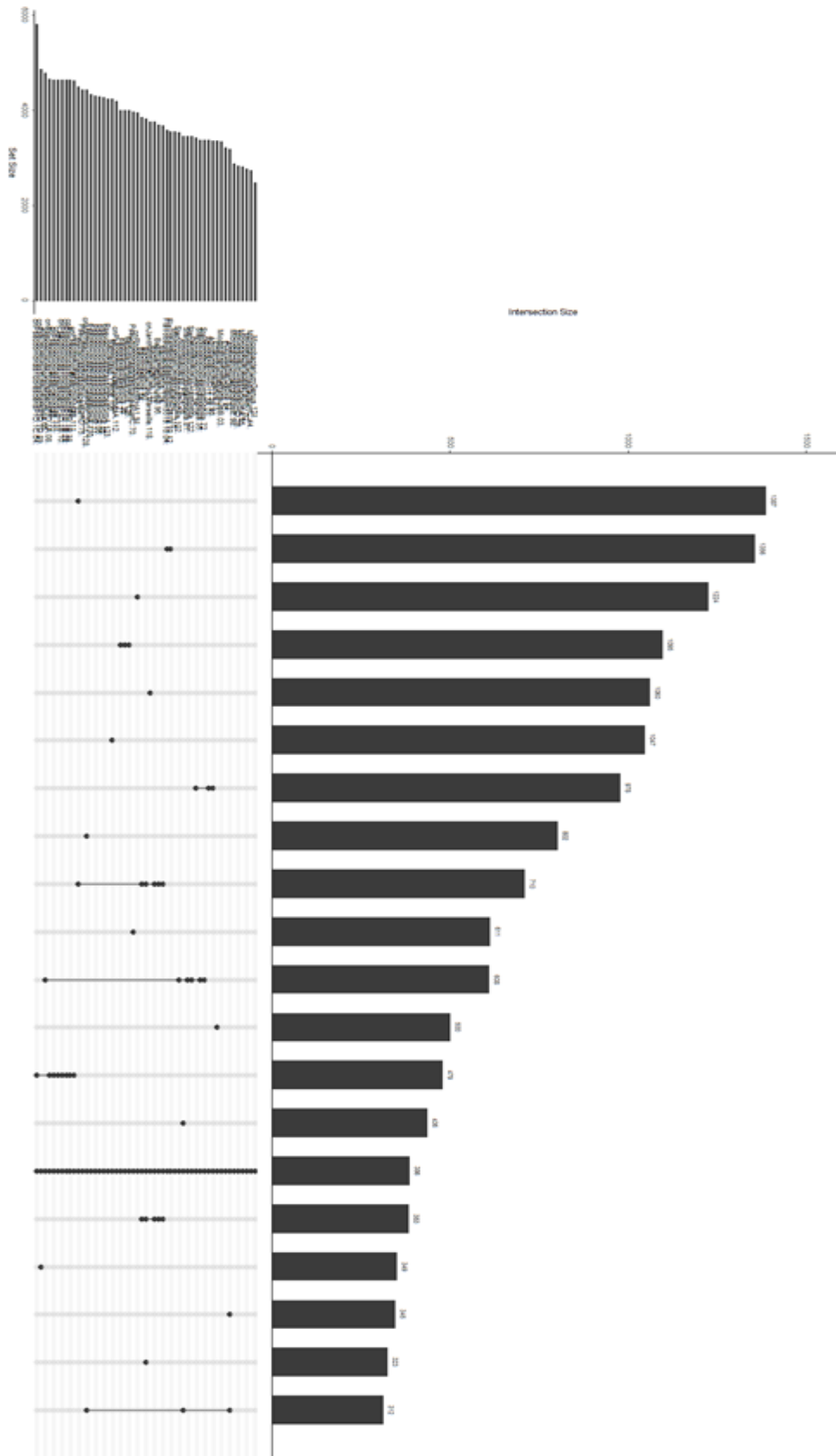
Figure S14 Proteome intersect for all bacterial isolates. *Intersection size denotes the number of proteomes shared by the isolates indicated by the corresponding circle below the intersection. The length of the coloured bar next to each isolate represents the length of the assembly.*
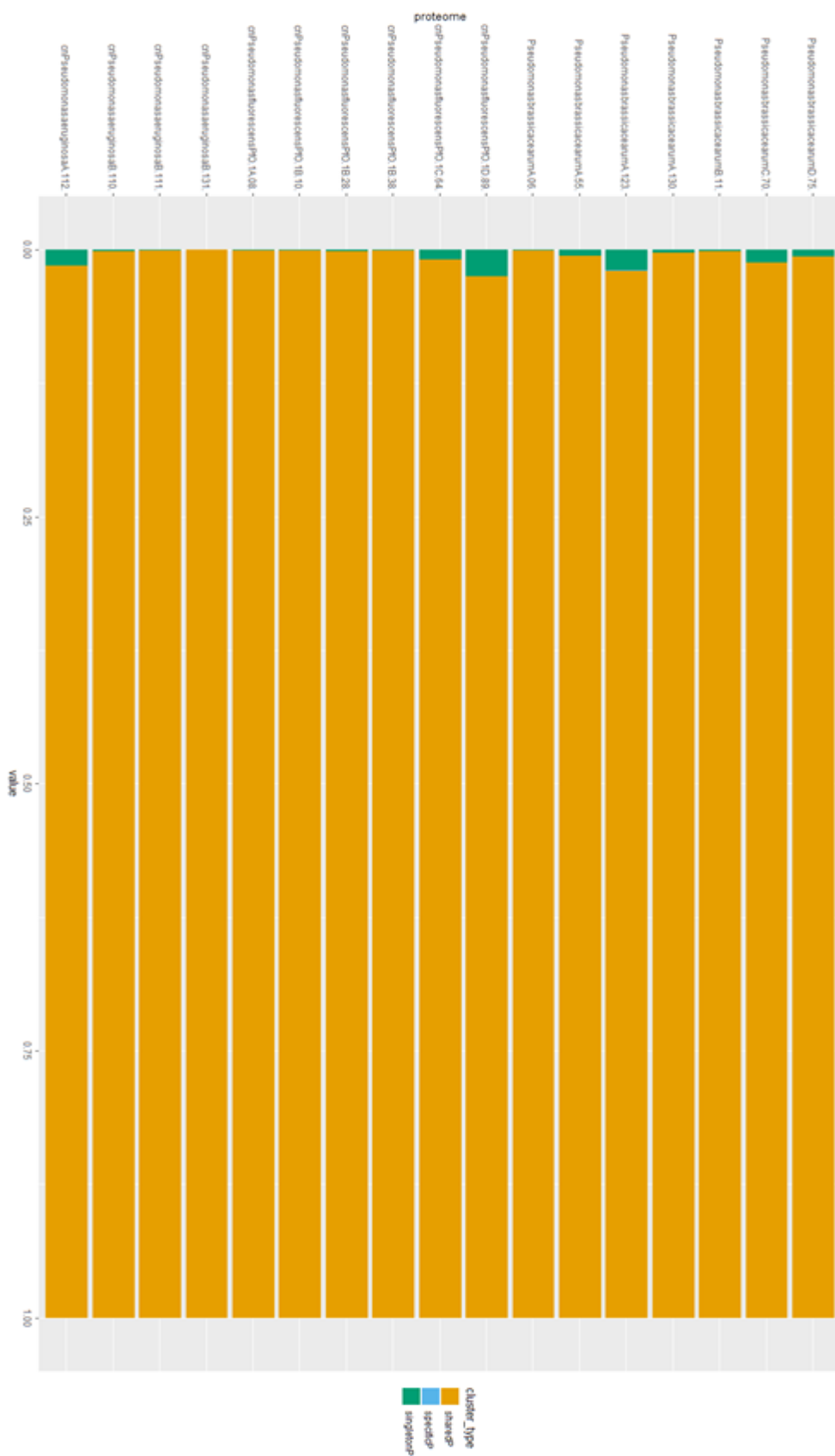
Figure S15 Single, shared and singleton proteomics of all Pseudomonas isolates. *Colours depict Single, shared or singleton proportions*
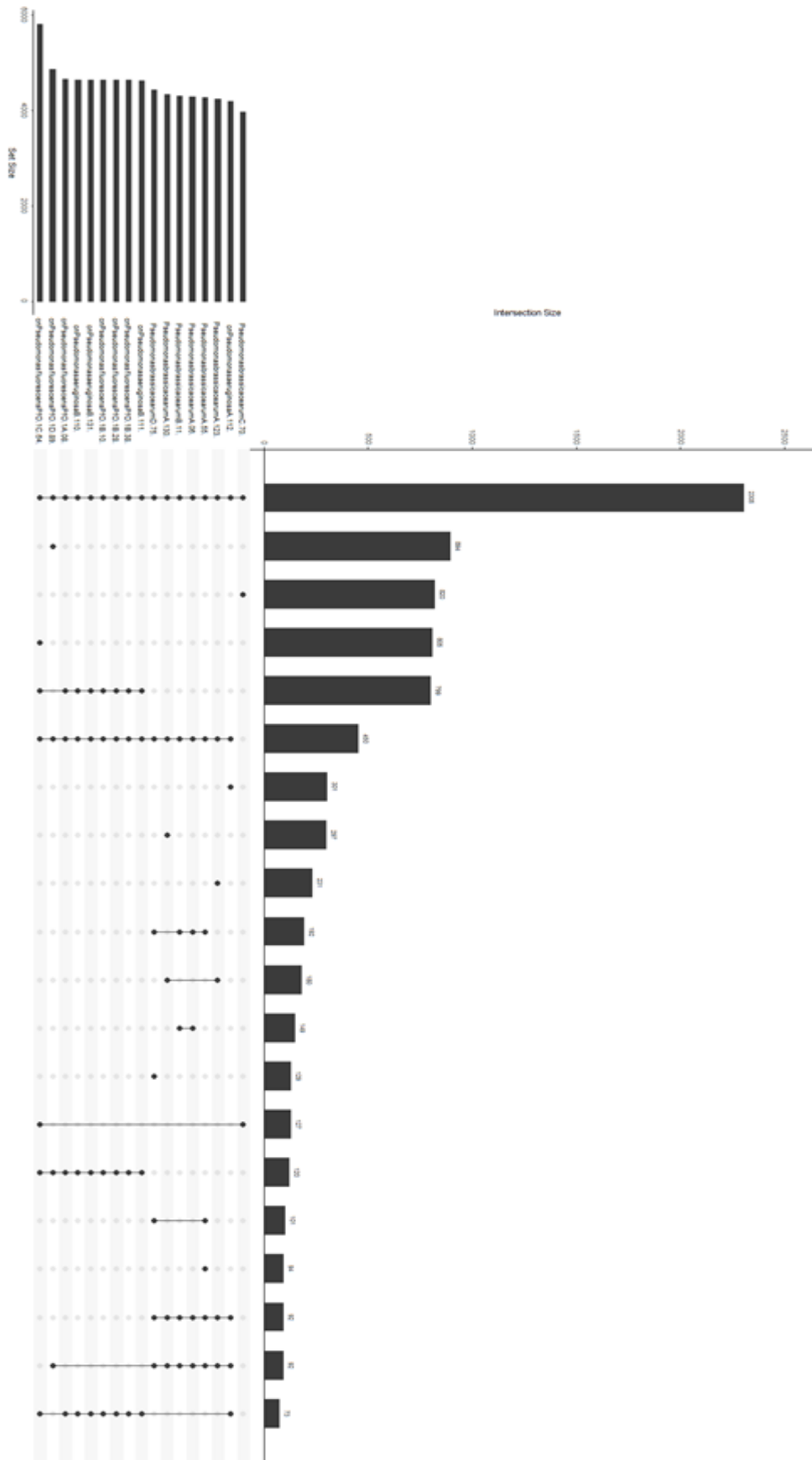
Figure S16 Proteome intersect for Pseudomonas isolates. Intersection size denotes the number of proteomes shared by the isolates indicated by the corresponding circle below the intersection. The length of the coloured bar next to each isolate represents the length of the assembly.
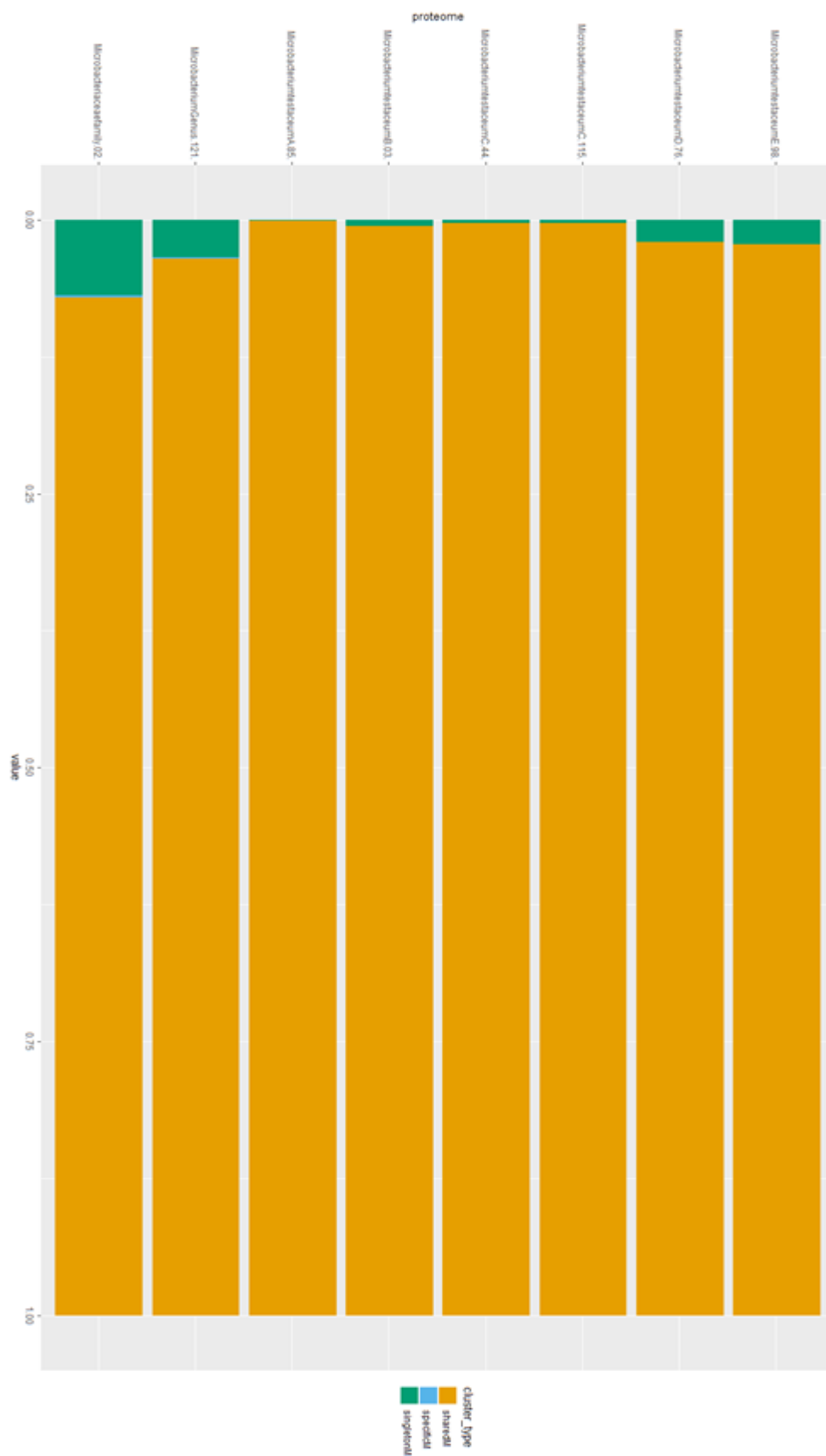
Figure S17 Single, shared and singleton proteomics of all Microbacterium isolates. *Colours depict Single, shared or singleton proportions*
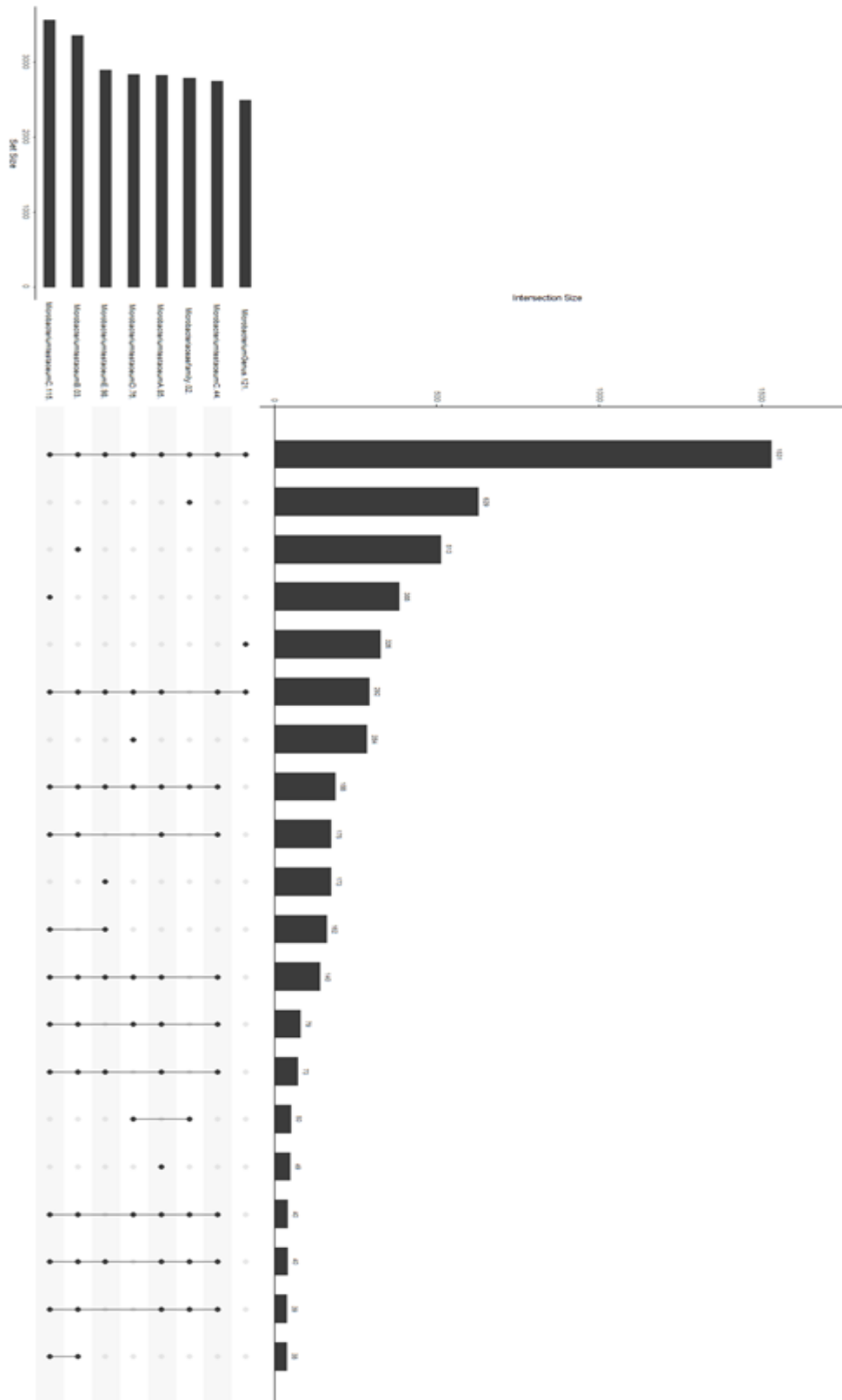
Figure S18 Proteome intersect for Microbacterium isolates. *Intersection size denotes the number of proteomes shared by the isolates indicated by the corresponding circle below the intersection. The length of the coloured bar next to each isolate represents the length of the assembly.*
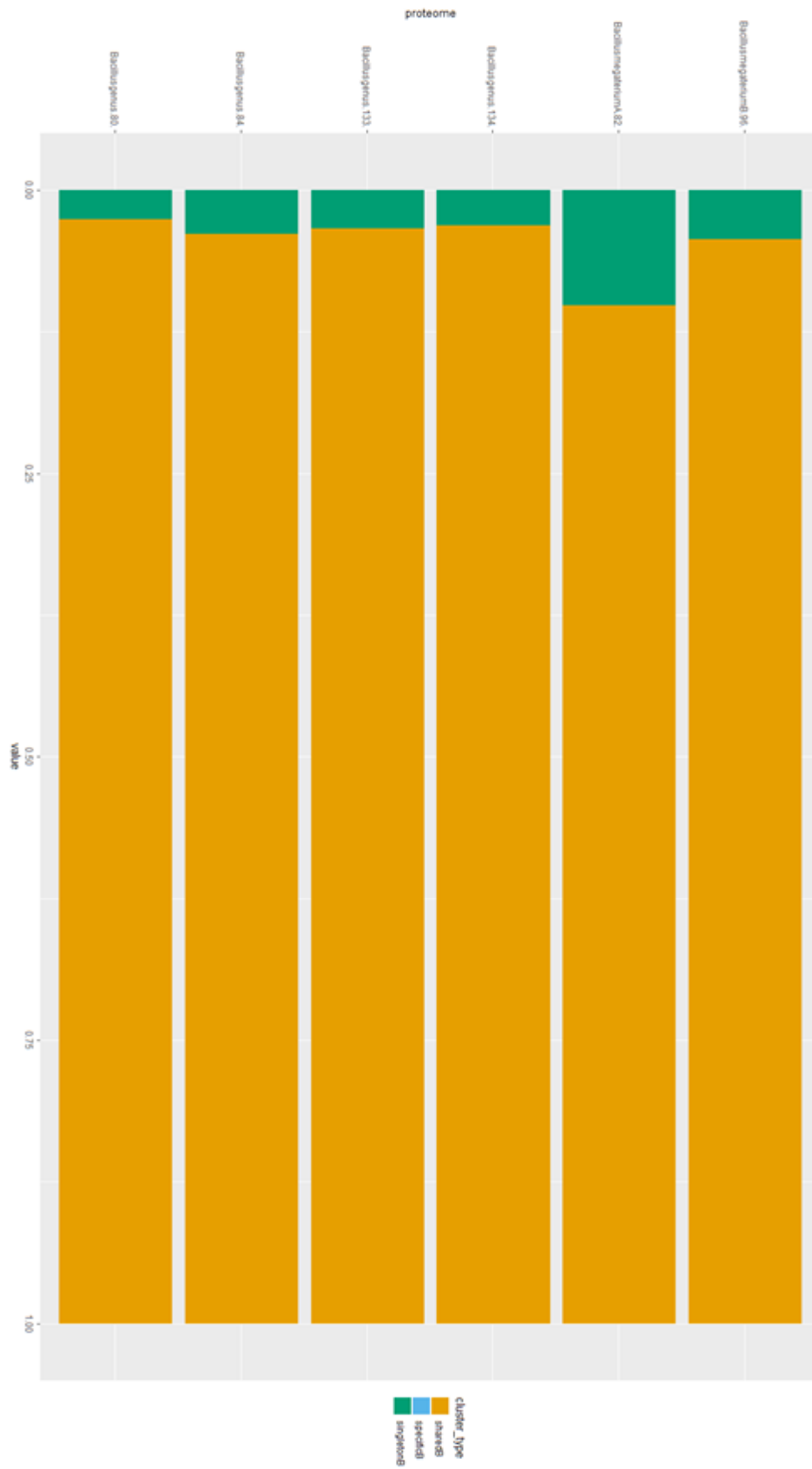
Figure S19 Single, shared and singleton proteomics of all Bacillus isolates. *Colours depict Single, shared or singleton proportions*
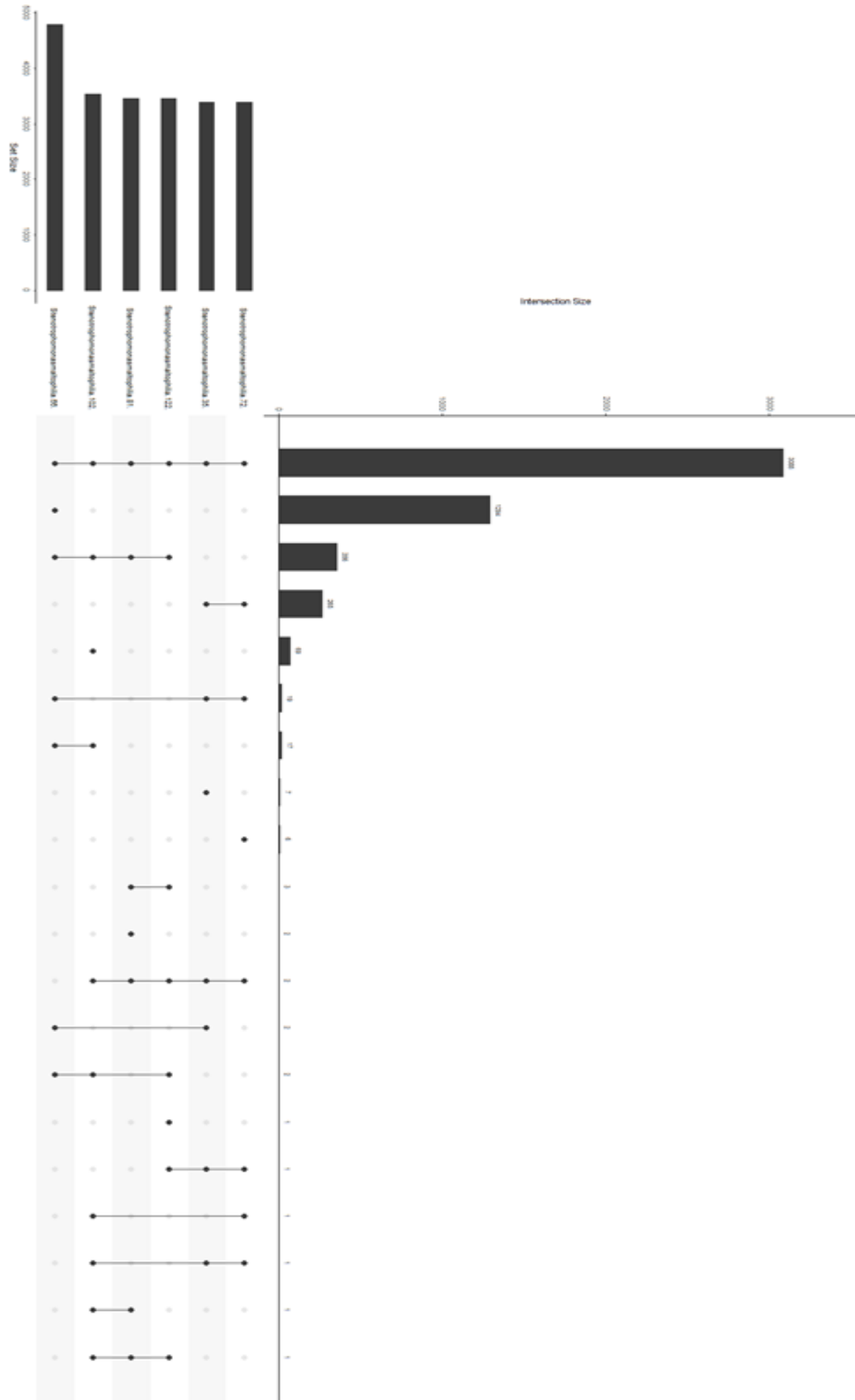
Figure S20 Proteome intersect for Bacillus isolates. *Intersection size denotes the number of proteomes shared by the isolates indicated by the corresponding circle below the intersection. The length of the coloured bar next to each isolate represents the length of the assembly.*
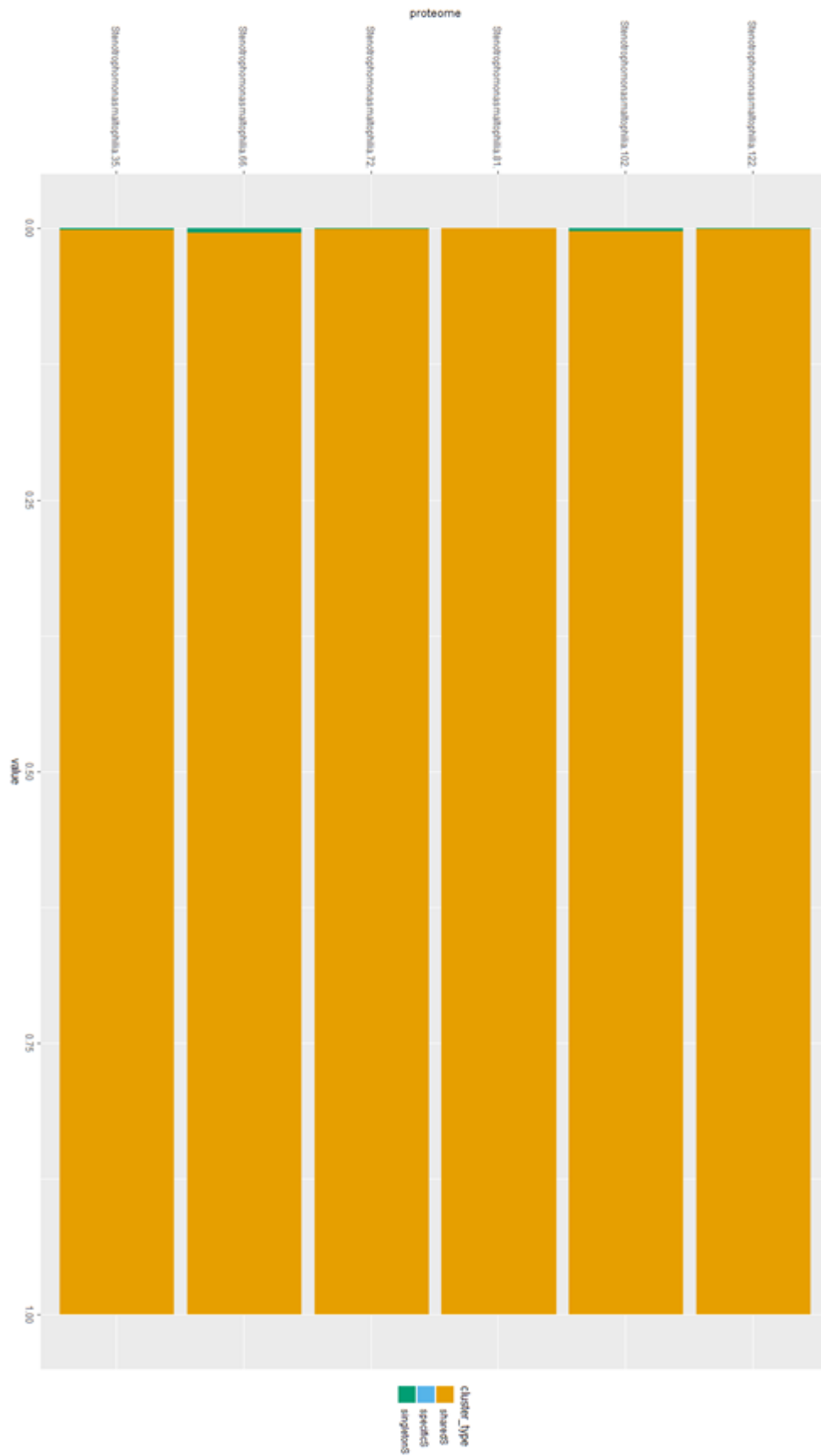
Figure S21 Single, shared and singleton proteomics of all Stenotrophomonas isolates. *Colours depict Single, shared or singleton proportions*
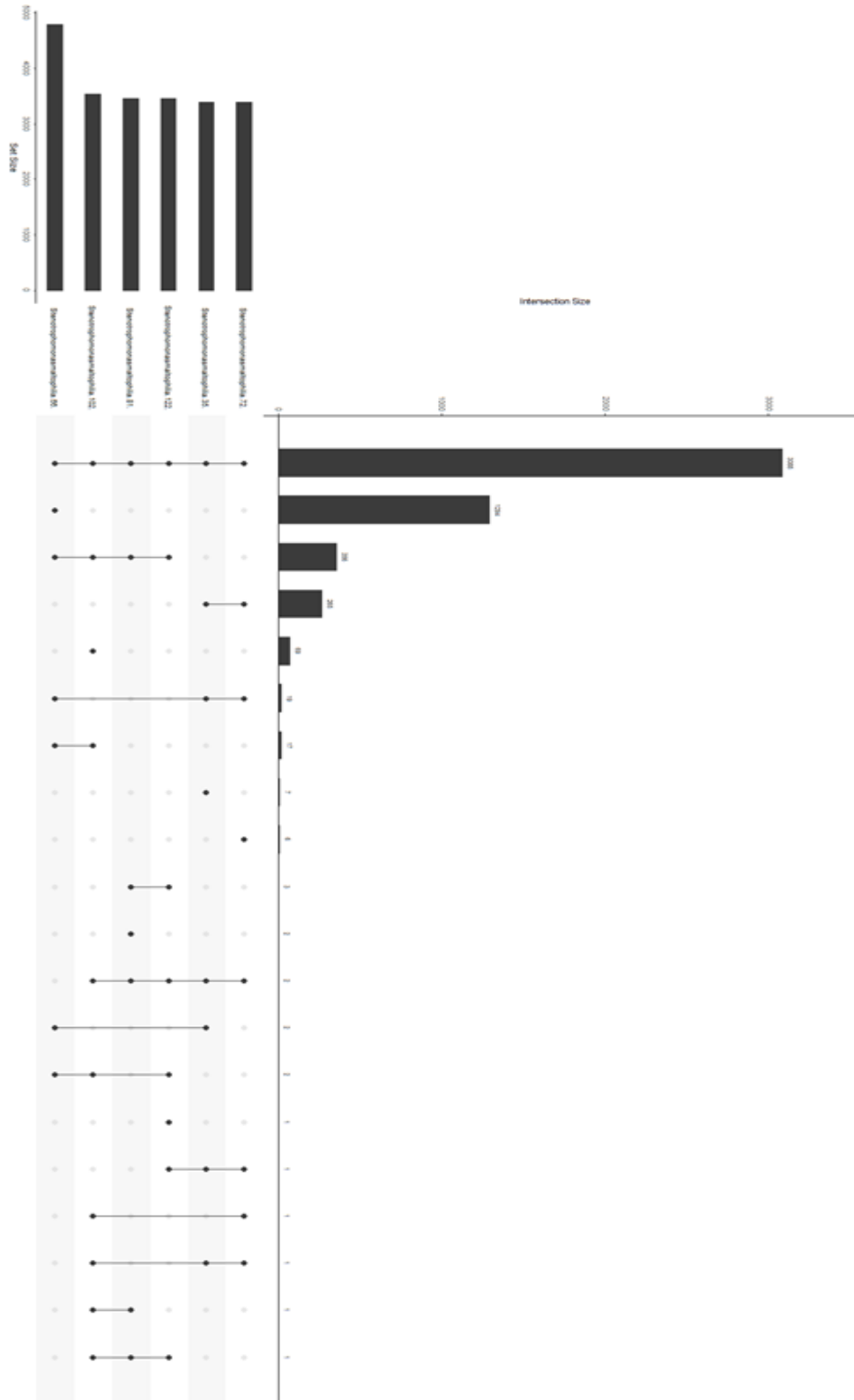
Figure S22 Proteome intersect for Stenotrophomonas isolates. *Intersection size denotes the number of proteomes shared by the isolates indicated by the corresponding circle below the intersection. The length of the coloured bar next to each isolate represents the length of the assembly.*
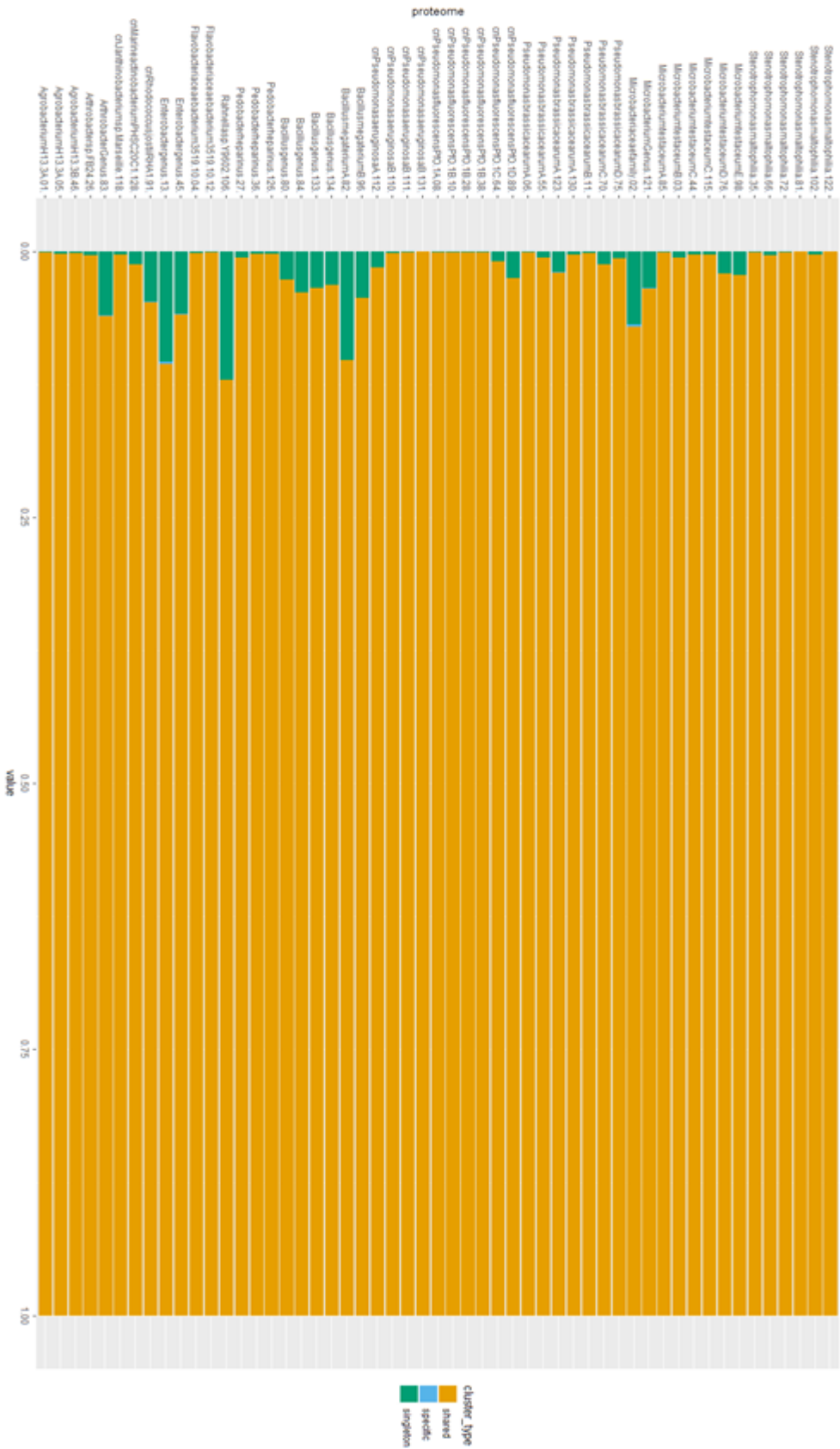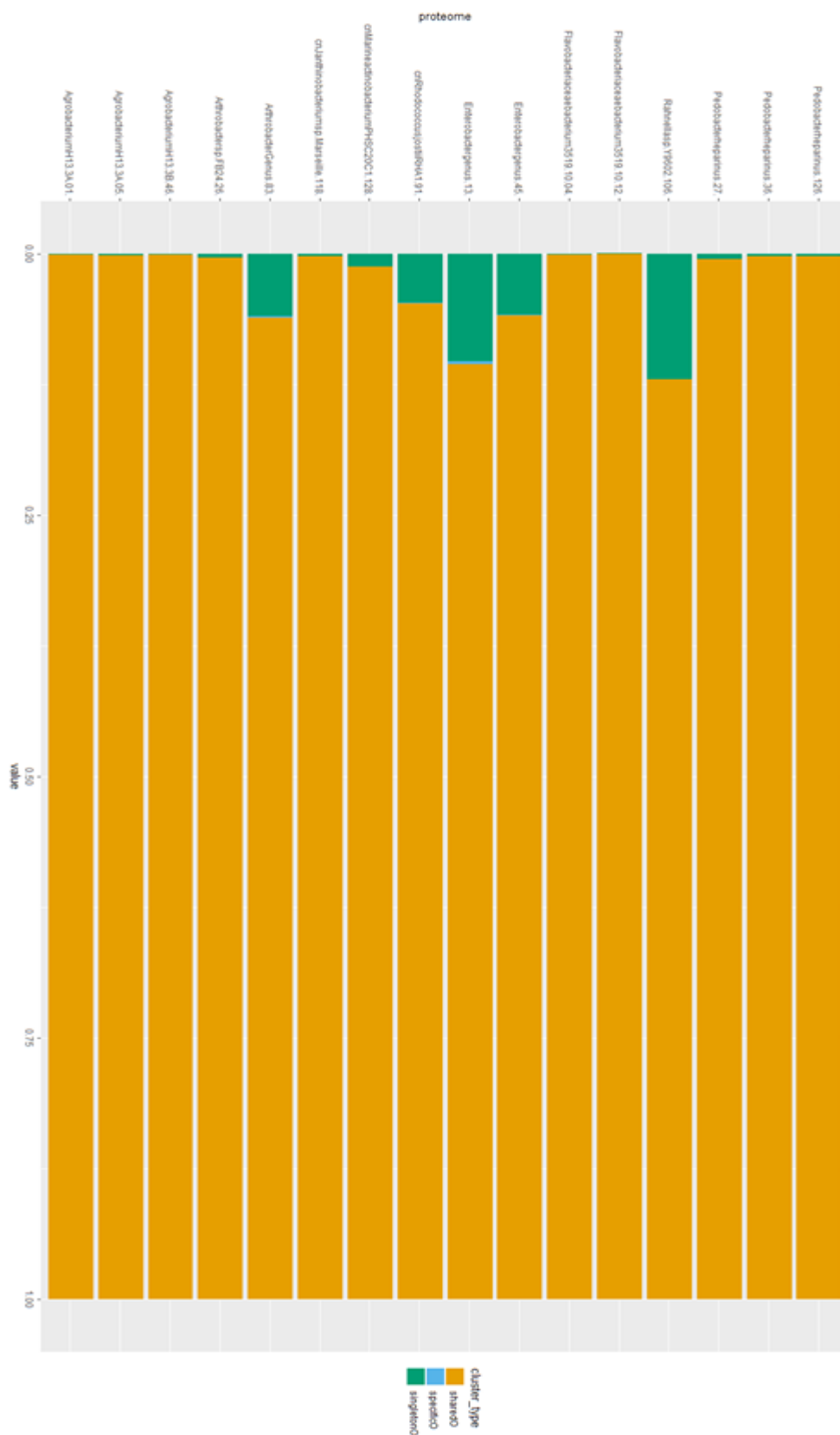
Figure S23 Single, shared and singleton proteomics of all "Other" isolates. *Colours depict Single, shared or singleton proportions*

8.4.4 Supplementary Tables

Table S1 Next generation sequence quality results for bacterial isolate sample analysis. Single GC peak indicates the presence or absence of a single peak, two or more peaks indicates the likely presence of two or more bacterial species in the sample. Size indicates the total genome length. N50 indicates the minimum contig length required to cover 50% of the genome and number of contigs defines the number of overlapping DNA segments that, together, represent a consensus region of DNA. Rows highlighted in red indicate samples that were identified as low quality based on the combination of the information detailed.

Sample ID	Single GC Peak	Size	N50	No. Contigs
bi01	Yes	5173015	417504	49
bi02	Yes	4006489	112363	107
bi03	Yes	4214240	63748	1539
bi04	Yes	5083280	280276	76
bi05	Yes	5215254	417533	145
bi06	Yes	6588387	68008	213
bi07	No	9626604	98749	266
bi08	Yes	6727906	94982	197
bi09	No	7466677	1207	9228
bi10	Yes	6709171	92401	178
bi11	Yes	6595069	99013	169
bi12	Yes	5071833	309596	46
bi13	Yes	4955784	383165	83
bi26	Yes	5131165	43594	258
bi27	Yes	6299567	908772	57
bi28	Yes	6706126	102722	155
bi30	No	11456784	16183	3422
bi33	Yes	12555304	15710	1744
bi35	Yes	4604127	67604	126
bi36	Yes	6308893	901067	74
bi38	Yes	6706263	111592	146
bi39	No	26111123	7071	13012
bi41	No	7792025	1074	9471
bi43	Yes	19669457	3631	11389
bi44	Yes	3554491	117718	101
bi45	Yes	4762104	697319	41
bi46	Yes	5167678	379239	34
bi47	Yes	8617286	86056	196
bi49	No	23146553	13004	12737
bi50	Yes	13684398	13502	11116
bi55	Yes	6166739	5298	1991
bi58	No	33055617	2081	29200
bi61	No	21552216	26330	9585
bi63	Yes	6475567	208747	2080
bi64	Yes	8153349	163868	2902
bi66	Yes	6038928	114998	2675
bi68	Yes	11430389	2658	7625
bi69	No	27033366	2195	20988

bi70	Yes	5661755	33755	1053
bi71	Yes	18065850	2822	13245
bi72	Yes	4611044	132292	80
bi73	Yes	3238068	1014	3890
bi74	Yes	9944606	4677	4049
bi75	Yes	6690850	148766	800
bi76	Yes	3918065	78635	339
bi78	No	13986295	15420	5641
bi79	No	6676413	1406	6750
bi80	Yes	5306717	7008	1330
bi81	Yes	4661483	99706	99
bi82	Yes	6045695	369445	594
bi83	Yes	4903204	76950	142
bi84	Yes	5634431	27035	563
bi85	Yes	3723547	95926	457
bi86	Yes	2436128	862	3358
bi87	Yes	5057432	83648	3153
bi89	Yes	6810734	165400	1494
bi91	Yes	5787904	276010	301
bi93	Yes	6184808	145197	4295
bi96	Yes	5525199	186917	84
bi97	No	17152363	26417	8452
bi98	Yes	3702041	247909	146
bi102	Yes	4744947	75776	300
bi104	No	10526689	122097	5437
bi105	Yes	20254762	1687	19801
bi106	Yes	5201418	3019	2672
bi110	Yes	6717616	160812	121
bi111	Yes	6707057	75930	198
bi112	Yes	6347610	28693	902
bi115	Yes	6913686	18468	1789
bi116	No	13274449	25072	1052
bi117	No	15045637	116031	6061
bi118	Yes	5796170	291100	81
bi120	No	8401094	24612	6312
bi121	Yes	3139027	392560	135
bi122	Yes	4657009	66023	134
bi123	Yes	6456319	250449	170
bi124	Yes	7990854	44741	373
bi126	Yes	6306567	1097689	66
bi127	No	19815708	8797	7100
bi128	Yes	7314863	4998	2937
bi130	Yes	6583757	239349	76
bi131	Yes	6716789	102807	166
bi132	Yes	4158080	1471	3767
bi133	Yes	5342657	42292	301

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CHAPTER 10

APPENDIX OF MANUSCRIPTS

- Caradonia, F., Ronga, D., Catellani, M., Giaretta Azevedo, C.V., Alegria Terrazas, R., **Robertson-Albertyn, S**., Francia, E., and Bulgarelli, D. (2019). Nitrogen Fertilisers Shape the Composition and Predicted Functions of the Microbiota of Field-Grown Tomato Plants. *Phytobiomes Journal*.Phytobiomes Journal 0 0:ja

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