# Bioprospecting for plant growth promoting microbes: Rich seams in long-term agricultural field experiments?

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Declaration:

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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***ABSTRACT***

Interactions between plants and microbes are believed to have facilitated the conquest of land and to this day profoundly affect the success of plants. Naturally occurring soil microbes – especially the subset that live associatively with plant roots – offer the potential to contribute to the sustainable intensification of agriculture by a variety of means, including the suppression of plant diseases and provision of nutrients. However, the effects of different agricultural management practices and cropping systems on the proliferation of beneficial microbes may determine the manifestation of these functions. The design of the Broadbalk winter wheat experiment at Rothamsted has ensured that a diverse range of biotic and abiotic selective pressures associated with different combinations of such treatments have acted continuously on resident soil microbes over the last 180 years, both directly and mediated via continuously cultivated crop plants. This thesis presents an investigation into the utility of this living experiment to advance our understanding of the plant microbiome under various environmental stressors principally surrounding nutrient limitations. The central role of inorganic nitrogen amendments in structuring the rhizosphere microbiome at Broadbalk was revealed by a bacterial 16S rRNA gene amplicon sequencing survey in tandem with robust soil chemical analyses. A high-throughput, culture collection pipeline was developed to integrate functional characterisations of resultant rhizosphere microbial communities by screening isolate libraries *in vitro*. The role of mineral nitrogen inputs dominated outcomes, and no enrichment of putatively plant growth promoting (PGP) microbial traits relevant to nutrient limitations in the plots

from which they were cultured were exhibited, potentially a result of thorough geochemical depletions through repeated cropping. This multifaceted approach towards understanding the nature of selection for PGP microbial associations holds significant promise and the data and biological resources generated can be further leveraged to develop microbial solutions to problems faced by contemporary agricultural systems.

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# CHAPTER 1 – INTRODUCTION

Contemporary agricultural productivity is largely sustained by the application of external chemical inputs, most notably in the form of synthetic macronutrient fertilisers. This state of affairs has facilitated massive human population growth over the last 60 years, perpetuated by the responsiveness of elite crop varieties to these practices post-Green Revolution (Pingali, 2012). The resultant demand for food for a global population expected to exceed 9 billion by the year 2050 is however set to outstrip current yield increases (FAO, 2009), which increasingly exhibit diminishing returns on further increases in fertiliser application (Tilman *et al.*, 2002; Zhang *et al.*, 2010). Concurrently, the sustainability of such high-input systems is being held to account, both in terms of natural resource depletion and the deterioration of the environment (Vitousek *et al*., 1997; Pingali, 2012). Therefore, the real challenge is to further increase agricultural productivity in a way that also minimizes reliance upon external chemical inputs (Adesemoye and Kloepper, 2009; Godfray *et al*., 2010; Foley *et al.*, 2011). To these ends, the biological potential harboured within historically neglected belowground components of crop production systems – namely roots and the pervasive soil microbiology with which they interact – is perfectly poised for exploitation.

### *MICROBIAL PLANT GROWTH PROMOTION*

Microbes are fundamental to the maintenance of life on Earth and are widely acknowledged for their role in the survival and fitness of the many higher organisms with which they associate (Berendsen *et al.*, 2012; Hacquard *et al.*, 2015; Sánchez-Cañizares *et al.*, 2017). Plant-Microbe interactions are believed to have facilitated the conquest of land itself (Selosse and Le Tacon, 1998; Delaux and Schornack, 2021), and to this day can profoundly affect the success of plants in both ecological and agricultural contexts (Compant *et al.*, 2010; Vandenkoornhuyse *et al.*, 2015). This is

reflected by the increasing prevalence of research into the plant microbiome - that is the ecological communities of microorganisms living associatively with all plant tissues. Indeed, the Food and Agriculture Organization of the United Nations has outlined further research and development of Plant Growth-Promoting (PGP) microbial associations as a priority for improving the sustainability of food production (FAO, 2019). The greatest diversity of microorganisms directly interacting with any plant occurs at the interface between its roots and the surrounding soil environment (Reinhold-Hurek *et al.*, 2015). This extremely rich microhabitat was described over 100 years ago by Lorenz Hiltner who coined the term 'rhizosphere' - from the Greek 'rhiza', meaning root, and 'sphere', meaning field of influence - to describe the highly microbially active region that he observed around plant roots (Hiltner, 1904; Hartmann *et al.*, 2008). Indeed, the plant's investment in the rhizosphere is not to be underestimated, with anything between 10% and 44% of the net carbon assimilation of the plant released from the roots (Bais *et al.*, 2006). These so called rhizodeposits - that is the nutrients, border cells, mucilage and other specific exudates supplied by the plant, provide the basis for the establishment of a plethora of plant-microbe interactions with varying degrees of intimacy (Morgan *et al.*, 2005; Turner *et al.*, 2013a).

The most well-known of these plant-microbe interactions lie at the more intimate end of this spectrum and see the provision of essential nutrients to the plant by bacterial and fungal mutualists permitted access to plant tissues. Indeed, residing in close proximity to the principal site of all nutrient uptake by the plant, rhizosphere microbes can hold great influence over the nutritional status of their 'host' and there exists an abundance of microbes that assist in plant nutrient acquisition (Wang *et al*., 2022). Such organisms, sometimes termed biofertilisers (Figure 1.1), may variously increase

the availability of nutrients or the efficiency of their uptake from the surrounding soils. In fact, the widely known bacterial and fungal endosymbioses alluded to above exemplify these two roles, respectively. In their endosymbiotic association with leguminous plants, bacteria capable of biological nitrogen fixation (BNF) primarily from the family *Rhizobiaceae* are able to transform atmospheric $N_2$ into bioavailable N inside root nodules (Gage, 2004). Mycorrhizal fungi also engage in symbiotic associations with plant roots, colonising root tissues either intra- or extra-cellularly and effectively extending the surface area available to plants for the uptake of soil nutrients by co-opting the vast networks of fungal hyphae (Marschner and Dell, 1994).

However, these roles are not unique to such highly specialised symbionts, which are exceptions rather than the rule for PGP rhizosphere microbes more generally. Indeed, a diversity of free-living microbes also carry out the same BNF reactions performed by rhizobia (Reed *et al.*, 2011; Smercina *et al.*, 2019), and the increased nutrient uptake efficiency afforded by mycorrhizal association may also be affected by several rhizobacterial species implicated in the stimulation of physiological and molecular changes in plant tissues that enhance nutrient uptake (Vacheron *et al.*, 2013). Additional mechanisms of biofertilisation critical for plant nutrition are exclusively undertaken by microbes engaged in much more commensal relationships with plants. Plants are in fact dependent on such microbes for the mobilisation of recalcitrant forms of soil nutrients including N, P, K, and S that are key for ecosystem functioning (van Der Heijden *et al.*, 2008; Jacoby *et al.*, 2017). Indeed, the widespread abilities of a diversity of free-living soil microbes that thrive in the rhizosphere to transform minimally available sources of these nutrients are entirely lacking in plants. The availability of P for example may variously be increased by ubiquitous rhizosphere microbes such as *Pseudomonas spp.* via the depolymerisation and mineralisation of

organic P sources such as phytate by various enzymes, and the production of extracellular organic acids that solubilise inorganic sources of P (Rodríguez *et al.*, 2006; Miller *et al.*, 2010; Alori *et al.*, 2017). This ubiquitous genus serves as an ideal example of the diversity of lifestyles exhibited by plant-associated microbes and the many PGP mechanisms (Figure 1.1) besides those that directly enhance plant nutrition.

Pseudomonads are generalist Gram-negative Gamma-proteobacteria found throughout almost all terrestrial habitats in a range of environmental niches (Loper *et al.*, 2012). Living saprophytically, *Pseudomonas spp.* commonly reside both on the surfaces of, and within the tissues of many higher organisms (epi- and endo-phytically, respectively) including almost all plant species (Silby *et al.*, 2011). The remarkable diversity and widespread distribution of the genus are often attributed to the metabolic plasticity demonstrated by its members (Silby *et al.*, 2011). This plasticity is exemplified perfectly by the elegant transcriptional regulation of primary carbon metabolism by the RpiR family transcription factors HexR and RccR which rapidly respond to carbon source availability by sensing a single key intermediate; 2-keto-3-deoxy-6-phosphogluconate (KDPG) (Daddaoua *et al.*, 2009; Campilongo *et al.*, 2017). This capacity to rapidly adapt to, and then utilise a range of carbon sources makes *Pseudomonas* highly successful in the exudate-rich rhizosphere compartment, with *Pseudomonas spp.* representing a major constituent of most plants' rhizosphere microbiomes (Mauchline and Malone, 2017).

However, as well as the numerous commensal pseudomonads that are sustained by nutrients secreted from (e.g. root exudates) or those that are present within (e.g. apoplast constituents) plants with no fitness effects on the host, the diversity of the

genus is spread right across the mutualism-parasitism continuum (Preston, 2004). The plethora of *P. syringae* pathovars for example, cause disease symptoms in a number of economically important crop plants via the secretion of a variety of hypersensitive response and pathogenicity (Hrp) effector proteins directly into host cells (Ichinose *et al.*, 2013). On the other hand, many other plant-associated pseudomonads - most notably those in the *P. fluorescens* species complex - promote the growth of their host by antagonising pathogenic microorganisms, synthesizing growth-stimulating hormones and promoting increased disease resistance (Haas and Defago, 2005). The significance of this diversity for plant health was strikingly demonstrated by Haney *et al.* (2015) via the 16S ribosomal RNA (rRNA) gene sequencing of the rhizobacterial communities associated with various *A. thaliana* accessions grown in natural soils. The authors found that between-accession differences in the rhizosphere microbiome were in fact largely restricted to strains belonging to a subset of the Pseudomonadaceae, with divergent consequences for plant health and productivity depending on the precise biotic and abiotic stresses faced (Haney *et al.*, 2015).

The intriguing capacity of certain members of the *P. fluorescens* complex to effectively suppress soilborne plant pathogens is of particular interest with some touted as 'biocontrol' agents (Haas and Defago, 2005; Santoyo *et al.*, 2012) - another major PGP mechanism besides biofertilisation (Figure 1.1). Characteristically rapid root colonisation and the ability to quickly swarm and form exopolysaccharide-based biofilms on root surfaces (Chin-A-Woeng *et al.,* 1997; Lugtenberg *et al.*, 2001) certainly contribute to the competitive exclusion of pathogenic strains via niche pre-emption and modification, respectively (Fukami, 2015). However, these remarkable abilities are in fact primarily the result of the action of a chemically diverse array of bioactive secondary metabolites and secreted proteins, the production of which characterises

the high taxonomic diversity of the pseudomonads (Loper *et al.*, 2012; Seaton and Silby, 2014). Accordingly, these molecules may antagonise pathogens through various means including siderophore-mediated competition for iron (Kloepper *et al.*, 1980), the production of relevant exoenzymes such as AHL lactonases that disrupt pathogenic quorum sensing (Jafra *et al.*, 2006), as well as classical antibiosis via the production of dedicated antifungal compounds such as pyoluteorin, pyrrolnitrin and phloroglucinols like 2,4-DAPG (Haas and Defago, 2005) alongside more broad-spectrum toxins like hydrogen cyanide (Blumer and Haas, 2000). Additional chemical diversity in this exemplar genus benefits plant growth via mechanisms other than biocontrol and biofertilisation per se (Figure 1.1), but which may in fact improve plant nutrition or preclude pathogenesis indirectly by enhancing root growth and 'priming' plant defences respectively (Glick *et al.*, 2012; Pieterse *et al.*, 2014). Indeed, pseudomonads may induce systemic resistance to certain phytopathogens by eliciting salicylic acid- as well as jasmonic acid and ethylene- mediated immune responses (Bakker *et al*., 2007). Moreover, a number of similarly rhizosphere-inhabiting microbes are known to directly synthesise cytokinins, gibberellins, and auxins such as indole-3-acetic acid (IAA) with growth-enhancing effects (Glick *et al.*, 2012; Vacheron *et al*., 2013). Such phytohormone-mediated functions (Figure 1.1) are also particularly relevant for the maintenance of growth in the face of various biotic and abiotic stresses. Indeed, widespread strains degrading stress hormones and their precursors such as 1-aminocyclopropane-1-carboxylic acid (ACC) act to limit the negative growth effects that would otherwise be incurred (Patten and Glick, 1996; Glick 2014). This *Pseudomonas*-centric overview of PGP mechanisms is not intended to suggest that this taxon is a holy grail of sorts, more so to emphasise the diversity of functions harboured by a single ubiquitous soil bacterial lineage, of which the Pseudomonads

are one of many notable genera implicated in PGP associations. These mechanisms, summarised in Figure 1.1, have significant potential to reduce reliance on chemical inputs in agriculture, however this has yet to be fully harnessed.

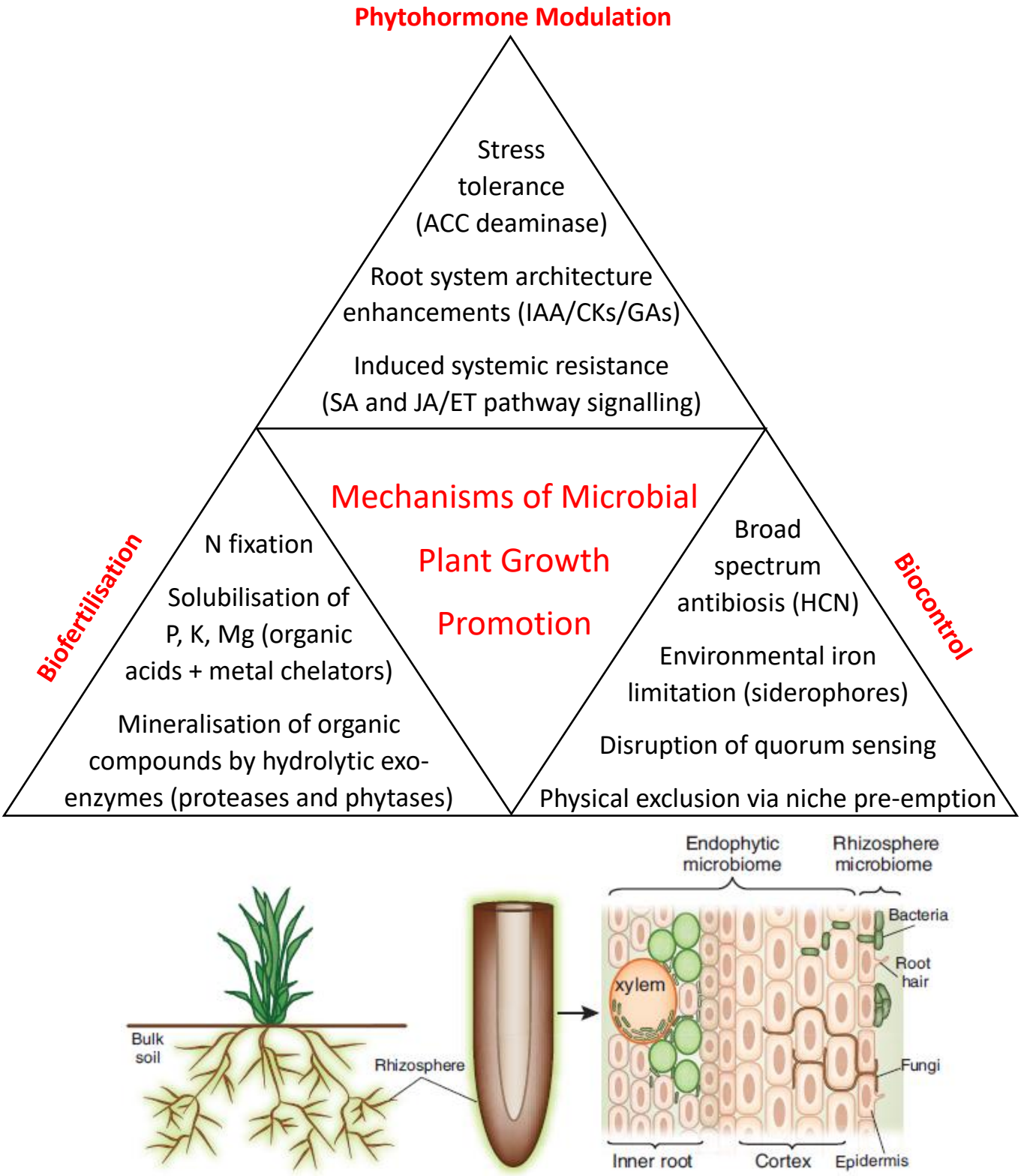



**Figure 1.1 – Mechanisms and locations of microbial plant growth promotion.** Schematic detailing the 3 primary categorisations of common mechanisms of plant growth promotion by microbes and their localisation belowground. Adapted from Hirsch and Mauchline (2012). ACC = 1-aminocyclopropane-1-carboxylic acid; IAA = indole-3-acetic acid, CKs = cytokinins, Gas = gibberellins; SA = salicylic acid, JA = jasmonic acid, ET = ethylene; HCN = hydrogen cyanide.

## *RHIZOSPHERE MICROBIOME ASSEMBLY*

The precise membership of the rhizosphere microbiome is thus of utmost importance to plant health and growth as well as the manifestation of a number of agriculturally relevant traits. However, whilst plants originate from seeds, the community of microorganisms present in the rhizosphere primarily assemble from the immensely diverse microbiota of the surrounding bulk soil. In contrast to the elevated microbial density in the rhizosphere, a substantial reduction of diversity is observed compared with the surrounding soil as a result of the abiotic selection pressures within this niche as well as specific plant-microbe interactions and intense competition between microorganisms for resources (Compant *et al.*, 2010; Philippot *et al.*, 2013). From a bacterial perspective, the increased dominance of the Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria and an underrepresentation of Acidobacteria characterise rhizobacterial assemblages when compared with the reservoir of diversity in the surrounding soil (Bulgarelli *et al.*, 2013). These observations are a reflection of a wider trend for the enrichment of so called 'rhizosphere competence' traits within these communities (Compant *et al.*, 2010; Barret *et al.*, 2011). Indeed, both the genetic potential for, and functional expression of the genes that control traits such as root exudate metabolism, type III secretion, polysaccharide degradation, motility, and chemotaxis correlate well with this rhizospheric enrichment of 'competent' microbes at the community level (Mark *et al.*, 2005; Ofek-Lalzar *et al.*, 2014). Such studies highlight the value of considering the functional significance of selection pressures imposed on the microbiota, especially when many important traits may be represented across a diverse range of taxa with different environmental distributions. This functional redundancy should theoretically ensure the assembly of a 'core functional microbiota' regardless of the edaphic microbial reservoir (Lemanceau *et al.*, 2017). Indeed, Louca

*et al.* (2016) describe such a similarity of functional microbial community structures between natural aquatic ecosystems contained within wild bromeliad foliage despite the high variability of the taxonomic composition of individual functional groups. Functional genes are reported as the units of selection in bacterial communities associated with plants such as the green macroalga *Ulva australis* (Burke *et al*., 2011), and more recently common weed species *Jacobaea vulgaris*, where functional genes, particularly those related to transporters, exhibited consistently significant enrichments in the rhizosphere (Yan *et al*., 2017). Relative to the diversity of the surrounding environment, these specialisations are indicative of the plethora of selective pressures exerted upon root-associated microbial communities by all plants.

Although principally determined by the properties of the surrounding soil and the requirement for general competence traits, the structure and function of the rhizosphere microbiome is at least in part also modulated by the identity of the host plant (Garbeva *et al.*, 2004; Berg and Smalla, 2009; Badri and Vivanco, 2009; Hartmann *et al.*, 2009). As such, rhizospheric microbial communities recruited from the same bulk soils are consistently shown to differ from species to species (Ladygina and Hedlund, 2010; Turner *et al.*, 2013b; Ofek-Lalzar *et al.*, 2014). Furthermore*,* different accessions of a single species can harbour distinct microbial communities in the rhizosphere *(*Micallef *et al.*, 2009a; Hardoim *et al.*, 2011; Bouffaud *et al.*, 2012; İnceoğlu *et al.*, 2012; Mahoney *et al.*, 2017), with further modifications coinciding with developmental transitions (Mougel *et al.*, 2006; Micallef *et al.*, 2009b; Chaparro *et al.*, 2013a). These findings are consistent with the two-step selection model for root microbiota differentiation from bulk soil synthesised from the literature by Bulgarelli *et al.* (2013) whereby initial competence-based community shifts converge on

increasingly host genotype-dependent fine-tuning of more closely root associated microbial communities.

The host plant's influence on the rhizosphere environment may be exerted through both its root architecture and the nature of rhizodeposition (Sasse *et al.*, 2018), with the specific chemical profile of root exudates proposed as the principal mechanism controlling microbiome composition (Bais *et al.*, 2006; Badri and Vivanco, 2009; Lareen *et al.*, 2016; Massalha *et al.*, 2017). For the microbes in the rhizosphere, the exudation of primary and more complex secondary metabolites can act as sources of carbon for the growth of microbes able to utilise them or further contribute to the complex network of molecular interactions in the rhizosphere by attracting, deterring, or directly antagonising individual microbial genotypes (Lareen *et al.,* 2016; Massalha *et al.*, 2017; Sasse *et al.*, 2018). The sheer complexity of this process is perfectly exemplified by the findings of Huang *et al.* (2019) who demonstrated that the purified products of a recently discovered specialized metabolic network capable of synthesizing more than 50 previously unknown *A. thaliana* root metabolites selectively modulate the growth of taxonomically diverse rhizosphere microbes, with examples of both positive and negative modulation in evidence. Excitingly the authors were also able to confirm that mutants impaired in the biosynthesis of these compounds exhibited shifts in the overall diversity and composition of their root microbiome compared with those of the wild type – specifically disrupting the portion of the microbiota specific to *A. thaliana* when compared with other plant species (Huang *et al.,* 2019). Moreover, studies examining root exudates alongside comparative analyses of the rhizosphere microbiota would appear to confirm that the aforementioned effects of host plant genotype (Micallef *et al.*, 2009a) as well as

developmental stage (Chaparro *et al.*, 2013b; Zhalnina *et al.*, 2018) can be attributed to differences in root exudate chemistry that modulate the microbiome.

These dynamic root exudation strategies may well represent mechanisms by which the growth of microbes that perform functions most beneficial to a particular plant accession at a particular growth stage can be favoured in the rhizosphere. Indeed, it is thought that millions of years of coevolution between plant and microbial partners could have yielded genetically optimised mechanisms for the recruitment of relevant microbiome functions that increase plant fitness (Bakker *et al.*, 2012; Hartman and Tringe, 2019, Liu *et al.*, 2020). However, whilst such preprogramed manipulations of the rhizosphere microbiota represent adaptations to challenges ubiquitous amongst individuals of a particular accession at a particular growth stage, plants are also at the mercy of an ever-changing barrage of context dependent biotic and abiotic stressors. Indeed, as sessile organisms the success of individual plants is directly dependent on their ability to respond to stress, and a growing body of evidence points towards the evolution of a variety of mechanisms by which plants may actively seek relationships with rhizosphere microbes (Pascale *et al.*, 2020).

Enrichments of rhizosphere microbes following pathogen defence activation are often reported, most commonly for known biocontrol strains such as pseudomonads with antifungal traits (Dudenhöffer *et al.*, 2016), but also novel consortia able to induce systemic resistance (Berendsen *et al.*, 2018). Plant root exudates are often implicated in such phenomena, consistent with the so called ‘cry-for-help’ hypothesis (Bakker *et al.*, 2018) by which stressed plants assemble protective microbiomes (Rolfe *et al.*, 2019). Indeed, mechanistic evidence of plants naturally recruiting specific microbes to the rhizosphere microbiome abound, seminal examples of which include the elegant

description of the selective stimulation of the beneficial rhizobacterium *Bacillus subtilis* FB17 mediated by pathogen-induced malic acid exudation from *A. thaliana* roots (Rudrappa *et al.*, 2008), and the increased accumulation of *Pseudomonas putida* KT2440 on maize roots exuding benzoxazinoids (Neal *et al.,* 2012). Less is understood about the relationships between biofertilisation-type microbial associations and plant nutrient status besides the particularly energetically costly rhizobial and mycorrhizal endosymbioses respectively supplying N and P only under otherwise growth-limiting supplies of these nutrients (Oldroyd and Leyser, 2020). Some recent examples do however exist linking soil nutrient availability to such traits whereby exogenous nutrient applications may reduce the abundance of rhizobacteria harbouring a variety of nutrient solubilisation traits (Reid *et al*., 2021). However, the roles of individual elemental nutrients within this fertilisation-effect have not yet been dissected as such regarding the alleviation of specific nutrient deficiencies via microbial PGP. Conceptually at least, it is feasible that the microbiota associated with any given plant likely comprises the microbial functions most beneficial to its survival and reproductive fitness under the conditions experienced in the particular environment in which it grows. Indeed, if the diversity of resident soil microorganisms is considered as a genetic reservoir of rhizosphere functions that may be incorporated into the 'extended genome' of the host plant (Turner *et al.*, 2013a), there exists a plethora of potentially PGP microbes that may alleviate the negative consequences of various biotic and abiotic stresses (Tkacz and Poole, 2015). Targeted investigations of microbial communities assembled under the presence or absence of particular stresses should thus reveal plant-microbe interactions that may have been initiated as such and define their relative importance as drivers of rhizosphere microbiome diversity in the field.

## *RESERVOIRS OF ROOT-ASSOCIATED MICROBIAL DIVERSITY*

Rhizosphere microbiomes are highly context dependent assemblages with structure and functions determined by a plethora of factors, some of which are introduced above, yet many remain poorly characterised. Our lack of understanding of these many factors and their effects on the rhizosphere microbiota, both directly and mediated via the plant – as well as any interactions between them – presents a major barrier to efforts towards manipulating the microbiota to encourage PGP microbial associations. Indeed, the limited successes typical of 'first generation' microbiome management strategies (French *at al*., 2021) are characterised by diminishing PGP effects of single strain inoculants from the lab to the greenhouse and again into the field (Bacilio *et al*., 2017). This trend reflects the increasing complexity of these settings and the number and strength of factors determining the specific context of the rhizosphere microbiota that is the target of manipulation (Sessitsch *et al*., 2019). By focusing on understanding the factors that may lead to this low level of integration of PGP associations in the complex contexts of field settings we may better guide future microbiome management strategies and uncover sources of microbes better capable of enhancing plant growth in agriculture. Thus, while we can make predictions about where we might identify hotspots of PGP associations (Santos *et al*., 2010) such as in arid regions for drought stress alleviating microbes (Kavamura *et al*., 2013), many such enrichment strategies neglect the environment in which these organisms will be deployed beyond plant-compatibility (Zachow *et al*., 2013; Teheran-Sierra *et al*., 2021). Surveying plant microbiomes in more well defined, experimentally tractable field settings should afford insights into the influence of agriculturally relevant factors and provide sources of microbes enriched as such. Moreover, as opposed to pipelines that culminate in a sink or swim test of simultaneous field survival, crop plant

association, and growth promotion, such approaches can start with organisms enriched in existing microbial communities that more closely reflect the ultimate focal environments for the stimulation of microbial plant growth promotion in all their complexity.

As alluded to above when discussing the small but significant influence of plant identity and growth stage on microbiome assembly, the main drivers of rhizosphere microbiome structure and function are various properties of the soil from which it formed. However, by controlling for these factors by looking only at one plant genotype grown in the same soils and sampling at specific growth stages, the focus can be put purely on the effects of experimental variables and plant responses to them such as any cries-for-help (Rolfe *et al.*, 2019). Indeed, in much the same way as field trials concerned principally with the yield implications of such plant responses to particular practices, the implications for the microbiota – which may in fact contribute to the agronomic results – may also be elucidated. In this context, comparisons of microbial communities assembled during agronomic field experiments investigating crop responses to relevant stressors of study could well be central to both the identification and extraction of novel PGP microbes and better understanding the drivers of PGP associations in field settings and perhaps more relevantly their constraints too. Pre-existing experiments thus offer a wealth of opportunity for defining the effects on the microbiota of agriculturally relevant variables that are by their very nature of the upmost importance having been studied agronomically.

***The Broadbalk long-term experiment***

The Broadbalk winter wheat experiment at Rothamsted Research is the oldest continuous agronomic experiment in the world, having been established by Sir John Lawes and Sir Henry Gilbert in 1843. The experiment is part of a set of classical

experiments at Rothamsted designed to demonstrate the effects of inorganic fertilisers containing nitrogen, phosphorus, potassium, and magnesium (N, P, K, and Mg) on the yields of different crops in comparison to organic manures as well as controls receiving no inputs at all (Macdonald *et al*., 2018). At Broadbalk, dissections of the individual elemental constituents of fertilisation are possible as inorganic compounds are tested both alone and in different combinations with nitrogen in particular also applied at several rates reflecting its significance for winter wheat yields. The aforementioned two-fold problem of increasing agricultural productivity with fewer inputs is perfectly exemplified by this globally important cropping system. Hexaploid bread wheat (*Triticum aestivum*) is the world's most important crop for direct human consumption and represents ~30% of global cereal production across an unrivalled geographic range of cultivation (Gustafson *et al.*, 2009; Shewry, 2009). Cultivated wheat has an unmatched nutritional profile when compared to other cereals and constitutes roughly 20% of all human caloric consumption and 25% of protein intake (www.fao.org/faostat). However, whilst the global demand for wheat is expected to increase by 60% by 2050 (www.wheatinitiative.org) due to population growth and shifting patterns of consumption (Lobell *et al.*, 2009), current yields appear to have plateaued (Grassini *et al.*, 2013). This stagnation is in fact reflected by the long-term trends at Broadbalk (Figure 1.2) where the stark yield increases of nutrient amended plots c.1968 highlight the heightened responses of modern short-strawed cultivars to fertilisation, on which the post-Green Revolution yield increases of the last 60 years are largely predicated (Pingali, 2012). Of further note are the wheat yield increases associated with the introductions of herbicides (c.1964) which replaced sequential bare-fallowing every fifth year (c.1920s), crop rotations (c.1968), and fungicides (c.1979). These modifications were incorporated to keep the experiment relevant to

contemporary farming practice, but importantly whenever introduced, were achieved via subdivision of the field such that certain sections remained unchanged (Poulton *et al*., 2024a). This served both to preserve the long-term integrity of the experiment and provides valuable comparisons such as that between the yields of continuous wheat and that of the first wheat in rotation, particularly across changes in wheat cultivars (Figure 1.2).

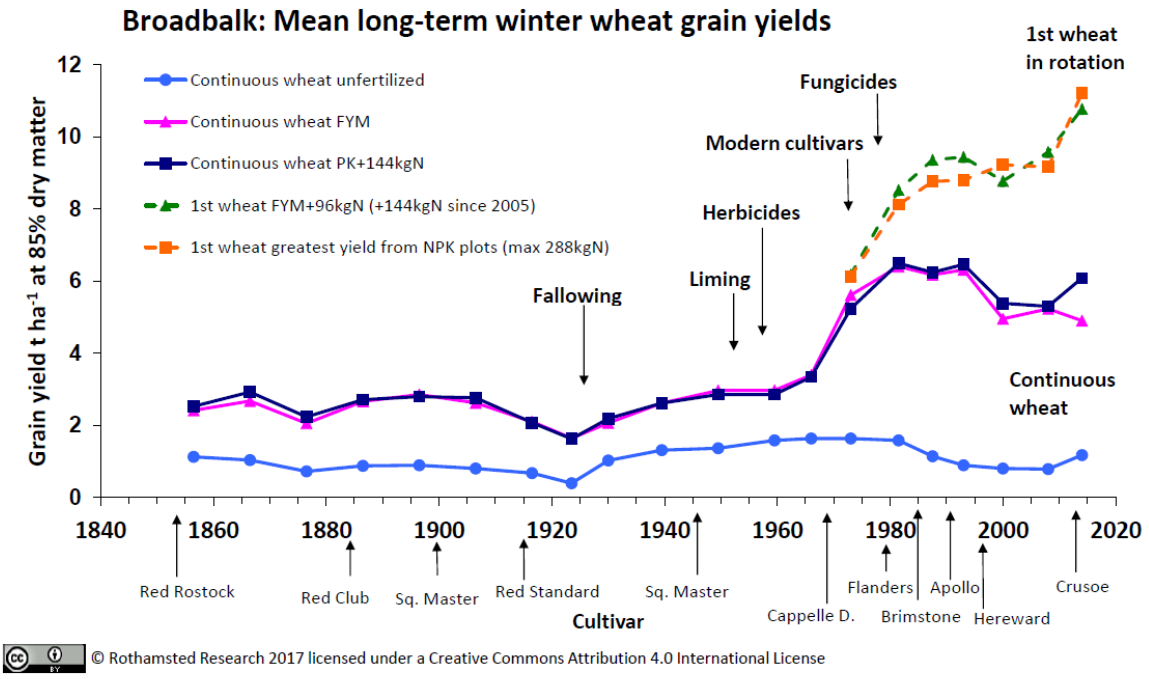



**Figure 1.2 – Broadbalk mean long-term winter wheat grain yields.** Yield data for selected treatments on the Broadbalk long-term experiment at Rothamsted from 1852 to 2016. Reproduced from Rothamsted Research (2017).

The resulting mosaic of treatment combinations applied to the Broadbalk long-term experiment may in fact have encouraged the establishment of the whole gamut of relevant plant-microbe interactions possible in this singular field setting. Moreover, through 180 years of continuous cultivation under specific combinations of fertilisation regimes and various incorporated management practices, any treatment-specific plant-microbe associations have likely become very robust due to their repeated

enrichments by the continuous cropping. In much the same way that rhizosphere microbiome composition has been demonstrated to become increasingly dissimilar between closely related crop accessions through successive cultivation (Cordovez *et al.*, 2021), the precise microbial communities associated with genetically identical wheat plants facing the specific combinations of biotic and abiotic stresses associated with these treatments may also be amplified as such. Indeed, under controlled conditions, successive plantings of a single wheat cultivar in soils with and without a fungal pathogen load assembled distinct bacterial community profiles in a multi-cycle selection system under controlled conditions (Yin *et al.,* 2021). Thus, this single field of genetically identical wheat plants grown under different combinations of relevant treatments applied with such continuity should enhance distinctions of the microbiota favoured by particular factors and provides a living resource for investigating PGP microbial associations under contemporary agricultural settings and the extraction of microbes of interest. Moreover, the potential relevance of any microbial associations specific to plots in which chemical inputs have been withheld and their potential for stress alleviation and the avoidance of associated yield penalties is increasingly realised in the context of more sustainable approaches to wheat cultivation and food production more generally.

***BIOPROSPECTING***

Wheat-associated rhizosphere microbes capable of enhancing plant growth in agricultural settings seem perfectly poised for exploitation in future crop production systems and Broadbalk stands out as a promising resource. ‘Bioprospecting’ is the term often given to the processes involved in the discovery and exploitation of novel, ultimately commercialisable products from such biological resources (Müller *et al*., 2016). When prospecting for other belowground resources in more well-established

industries, an understanding of the major drivers of the diversity of such resources is a critical requirement. For mineral or hydrocarbon exploration, this might comprise a map of the underlying rock formations informed by geophysical surveys which can be used to outline potentially rich seams of valuable resources within the strata that may be exploited. To bio-prospect for agriculturally relevant PGP microbes, this exploratory phase will involve utilisation of methodologies with which to survey the major drivers of the diversity of rhizosphere microbiomes. Indeed, simply extracting potentially PGP rhizosphere microbes without the relevant knowledge of the environmental contexts in which they are identified is of limited benefit for many downstream applications and therefore thorough characterisations of the selective pressures acting on the source microbiota and indeed the plant are essential. Thus, bioprospecting on Broadbalk will comprise several approaches across various scales to best take advantage of the defined long-term treatments. In this project I will develop a microbial isolate library from a subset of plots as well as broader, culture independent amplicon sequencing datasets to define the structure and nutrient cycling functional potential of the Broadbalk rhizosphere microbiome. The current chemical status of soils from which these microbes assemble will be comprehensively assessed to fully contextualise these findings from soils with such lengthy treatment histories. By comprehensively surveying the microbiota associated with genetically identical wheat plants under the diversity of different combinations of treatments on Broadbalk, robust PGP associations and the particular conditions under which they manifest may be identified and selectively investigated with a view to subsequent exploitation. Indeed, elucidation of the roles of groups of microbes in the alleviation of biotic or abiotic stresses is often facilitated by studies of compositional shifts in the plant microbiome under such stressors (Berendsen *et al*., 2018; Li *et al*., 2021).

Whilst not all of the microbes which increase in abundance under plant stressors are directly beneficial to plant performance, as consortia termed ‘DefenseBiomes’ by Liu *et al.* (2020) they seemingly possess properties that enable stable interactions with the plant under these conditions that are required to confer relevant stress-mitigating benefits. Indeed, this top-down method of deriving PGP microbial consortia solves some of the same problems of ‘first generation’ microbiome manipulations (French *et al*., 2021) as PGP synthetic communities (SynComs) designed from the bottom-up – both by leveraging microbe-microbe interactions. However, it is the known stability and stress specificity of such pre-existing associations that offers key benefits over other such pipelines and accordingly it is these treatment-specific consortia that are of particular interest for bioprospecting here. In this way, Broadbalk can be seen as a living example of a top-down ‘directed evolution by plant-mediated indirect selection’ experiment with agriculturally relevant variables that make the resulting microbial communities a particularly valuable resource.

This is not to say that (inherently ‘bottom-up’) approaches predicated on the isolation of individual microbes are not still central to bioprospecting efforts. Indeed, the collection of pure microbial cultures is still critical for the actual validation of plant growth-promotion and subsequently the development of any potential applications as inoculants. Moreover, such *ex situ* experimentation allows thorough interrogation of potentially PGP microbial physiologies by phenotypic assessment of the responses of cultures to a multitude of different *in vitro* growth conditions including putatively PGP phenotypes. Returning to the pseudomonads as an example, the extensive diversity of PGP functions conferred by members of the *P. fluorescens* complex is reflected by an enormous pan-genome totalling approximately 30,000 coding sequences, within which as few as 1,334 are conserved among all members (Garrido-Sanz *et al.*, 2016).

Such pseudomonads thus highlight the importance of within-species strain-level diversity to the functional profile of the microbiota and the intrinsic limitations of the operational taxonomic units (OTUs) or amplicon sequence variants (ASVs) routinely relied upon by culture-independent microbiome surveys which may mask important properties of microbial communities (Jaspers and Overmann, 2004; García-García *et al.*, 2019). Indeed, within the same plant-associated OTU investigated by Karasov *et al.* (2018) co-existed *Pseudomonas* strains that diverged ~300,000 years ago, differing significantly in their gene content and functional capabilities. Thus, particularly for important PGP traits such as phosphorus mobilisation which are known to vary dramatically between *Pseudomonas* strains (Browne *et al.*, 2009; Lidbury *et al*., 2016), such arbitrary taxonomic groupings cannot be used to infer shared functions. This variation is however of particular relevance to cereal crops that exhibit 'pseudomonas blooms' (Walters *et al.*, 2018) during which single OTUs recently shown to comprise large numbers of distinct pseudomonas lineages come to dominate the rhizosphere microbiome (Chiniquy *et al.*, 2021). Therefore, thorough dissections of the functional potential of agricultural rhizosphere microbiomes should not neglect this wealth of micro-diversity in this and other microbial taxa and will benefit hugely from the functional characterisation of individual microbial isolates to complement exploratory amplicon sequencing efforts. The approach taken to bioprospecting here thus seeks to combine the benefits of top down and bottom-up approaches to capitalise on both the top-down nature of the various Broadbalk treatments as directed evolution by plant-mediated indirect selection experiments as well as leveraging bottom-up culture collection methodologies necessary to understanding microbial functions.

## *CONCLUSIONS AND RESEARCH GOALS*

Much like when prospecting for other precious belowground resources, an understanding of the major drivers of the diversity of such resources is key, in this case obtained via an amplicon survey contextualised by soil chemical analyses. When subsequently combined with culture-based characterisations of the PGP potential in selected plots – analogous to the analysis of ore samples – this approach may well reveal rich seams across Broadbalk. Moreover, this approach enables the dissection of the effects of the individual elemental nutrients applied at Broadbalk on specific nutrient-mobilising traits amongst rhizosphere microbes. Investigation of specific treatments under the agriculturally relevant management practices incorporated over the years across Broadbalk should reveal how PGP associations might be modulated in field settings. Indeed, the manifestation of particular plant-microbe associations may well be precluded by certain agricultural practices or enhanced by others – information that may guide best practises for maximising plant growth enhancements from native microbes and further refine the search for agriculturally relevant PGP microbes.

In the investigative chapters of this project the research aims are as follows:

**Define the contemporary abiotic selection pressures on Broadbalk:**

Establishing the current chemical status of Broadbalk soils characterised by each of the principal nutrient treatments after ~180 years of continuous wheat cultivation is key to underpinning all future work here.

**Survey the microbiota across Broadbalk to identify major drivers of diversity:**

A high-throughput bacterial amplicon sequencing approach will help develop our understanding of the impacts of agronomy on the structure of bulk soil and rhizosphere microbiomes by surveying the diversity across Broadbalk treatments.

**Establish and interrogate Broadbalk microbial culture collections for PGP traits:**

A partially automated microbial isolation pipeline will be developed to establish large microbial libraries from selected Broadbalk plots with which to assess the prevalence of PGP traits related to improving plant nutrient status by *in vitro* screening.

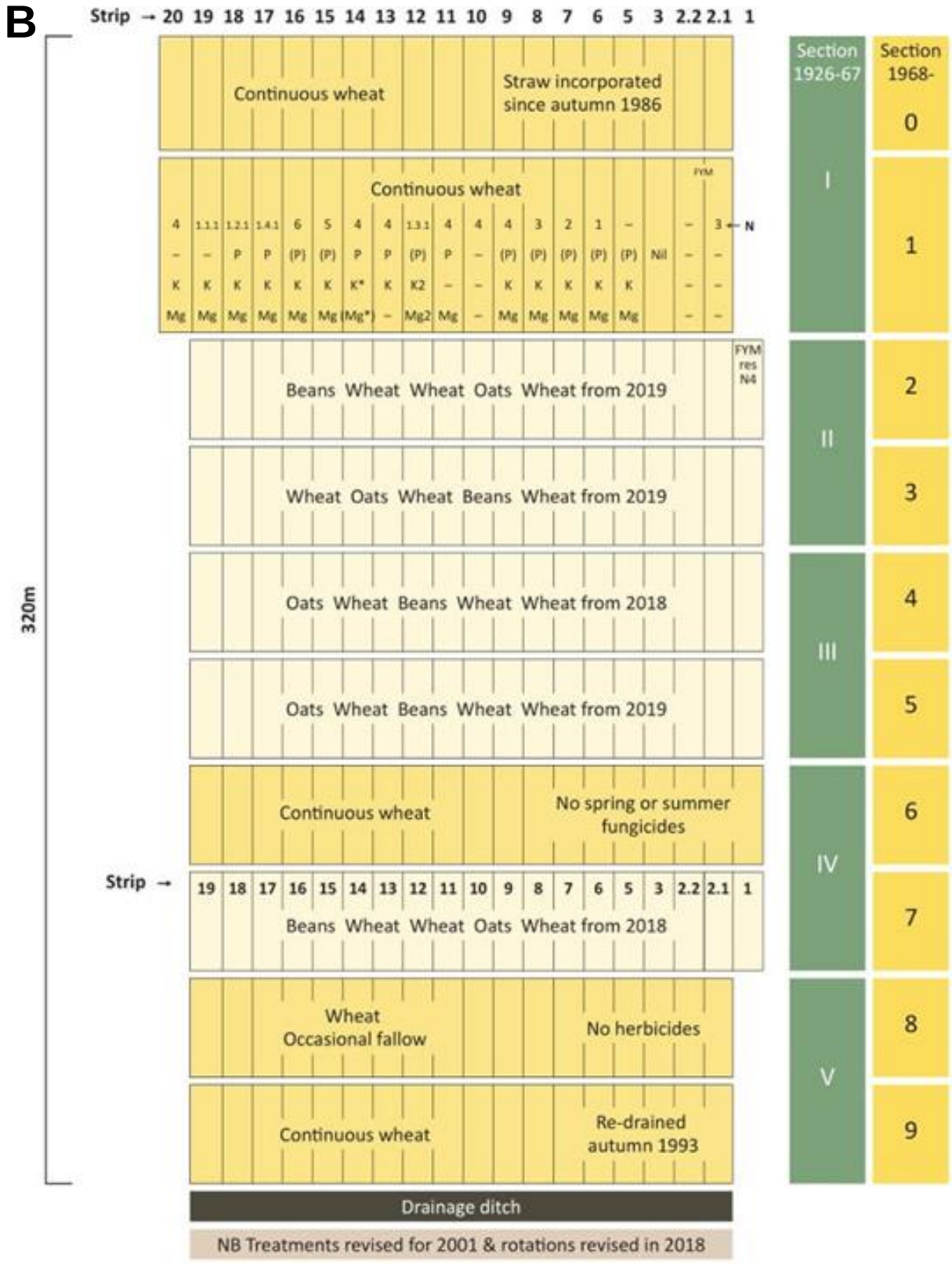
My overall hypothesis is that the long-term treatments at Broadbalk will be clearly evidenced through bulk soil chemical analysis (Chapter 2), and these treatments influence microbiome community structure and function (Chapters 3 and 4, respectively). More specifically, I hypothesise that differential nutrient applications are more influential than crop rotation and agrochemical application in structuring the wheat rhizosphere microbiome (Chapter 3) and that long-term omissions of principal elemental nutrients enrich for relevant microbial functions relating to the alleviation of associated plant growth limitations (Chapter 4).

# CHAPTER 2 - DEFINING THE LANDSCAPE OF SELECTION PRESSURES AT THE BROADBALK LONG-TERM EXPERIMENT

## *INTRODUCTION*

As described in the general introduction Chapter, Broadbalk is a long-term field experiment, started in 1843, which explores the impacts on wheat yields of organic manures, inorganic fertilisers, and other agrochemicals (herbicides and fungicides) as well as crop rotations. The design of the experiment consists of a range of sections in which wheat is grown either continuously with and without the application of herbicides and fungicides or incorporated in a crop rotation cycle (currently wheat, wheat, oats, wheat, beans). Each section is divided in up to at least 18 treatment plots, each belonging to a strip that runs the length of the field which receives a bespoke fertilisation regime ranging from no inputs to very high levels of inorganic inputs as well as organic amendments (Figure 2.1). Strips 2.2 and 3, respectively receiving farmyard manure (FYM) and nothing at all (Nil) besides atmospheric N deposition have visually striking effects in the field, the former even apparent in bare soils over winter due to the continuous accumulation of organic matter. These treatments are the most disparate from the majority of strips under various combinations of inorganic amendments delivering N, P, K, and Mg. Inorganic nitrogen amendments as ammonium nitrate are of particular focus at Broadbalk, where treatments with various rates of application and even multiple split applications are in evidence. For the purposes of the present studies, here I focus on a selection of these strips which in comparison to a ‘conventionally’ fertilised strip (9) describe a gradient of N application rates from none to half and double that in strip 9 (5, 7, and 16, respectively), as well as the omission of P, K, and Mg both individually (20/19, 11, and 13, respectively) and all at once (10). Strips 5, 20/19, 11, and 13 are particularly relevant here as sources of nutrient ‘drop-out’ plots lacking only N, P, K, and Mg applications, respectively.


**Figure 2.1 – The Broadbalk long-term experiment: (A) Aerial photograph (2018) and (B) Treatment plan [c. 2020] adapted from Macdonald *et al.* (2018).** Details of the applications of fertilisers supplying the four principal elemental nutrients (N, P, K, and Mg) to strips running the length of the field as well the cropping regimes and agrochemical omissions associated with each of the 10 contemporary transverse sections (0-9). Numbering associated with N, P, K, ang Mg notations correspond to simplified application rates, multiple numbers (e.g. 1.3.1) indicate the rates of each split application, asterisks (*) indicate alternative compounds not containing S are applied, and brackets (e.g. (P)) indicate that applications have not been made since the year 2000 whilst residual levels remain high.


B
Strip → 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 3 2.2 2.1 1
Continuous wheat
Straw incorporated since autumn 1986
Continuous wheat
FYM
4 – K Mg
1.1.1 – K Mg
1.2.1 P K Mg
1.4.1 P K Mg
6 (P) K Mg
5 (P) K Mg
4 P K* (Mg*)
4 P K –
1.3.1 (P) K2 Mg2
4 P – Mg
4 – – –
4 (P) K Mg
3 (P) K Mg
2 (P) K Mg
1 (P) K Mg
– (P) K Mg
Nil
– – – –
3 ← N – –
Beans Wheat Wheat Oats Wheat from 2019
FYM res N4
Wheat Oats Wheat Beans Wheat from 2019
Oats Wheat Beans Wheat Wheat from 2018
Oats Wheat Beans Wheat Wheat from 2019
Continuous wheat
No spring or summer fungicides
Strip → 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 3 2.2 2.1 1
Beans Wheat Wheat Oats Wheat from 2018
Wheat Occasional fallow
No herbicides
Continuous wheat
Re-drained autumn 1993
320m
Drainage ditch
NB Treatments revised for 2001 & rotations revised in 2018
Section 1926-67
Section 1968-
I
II
III
IV
V
0
1
2
3
4
5
6
7
8
9

Here I focus on characterising the nutrient status of soils from a selection of these fertilisation strips in one continuous wheat section (section 1), as well as two sections which were sown to wheat in the year of sampling but had been under bean (section 2) or oat (section 5) cultivation in the previous year as part of identical 5-year crop rotation schemes. In the two sections under rotation (2 and 5) it is important to note that in oat cultivation years the N application rate is half of what is applied to wheat. For example, where an application rate of 192 kg N per hectare would be applied under wheat cultivation, mineral N amendments supplying 96 kg N/ha would apply when sown with oat. Additionally, in bean cultivation years there is no application of inorganic N or FYM. Moreover, it should also be noted that the current five-year rotation only replaced the previous rotation scheme (wheat, wheat, wheat, oats, maize) of 20+ years in 2018.

In addition, the wheat crop grown in the year of sampling here was a spring wheat (cv. Tybalt) sown in exception to the winter wheat convention on Broadbalk because wet autumn and winter conditions prevented sowing. As a result, spring applications of fertilisers supplying Mg and K were not made until one week before and two weeks after the sampling date, respectively. However, given the length of treatment histories on Broadbalk it can be assumed that trends reflective of these treatments will be distinguishable at least in terms of total elemental concentrations if not so much for measures of currently available forms.

It should also not be ignored that since the year 2000 applications of P have been withheld from certain plots (marked (P) in Figure 2.1b) that had been under long-term inorganic P fertilisation. This was not however, intended to introduce a limitation for this nutrient (Poulton *et al*., 2024a), but instead to reduce the high levels of soil P

accumulated through the repeated applications of an excess of P needed to ensure that yields would not be limited by this nutrient, and which are not reflective of wider farming practise/agricultural soils more generally. Indeed, in these Broadbalk plots, plant-available P (Olsen *et al*., 1954) was, and indeed still is well above the level at which arable soils are recommended to be maintained at in the UK (AHDB, 2023) in order to limit surface water eutrophication mediated by phosphate leaching (Heckrath *et al*., 1995). It will be interesting to determine to what extent these plots can be distinguished from those that continued to receive P applications (plots 11 and 13) during 20 years of this temporarily introduced modification, and after which applications ceased in these plots too.

It should be noted that at Broadbalk the treatment plots are not randomised or replicated in a split plot design, as the experiment was initiated before the dawn of modern statistical design. However, a recently published comprehensive review of the experiment by Poulton *et al.* (2024b) encourages confidence that the findings are real and that the trends observed are a result of particular treatments rather than an artifact of their position in the field.

The primary aim of this chapter is to establish the bulk soil macro and micronutrient status of selected sections and plots at Broadbalk at the time of sampling. It can be anticipated that lower concentrations of particular nutrients will be observed in plots that have not received applications of compounds containing these nutrients. This data will then be used as metadata for subsequent Chapters in this project, namely the high-throughput amplicon-based survey as well as the culture-dependent functional screening to determine the impacts of fertilisation regime as well as cropping cycles on microbiome structure and function. Moreover, this will enable the formulation of

hypotheses relating to the selective pressures acting on various PGP microbial functions that may act to alleviate the specific nutrient limitations defined here.

## *MATERIALS AND METHODS*

### *Experimental site*

The Broadbalk long-term experiment at Rothamsted Research (Harpenden, UK, 51°48′33″ N; 0°22′19″ W, 128 m a.s.l.) is the oldest continuing field experiment in the world, principally concerned with winter wheat yields under defined nutrient regimes through continuous cultivation since 1843. Broadbalk soils are slightly calcareous, silty, clay loams of the Batcombe series classified as Chromic Luvisol (FAO) or Aquic Paleudalf (USDA) internationally, with clay contents varying between 19 – 39 % and an abundance of flints in evidence (Watts *et al*., 2006). The site is freely draining with a downslope of 1°, West to East, along the length of the 320 m by 150 m cultivated area (4.8 ha) sown principally to winter wheat. Various combinations of inorganic fertilisers (supplying N, P, K, and Mg) and farmyard manure (FYM) are applied to 20 strips (typically 6 m wide) most of which run the full length of the field separated by paths (typically 1.5 m wide). The field is further divided transversely into 10 cropping sections (typically 23 m long) numbered 0 – 9 (Figure 2.1b), half of which are in a five-year crop rotation (currently: wheat, wheat, oats, wheat, beans) such that each phase is evidenced every year, the other five cropping sections growing winter wheat continuously. The portion of each strip within a given section is referred to hereafter as a treatment plot, each of which were subdivided transversely into 4 equally sized ‘replicate’ subplots for the purpose of this study.

The 11 treatment strips studied here are listed in Table 2.1 with details of their bespoke fertilisation regimes along with a description of their relation to a ‘conventionally’ fertilised strip. These treatment strips are investigated in one continuous wheat section (section 1) last fallowed in 1966, and two sections grown in rotation (sections 2 and 5 in 2020) such that they were preceded by bean and oat cropping years, respectively.

Table 2.1. Fertiliser and organic manure treatments for selected Broadbalk strips

| Strip number | Strip name | Treatment c.2020 | Description |
|---|---|---|---|
| 2.2 | FYM | FYM | Farmyard manure |
| 3 | Nil | Nil | Nothing |
| 5 | noN | (P) K Mg | No N |
| 7 | ½N | N2 (P) K Mg | 0.5x N |
| 9 | conv | N4 (P) K Mg | 'Conventional' fertilisation |
| 10 | onlyN | N4 | No P, K, or Mg (i.e. Only N) |
| 11 | noK | N4 P Mg | No K |
| 13 | noMg | N4 P K | No Mg |
| 16 | 2xN | N6 (P) K Mg | 2x N |
| 19 | noP* | N1+1+1 K Mg [C] | No P proxy* |
| 20 | noP | N4 K Mg | No P |

FYM: Farmyard manure at 35 t/ha in autumn
N: Ammonium nitrate (Nitram, 34.5% N) in single (N2, N4, N6 : 96, 192, 288 kg N/ha in mid-April) or split (N1+1+1 : 48+48+48 kg N/ha in mid-March, mid-April, mid-May) applications. (n.b. Sections under rotation growing oats receive N at ½ rate, and beans do not receive any N or FYM)
P: 35 kg P/ha as triple superphosphate in autumn
(P): 35 kg P/ha as triple superphosphate until 2000 (to be reviewed 2025)
K: 90 kg K/ha as potassium sulphate in spring
Mg: 12 kg Mg/ha as Kieserite in spring
[C]: Castor bean meal to supply 96 kg N/ha until 1988
*strip 19 used for comparisons outside of section 1 where strip 20 does not exist

### *Soil sampling and processing*

Soil sampling from Broadbalk in 2020 occurred concurrently with the harvesting of rhizosphere samples from wheat plants (cv. Tybalt sown 25/03/2020) at flowering stage (Zadok's growth stage 65) over two consecutive days (30th June & 1st July). Bulk soils were sampled across sections 1, 2, and 5 from four 'replicate' subplots per treatment plot from each of strips 2.2, 3, 5, 7, 9, 10, 11, 13, 16, 19, (and 20 only in section 1). From each 'replicate' subplot three 2.5 cm diameter bulk soil cores were taken to a depth of 20 cm (within the ~23 cm cultivated layer) roughly 1.5 m from the

path boundaries between strips, just either side of the central 2.1 m wide 'yield strip', one from one side, two from the other, spaced equidistantly along the subplot in a wide 'V' formation. All three soil cores from each 'replicate' subplot were pooled, crumbled and mixed together thoroughly inside polythene bags by crushing and shaking manually before storing at -20 °C prior to soil chemical analyses.

***Soil chemical analyses***

Flint-free portions of thawed, field-moist soils were subject to potassium chloride (KCl) extraction of mineral nitrogen using a 1:2.5 ratio of soil to 2 M KCl with 2 hours of reciprocal shaking (100 rpm), as well as oven drying (105 °C overnight) to determine % dry matter. Concentrations of nitrate ($NO_3$-N) and ammonium ($NH_4$-N) in KCl extracts immediately filtered through Whatman no.1 filter papers (11 µm particle retention) were determined colourimetrically using a Skalar SAN$^{PLUS}$ System continuous flow analyser (Skalar Analytical BV, Netherlands) in the Analytical Chemistry Unit at Rothamsted Research, where all subsequent extractions and analyses were also conducted. The remainder of thawed soils were air-dried and sieved to < 2 mm, removing flints. Soil pH was determined in a 1:2.5 (w/v) suspension of soil in boiled and cooled 18MΩ deionised water using a temperature compensated Jenway 3540 pH meter (Jenway Ltd., UK) with a VWR 662-1782 combined pH electrode (VWR International Ltd., UK) after 1 hour equilibrating time with intermittent shaking. 'Plant-available' phosphorus was determined by colorimetric analysis after extraction with sodium bicarbonate as described by Olsen *et al.* (1954). Similarly, 'plant-available' major and trace elements extractable with the Mehlich 3 extractant (Mehlich, 1984) were quantified by Inductively Coupled Plasma – Optical Emission Spectrometer (ICP-OES) analysis using an Agilent 5900 SVDV ICP-OES (Agilent Technologies, California, USA). Air-dried 2 mm soils additionally finely milled to a

powder using a Retsch PM400 planetary agate ball mill (Retsch GmbH, Germany) were used to determine total N and C as well as Inorganic C by Dumas dry combustion using a Leco TruMac CN combustion analyser (LECO Corporation, Michigan, USA). Finally, (pseudo-) total concentrations of major and trace elements were determined by aqua regia (hydrochloric acid:nitric acid; 80:20 v/v) digestion (McGrath and Cunliffe, 1985) of soils finely milled as above, quantified similarly to their Mehlich 3 extractable equivalents by ICP-OES analysis using an Agilent 5900 SVDV ICP-OES (Agilent Technologies, California, USA). Principal coordinates analysis (PCA) was conducted on all soil chemical data from all plots using PAST v4.14 (Hammer *et al*., 2001) with normalised var-covar settings specified to normalise the variance of all variables via division by their standard deviations.

## *RESULTS*

### Available and Total percentage N:

As expected, addition of increasing amounts of mineral N fertiliser in the form of ammonium nitrate resulted in generally increased concentrations of soil $NO_3$- and $NH_4$-N regardless of section for plots receiving mineral N, especially when compared to plots 2.2, 3 and 5 which did not receive any mineral N fertilisation (Figure 2.2a and b). However, in plot 2.2 which received FYM, a much higher total % N was recorded compared to all other plots (Figure 2.2c). Moreover, it seems to be the case that for total % N, section 1 exhibits a generally elevated level compared with sections 2 and 5 for any given plot.

### Total and Available P:

As expected, plots that have not received P (3,10,19/20), have a lower available (Olsen) as well as total P level in bulk soil (Figures 2.2d and e) compared with those that have always received P (11,13) as well as those that have not received P since 2000 (5,7,9,16). In terms of section effects between plots, these are minimal, however, section 5 plots (after oats) are associated with a higher level of total and available P in plot 10.

### Total and Available K:

Plots which have not received K: 3, 10, 11 (Nil, only N, -K) have a lower K level as determined by traditional total elemental K determination (Figure 2.2g) as well as using the Mehlich-III methodology of available K (Figure 2.2f) compared with plots receiving K. In addition, there is very little difference in the Mehlich-III available K amount of K between sections for a given plot, whereas for the traditional total K determination methodology section 1 plots tended to have a higher K concentration than the equivalent plots in sections 2 and 5.

### Total and Available Mg:

In terms of Mg levels in soil it was found that for available Mg (Mehlich III extraction) plots which have not received Mg amendments (3, 10 and 13) had obviously lower Mg levels than the other plots (Figure 2.2h). However, this pattern was not observed in

the total Mg estimates (Figure 2.2i) where the Mg estimates were not discernibly lower than other treatments. When comparing Mg levels between sections it was found that there were very little differences in detected available Mg levels. However for total Mg estimates in most cases section 5 Mg measurements (after oats) were noticeably lower than sections 1 and 2. This was most apparent for plots 2.2, 3, 5, 7, 9, 10, 19 and 20. Furthermore, it was also observed that total Mg level was higher in section 1 when compared with section 2 for plots 7, 9, 10, 11, 13, 16, 19 and 20.

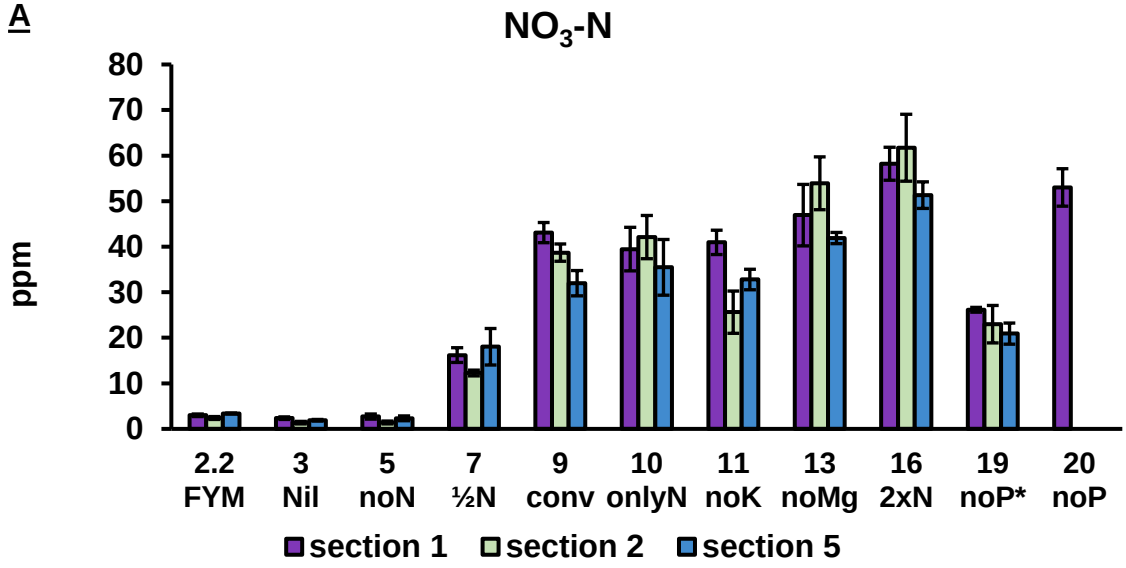
**% Organic C (= total % C - % IC):**

As expected, the plot receiving FYM had a higher carbon percentage than the other plots (Figure 2.2j). Aside from this highly disparate strip, when comparing a given plot between sections it was consistently found that organic C was slightly higher in section 1, relative to sections 2 and 5.

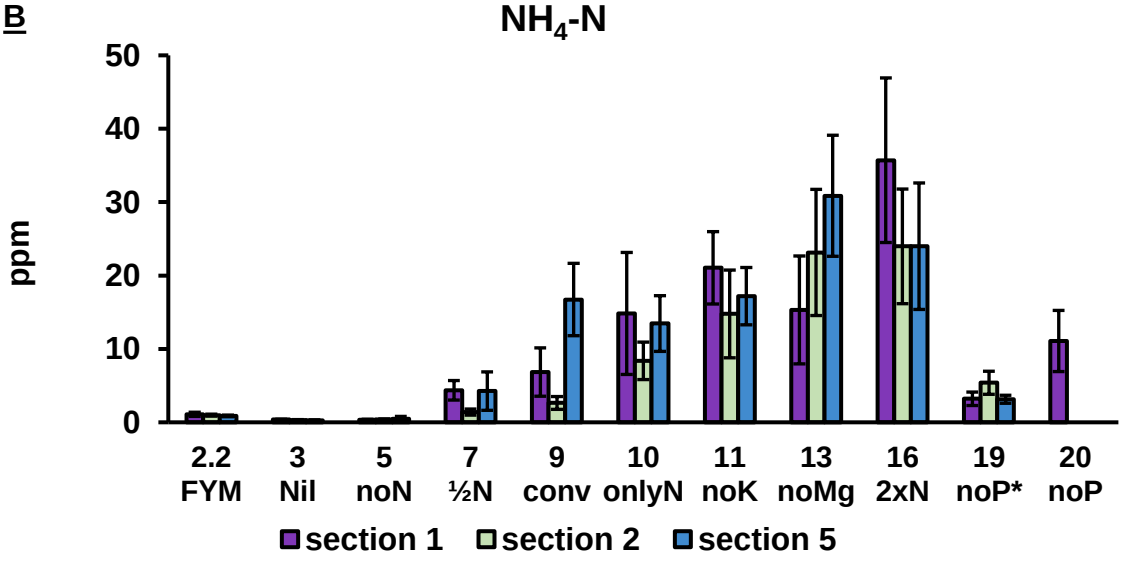
**pH:**

Measurements of pH (Figure 2.2k) confirmed that the periodic liming of plots has largely controlled the pH (between pH 7-8) levels, even though there were expected observed decreases in pH in response to acidification following inorganic fertilisation (plots 7, 9, 10, 11, 13, 16), with plot 16 which received the highest amount of mineral N fertiliser having the lowest average pH across all samples. In addition, very small differences in pH were observed between sections for a given plot, with plots 9 and 13 being the exceptions where in both cases section 5 (after oats) there was a trend for reduced pH.

A


NO3-N
ppm
80
70
60
50
40
30
20
10
0
2.2 FYM
3 Nil
5 noN
7 ½N
9 conv
10 onlyN
11 noK
13 noMg
16 2xN
19 noP*
20 noP
section 1
section 2
section 5

B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40
30
20
10
0
2.2 FYM
3 Nil
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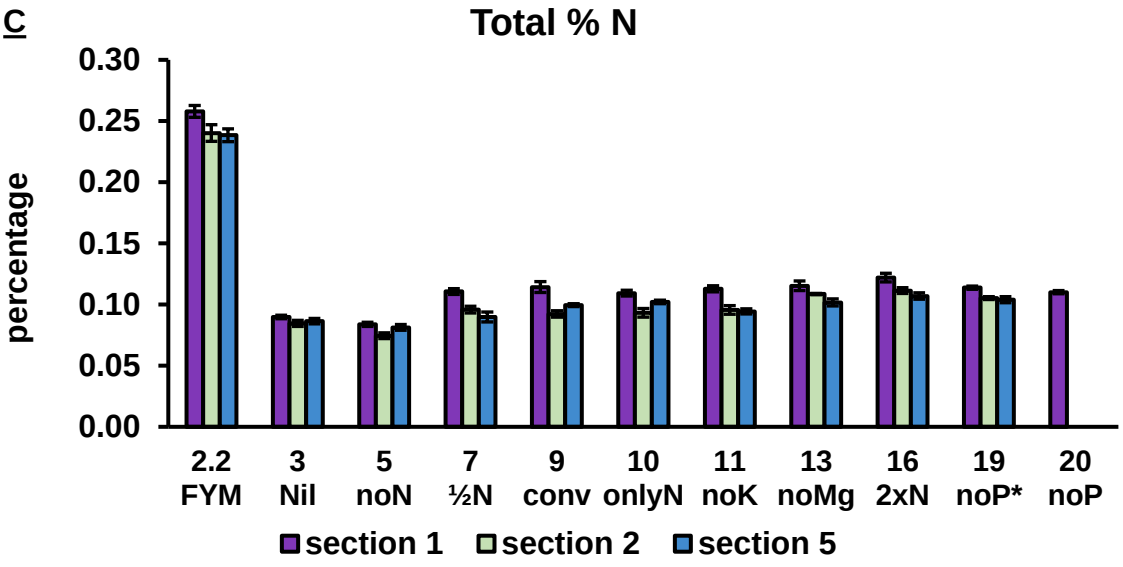
7 ½N
9 conv
10 onlyN
11 noK
13 noMg
16 2xN
19 noP*
20 noP
section 1
section 2
section 5

C


Total % N
percentage
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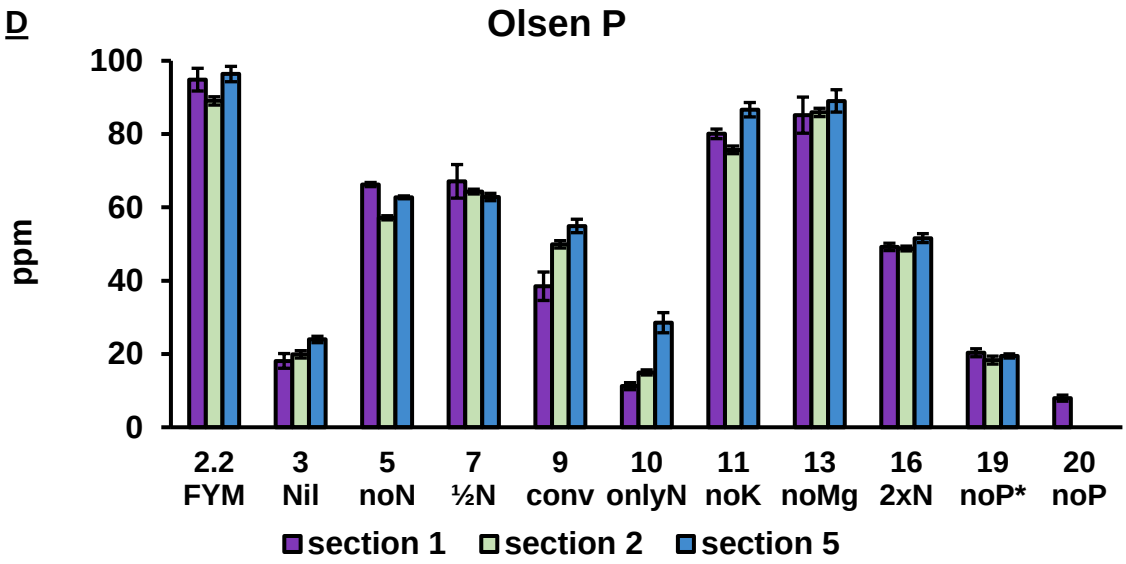
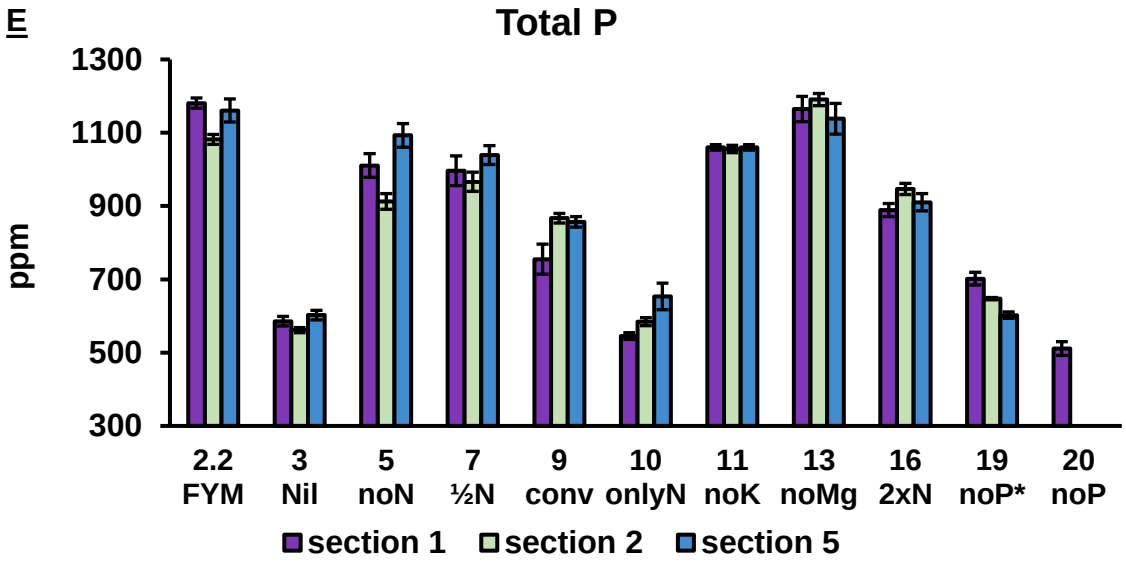
0.25
0.20
0.15
0.10
0.05
0.00
2.2 FYM
3 Nil
5 noN
7 ½N
9 conv
10 onlyN
11 noK
13 noMg
16 2xN
19 noP*
20 noP
section 1
section 2
section 5

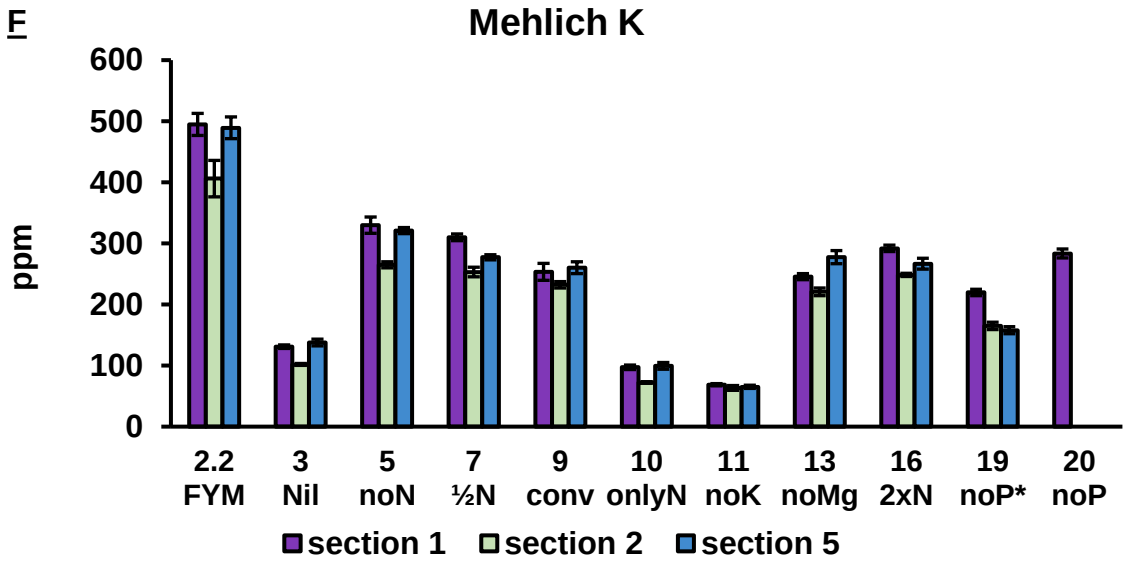
D


Olsen P
ppm
100
80
60
40
20
0
2.2 FYM
3 Nil
5 noN
7 ½N
9 conv
10 onlyN
11 noK
13 noMg
16 2xN
19 noP*
20 noP
section 1
section 2
section 5

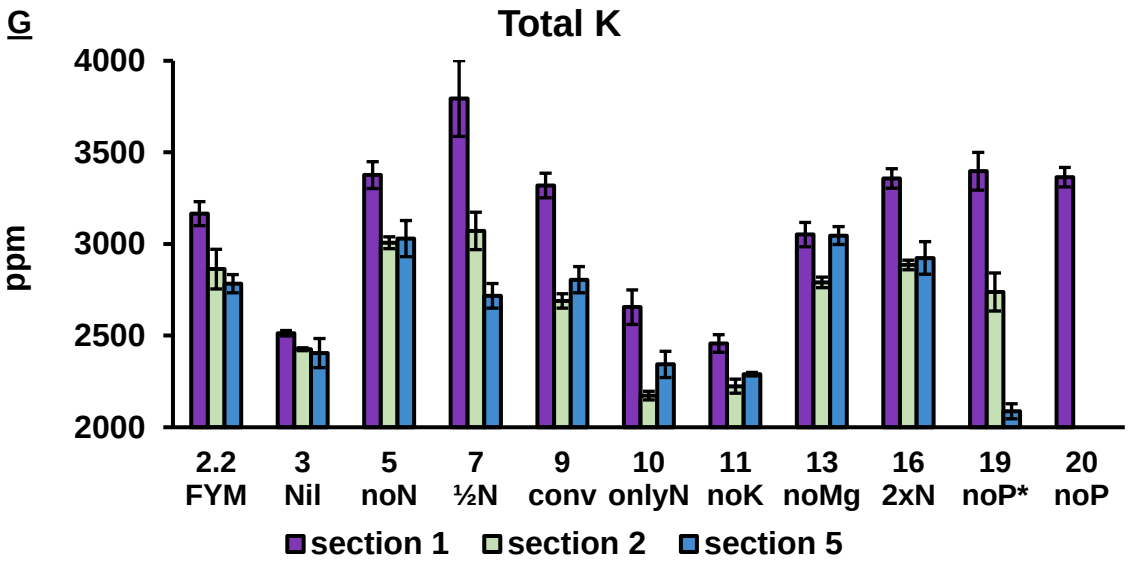
E


Total P
ppm
1300
1100
900
700
500
300
2.2 FYM
3 Nil
5 noN
7 ½N
9 conv
10 onlyN
11 noK
13 noMg
16 2xN
19 noP*
20 noP
section 1
section 2
section 5

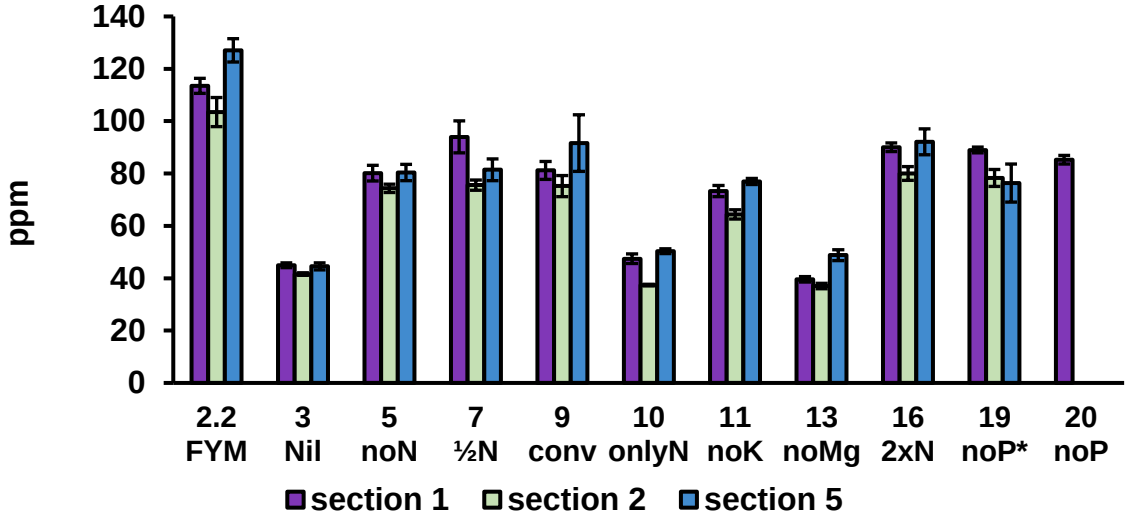
F


Mehlich K
ppm
600
500
400
300
200
100
0
2.2 FYM
3 Nil
5 noN
7 ½N
9 conv
10 onlyN
11 noK
13 noMg
16 2xN
19 noP*
20 noP
section 1
section 2
section 5

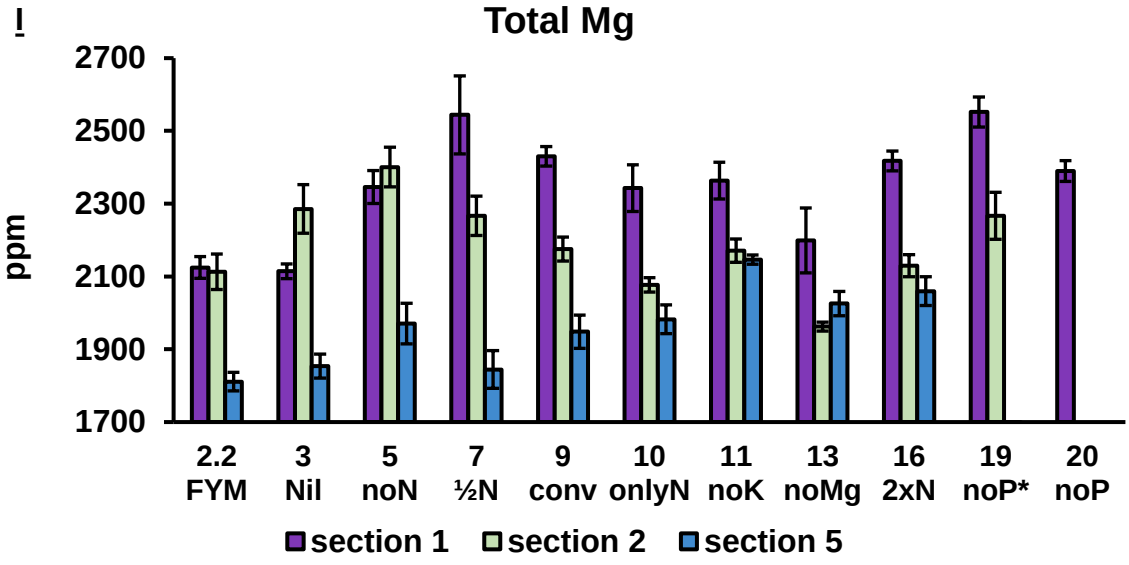
G


Total K
4000
3500
3000
2500
2000
ppm
2.2 FYM
3 Nil
5 noN
7 ½N
9 conv
10 onlyN
11 noK
13 noMg
16 2xN
19 noP*
20 noP
section 1
section 2
section 5

H


Mehlich Mg
140
120
100
80
60
40
20
0
ppm
2.2 FYM
3 Nil
5 noN
7 ½N
9 conv
10 onlyN
11 noK
13 noMg
16 2xN
19 noP*
20 noP
section 1
section 2
section 5

I


Total Mg
2700
2500
2300
2100
1900
1700
ppm
2.2 FYM
3 Nil
5 noN
7 ½N
9 conv
10 onlyN
11 noK
13 noMg
16 2xN
19 noP*
20 noP
section 1
section 2
section 5

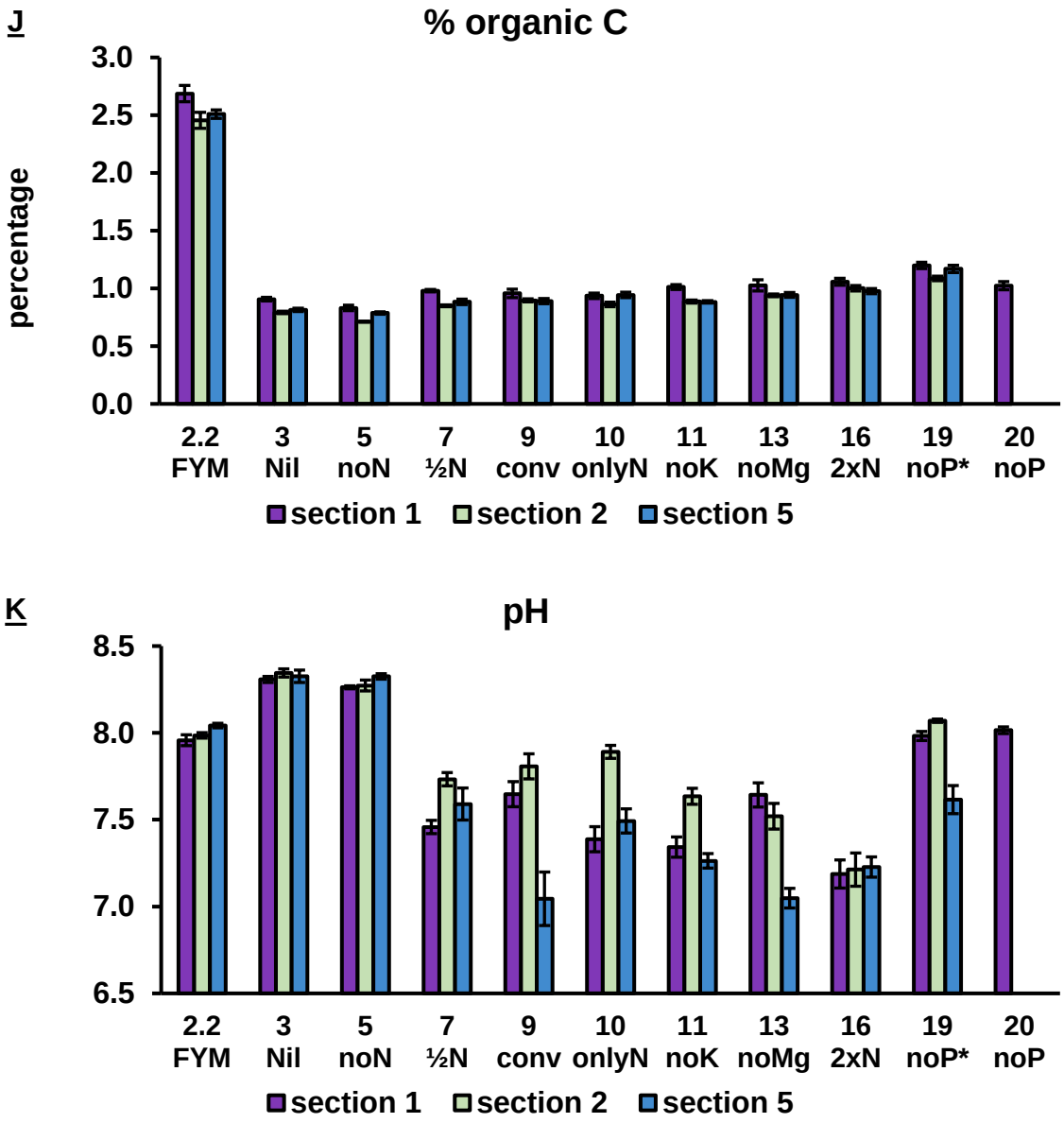



**Figure 2.2 – Comprehensive soil chemical characterisations of Broadbalk soils samples from across 11 nutrient amendment strips in 3 sections.** Measurements of (A) $NO_3$-N, (B) $NH_4$-N, (C) Total % N, (D) Olsen P, (E) Total P, (F) Mehlich K, (G) Total K, (H) Mehlich Mg, (I) Total Mg, (J) % organic C*, and (K) pH, are presented with error bars ± SEM, and all concentrations represent as ppm in dry matter. (*calculated as total % C minus % inorganic carbon)

Multivariate PCAs of all soil chemical properties revealed clear separations of Broadbalk soils under distinct managements, however the inclusion of samples from strip 2.2 receiving FYM obscured further interpretations (see Figure S1 in Appendix) because of highly disparate measures of total % N and C as outlined above and were thus removed from the analysis presented in Figure 2.3.

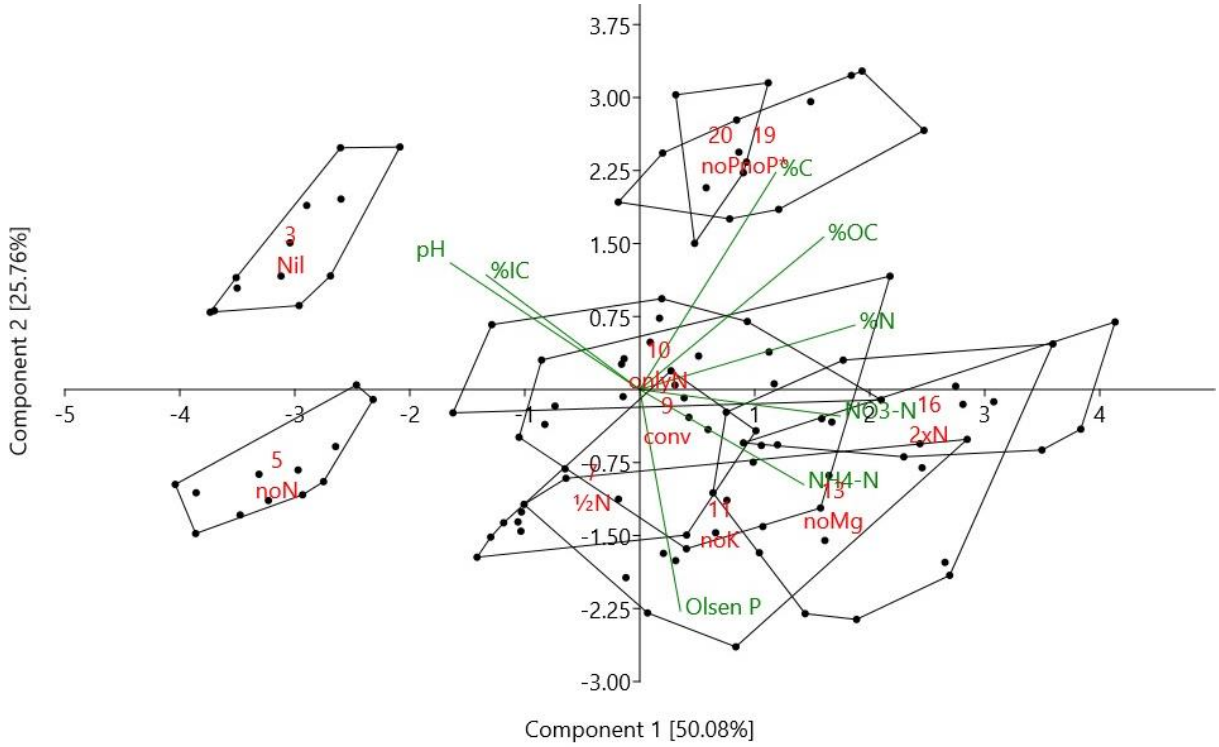



**Figure 2.3 – Principal components analysis (PCA) of all analysed soil chemical properties for Broadbalk soil samples across 10 nutrient amendment strips in 3 sections.** The main measurements responsible for observed variability are included in green. The most evident cluster of samples is coloured blue and represents strips never having received P inputs.

The first two components of this biplot (Figure 2.3) together explained over 75% of all variation, and separations of samples from several treatment strips were evidenced. This was most clearly seen when examining the x axis (component 1) with which measures of total and available N seemed to positively correlate, and where samples from strips never having received applications of ammonium nitrate N were most easily distinguished at a baseline level. Interestingly, the gradient of mineral N application

rates was also evidenced, with samples from strips that received the highest dosage of N in strip 16 (at 288 Kg N/ha) being most readily being discriminated along this first component. Available (Olsen) P was the principal means of discrimination on the second, orthogonal axis (component 2) where samples from the no P drop-out plots in strips 19 and 20 clearly separated from all others more positively associated with the Olsen P variable (Figure 2.3). It is also worth noting the clustering of samples from strip 9, which is fully fertilised, around the centre of this biplot (Figure 2.3), reflecting the commonality of the treatments it receives with many other plots that differ only in the omission of single amendments.

## *DISCUSSION*

The work in this Chapter is focussed around establishing the chemical status of soils at the Broadbalk experiment at time of sampling, 177 years after the principal experimental treatments were established. These treatment strips, variously subdivided and amended over the years, are cultivated under different fertilisation regimes, ranging from no inputs to high levels of mineral fertiliser amendments as well as organic manure applications. In addition, the soil chemical status of Broadbalk sections subjected to crop rotations in various subdivisions of these strips, namely those in phases following bean and oat cultivation was also assessed alongside plots under continuous wheat cultivation across the same set of nutrient regimes. The data presented in this Chapter will be useful metadata to apply to subsequent thesis chapters centred around microbiome structure and function of wheat rhizospheres at Broadbalk.

As expected, the predominance of fertilisation regime in determining soil chemical status was clearly observed, and this factor was apparently more important than crop rotation for all chemical analyses (Figures 2.2 and 2.3). Multivariate analysis of all soil chemical analyses conducted here revealed that variability across all soils sampled was largely explained by measures of two of the principal elemental nutrients added to Broadbalk plots: N and P (Figure 2.3). As predicted, samples from several individual nutrient drop-out plots separated readily in this analysis, allowing comparison with samples from conventionally fertilised plots that clustered around the centre of this biplot (Figure 2.3) acting as a baseline of sorts. Of particular note in this ordination is the relative absence of pH as a relevant explanatory variable other than for soils not subject to the acidification associated with inorganic N amendments as ammonium

nitrate in strips 3, and 5 which as expected had slightly higher pH values (Figure 2.2k). Indeed, otherwise there is very little variation in evidence, testament to periodic liming at Broadbalk (Glendining *et al*., 2022) carried out to maintain soil pH above 7.5 and minimise the range of pH values within sections through differential applications of chalk. As such, the well-established role of pH in shaping the soil microbiome (Fierer and Jackson, 2006; Griffiths *et al*., 2011) is relatively controlled for here. This should allow the influences of the treatments of interest to be disentangled from the overarching effect of pH in structuring these microbial communities which may otherwise also further modulate the impacts of these management factors (Mombrikotb *et al*., 2022).

The impacts of long-term mineral fertilisation were most evident in plots not receiving certain nutrients. Indeed, the so-called drop-out plots in which single elemental nutrients are withheld were clearly evident in corresponding measurements of plant-available P, K, and Mg (strips 19/20, 11, and 13, respectively) alongside the nil plots (strip 3) and strip 10 receiving only N (Figures 2.2d, f, and h). In the case of available K, this trend was observed despite the applications of potassium sulphate supplying K having not yet occurred in the cropping year in which samples were taken. This pattern reflects that observed for total K (Figure 2.2g), indicative of the equilibration of total and available pools of K in all plots (Blake *et al*., 1999) via potentially microbially facilitated processes. Indeed, such a process is suggested to sustain crop yields in soils not receiving K inputs by ‘topping up’ of available K from less available forms to the low levels at which they have largely remained at throughout the experiment (Poulton *et al*., 2024b). This relationship is even clearer for P where the availability of P (Figure 2.2d) very faithfully reflects total P measurements (Figure 2.2e), whereby even the effects of withholding P since the year 2000 (Johnston *et al*., 2016) from

selected strips with a history of P amendments (strips 5, 7, 9, and 16) are distinguishable from those still receiving P (strips 11 and 13).

However, in terms of total elemental measurements it was sometimes found, as for the example of Mg, that such trends were much less clearly observed. Whilst in sections 1 and 2 at least, plot 13 receiving no Mg exhibited lower total elemental concentrations of Mg and for section 2, plot 10 receiving only N was second lowest, the overall trend reflected the trends observed for available forms much less consistently than for P, and K, and varied between sections (Figure 2.2h). Indeed, the Nil plot was not appreciably lower in terms of total Mg in section 2, and neither was plot 10 (only N) in section 1. Moreover, where clear differences were observed for available Mg these could well have been accentuated by the timing of sampling just one week after the application of Kieserite Mg fertiliser, before much could be either utilised by the crop or sequestered in soils, similarly restoring equilibrium between available unavailable pools represented by the total measurements, despite those trends not appearing to be driven by Mg application alone.

Total % N was lowest in plots not receiving any N inputs (strips 3 and 5), reflecting the negligible concentrations of available $NO_3$- and $NH_4$-N also observed in these plots (Figures 2.2a and b). In contrast, total % N was highest in strip 2.2, likely as a direct result of the FYM amendments rich in organic N, even though very little available N was measured here either. An abundance of N must however be made available for plant growth in this strip which yields comparably to those receiving 144 kg N/ha (N3) of inorganic N (Poulton *et al*., 2024b). As such these supressed measurements may well be an artefact of the bottleneck on N mineralisation caused by the hydrolysis of proteins (Jan *et al*., 2009) meaning that any available N is likely utilised by the plant

quicker than it is made available as such, and thus is not measured here. Microbial contributions to such processes are a possible area of exploitation for the soil microbiome which has potential to unlock these and other nutritional resources through the secretion of organic acids and metal-chelating compounds or in this case, extracellular enzymatic activity. Subsequent investigation of the distribution of these important microbial traits on Broadbalk should start to illuminate the main drivers of such agriculturally relevant PGP associations in the field, such as the soil chemical contexts in which they are found under long-term experimental treatments.

The concentrations of plant-available mineral N from the remaining inorganically fertilised plots reflected the gradient of N rates applied to strips 7, 9, and 16 (96, 192, and 288 kg N/ha, respectively) (Figure 2.2a and b). These amounts of mineral N, measured after the majority of N uptake has occurred pre-anthesis (Pask *et al*., 2012) may therefore reflect trends in the balance of N able to be immobilised in or leached from the soil, the former contributing to the patterns of total % N observed through repeated accumulations in the long-term. Indeed, aside from the highly inflated values in plot 2.2, the highest total % N values were similarly found in plot 16 receiving the highest rate of N amendment, whilst plots 3 and 5 not receiving any exogenous N were also the lowest (Figure 2.2c). The more marginal differences in total % N between plots with different N application rates (Figure 2.2c) compared with those observed for available measures of N (Figure 2.2a and b) may in fact reflect the saturation of N in soil organic matter in plots with N applied at higher rates through repeated immobilisation of N as such. Indeed, with diminishing returns for continued amendments as these soils reach their new equilibria (Poulton *et al*., 2024b) and plots receiving N at lower rates may appear to ‘catch up’.

In terms of carbon stocks, it was also unsurprising to find that the FYM treatment led to an increase in total % organic C levels (Figure 2.2j) through its directly stimulating effects on soil organic matter. The divergent nature of these and other interrelated measurements of this plot are shared to a lesser degree by soils in plot 19 which also has a legacy of organic amendments. The use of this plot as a proxy for the plot not receiving mineral P is obscured by this treatment history, as despite also never having received inorganic P amendments, the annual castor bean meal applications supplying 96 kg N/ha until 1988 also supplied relatively substantial inputs of other nutrients including P. Thus strip 19 will not be investigated further, and the utilisation of plot 2.2 will be treated as more of contrasting outgroup than for the dissection of specific treatment effects.

Although crop rotation was of secondary importance in determining soil nutrient status, there were examples where differences between sections for a given fertilisation treatment were apparent. For example, the clearest trend was for section 1 plots (continuous wheat) to have higher total N percentages than sections 2 (wheat after beans) and 5 (wheat after oats) across all strips receiving N applications (Figure 2.2c). It is highly likely that this is due to the lower cumulative amount of N applied to crop rotation sections throughout their treatment histories. Rotation sections receive no N and half the rate of N application under bean and oats cultivation, respectively, in two out of every 5 years in the current crop rotation sequence and have always included at least 1 year without any N applications in every 3- or 5-year rotation scheme previously adopted since their introduction in 1968. Indeed, in terms of soil organic matter, measurements of % N and % organic C from sections under rotation are considered as a separate, divergent dataset for precisely this reason (Poulton *et al*., 2024b), in addition to the higher number of fallow years used to control weeds before

the introduction of herbicides c. 1964 in the corresponding old sections (I-V in Figure 2.1b). As such, the cumulative reduction in organic residues contributed by crops grown with fewer N inputs, and their total absence in fallow years have driven the only differences consistently observed between sections here in terms of total % N (Figure 2.2c) and % organic C (Figure 2.2j) whilst none were detected between sections 2 and 5 with comparable cropping histories.

Moreover, measures of available mineral N cannot be meaningfully compared between the sections studied here because of the confounding effects of the modified amounts of N applied to each rotation section during the previous growing season. However, it is interesting to note that the levels of available N are fairly comparable between sections within given strips (Figures 2.2a and b), indicating that the modified amounts applied to these crops in the previous year did not have a distinguishable effect on the amount available in the year of sampling, albeit measured towards the end of the growing season when majority of such N may already have been utilised as mentioned above (Pask *et al.*, 2012).

Similarly, other section-specific trends evidenced in the data were only reliably detected in total elemental measurements, and very rarely did plots from the same strip differ significantly in terms of available nutrients between sections. One such phenomenon potentially relating to the legacy effect of the preceding crop was however observed in strip 10 (only N) whereby Olsen P was higher in section 5 following oat cultivation, than sections 1 and 2 following wheat and beans, respectively (Figure 2.2d). This may contribute towards the trend observed in plots which receive inadequate N applications (plots 3, 5, and 7) for total P to similarly be highest in section 5 (post oats), and also lower in section 2 (post beans). Moreover, these reduced levels

of total P in soils in section 2 might well be expected after legume cropping due to a high requirement for P in the atmospheric N fixation process in their endosymbiotic *Rhizobium* mutualism (Israel, 1987; Al-Niemi *et al*., 1997). However, whether such changes to total P would be evident after just one crop of winter beans (new rotation only started in 2019) seems unlikely, especially without any similar trends reflected in Olsen P measurements (Figure 2.2d) persisting from the previous cropping year. It could however be the case that any changes in the availability of nutrients following the cultivation of different crops grown in rotation may be largely masked by their subsequent use by the wheat crop grown in the year of sampling, instead contributing to the increased yields of these 'first wheat' crops in rotation (Figure 1.2) alongside their more well-established roles as disease breaks (Kirkegaard *et al*., 2008).

Section-specific differences in the total elemental concentrations of K and Mg were in fact much more pronounced. In terms of total K measurements, it was found that section 1 plots had consistently higher total K levels than sections 2 and 5 for any given strip (Figure 2.2g), reflecting a lower K requirement in wheat than for the other crops in rotation. Indeed, according to the RB209 Nutrient Management Guide, winter wheat grain and straw have a potash concentration of just 10.5 kg/T compared with 16.5 kg/T for oats (AHDB, 2023) which have been incorporated in rotations since 1996. Furthermore, total soil Mg stocks could also be linked to the cropping regime, with the section not under crop rotation (section 1) similarly having notably higher reserves of this element than section 2, as well as most strips in section 5, bar those not receiving any N inputs (Figure 2.2i). Taken together these observations could well reflect the bespoke nutrient demands of the non-wheat break crops included in the rotations applied throughout the treatment histories of these plots. However, the trends observed here provide no evidence of the identity of the different crops cultivated

immediately prior to the year of sampling being responsible for the concentrations of any nutrients measured. Indeed, it seems more likely that the patterns displayed reflect a gradual accumulation of the effects of different break crops on nutrient availability, which are compounded in the differences observed in the total elemental concentrations between sections under rotation and continuous wheat cultivation through lengthy treatment histories.

Using these bulk soils as well as corresponding wheat rhizosphere samples for microbial community analyses it will be interesting to see whether we can capture any more of an effect of the previous crop than we were able to detect using soil chemical analyses here. Indeed, changes in microbial communities reflecting these managements as well as any recruitments of functional microbes under transient plant stressors including nutrient deficiencies developed during the growing season may persist even when the selection pressure is lifted. Moreover, it will be interesting in subsequent chapters of this thesis to see if the patterns of soil nutrient status that were observed can be correlated with soil and rhizosphere microbiome status using a culture independent methodology as well as microbial function through an isolate phenotype-based approach. Indeed, here I have characterised the geochemical implications of the principal treatments at Broadbalk on these otherwise similar agricultural soils that may now be used to develop our understanding of the main drivers of microbial community assembly in agricultural microbiomes, and test hypotheses of functional enrichments under defined nutritive circumstances.

# CHAPTER 3 - EXPLORATORY BROADBALK-WIDE BACTERIAL AMPLICON SEQUENCING SURVEY

## *INTRODUCTION*

The Broadbalk experiment at Rothamsted Research (Harpenden, UK) was established in 1843, making it the oldest continuing scientific experiment in the world. Experimentation at Broadbalk has principally concerned factors influencing the yield of wheat, such as fertilisation regime, agrochemical application as well as crop rotation. However, this living resource has found a multitude of other uses, including monitoring of multiple soil biogeochemical processes under various long-term treatments and their effects on soil physicochemical properties (Poulton *et al*., 2024b). Such edaphic factors are well known drivers of microbial diversity, but the effect of changes in soil characteristics due to different agricultural managements which in turn alter microbial structure, diversity, and functionality are still poorly investigated for agricultural systems. The continuity of the various well-defined treatments at Broadbalk lends itself to investigations of microbial communities *in natura* over those using soils with less characterised treatment histories. Indeed, the strength of treatment effects on soil microbial communities, mediated both directly and indirectly via the crop are likely reinforced through repeated cropping at Broadbalk. Prior studies of the microbiology of Broadbalk soils have included work centred around microbial community dynamics utilising both amplicon sequencing (Ogilvie *et al*., 2008; Zhalnina *et al*., 2013) and shotgun metagenomics (Neal *et al*., 2023), as well as bacterial 16S rRNA gene amplicon sequencing of the wheat rhizosphere microbiome for a limited number of fertilisation regimes (Kavamura *et al*., 2018). Although high resolution shotgun metagenomics is undoubtedly a more thorough tool to investigate microbiome structure and potential function, for the hundreds of samples being processed in this Chapter it is prohibitively expensive. An alternative, higher throughput option is the use of increasingly low-cost amplicon sequencing. The aforementioned bacterial 16S

rRNA gene is used routinely and affordably to gain taxonomic insights into microbial communities on large number of samples and for this reason will be adopted in this Chapter. I favour the 16S rRNA gene over amplification of fungal ITS sequences due to the greater phylogenetic diversity in soil bacterial communities compared to fungal sequences, and the fact that the vast majority of soil DNA is derived from bacterial cells.

The aim of this chapter was to assess the impact of long-term fertilisation, specific nutrient amendments, as well as crop rotation on rhizosphere microbiome assembly and diversity *in situ* at the long-term Broadbalk experiment at Rothamsted Research. In order to study the impacts of these factors on microbial community structure I will utilise a bacterial amplicon sequencing approach to capture the full extent of diversity in underlying bulk soils as well as wheat rhizosphere samples. It is expected that similar patterns will be evidenced across both niches sampled, and that a certain degree of convergence will have occurred for rhizosphere and bulk soil samples from any given subplot given the repeated cycles of selection for these wheat associated microbes. Across 7 treatment strips in 3 sections, I will examine impacts of inorganic N fertilisation level, mineral P, K, and Mg application and in terms of crop rotation, the impacts of oat and bean cropping in the preceding year. Inclusions of the aforementioned nutrient drop-out plots from strips in which single elemental nutrients are individually withheld from the full suite of fertilisers are thus particularly relevant to the dissection of the effects of long-term inorganic N, P, K, and Mg amendments. Indeed, soils from Broadbalk plots not receiving N inputs as such were previously utilised within our group in *ex situ* wheat cultivations investigating the role of inorganic N amendments. Accordingly, clear impacts of Broadbalk N applications on the bacterial community structures of both bulk soil and wheat rhizosphere microbiomes

were demonstrated (Kavamura *et al*., 2018). It is anticipated that similar effects may be recaptured for the other elemental nutrients that constitute the principal nutrient treatments at Broadbalk in this Chapter.

Additionally, it is hypothesised that crop rotation will also impact rhizosphere microbiome structure, and as such samples from two sections under rotation but currently sown to wheat (post-oats and post-beans) are investigated alongside those under continuous wheat cultivation. Further dissections of the impact of the previous crop in the rotation sequence may however be precluded by the likely highly influential role of N, which is applied at half the specified rate for oats and not at all for beans, potentially masking any such effects. Moreover, it should also be noted that the current five-year rotation (wheat, wheat, oats, wheat, beans) only replaced the previous rotation scheme (wheat, wheat, wheat, oats, maize) of 20+ years in 2018. As such, I will primarily aim to ascertain the impact of crop rotation as a management practice when compared to continuous wheat cultivation.

Furthermore, the effects of withholding P will not be investigated under crop rotation as the ‘no P’ strip 20 does not exist in the sections studied here (Figure 2.1). Whilst an adjacent strip not receiving P does extend across all sections (strip 19), its fundamentally disparate treatment history of organic amendments until 1988 was clearly observed for multiple soil characteristics in Chapter 2 which are likely to fundamentally alter the resident microflora. Nonetheless, the importance of P inputs for structuring microbial communities should not be overlooked and it may well be expected that under continuous wheat cropping (section 1) at least, their omission may well be of similar importance to N as per the results of the ordination (Figure 2.3) in Chapter 2. Indeed, it is hypothesised that the lack of individual nutrients will significantly impact rhizosphere microbiome structure, and that that the order of

significance will be N>P>K>Mg, this order being determined by the relative contribution of each to microbial and indeed plant cellular requirements.

## *MATERIALS AND METHODS*

### *Experimental site*

The Broadbalk long-term experiment at Rothamsted Research (Harpenden, UK, 51°48′33″ N; 0°22′19″ W, 128 m a.s.l.) is the oldest continuing field experiment in the world, principally concerned with winter wheat yields under defined nutrient regimes through continuous cultivation since 1843. Broadbalk soils are slightly calcareous, silty, clay loams of the Batcombe series classified as Chromic Luvisol (FAO) or Aquic Paleudalf (USDA) internationally, with clay contents varying between 19 – 39 % and an abundance of flints in evidence (Watts *et al*., 2006). The site is freely draining with a downslope of 1°, West to East, along the length of the 320 m by 150 m cultivated area (4.8 ha) sown principally to winter wheat. Various combinations of inorganic fertilisers (supplying N, P, K, and Mg) and farmyard manure (FYM) are applied to 20 strips (typically 6 m wide) most of which run the full length of the field separated by paths (typically 1.5 m wide). The field is further divided transversely into 10 cropping sections (typically 23 m long) numbered 0 – 9 (Figure 2.1b), half of which are in a five-year crop rotation (currently: wheat, wheat, oats, wheat, beans) such that each phase is evidenced every year, the other five growing winter wheat continuously. The portion of each strip within a given section is referred to hereafter as a treatment plot, each of which were subdivided transversely into 4 equally sized ‘replicate’ subplots for the purpose of this study.

The 7 treatment strips studied here are listed in Table 3.1 with details of their bespoke fertilisation regimes along with a description of their relation to a ‘conventionally’ fertilised strip. These treatment strips are investigated in one continuous wheat section (section 1) last fallowed in 1966, and two sections grown in rotation such that they

were preceded by bean and oat cropping years, respectively (sections 2 and 5 in 2020).

Table 3.1. Fertiliser and organic manure treatments for selected Broadbalk strips

| Strip number | Strip name | Treatment c.2020 | Description |
|---|---|---|---|
| 3 | Nil | Nil | Nothing |
| 5 | noN | (P) K Mg | No N |
| 9 | conv | N (P) K Mg | 'Conventional' fertilisation |
| 10 | onlyN | N | No P, K, or Mg (i.e. Only N) |
| 11 | noK | N P Mg | No K |
| 13 | noMg | N P K | No Mg |
| 20 | noP | N K Mg | No P |

FYM: Farmyard manure at 35 t/ha in autumn
N: 192 kg N/ha (N4) as ammonium nitrate (Nitram, 34.5% N) in mid-April (n.b. rotation sections receive N at ½rate under oats, and do not receive any N or FYM under beans)
P: 35 kg P/ha as triple superphosphate in autumn
(P): 35 kg P/ha as triple superphosphate until 2000 (to be reviewed 2025)
K: 90 kg K/ha as potassium sulphate in spring
Mg: 12 kg Mg/ha as Kieserite in spring

***Rhizosphere and bulk soil sampling and processing***

Sampling of Broadbalk wheat rhizosphere and bulk soils in 2020 occurred over two consecutive days (30$^{th}$ June & 1$^{st}$ July) to coincide with flowering (Zadok's growth stage 65) of wheat (cv. Tybalt) – a spring wheat sown 25/03/2020 in exception to the winter wheat convention as wet autumn and winter conditions prevented sowing. Samples were taken across sections 1, 2, and 5 from four 'replicate' subplots per treatment plot from each of strips 3, 5, 9, 10, 11, and 13, as well as 20 in section 1 for a total of 76 samples of rhizosphere and bulk soil each.

From each 'replicate' subplot, rhizosphere soils from three individual wheat plants spaced equidistantly along the subplot, roughly 1.5 m from the path boundaries between strips were sampled in a 'V' formation, one from one side and two from the other of the central 2.1 m wide 'yield strip'. Whole wheat plants were uprooted with the

use of 70% EtOH sterilised hand trowels and forks, such that the crown roots and a proportion of the primary root, seminal, and lateral roots were attached, before pooling with the other two whole wheat plants from each subplot in large clear polythene bags, secured with rubber bands around the stems to contain the roots. Concurrently, bulk soils sampled using a 2.5 mm diameter auger similarly sterilised with 70% EtOH between treatment plots were taken to a depth of 20 cm within the cultivated layer (plough depth ~23cm) of bare soils adjacent to each of the corresponding wheat plants sampled and also pooled in clear polythene bags. As such, a total of four rhizosphere as well as bulk soil samples were taken for each treatment plot corresponding to each 'replicate' subplot. All samples were transported to a 4 °C cold room within 1 hour of being taken, with all subsequent processing at RT occurring on top of cool-packs in the order in which they were sampled. Pooled whole-plant rhizosphere samples were manually separated into their constituent tillers at the root crown, dividing the root mass and dislodging any soil not directly attached to individual roots which was then discarded. Finally, whole plant samples were shaken vigorously by their stems to detach as much of the remaining root-adhering soil as possible before removing all plant parts from the bag, leaving just the rhizosphere soil sample. Thoroughly mixed 5 g portions of both rhizosphere and bulk soils were separated into sterile 7 ml bijou tubes after manual homogenisation including crushing of occasionally intact bulk soil cores inside bags, before storing at -80 °C prior to nucleic acid extraction.

***DNA extraction and quantification***

For each sample, DNA was extracted from 0.25 g of soil using the MoBio PowerSoil™ DNA Isolation Kit (Carlsbad, CA, USA). Extractions were performed according to the manufacturer's instructions, and the bead-beating step was performed with a MP Biomedicals FastPrep-24 machine twice for 30 s at 5.5 m $s^{-1}$. DNA purity and

concentration were determined by NanoDrop spectrophotometry (Thermo Scientific, Wilmington, DE, USA) as well as a Qubit 2.0 Fluorimeter using the ds DNA HS assay kit (Thermo Fisher).

***16S rRNA gene amplicon sequencing and bioinformatic processing***

Bacterial 16S rRNA genes were amplified from bulk soil and rhizosphere DNA samples, using barcoded universal primers 515F (5'-GTG CCA GCM GCC GCG GTA A-3') and 926R (5'-CCG YCA ATT YMT TTR AGT TT-3') for paired-end microbial community amplification. The amplicons were subjected to Illumina® sequencing using the NovaSeq6000 platform to generate 2 x 250 bp paired-end reads at Novogene (China). 16S rRNA gene amplicon sequences were analysed using the pipeline proposed by Quantitative Insights Into Microbial Ecology (QIIME2) (version 2022.11) (Bolyen *et al*., 2019). DADA2 (Callahan *et al*., 2016) was performed on reads which had their barcodes and primers previously removed. Feature table, taxonomy table, metadata file and tree were uploaded into RStudio (version 3.5.0) and the package phyloseq (McMurdie and Holmes, 2014) was used for downstream analysis. Unclassified ASVs, archaeal, chloroplasts and mitochondrial sequences were removed from the dataset. Prevalence filtering was performed, where reads present in less than 5% of samples were removed. Bacterial alpha diversity indices related to richness (Chao1) and diversity (Shannon) were calculated based on the rarefied ASV table (17,712 reads). For beta diversity analyses, data was normalised using DESeq2. Bray-Curtis distance was calculated from the resulting normalized ASV table using the "ordinate" function on "phyloseq" package. The resulting matrix was used for Principal Coordinates Analysis (PCoA). Permutational Multivariate Analysis of Variance (PERMANOVA) was used to detect the significance of different factors (niche, sections

and plots) in structuring the bacterial communities, using the “adonis2” function (R package: vegan v2.6-4) with 9999 permutations.

***RESULTS***

Principal Coordinate Analysis (PCoA) showed clustering of all samples by niche, with bulk soil samples separating from rhizosphere samples along the first axis (Figure 3.1), and which themselves varied to lesser extent than bulk soils on the second axis. PERMANOVA results indicated that niche explains 8.92% of the total variability in the data, while section explains 5.84% and plot 7.27% (Table 3.2). The interactions of "niche:section" and "section:plot" were also significant ($p < 0.05$), whereas a large proportion of the total variation was residual (59.8%), remaining unexplained by the factors studied in this analysis.

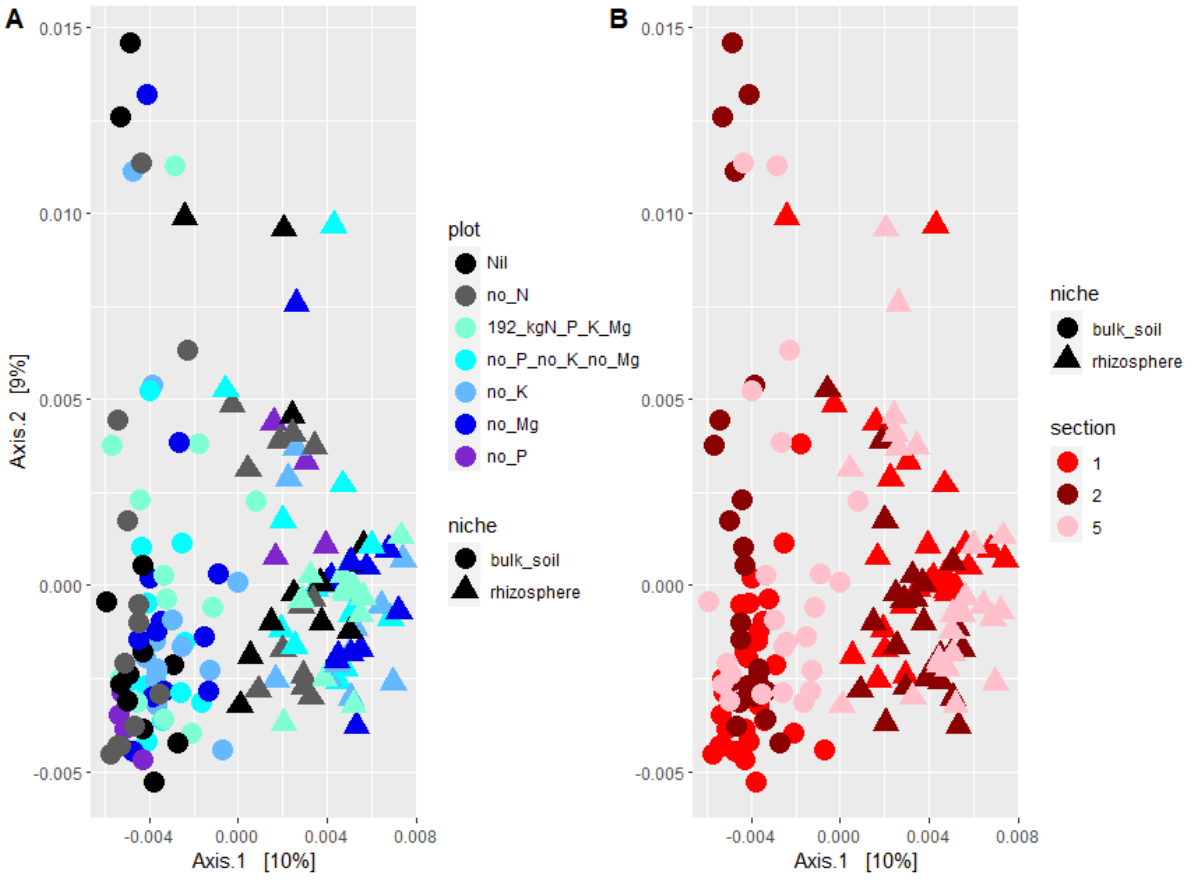



**Figure 3.1 – PCoA plot based on Bray-Curtis similarity distance matrix showing the structure of rhizosphere and bulk soil bacterial communities** (triangles and circles, respectively) from plots receiving different fertilisers (A), obtained from sections 1 (red), 2 (dark red) and 5 (pink) (B), at Broadbalk.

Table 3.2. PERMANOVA results of beta diversity analysis of bacterial communities from all bulk soil and rhizosphere samples across sections 1, 2 and 5.

| **Sources of variation** | **$R^2$** | **F** | **Pr(>F)** |
|---|---|---|---|
| Niche | 0.08922 | 17.0074 | p=0.001 |
| Section | 0.05841 | 5.5674 | p=0.001 |
| Plot | 0.07272 | 2.3104 | p=0.001 |
| Niche:section | 0.02252 | 2.1469 | p=0.001 |
| Niche: plot | 0.03012 | 0.9570 | p=0.682 |
| Section:plot | 0.07466 | 1.4232 | p=0.001 |
| Niche:section:plot | 0.05431 | 1.0352 | p=0.285 |
| Residual | 0.59804 | | |

As the bacterial communities were clearly influenced by niche, the data was split by niche and all further analyses were conducted on rhizosphere samples. For section 1, the different fertiliser treatments explain 28.79% of variability (F=1.4148, p=0.001), whereas for section 2, they explain 27.18% of variability (F=1.3449, p=0.001) and the different fertiliser treatments explain 27.13% of variability (F=1.3405, p=0.004) in section 5 (Figures 3.2a, b and c, respectively) (Table 3.3). In sections 2 and 5, the separation of plots which have received nitrogen versus the plots which haven't received any inorganic N is evident (Figure 3.2b and c). In addition, for section 2 there is a further separation of a cluster of samples from plots which do not receive Mg or K inputs (Figure 3.2b). In section 1, the only clear separation of plots was for those from strip 20 that has never received inorganic P inputs (Figure 3.2a).

Table 3.3. PERMANOVA results of beta diversity analysis of bacterial communities from rhizosphere samples of sections 1, 2 and 5, separately.

| Sections | **Sources of variation** | **$R^2$** | **F** | **Pr(>F)** |
|---|---|---|---|---|
| Section 1 | Plot (treatment) | 0.2879 | 1.4148 | p=0.001 |
| Section 2 | Plot (treatment) | 0.2718 | 1.3449 | p=0.001 |
| Section 5 | Plot (treatment) | 0.2713 | 1.3405 | p=0.004 |

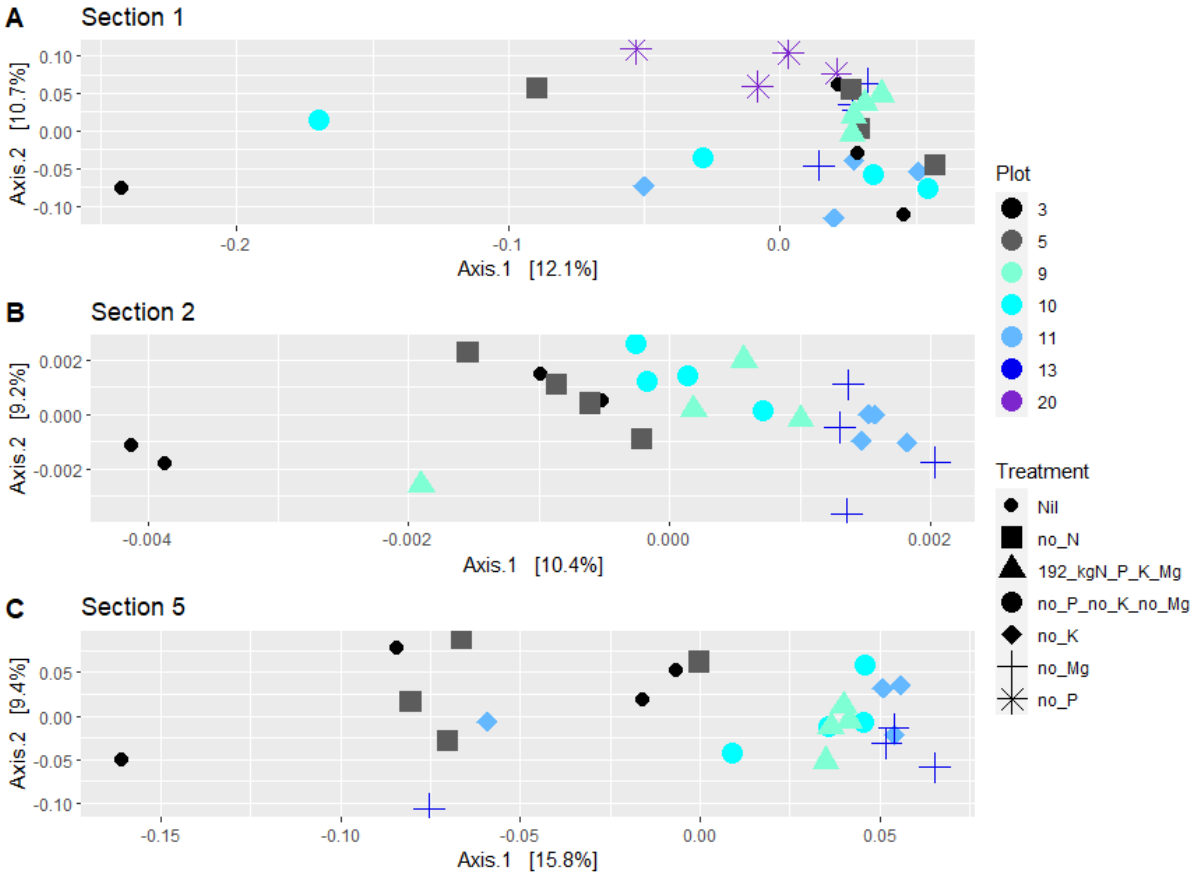



**Figure 3.2 – PCoA plots based on Bray-Curtis similarity distance matrix showing the structure of rhizosphere bacterial communities obtained from sections 1, 2 and 5, separately, from plots receiving different fertilisers at Broadbalk.**

Regarding the contribution to this treatment variation of each principal nutrient individually, the results of further section-specific beta diversity analyses considering the application of N, P, K, and Mg to the plots from which samples were taken are presented in Table 3.4. These results, along with an additional PERMANOVA of all rhizosphere samples across these three sections (Table 3.5) confirm the overarching significance of N additions, albeit to a lesser extent in section 1, as well as a generally decreasing proportion of total variation in the bacterial communities attributable to exogenous nutrients in the order N > P > K > Mg.

Table 3.4. PERMANOVA results of beta diversity analysis of bacterial communities from rhizosphere samples of sections 1, 2 and 5, separately.

| | Section 1 | Section 2 | Section 5 |
|---|---|---|---|
| N_treatment | $R^2$=0.05223, F=1.5403, p=0.013 | $R^2$=0.07813, F=1.9318, p=0.001 | $R^2$=0.11285, F=2.7878, p=0.001 |
| P_treatment | $R^2$=0.06245, F=1.8417, p=0.003 | $R^2$=0.04998, F=1.2358, p=0.061 | $R^2$=0.04109, F=1.0150, p=0.371 |
| K_treatment | $R^2$=0.07698, F=2.2700, p=0.001 | $R^2$=0.04540, F=1.1226, p=0.164 | $R^2$=0.03941, F=0.9735, p=0.482 |
| Mg_treatment | $R^2$=0.03063, F=0.9031, p=0.634 | $R^2$=0.04741, F=1.1722, p=0.113 | $R^2$=0.04371, F=1.0798, p=0.296 |

Table 3.5. PERMANOVA results of beta diversity analysis of bacterial communities from all rhizosphere samples across sections 1, 2 and 5.

| **Sources of variation** | **$R^2$** | **F** | **Pr(>F)** |
|---|---|---|---|
| N treatment | 0.03929 | 3.1313 | p=0.001 |
| P treatment | 0.02679 | 2.1354 | p=0.001 |
| K treatment | 0.02584 | 2.0593 | p=0.001 |
| Mg treatment | 0.01530 | 1.2191 | p=0.101 |

When comparing bacterial communities at the section level, the structure of bacterial communities from plots 3 (Nil) and 5 (No_N) were not significantly different from each other but were from those plots that had received mineral N (p=0.159 fertiliser and p=0.074, respectively) (Figures 3.3a and b, respectively). For the other plots in which mineral nitrogen is applied, it is possible to observe differences in bacterial communities across different sections, showing that the previous crop was important in shaping bacterial communities from plot 9 (192_kgN_P_K_Mg) (Figure 3.3c), in which they account for 30.06% of variability (F=1.9343, p=0.001), plot 10 (no_P_no_K_no_Mg) (Figure 3.3d) (25.53%, F=1.5425, p=0.001), plot 11 (no_K) (Figure 3.3e) (24.28%, F=1.4432, p=0.003) and plot 13 (no_Mg) (Figure 3.3f) (25.72%, F=1.5588, p=0.002).

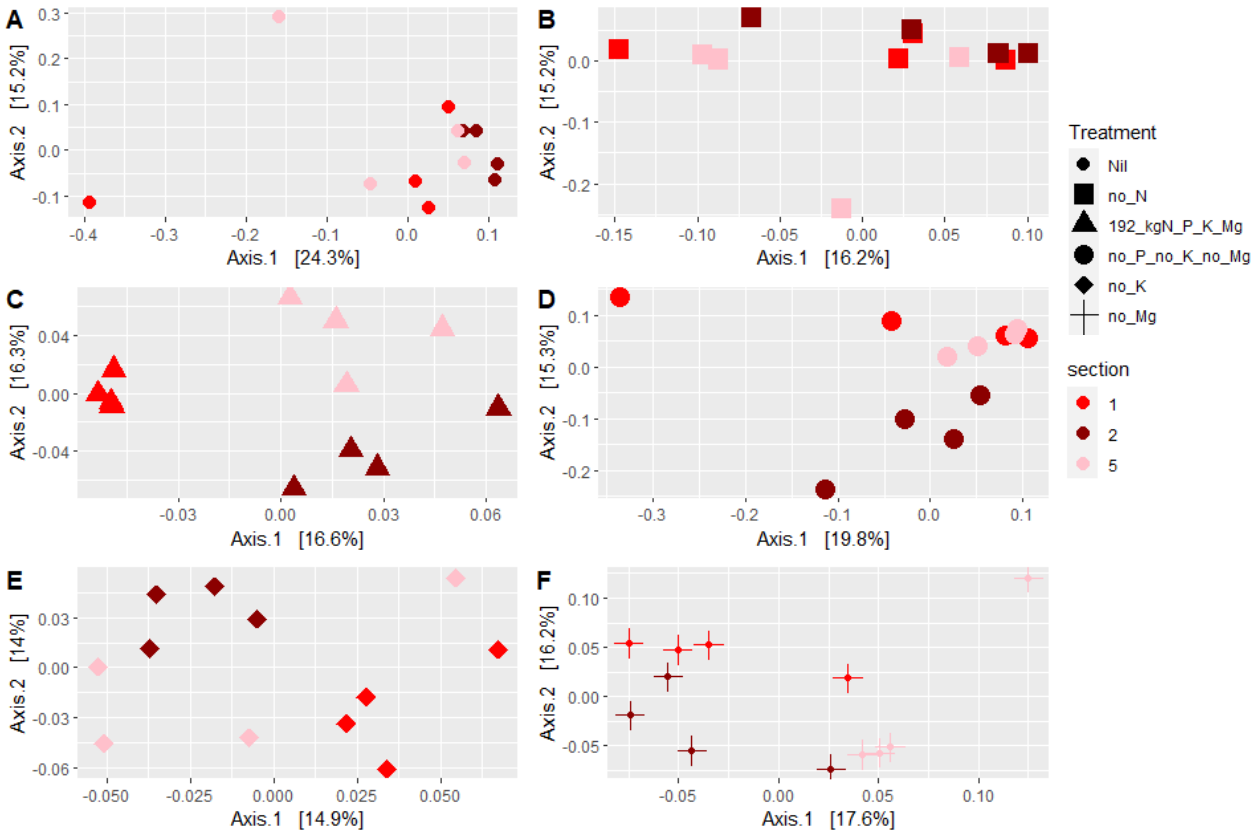



**Figure 3.3 – PCoA plots based on Bray-Curtis similarity distance matrix showing the structure of rhizosphere bacterial communities obtained from plots receiving different fertilisers across different sections.** A – plot 3 (nil), B – plot 5 (no_N), C – plot 9 (192_kgN_P_K_Mg), D – plot 10 (no_P_no_K_no_Mg), E – plot 11 (no_K), F – plot 13 (no_Mg).

Overall, the different fertilisers did not affect the richness (Chao1) and diversity (Shannon) of rhizosphere samples collected from sections 1, 2 and 5 (Figures 3.4 and 3.5). Although no statistical significances have been found (p>0.1), it is possible to highlight some trends, such as in the continuous wheat section where the plot which has never received any mineral P exhibits a slightly lower richness (Figure 3.4a) and diversity (Figure 3.5a) when compared to the plots in which P is applied.

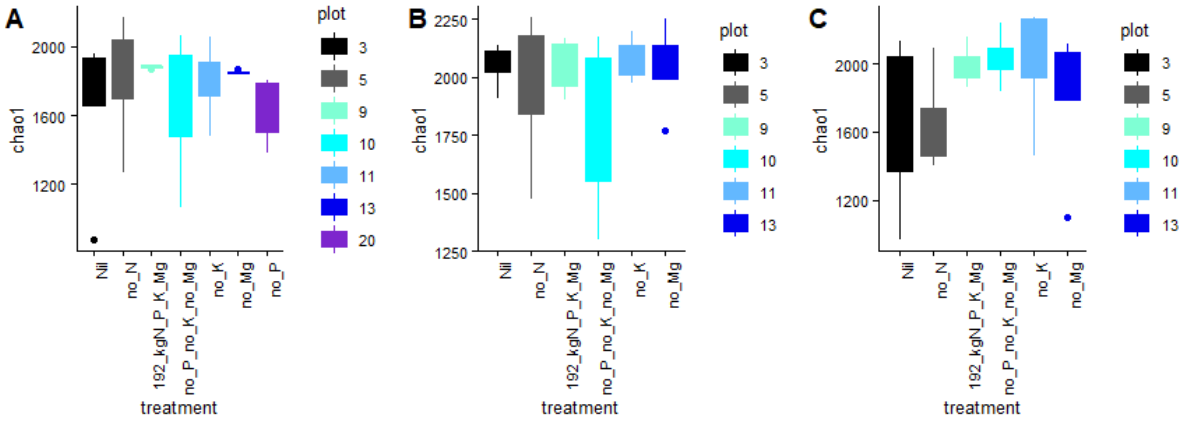



**Figure 3.4 – Chao1 index obtained for rhizosphere samples from plots 3, 5, 9, 10, 11, 13 and 20* from sections 1 (A), 2 (B) and 5 (C).**

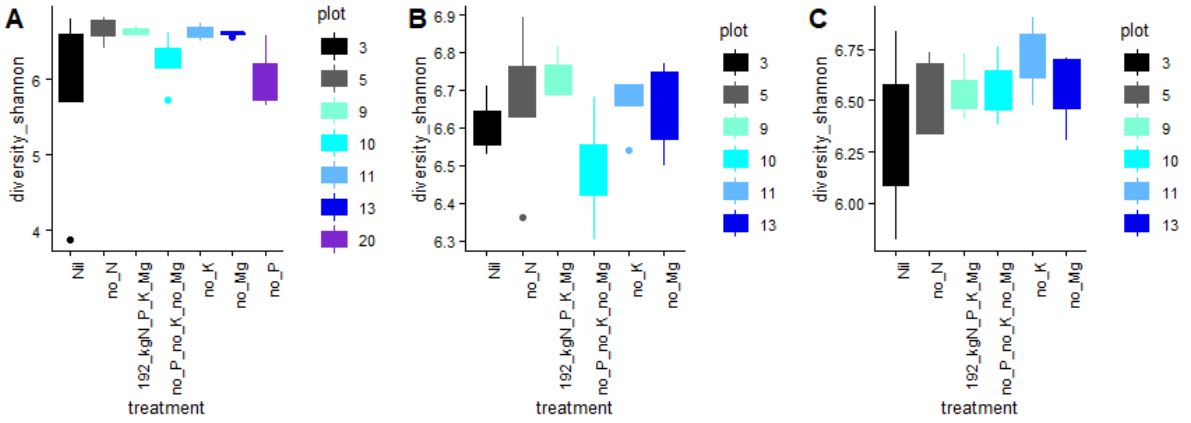



**Figure 3.5 – Shannon index obtained for rhizosphere samples from plots 3, 5, 9, 10, 11, 13 and 20* from sections 1 (A), 2 (B) and 5 (C).**

Cropping regime did not significantly affect the richness (p=0.388) and diversity (p=0.483) of rhizosphere samples from plot 3 where no fertiliser is added, across sections 1, 2 and 5 (Tables 3.6 and 3.7). Although no significant differences were observed for plot 5 where no nitrogen was applied, lower richness and diversity indices were observed when oats were grown following wheat (section 5). For strip 9 where all nutrients were supplied, although not significant, richness was higher for the wheat plots following beans (section 2) and oats (section 5). Additionally, measures of diversity were higher in the rhizosphere of wheat post-beans for strip 9, although these did not differ significantly from those found for wheat cultivated either continuously (section 1) or following oat cropping (section 5). In general, it is also possible to identify a trend for the bacterial richness metrics to be higher in the wheat rhizosphere in section 2 following bean cultivation, except for plot 10 in which no P, K, or Mg is applied (Table 3.6). Individual omissions of these nutrients did not obscure this effect, which was recaptured in strips not receiving Mg amendment, whilst under K omissions both sections under rotation were more diverse, though none of which differed significantly so.

Table 3.6. Chao1 index obtained for rhizosphere samples from plots 3, 5, 9, 10, 11 and 13 from sections 1, 2 and 5. Different letters in each column denote statistical differences as indicated by Tukey test ($p < 0.05$), across the three sections for each individual plot/treatment.

| | **Plot 3** | **Plot 5** | **Plot 9** | **Plot 10** | **Plot 11** | **Plot 13** |
|---|---|---|---|---|---|---|
| **Section 1** | 1,672.90 ± 564.37 a | 1,820.12 ± 355.34 a | 1,894.31 ± 12.18 a | 1,662.03 ± 430.23 a | 1,809.51 ± 247.97 a | 1,836.73 ± 33.80 a |
| **Section 2** | 2,036.07 ± 108.26 a | 1,941.10 ± 343.61 a | 2,048.17 ± 127.63 a | 1,792.41 ± 399.22 a | 2,099.09 ± 79.21 a | 2,052.53 ± 192.30 a |
| **Section 5** | 1,686.28 ± 567.58 a | 1,662.77 ± 321.47 a | 2,005.53 ± 116.28 a | 2,056.20 ± 163.88 a | 1,997.97 ± 374.37 a | 1,815.90 ± 485.64 a |

Table 3.7. Shannon index obtained for rhizosphere samples from plots 3, 5, 9, 10, 11 and 13 from sections 1, 2 and 5. Different letters in each column denote statistical differences as indicated by Tukey test ($p < 0.05$), across the three sections for each individual plot/treatment.

| | **Plot 3** | **Plot 5** | **Plot 9** | **Plot 10** | **Plot 11** | **Plot 13** |
|---|---|---|---|---|---|---|
| **Section 1** | 5.90 ± 1.35 a | 6.67 ± 0.17 a | 6.65 ± 0.06 a | 6.24 ± 0.39 a | 6.62 ± 0.12 a | 6.59 ± 0.05 a |
| **Section 2** | 6.60 ± 0.07 a | 6.67 ± 0.23 a | 6.72 ± 0.07 ab | 6.49 ± 0.15 a | 6.66 ± 0.10 a | 6.65 ± 0.11 a |
| **Section 5** | 6.33 ± 0.45 a | 6.52 ± 0.22 a | 6.55 ± 0.12 b | 6.56 ± 0.16 a | 6.71 ± 0.15 a | 6.55 ± 0.19 a |

## *DISCUSSION*

In this Chapter, a bacterial amplicon sequencing approach was taken to assess the main drivers of microbial community structure amongst the principal Broadbalk treatments. As expected, the main factor separating all sequenced samples was the niche targeted by samples of the bulk soil and rhizosphere compartments, confirming the key role of the crops planted in shaping bacterial communities. Indeed, even after 177 years of divergent treatments at Broadbalk, the background soil communities have not converged with that of rhizosphere communities enriched every growing season. This indicates that the assembly of treatment specific rhizosphere communities is still a fairly transient occurrence and must be reactive to the growing plant and the challenges it currently faces. The plant may exert this influence both physically and chemically (Sasse *et al.*, 2018) through the architecture of their root systems and associated exudation of compounds that enrich a subset of the microbial diversity present in the bulk soil. This selection is reflected by the lower extent of separation of rhizosphere samples on the second axes of Figure 3.1 when compared with those of bulk soils.

Whilst the niche was identified as the strongest source of variation in in the PERMANOVA with the largest F statistic Table 3.2, several other experimental factors had notable, yet weaker effects. Of the other statistically significant factors in this beta diversity analysis it is interesting to note that the interaction of section and plot explained a similar amount of variation to both section and plot individually, reflecting the strong modulating effect of their different managements on the principal nutrient treatments. However, the total amount of variation explained in this analysis left the majority (59.8%) unaccounted for by the factors studied here. This may well be indicative of the nature of these *in situ* field observations wherein many environmental

variables may confound the experimental treatments. For example, the proximity of certain plots in section 1 to the wooded portion of the Broadbalk 'wilderness' left to revert to an unmanaged state (Harmer *et al*., 2001) and also the unevenly distributed variance in the percentage of clay in soils across the field (Watts *et al*., 2006), before even considering the non-randomised design of the plot layout, conceived before the dawn of modern statistical design. Indeed, ordination analysis in a previous study by Kavamura *et al.* (2018) utilising Broadbalk soils in a glasshouse pot experiment explained a far higher proportion of the community variation in the first two principal components than in the present study. This suggests that environmental factors at this site reduced the ability to determine the impact of nutrients as well as crop rotation on the microbiome structure in the present study. As such, I suggest that the use of field soil in pot trials is a good strategy to ascertain the importance of these as well as other management factors in future studies.

Moreover, this prior work identified similar structuring of both bulk and rhizosphere soil bacterial communities across nutrient treatments with stronger temporal shifts in the rhizosphere indicative of plant selection (Kavamura *et al*., 2018). As such I focused all further analyses on the bacterial rhizosphere microbiome as opposed to the bulk soil communities. Indeed, given the ultimate goal of understanding extant PGP interactions and prospecting the microbes involved, it is logical to turn the focus of this chapter towards samples in which microbes exhibiting rhizosphere competence and wheat-associated lifestyles prevail.

Considering each section separately, the amount of variability explained by the different fertilisation treatments applied to plots was similar (~27-28%), however the expected clustering of rhizosphere samples as such in the ordinations in Figure 3.2 were not equally distinguishable across these 3 sections. Indeed, the anticipated

separations of sequenced rhizosphere bacterial communities according to different N input levels at Broadbalk as described by Kavamura *et al.* (2018) were not universally evidenced. Most notably, for section 1 in which wheat has been cultivated continuously, similarly to the soils used for those *ex situ* cultivations, no clear separations of samples from the principal nutrient treatments strips were exhibited (Figure 3.2a) aside from samples from the plot never having received P inputs (strip 20) which are unique to this section. Indeed, these samples clustered together away from the other samples on the second axis, providing an indication of the wider importance of the provision of inorganic P in structuring bacterial communities associated with wheat roots at Broadbalk. Accordingly, we also observed slightly lower measures of richness and diversity in the no P plot (Figure 3.4a and 3.5a) – the only result of note from alpha diversity comparisons across strips within sections, albeit not a statistically significant one. This is consistent with some but not all (Tan *et al*., 2013; Silva *et al*., 2017) similar assessments under long-term omissions of P inputs, but still may well reflect the dominance of taxa specifically enriched in the utilisation of insoluble sources of P (Silva *et al*., 2017). Alternatively, this may be symptomatic of a reduction in ability of the crop to recruit a more diverse microbiome mediated by the highly limited plant growth in this plot, for which yields have collapsed to below the level of plots receiving no external inputs at all (Poulton *et al*., 2024b).

The overarching N-driven effect was however more clearly observed in sections 2 and 5 (Figure 3.2b and c) under crop rotation, where samples from plots not receiving any inorganic N inputs (3 and 5) clustered away from fertilised plots. This similarity of samples from plots not receiving mineral N inputs (3 and 5) reflects the important role of nitrogen in driving differentiation of the microbial communities associated with wheat roots. Furthermore, the clustering together of samples from plots in strips 9 and 10

which differ in terms of all three other principal nutrient inputs besides N was particularly conspicuous, although similarly more so in sections 2 and 5, consistent with the above findings. The results of further beta diversity analyses considering the relative contribution of each of the principal nutrients (N, P, K, and Mg) individually confirmed this pre-eminence of N applications (Table 3.5) whilst reflecting the inconsistency of this N-driven pattern in section 1 (Table 3.4) consistent with the less defined clustering in Figure 3.2a outlined above.

The observations that this N-driven effect was more clearly detected in plots 2 and 5 may be further related to the less nutrient-depleted nature of these plots which, as mentioned in Chapter 2, have been subject to fewer wheat cropping years through fallowing and now crop rotation. Indeed, with more potentially available resources remaining for plant growth, the extent to which the associated microbiome may be modulated is greater. Yields of such first wheat crops in rotation have been notably elevated since the introduction of rotations c.1968 (Figure 1.2), potentially indicating an increased capacity with which to modulate the microbiome afforded by this growth benefit. The possibility also exists that these trends represent the legacy of the identity of different crop plants cultivated in the previous cropping year, or more generally the accumulated effects of the inclusion of these plants in the rotation scheme. In non-wheat cropping years these other plants may have been better able to drive microbial communities in different directions under various nutrient limitations, especially in comparison to the modern wheat cultivars grown at Broadbalk which are selected primarily for their yield potential. Indeed, through the 60 years of breeding elite wheat germplasm under optimal nutrient conditions post-Green Revolution, it is posited that modern cultivars are less well equipped to interact with the microbiota in times of nutrient limitation (Pérez-Jaramillo *et al*., 2016; Hassani *et al*., 2019). Accordingly,

investigation of the dwarfing genes central to Green Revolution crop improvements by Kavamura *et al.* (2020) revealed the rhizosphere microbiomes of modern semi-dwarf wheat cultivars to be more similar to the bulk soil than corresponding tall cultivars.

In terms of the effects of sections under different managements on individual treatment strips, the importance of N in driving rhizosphere microbial community assembly was further underscored. Indeed, only under adequate N fertilisation (192 kg N/ha) and normal doses of other nutrients (P, K and Mg) did samples from different sections clearly cluster according to the crop previously cultivated (Figure 3.3c), potentially indicative of their necessity for those crops to exert an influence on the persisting microbial community structure, most likely through their growth-limiting effects. Moreover, no significant differences between the structure of bacterial communities from strips not receiving N inputs (3 and 5) were detected between sections. Whilst a certain amount of separation was still observed for the other plots receiving N inputs, a lower percentage of variability was explained by the previous crop grown in these sections in our analyses. Furthermore, although not significant, analyses of alpha diversity revealed generally higher taxonomic richness in rotation sections, apart from for strip 10 lacking P, K, and Mg simultaneously. This effect was particularly evident following bean cultivation, for which diversity was also slightly elevated under fully-fertilised conditions (Table 3.7) potentially indicative of the legacy of the selection for a more disparate microbiota structured around N-fixing mutualist species in leguminous root nodules in the previous year.

Indeed, this also serves to highlight the relative importance of other non-N nutrients in sustaining interactions with the microbiome, over and above the modulating effects of the previous crops, which were only visualised clearly for samples in the fully-fertilised plot 9 (Figure 3.3c), in which differences such as the admittedly insignificantly elevated

levels of diversity following bean cultivation additionally became apparent. The presence of limitations for these other nutrients seems to obscure the effects of the previous crop in terms of variation in the bacterial rhizosphere microbiome. This is most likely mediated by their plant growth-limiting effects through continuous cropping, potentially restructuring the microbiota towards a state best able to help deal with these pressures.

A hierarchy of these effectors may be becoming apparent wherein N amendments are the most important factor, followed by the other principal elemental nutrients applied at Broadbalk, and finally the management practices applied to the different sections. In plots without N amendments, the effects of other nutrients are not discernible, and only in fully fertilised plots are the effects of prior cropping clearly realised. The results presented here highlight the central role of nitrogen as a primary determinant of growth in agricultural settings such as these, and without which the extent of manipulation of the rhizosphere microbiome is limited. Indeed, the plant needs the nitrogen to drive growth with which they extend their influence on the associated rhizosphere microbiome, be that with or without other nutrient limitations or the legacy effects of different cropping regimes.

# CHAPTER 4 – SCREENING FOR PGP FUNCTIONS RELEVANT TO BROADBALK CULTURE COLLECTIONS *IN VITRO*

## *INTRODUCTION*

Microbial culture collections provide the basis for the discovery and validation of PGP microbes and are increasingly utilised as resources for investigations of the interactions of complex microbial communities and plants (Finkel *et al.*, 2017). Successful cultivation and subsequent investigation of environmental isolates enables the formulation of hypotheses to which data from culture-independent surveys may be linked and later tested in experimental plant-microbiome systems (Gutleben *et al.*, 2018). Indeed, establishing causality in agriculturally relevant PGP microbial associations requires the integration of data from culture-independent surveys of naturally occurring microbial communities under various conditions with hypothesis-driven *ex situ* experimentation (Zengler, 2009).

The basis of such study is the isolation of pure microbial cultures such that physiology can be interrogated, allowing insight into the functional repertoire of microbial communities. The isolation of pure microbial cultures is however far from a perfect sampling method given the oft-cited 'great plate-count anomaly' (Staley and Konopka, 1985) between the number of microbial cells directly observed in a sample and the fraction that form propagation colonies on standard laboratory growth media solidified with agar. Whilst the veracity of this phenomena is debated (Martiny, 2019; Steen *et al*., 2019) it is certainly true that microbiome research over the last decade has been dominated by high resolution culture-independent amplicon studies for this very reason, neglecting the central role of *in vitro* study of pure cultures (Sarhan *et al*., 2019). Moreover, conventional 'colony-based culture' (CBC) collection involves labour intensive spread-plating and colony picking which along with the common requirement to re-streak colonies picked from solid media which are often non-clonal (Armanhi *et*

*al*., 2016) often precludes the establishment of sufficiently large, and therefore representative culture collections.

The occurrence of such mixed cultures can be minimised to its logical conclusion by further dilution of the initial inoculum up to the point at which only one pure colony is cultured from a single viable cell – the 'limiting dilution'. Doing so with agar plates would obviously be immensely inefficient but if the units of cultivation are reduced to the size of individual wells in microwell plates for example, large numbers of clonal cultures may be isolated simultaneously from the same inoculum. Methodologies known as dilution-to-extinction (D2E) culturing are based on this premise, such that the purity of cultures in each microwell can be ensured by maximising the statistical likelihood of an individual microwell receiving a single viable cell from an aliquot of an inoculum of known concentration as opposed to more than one cell. Indeed, assuming the distribution of viable cells into microwells follows a Poisson distribution (Haas, 1989), Goodman *et al.* (2011) elegantly describe maximising the proportion of clonal to non-clonal cultures by inoculating 384-well plates with an inoculum that left 70% of wells empty at the end of their incubation (Figure 4.1).

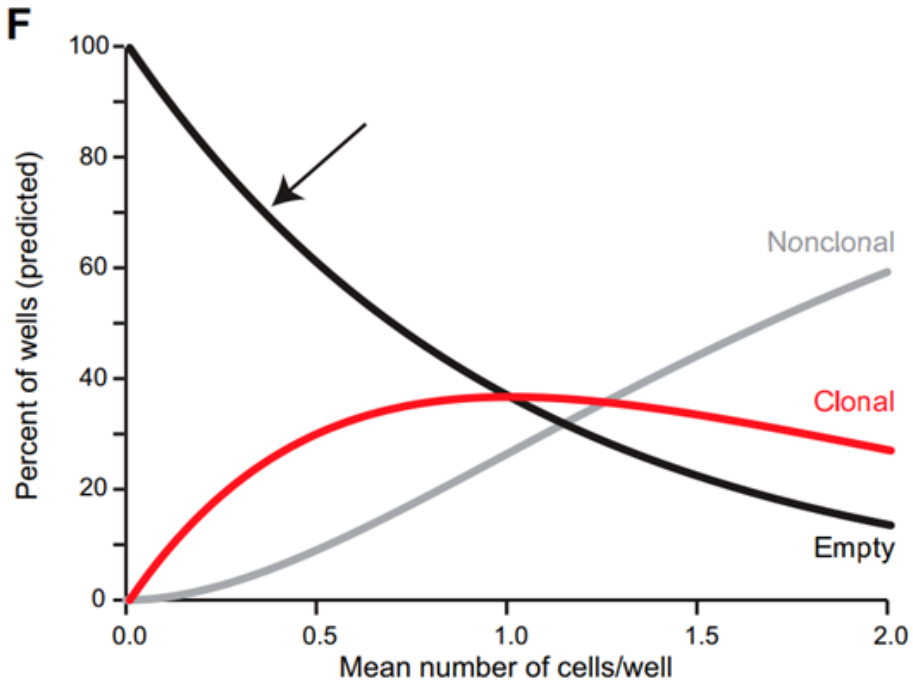



**Figure 4.1 – Communities of cultured microbes clonally archived in multi-well plate format by limiting dilution.** At a dilution of a sample that produces 70% empty wells (arrow), Poisson distributions predict that ~25% of wells will contain a clonal population of cells and that ~5% of wells will be nonclonal. Reproduced from Goodman *et al.* (2011).

Large-scale cultivation efforts have recently found success with these methodologies, particularly regarding the high proportion of rhizosphere OTUs cultivated by Zhang *et al.* (2019) from an agricultural setting. Indeed, using a single growth medium in 96-well plate format ~70% of OTUs reproducibly detected by culture-independent methods in the rhizosphere of rice plants grown in agricultural soils were isolated, including representatives of all four bacterial phyla and 27 bacterial families typically associated with rice roots. Based on these successes, the same simple, dilute growth medium (1/10$^{th}$ concentration Tryptone Soya Broth) and scalable 96-well plate format subsequently formalised by Zhang *et al.* (2021) seem a suitable starting point for the development of representative culture collections from agricultural rhizosphere microbiomes.

Furthermore, the ability to conduct such liquid media-based isolations in the wells of standardised laboratory microwell plates enables automation of the repetitive liquid handling tasks during dilutions, well inoculations, and the consolidation of cultures from D2E plates occupied mostly by empty wells into more efficient long term storage vessels. Such tasks are truly the low hanging fruit of laboratory automation which not only reduces workload but also human errors which are of particular note during the consolidation of D2E plates, where cultures must be cherrypicked using unintuitive well coordinates. Recently popularised low-cost liquid handling robotics running open-source software have significantly lowered the barrier of entry for automation in research settings, facilitating viable alternatives to conventional methods for culture-dependent characterisations of microbial communities here. This development, along with the lack of time-consuming spread-plating and colony picking involved in CBC should enable the creation of culture collections large enough to be much more representative of their source microbial communities, as well as much broader sampling campaigns.

This Chapter aims to give a functional context to the fertilisation driven trends of microbial diversity identified by the Broadbalk-wide amplicon survey in the previous chapter and what this means for the functional repertoire of these agricultural microbiomes. That work identified the principal nutrient treatments on Broadbalk as the main drivers of microbial community structure, and so here I focus on the central role of inorganic N fertilisation as well as individual nutrient dropouts in selected plots across a single section under continuous wheat cultivation (section 1). Specifically, I test hypotheses that withholding specific nutrients results in the selection of a greater proportion of microbes in the rhizosphere with the capacity to increase the availability of these nutrients. Previous work by Reid *et al.* (2021) indicated that the wholesale

addition of a high dosage of nutrients to a low nutrient soil resulted in a clear reduction in the selection of putative plant growth promoting bacteria in the rhizosphere and rhizoplane of wheat in comparison to wheat grown in the unamended nutrient-poor soil.

This Chapter will further the findings of that study by screening culture collections of microbes associated with Broadbalk-grown wheat for nutrient-liberation potential and ascertain whether the effects of individual elemental nutrient amendments can be dissected rather than comparing unfertilised treatments with fully fertilised ones. To do this I develop high throughput plate-based bioassays using previously established specialised growth media for the detection of visible *in vitro* phenotypes. Eight putatively PGP functions were tested as such, including several implicated in improved plant-availability of the four elemental nutrients (N, P, K, and Mg) that constitute the principal mineral amendments on Broadbalk, and for which individual drop-out plots exist. The ability of individual microbial cultures to increase the availability of nitrogen is assessed via their capacity for N mineralisation by protein-hydrolysing exoenzymes (Jan *et al*., 2009) degrading casein *in vitro*. Similarly, the microbial degradation of single, defined sources of insoluble P, K, and Mg, namely tricalcium phosphate (Pikovskaya, 1948), potassium aluminosilicate (Zhang and Kong, 2014), and magnesium trisilicate (Kang *et al*., 2017) is indicative of their capacity to increase the plant-available pools of these nutrients. Additionally, bioassays targeting specific mechanisms also implicated in the enhanced bioavailability of these nutrients were conducted. Extracellular acidification is understood as a principal mechanism of direct nutrient mobilisation via the production of pH-lowering organic acids and ammonium assimilation mediated proton extrusion (Alori *et al*., 2017), increasing the availability and uptake of most plant macro- and micro-nutrients via changes to their solubility,

ionic form, adsorption and mobility (Penn and Camberato, 2019). More indirectly, chelates and complexes of microbial siderophores and exopolysaccharides strongly binding metal cations (namely $Fe^{3+}$, $Fe^{2+}$, $Al^{3+}$, and $Ca^{2+}$) may also mediate the provision of limiting nutrients to plants (Rawat *et al*., 2021). The production of Fe-binding siderophores in particular may also alleviate plant iron deficiencies and control the virulence of other potentially pathogenic microbes by complexing iron for uptake by roots and limiting environmental iron concentrations (Kloepper *et al*., 1980; Crowley *et al*., 1991; Crowley, 2006; Vansuyt *et al*., 2007). Moreover, exopolysaccharide production is critical for the formation of microbial biofilms, which amongst myriad other roles increase the efficiency of many PGP mechanisms by concentrating secreted molecules around both insoluble substrates and the plant roots which benefit from these functions (Ahmed and Holmström, 2015; Ghosh *et al*., 2019; Li *et al*., 2023a). Finally, the production of hydrogen cyanide (HCN), which has a well characterised role in the biocontrol of fungal plant pathogens (Blumer and Haas, 2000), is also similarly implicated in geochemical processes in the substrate (i.e. chelation of metals), indirectly increasing the availability of phosphate (Rijavec and Lapanje, 2016).

Whilst it would be inappropriate to define isolates as PGP microbes based on the results of these simplified, single-substrate screens assessing individual *in vitro* traits (Bashan *et al*., 2013), these data help build an understanding of the functional repertoire of Broadbalk microbial communities in order to both elucidate the drivers of agriculturally relevant PGP associations *in situ* and inform future bioprospecting efforts. As such, the breadth of tests conducted, although not exhaustive, is broad enough to assess macro- and micro-nutrient cycling potential of culture collections and can be used to address the central hypothesis that nutrient depletion results in an

enhanced selection for microbes capable of solubilising and mineralising nutrients in the root zone. Here, the establishment of isolate libraries from selected Broadbalk treatment plots under continuous wheat cultivation (section 1) enables contextualisation of the fertilisation-driven gradients of soil chemistry and amplicon sequencing-derived microbial diversity in terms of microbial functionality. Indeed, now that the main drivers of microbial community structure identified in the amplicon survey have outlined the microbial ‘strata’ on Broadbalk, we now need to identify the value that lies within any of these potentially rich seams by assessing the functional repertoire of microbiomes across these gradients of diversity.

## *MATERIALS AND METHODS*

### *Experimental site*

The Broadbalk long-term experiment at Rothamsted Research (Harpenden, UK, 51°48′33″ N; 0°22′19″ W, 128 m a.s.l.) is the oldest continuing field experiment in the world, principally concerned with winter wheat yields under defined nutrient regimes through continuous cultivation since 1843. Broadbalk soils are slightly calcareous, silty, clay loams of the Batcombe series classified as Chromic Luvisol (FAO) or Aquic Paleudalf (USDA) internationally, with clay contents varying between 19 – 39 % and an abundance of flints in evidence (Watts *et al*., 2006). The site is freely draining with a downslope of 1°, West to East, along the length of the 320 m by 150 m cultivated area (4.8 ha) sown principally to winter wheat. Various combinations of inorganic fertilisers (supplying N, P, K, and Mg) and farmyard manure (FYM) are applied to 20 strips (typically 6 m wide) most of which run the full length of the field separated by paths (typically 1.5 m wide). The field is further divided transversely into 10 cropping sections (typically 23 m long) numbered 0 – 9 (Figure 2.1b), half of which are in a five-year crop rotation (currently: wheat, wheat, oats, wheat, beans) such that each phase is evidenced every year, the other five growing winter wheat continuously. The portion of each strip within a given section is referred to hereafter as a treatment plot, each of which were subdivided transversely into 4 equally sized ‘replicate’ subplots for the purpose of this study.

The 8 treatment strips studied here are listed in Table 4.1 with details of their bespoke fertilisation regimes along with a description of their relation to a ‘conventionally’ fertilised strip. These treatment strips are investigated in one continuous wheat section (section 1) last fallowed in 1966.

Table 4.1. Fertiliser and organic manure regimes for selected Broadbalk strips

| Strip number | Strip name | Treatment c.2020 | Description |
|---|---|---|---|
| 2.2 | FYM | FYM | Farmyard manure |
| 3 | Nil | Nil | Nothing |
| 5 | noN | (P) K Mg | No N |
| 9 | conv | N (P) K Mg | 'Conventional' fertilisation |
| 10 | onlyN | N | No P, K, or Mg (i.e. Only N) |
| 11 | noK | N P Mg | No K |
| 13 | noMg | N P K | No Mg |
| 20 | noP | N K Mg | No P |

FYM: Farmyard manure at 35 t/ha in autumn
N: 192 kg N/ha (N4) as ammonium nitrate (Nitram, 34.5% N) in mid-April.
P: 35 kg P/ha as triple superphosphate in autumn
(P): 35 kg P/ha as triple superphosphate until 2000 (to be reviewed 2025)
((P)): 35 kg P/ha as triple superphosphate until 2020 (to be reviewed 2025)
K: 90 kg K/ha as potassium sulphate in spring
Mg: 12 kg Mg/ha as Kieserite in spring

***Rhizosphere sampling and processing***

Rhizosphere samples of winter wheat cv. Zyatt (sown 28/09/2020) were collected at flowering stage (Zadok's growth stage 65) within one morning after at least 48 hours of dry weather on 2nd July 2021. Samples were taken across section 1 of Broadbalk from four 'replicate' subplots per treatment plot in each of strips 2.2, 3, 5, 9, 10, 11, 13, and 20. From each 'replicate' subplot, rhizosphere samples were pooled from three individual wheat plants spaced equidistantly along the subplot, roughly 1.5 m from the path boundaries between strips were taken in a 'V' formation, one from one side and two from the other of the central 2.1 m wide 'yield strip'. Whole wheat plants were uprooted with the use of 70% EtOH sterilised hand trowels and forks, such that the crown roots and a proportion of the primary root, seminal, and lateral roots were attached, before pooling with the other two whole wheat plants from each subplot in large clear polythene bags, secured with rubber bands around the stems to contain

the roots. As such, a total of four rhizosphere samples comprising 3 whole wheat plants each were taken for each treatment plot corresponding to each 'replicate' subplot. All samples were transported to a 4 °C cold room within 1 hour of being taken, with all subsequent processing at RT occurring on top of cool-packs in the order in which they were sampled. Pooled whole-plant rhizosphere samples were manually separated into their constituent tillers at the root crown, dividing the root mass and dislodging any soil not directly attached to individual roots which was then discarded. From each separated plant, a variety of root types listed above were excised into 50 ml falcon tubes using 70% EtOH sterilised scissors and mechanically agitated using an IKA Vibrax VXR (IKA-Werke GmbH, Germany) with 35 ml of 4 °C sterile phosphate buffered saline (PBS) for 5 minutes at 1500 rpm. After settling for 10 minutes, 1 ml aliquots of clear supernatant were removed from root wash tubes for immediate assessment of microbial load and subsequent culturing.

***Microbial culture collection***

Unless stated otherwise, all microbial culturing for the assessment of microbial load and subsequent isolation and subculturing utilised $1/10^{th}$ concentration Tryptone Soya Broth (Oxoid, UK) hereafter 0.1xTSB (0.17% pancreatic digest of casein, 0.03% enzymatic digest of soya bean, 0.025% D-glucose, 0.025% $KH_2PO_4$, 0.05% NaCl), and all incubations were conducted in darkness at 25 °C. From each rhizosphere suspension, cultures supplemented with the redox dye resazurin to a final concentration of 100 µM were prepared in four replicate 2-fold dilution series starting from a $10^{-4}$ dilution, all the way to a ~$10^{-7}$ dilution in 11 sequential transfers across 96-well plates. For each sample, the 'limiting' dilution beyond which metabolically induced, reductive colour changes of resazurin (blue to pink) did not occur across all 4 replicate dilution series 2 days post inoculation (dpi) was used to calculate an optimal

dilution factor for D2E cultures receiving ~0.3 viable cells/well using a previously empirically determined transformation.

D2E cultures were prepared by diluting remaining aliquots of PBS rhizosphere suspensions stored at 4 °C in the interim to these bespoke optimal dilutions and dispensed into 96-well plates such that 26 - 49% of wells contained microbial cultures 14 dpi as determined by measurements of optical density (OD) at 600 nm in each well using a Varioskan microplate-reader (Thermo Scientific, UK) after orbital shaking to resuspend cultures where present. Automated protocols for the consolidation of these cultures into 96-well plates without empty wells were generated using this microplate format OD data and executed by the Opentrons OT-2 liquid handling robot (Opentrons, New York, USA) with HEPA Filter Module fitted. For each treatment plot, collections of isolates (n=382-543) from 3 of the 4 subplot samples per plot with proportions of wells containing microbial growth 14 dpi closest to 30% were indexed as such in 96-well format (inclusive of negative control wells) for storage at -80 °C after the addition of glycerol to a final concentration of 15% (v/v) 2 dpi.

***Bioassays***

A total of 3827 wheat rhizosphere isolates were screened for 8 *in vitro* phenotypes indicative of putatively PGP functionality in the rhizosphere, including several relating to the plant-availability of each of the four elemental nutrients (N, P, K, and Mg) that constitute the principal mineral amendments on Broadbalk. High throughput bioassays utilising previously established specialised growth media solidified with agar allowed up to 576 third generation liquid cultures incubated at 25 °C for 48 hours to be spotted onto each large 25 x 25 cm square plate with a microplate replicator, retaining their indexed 96-well format. Unless otherwise stated, all agar plates were incubated in darkness at 25 °C. To assess the hydrolysation of peptide bonds by extracellular

proteases, colonies grown on casein agar (5% skimmed milk powder, 0.5% pancreatic digest of casein, 0.25% yeast extract, 0.1% D-glucose, 1.25% agar) were assessed for the presence of clear degradation halos 2 dpi (Frazier and Rupp, 1928). Microbial solubilisation of insoluble sources of phosphate (tricalcium phosphate), potassium (potash feldspar), and magnesium (magnesium trisilicate) was detected by the presence of clear degradation halos around colonies grown on Pikovskayas (PVK) agar (0.5% $Ca_3(PO_4)_2$, 1% D-glucose, 0.05% yeast extract, 0.05% $(NH_4)_2SO_4$, 0.02% KCl, 0.01% $MgSO_4 \cdot 7H_2O$, 0.00001% $MnSO_4 \cdot H_2O$, 0.00001% $FeSO_4 \cdot 7H_2O$, 1.5% agar), Aleksandrov agar (0.2% potash feldspar (Bath Potters, UK), 0.5% D-glucose, 0.05% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCO_3$, 0.0005% $FeCl_3 \cdot 6H_2O$, 0.2% $Ca_3(PO_4)_2$, 2% agar, pH 7.0–7.2), and magnesium trisilicate agar (0.25% $Mg_2O_8Si_3$, 1% D-glucose, 2% agar) at 3, 5, and 6 dpi, respectively (Pikovskaya, 1948; Aleksandrov *et al*., 1967; Kang *et al*., 2017). Extracellular acidification of the growth medium was detected by a purple to yellow colour change around acidifying isolates growing on pH indicator agar (0.15% urea, 1.5% D-glucose, 0.1% $KH_2PO_4$, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, 1.5% agar, pH 7.0, with 0.35% (v/v) bromocresol purple solution (925 μM)) 2 dpi. Siderophore production was detected by a colour change from blue to orange upon removal of iron from the chrome azurol S (CAS) and hexadecyltrimethylammonium bromide dye complex in CAS agar (prepared after Louden *et al*., 2011) around producing isolates observed 2 dpi (Schwyn and Neilands, 1987). The production of exopolysaccharides binding the Congo red dye from Congo red agar (0.1xTSB with 0.005% Congo red, 1.5% agar) was detected by red colony pigmentation 2 dpi. Finally, microbes producing hydrogen cyanide (HCN) were detected in liquid culture 2 dpi following a modification of the method of Castric and Castric (1983) utilising 96-well plates with a 0.1xTSB growth medium. Briefly, 96-well plates are overlaid with

Whatman 3MM filter paper pretreated with a HCN-sensitive solution of tetra base (4,4'-methylenebis-(N,N-dimethylaniline)) and copper(II) ethylacetoacetate in chloroform (Feigl and Anger, 1966), which develops a dark blue colour above cyanogenic isolates during growth. Exemplar screening plates depicting positive and negative phenotypes for each of the above bioassays are included in Figure S2 (in Appendix).

The relationships between these microbial phenotypes was probed using Spearman's rank correlation coefficients, visualised in a correlation matrix using the 'corrplot()' R function (Wei and Simko, 2021) using RStudio (version 3.5.0). The proportions of isolates from the 8 treatment plots exhibiting positive phenotypes for each of these bioassays individually, as well as the proportions exhibiting at least one of these traits across all screens were compared using Chi-square tests (d.f. = 7). Post-hoc pairwise Chi-square comparisons (d.f. = 1) were used to determine the significance of differences in the distributions of positively and negatively responding isolates between specific treatment plots, with *p* values adjusted using the Bonferroni correction for multiple comparisons.

***RESULTS***

Collections of wheat rhizosphere microbes from each of the 8 Broadbalk treatment plots were isolated in parallel from 3 subplots each using a partially automated D2E pipeline as outlined above. High-throughput screens for 8 *in vitro* phenotypes indicative of putatively PGP functionality in the rhizosphere generated functional profiles for each of a total of 3827 wheat rhizosphere isolates. Across the whole culture collection 2109 isolates (55.1%) exhibited at least one of these phenotypes, and of these just 568 possessed only one functional trait – 26.9% of these positive isolates (Figure 4.2a). After those isolates with just one phenotype, more isolates (n = 350) exhibited 6 out of the 8 phenotypes assessed here (16.6% of 2109) than any other number of traits (Figure 4.2a). In fact, other than those with no functions tested, the single most frequent (n = 329) permutation of phenotypes (i.e. 'functional profile') observed lacked only the HCN production and Congo red-binding traits. No isolates responded positively to all 8 bioassays tested, and just 24 cultures (1.1% of 2109) exhibited 7 of the 8 functional traits assessed. This represents a sharp drop-off from the 11.8-16.6% of positive isolates responding positively to 2, 3, 4, 5, and 6 bioassays, respectively (Figure 4.2a), likely because 2 of the 8 traits assessed here were much less commonly observed. Indeed, compared with the 20.9-35.6% of all isolates responding positively to the 6 other screens, HCN production and Congo red-binding were not particularly prevalent (< 5% and < 10% of isolates, respectively) and exhibited weaker correlations scores compared with the six more commonly observed phenotypes (Figure 4.2b). Results from these other screens revealed consistently positive correlations amongst all six phenotypes, albeit to a slightly lesser extent for casein hydrolysis (Figure 4.2b).

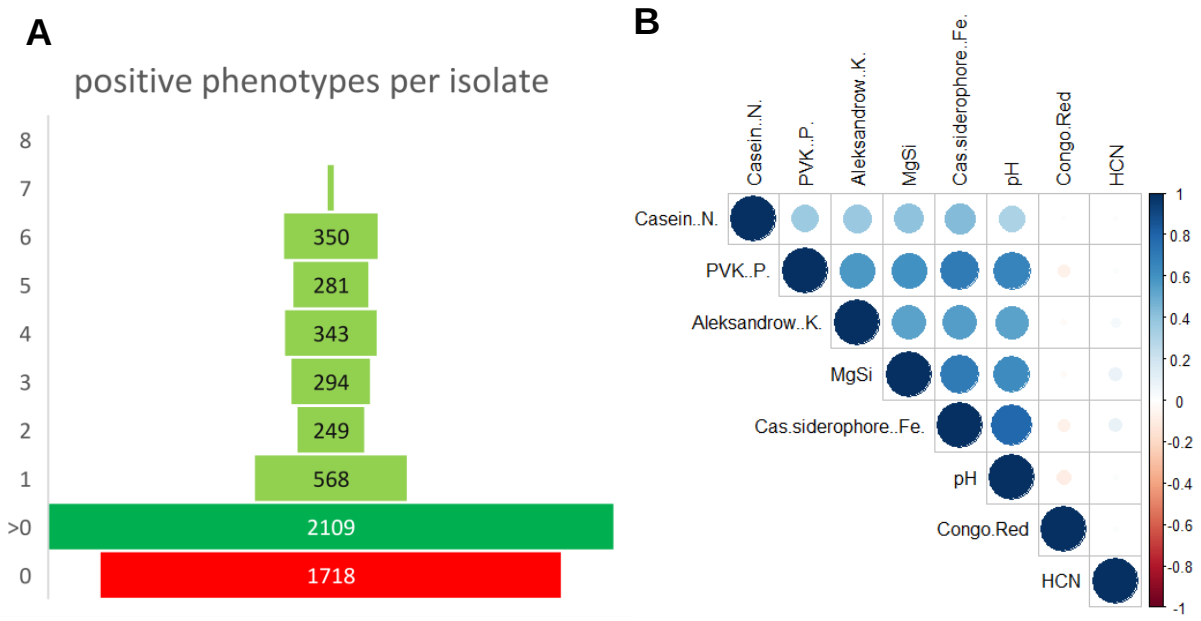



**Figure 4.2 – Numerical breakdown and correlative analysis of microbial isolates exhibiting putatively plant growth promoting phenotypes revealed by *in vitro* screening.** Graphical representations of (A) the number of isolates exhibiting different number of positive phenotypes in a microbial culture collection comprising 3827 wheat rhizosphere isolates from diverse Broadbalk treatments (n.b. '>0' indicates the sum of all isolates with one or more phenotypes), and (B) Spearman's rank correlation coefficients between the 8 *in vitro* phenotypes screened for.

The proportion of isolates with at least one putatively PGP phenotype was highest amongst cultures collected from the conventionally fertilised plot 9 (Figure 4.3). This reflects the general trend across all screens (Figure 4.4) for culture collections from plot 9 to have the highest proportion of positively responding isolates, except for phosphate solubilisation for which plot 20 where P is withheld was slightly higher (Figure 4.4a). At the other end of the scale, the lowest proportions of positively responding isolates always came from plots 3 and 5 that do not receive any inorganic N amendments (Nil and no N, respectively), whilst functional cultures from the organically fertilised plot 2.2 were represented at a roughly intermediate level (Figures 4.3 and 4.4).

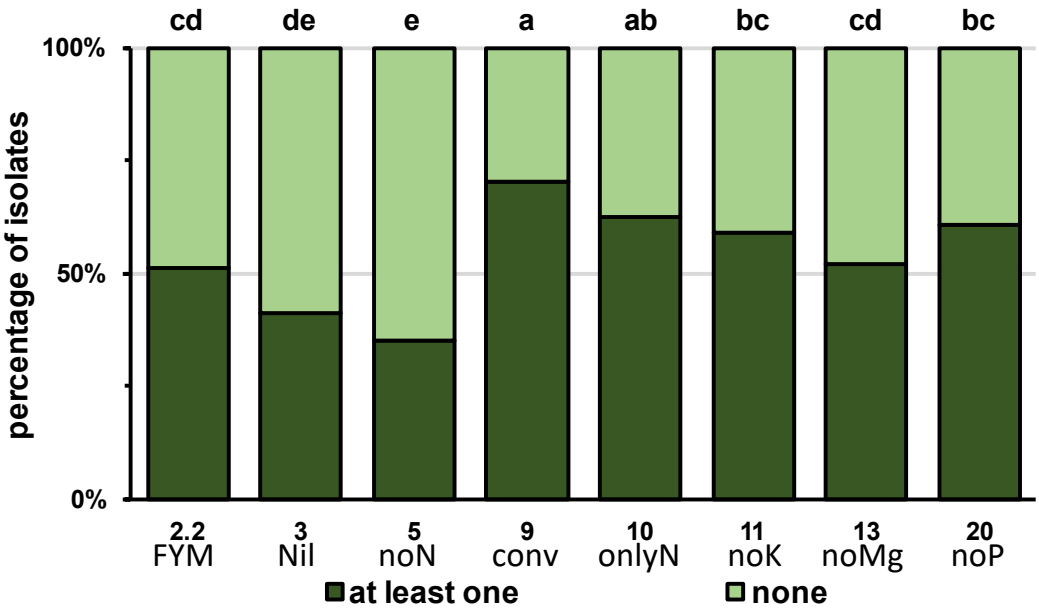



**Figure 4.3 – Proportions of wheat rhizosphere isolates from 8 Broadbalk treatment plots exhibiting at least one positive phenotype across 8 *in vitro* bioassays.** Treatment plots annotated with the same letter at the top of the graphs do not differ significantly as per Bonferroni corrected pairwise Chi-square comparisons ($p > 0.05$).

Chi-square tests revealed statistically significant variation amongst the proportions of isolates in culture collections from different treatment plots displaying both at least 1 positive phenotype ($\chi^2 = 176.4$, $p < 0.001$, d.f. = 7) across all bioassays (Figure 4.3), as well as each functional screen individually ($p < 0.004$). Comparisons between cultures collected from different treatment plots for HCN production and CR-binding screens were not appropriate because of the low number of positive isolates recovered (<5 for some individual subplot isolations n=100-188) and are not presented here. Across the remaining 6 screens (Figure 4.4) pairwise Chi-Square comparisons revealed that the proportions of positively responding isolates in plots 5 and 9 were always significantly different from one another (adjusted $p < 0.005$), whilst plots 3 and

5 never differed significantly (adjusted $p > 0.706$). To a lesser extent culture collections from plots 11 (no K) and 13 (no Mg) also differed significantly from plot 9 in terms of casein hydrolysation, extracellular acidification and siderophore production, as well as magnesium solubilisation for plot 13 (Figure 4.4c, e, f, and b). Indeed, the proportions of isolates exhibiting these four functions individually across each of the 8 treatment plots followed a very similar trend to the pattern described by isolates responding positively to at least one bioassay across all screens in Figure 4.3. This was not the case for screens using PVK and Aleksandrov media for which there was less variation between plots as evidenced by their lower chi-square statistics ($\chi^2 = 47.995$ and $\chi^2 = 20.891$, respectively) compared with this overall trend (Figure 4.4a and d), however the significant differences between plots 9 and 5 were still evident here.

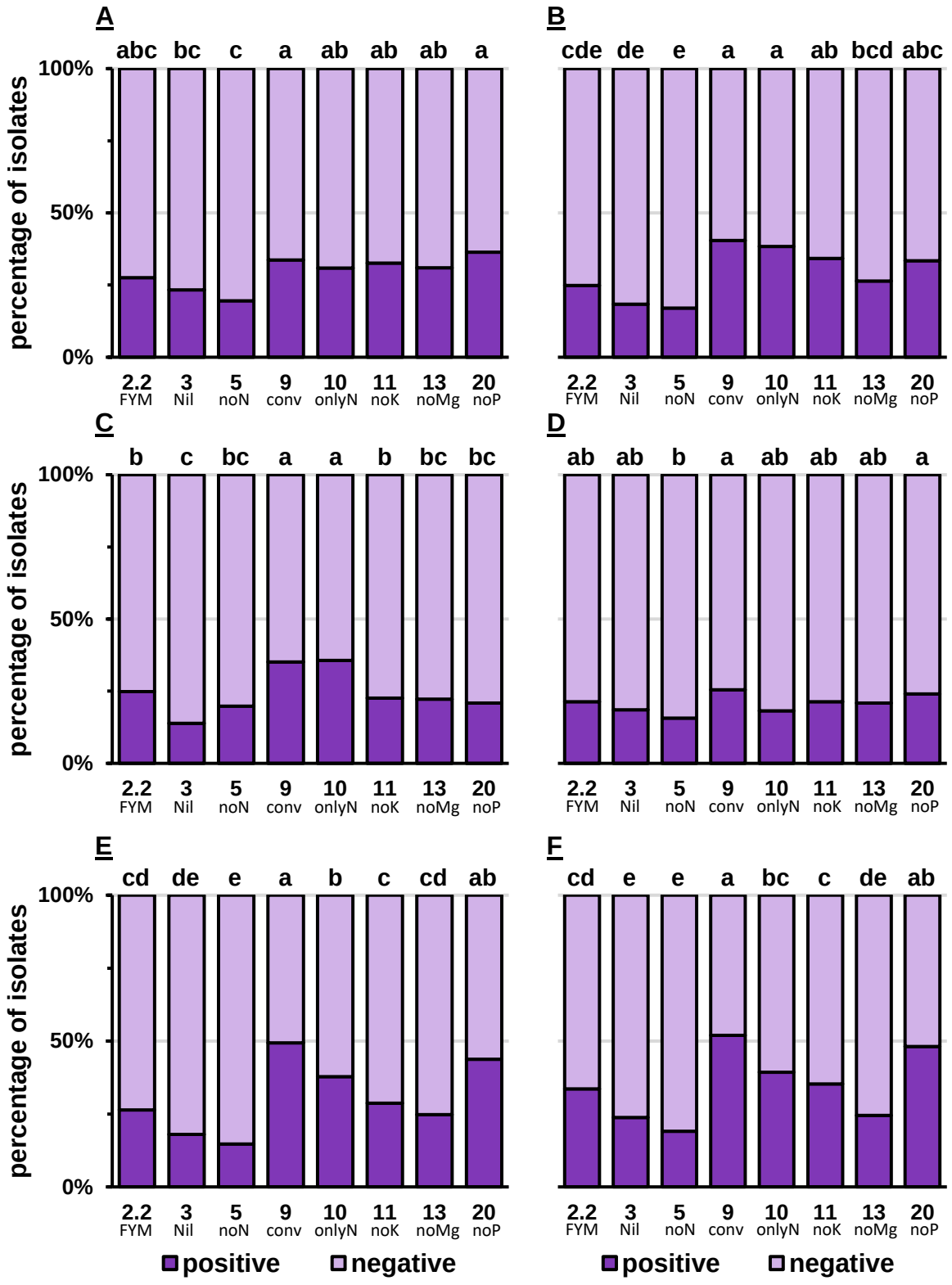



**Figure 4.4 – Proportions of wheat rhizosphere isolates from 8 Broadbalk treatment plots exhibiting positive phenotypes for each of 6 *in vitro* bioassays.** (A) phosphate solubilisation; (B) magnesium solubilisation; (C) casein hydrolysation; (D) potassium solubilisation; (E) extracellular acidification; (F) siderophore production. Treatment plots annotated with the same letter at the top of the graphs do not differ significantly as per Bonferroni corrected pairwise Chi-square comparisons ($p > 0.05$).

## *DISCUSSION*

The functional repertoire of microbial communities associated with the roots of wheat plants at Broadbalk was assessed by screening 3827 microbial isolates for 8 *in vitro* phenotypes relevant to the treatment plots from which they were cultured. The generally positive correlations between traits (Figure 4.2b) reflects the large number of isolates that responded positively to multiple bioassays. This may be attributed to the shared mechanisms by which many of these *in vitro* phenotypes are mediated. Indeed, the extracellular acidification trait screened for here is understood to be the main mechanism by which many recalcitrant nutrients are solubilised by microbes secreting organic acids (Barker *et al*., 1997; Alori *et al*., 2017). As such, the slightly lower correlation scores attributed to the casein hydrolysation trait (Figure 4.2b) may well reflect the more monogenic nature of this comparatively specific phenotype, likely principally determined by the synthesis of a single exoenzyme. Nonetheless, the non-perfect correlation of nutrient solubilisation traits with extracellular acidification suggests that the degradation of the insoluble nutrient sources in these bioassays was not entirely explained by microbially-mediated changes in extracellular pH. Moreover, it could also be true that the mechanisms underlying some of the more generic *in vitro* traits screened here may also be under selection for multiple reasons. The secretion of gluconic acid for example may in fact be under selection for its central role in localised suppression of flg22-induced root immune responses necessary for effective colonisation of the rhizosphere niche (Yu *et al*., 2019) as well as in nutrient mobilisation, for which it is the most frequent agent of mineral phosphate solubilisation (Alori *et al*., 2017) conferred by the well-characterised *gcd* and *pqq* genes (Rawat *et al*., 2021).

Considering the treatment histories of the Broadbalk plots studied here, it is interesting that the results generated in this chapter do not follow the expectation that wheat continuously cultivated in soil with zero fertiliser inputs (plot 3) selects for a greater proportion of PGP isolates than wheat grown under adequately fertilised conditions (i.e. plot 9). These findings seem to directly contradict previous work in our group (Reid *et al*., 2021) which developed this understanding by culturing rhizosphere microbes from wheat grown in a low nutrient bare fallow soil with and without high NPK fertiliser amendment. In these *ex situ* wheat cultivations, it was found that there was a profound difference in the absolute and relative abundance of PGP microbes in a fertilisation dependent manner, with nutrient amendment drastically reducing their prevalence in both the rhizosphere and rhizoplane compartments (Reid *et al*., 2021). Furthermore, it was also not the case here that individually withholding particular nutrient amendments selected for microbes capable of solubilising these nutrients specifically. Indeed, for Mg solubilisation, the proportion of magnesium trisilicate degrading isolates from plot 13 not receiving Mg was actually significantly lower than for those isolated from the fully fertilised plot 9 ($\chi^2$ = 19.892, adjusted $p$ < 0.001, d.f. = 1). Moreover, even the proportion of P solubilising isolates from the plot receiving no P (plot 20) which was the highest observed here, in contrast to the overall trend outlined in Figure 4.3, was in fact comparable to the level observed in most other plots receiving P, including plot 9 ($\chi^2$ = 0.8608, adjusted $p$ = 1, d.f. = 1) from which it differs only in the omission of P amendments. It is however possible that the bioassays employed here do not reflect phenotypes relevant to the alleviation of nutrient deficiencies in these particular soils, and thus alternative substrates more relevant to their physicochemical properties should instead be employed in similar assays (Bashan *et al*., 2013) as these phenotypes may well be under selection as such.

However, it appears to be the case that the fully fertilised Broadbalk strip 9 selected the highest proportions of PGP isolates regardless of function (Figure 4.4), as well as across all functional isolates (Figure 4.3). Interestingly, it was found that the highest proportions of PGP microbes isolated here were selected for in the rhizosphere following mineral N applications. Indeed, the general trend observed for isolates with at least one functional trait in Figure 4.3 which most individual screens adhered to appears to be driven primarily by inorganic N amendment, much like microbial community structure was in the previous chapter. Similarly, there also seems to be a hierarchy of importance for nutrient treatments, with inorganic N application followed by other mineral amendments. Indeed, whilst in comparison to microbial culture collections from the nutrient replete plot 9, plots not receiving any N (plots 3 and 5) always had significantly lower proportions of functional isolates (Figure 4.4), plots 13 (no Mg) and 11 (no K) only differed significantly for casein hydrolysation, extracellular acidification and siderophore production, as well as magnesium solubilisation for plot 13 only. The minimal differences in the proportions of functional isolates between culture collections from the fully fertilised plot 9 and plot 10 which only receives mineral N (i.e. no P, K, Mg) , as well as the lack of any significant differences between the Nil treatment (plot 3) and plot 5 receiving P, K, and Mg but no N (Figure 4.4) further indicates that the ubiquitous significant differences between fertilised and non-fertilised treatments can be mostly attributed to N applications. It is also worth noting that the organic amendments applied to plot 2.2 supplying N at a similar rate (35 t/ha FYM containing approximately 225 kgN vs 192 kgN as ammonium nitrate otherwise) appeared to partially recapture this mineral N-driven effect of fertilisation (Figure 4.4).

The rate at which mineral N is applied to the Broadbalk plots studied here may in fact explain the contradictory nature of these findings compared with our expectations from

the work of Reid *et al.* (2021) that fertilisation suppresses PGP microbe recovery. Kang *et al.* (2022) describe a signalling effect of denitrification-produced nitric oxide (NO) under N fertilisation that stimulates microbially mediated growth-promotion at intermediate levels of fertilisation rather than with insufficient or excessive N inputs. For the PGP *Bacillus* sp. studied, rhizosphere colonisation and plant growth benefits were maximised at N input rates around 0.15 g N/kg soil (corresponding to 337.5 kg N/ha), which is considerably lower than the ~0.75 g N/kg soil applied by Reid *et al.* (2020), although this was in a controlled release format (Osmocote®) as opposed to the urea solution applied by Kang *et al.* (2022). Perhaps at a higher rate of N application on Broadbalk, the suppression of PGP functions expected from Reid *et al.* (2021) would be observed as such, although different soils may have different thresholds for NO production and subsequent growth promotion maxima. Future work should include isolations from Broadbalk plots receiving half and double (plots 7 and 16) the rate of N applied to plots studied here to dissect this phenomenon further, as well as *ex situ* fertilisations of Broadbalk soils at even higher levels if necessary. Nonetheless, here there was a consistently identifiable trend whereby withholding specific nutrients resulted in a reduction in the prevalence of PGP microbes with the absence of N application being most important, followed by Mg and K, indicating that all of these elements are crucial for optimal microbiome functioning, though N is most important.

I propose that the lower proportion of microbes with relevant functions in nutrient depleted soils at Broadbalk actually reflects the lack of these nutrients not in their available forms, but in recalcitrant pools not directly available to the plant, but able to be acted upon by such microbes. In plots with long-term omissions of fertilisers, where these pools have been depleted, as can be inferred from their total elemental

concentrations determined by soil digestions in Chapter 2, effective selection for the microbial nutrient liberation traits that likely caused this depletion can no longer occur. As such the proportion of nutrient solubilising microbes able to act on these recalcitrant pools appears higher in nutrient replete plots, despite the fact crops grown here have most of their nutritive requirements met through exogenous fertilisation. This opposite trend in the selection of PGP microbes at Broadbalk in comparison to the work of Reid *et al.* (2021) is indicative of an exhausted system, where the continuous cropping since 1843 has depleted these nutrient reserves in plots with sub-optimal fertilisation regimes. This is the key difference from the work of Reid *et al.* (2021) in which wheat was cultivated in a continually bare fallow soil that had been uncropped for over 50 years. I argue that although the soluble nutrient stocks were low in this soil, the recalcitrant pools of these nutrients have not been exhausted as they have on Broadbalk by microbes repeatedly under selection from nutrient-limited plants continuously cultivated in this system. As such, in their experimental cultivations the assembled microbiome was still able to act upon these substrates and so the relevant PGP functions may accordingly be selected for in the root zone.

In contrast, the results presented here seem more indicative of the absence of selection for PGP microbes under nutrient exhaustion rather than providing any evidence of the selection for any PGP traits in adequately fertilised plots *per se*. Given this apparent lack of selection for microbes able to alleviate nutrient limitations under fertiliser drop-outs, it is possible that the trends observed at Broadbalk simply reflect the continued presence of such recalcitrant sources of nutrients in adequately fertilised plots that may be acted upon by microbes with the traits tested for here. The mineralosphere concept (Uroz *et al*., 2015) proposes that the chemical properties of soil minerals favour the development of specific microbial communities and as such,

microbes associated with the solubilisation of recalcitrant pools of nutrients from minerals that have been exhausted would no longer be expected to be recovered from such soils. It would be interesting to see whether the trends observed here are mirrored in bulk soils in the absence of any selective pressures exerted by the plant, supporting this notion.

Additional experiments to investigate this theory of nutrient exhaustion could continuously culture wheat in the bare fallow soil used by Reid *et al.* (2021) and measure the proportion of PGP microbes after each cropping cycle in parallel with total elemental analyses to determine if a tipping point is eventually reached after which selection for such traits stops as the soil becomes exhausted of its recalcitrant nutrients. Furthermore, this approach could also be taken for fertilised soils at Broadbalk, for example plots receiving different rates of N application under long-term inorganic fertilisation as well as those with organic input regimes. Indeed, such a tipping point may well occur at different levels of total nutrient measurements in soils with different physicochemical characteristics influenced both by their long-term treatment history as well as their origin. Moreover, it is unknown whether amendment of exhausted soils with relevant recalcitrant nutrient stores is sufficient to rescue the situation at Broadbalk, or whether a tipping point has been reached in terms of the structure of the microbiome and it is beyond repair after such a long time after total depletion. In hindsight, Broadbalk may very well have been the optimal location to bioprospect for relevant PGP microbial interactions before these points of exhaustion had been reached, whilst microbes selected as such were in the process of acting on these nutrient pools. However, such lengthy treatment histories at Broadbalk have brought such nutrient-stripping practices to their logical conclusions through

continuous cultivations that are both agriculturally inadvisable and for that reason largely uncommon.

Even in plot 2.2 where the nutrient-exhaustion mediated collapse in the proportion of PGP microbes was partially prevented by FYM applications, we were unable to observe any selection for casein hydrolysing isolates (Figure 4.4c) that could mediate mineralisation of the significant organic N inputs in this plot. This was despite the low levels of available N and, in contrast to other totally depleted plots, high total % N as revealed in chapter 2 that should encourage their selection. Whilst the low measures of available N could well be an artefact of the rate-limiting nature of this microbially-mediated protein hydrolysis step in N mineralisation (Jan *et al*., 2009) meaning that N made available to the plant as such is likely utilised immediately, this does not mean that this function is not under selection as such. Indeed, it could be that all the N required by the crop had already been taken up by the time of sampling, removing the selective pressure for microbial N mineralisation. Indeed, only a small amount of N uptake occurs after flowering, with that required for grain filling mostly redistributed from pre-anthesis N 'sinks' (Pask *et al.*, 2012). Moreover, the FYM applied in the autumn of the previous year (22/09/2020) may already have been stripped of its N available for mineralisation as such, similarly rendering the potentially transient enrichment of microbes associated with this process imperceptible at the time of sampling. Besides the fact that the sole substrate (casein) used to assess this function here may also be too specific to reflect the microbial capacity for N mineralisation *in natura*, these potential explanations serve to highlight the 'perfect storm' required to effectively bioprospect agriculturally relevant PGP associations.

Specifically, there are numerous, sometimes temporal considerations to capturing microbes with specific PGP functions actively under selection by plants growing in

these soils with low plant-available nutrients, yet large total nutrient pools indicative of unexhausted reserves of recalcitrant nutrients able to be acted upon by such microbes. To address this, future work could investigate the utility of resources such as the UK Crop Microbiome Cryobank (Ryan *et al*., 2023), established using numerous characterised soils, which may include those with chemical profiles of interest. Alternatively a more reductionist approach may be taken, for example utilising the washed compost system developed in our group (Masters-Clark *et al*., 2020) as such, adding defined recalcitrant nutrients to a nutrient depleted soil-like substrate to enrich for solubilisers. Such *in planta* enrichments could also integrate an iterative approach to these *ex situ* cultivations as in De Zutter *et al.* (2021) to recapture the benefits of repeated selection cycles that attracted us to Broadbalk in the first place, and should also consider the soil physicochemical properties of the intended region of deployment when choosing the insoluble sources of these nutrients (Bashan *et al*., 2013).

The very nature of the novel isolation methodology utilised in the high-throughput culture collection pipeline developed here may additionally provide some reasoning for the results obtained here, which initially confounded our expectations from prior works (e.g. Reid *et al*., 2021). The separation of individual microbes from fellow community members that is central to the D2E methodology may in fact affect the amount and identity of microbes in the 'cultivable fraction' of a sample compared to conventional spread-plating and colony-picking. Minimisation of the inhibitive effect of fast-growing, 'weed-like' *r*-strategist species on the subsequent emergence of slower growing colonies is an often-cited justification for D2E culturing efforts (Button *et al.*, 1993; Connon and Giovannoni, 2002; Buerger *et al.*, 2012). However, methods based on the premise that certain 'co-culture-dependent' isolates lack the ability to perform essential growth processes such as siderophore-mediated iron scavenging

themselves and cannot be cultured individually have also been successful (Nichols *et al.*, 2008; D'Onofrio *et al.*, 2010). Indeed, the successes of the 'community-based' approach to CBC of Armanhi *et al.* (2016) wherein the majority of colony-based cultures comprise more than one OTU (Armanhi *et al*., 2016; Armanhi *et al*., 2018) may well leverage this phenomenon. Moreover, the relative contributions of cooperation as well as competition between microbes in the particular microbial communities sampled may well determine the extent of any taxonomic or functional differences observed as such in resultant culture collections (Hassani *et al*., 2018). Comparing D2E methods with CBC for the lettuce root endosphere microbiome using bacterial 16S amplicon sequencing, Persyn *et al.* (2022) found that whilst species richness and novelty did not differ, the diversity covered was different, creating the potential for differences in the functionality of the microbes cultured. CBC methods are however amenable to discriminatory colony-picking steps (Huang *et al.,* 2023) that may increase taxonomic diversity of isolate libraries over those created using D2E methods which often repeatedly isolate dominant strains. However, all these findings consider just taxonomic, not functional diversity, much of which may be harboured within individual ASVs as previously discussed. Indeed, only 54 unique functional profiles were shared by 5 or more isolates in the culture collection established here. However, besides those exhibiting no functions, the top 7 most frequently observed functional profiles were represented by 75 or more isolates each. Cumulatively these 1000+ cultures represented over half (50.6%) of all functional isolates in the collection, reflecting the potential for such strain-level redundancy, or perhaps just indicating the necessity of these combinations of functions in the wheat rhizosphere at Broadbalk. Further functional screening may resolve this redundancy by further differentiating isolates based on additional diverse phenotypes.

To investigate any potential taxonomic redundancy in the culture collection established here future work should develop a phylogenetic identification of isolates, which is also the first step towards elucidating the extent of functional redundancy of taxonomically distinct microbes in terms of nutrient cycling at Broadbalk. Indeed, sequencing of bacterial amplicons for a subset of isolates cultured by Reid *et al.* (2021) enabled integrations with culture-independent microbiome data, as did Armanhi *et al.* (2016 and 2018) using their mixed-culture approach, further justifying a similar strategy here. As such, the function as well as the taxonomy of the wheat root microbiome may be similarly achieved, albeit acknowledging the limitations of microbial isolations from a single growth medium.

Whilst CBC methodologies are tractable using additional media types (Reid *et al*., 2021) including additions of plant extracts (Sarhan *et al*., 2019) to enrich for plant-associated microbes as per Armanhi *et al.* (2018), the scope of such efforts has so far been limited. The streamlined D2E protocol developed here is however much more amenable to massively parallelised cultivations using diversified combinations of various growth media, culturing conditions, atmospheres and times of incubation, consistent with a 'culturomics' approach (Sarhan *et al*., 2019). Indeed, moving forwards towards bioprospecting proper where throughput is paramount, I believe D2E methodologies should thus be favoured, hence the development of the streamlined isolation pipeline used here. Indeed, similarly high-throughput designs for CBC pipelines using robotics are constrained by much higher set-up costs associated with the complexity of colony-picking – c.$250,000 for Huang *et al.* (2023). Moreover, the function-first approach taken here allows discriminatory processes to be integrated into culture collection pipelines, effectively 'weeding-out' isolates with highly similar or less desirable functional profiles in an equivalent manner to colony-picking strategies

based on capturing diverse colony morphologies to increase taxonomic diversity Huang *et al.* (2023).

Nonetheless, the data generated thus far from the interrogation of microbial isolates *in vitro* provides a snapshot of functional diversity in Broadbalk wheat rhizosphere microbiomes that is complementary to culture-independent assessments. Such culture collection exercises provide the basis for integration of observed functional data with the broader view of the factors affecting microbial community structure and diversity afforded by exploratory amplicon sequencing surveys in particular. Moreover, the partially automated, high-throughput microbial isolation pipeline developed here represents a valuable tool with which future targeted bioprospecting efforts may be undertaken based on such interpretations.

# CHAPTER 5 - GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Modern agricultural systems are the subject of a multitude of concerns principally surrounding their sustainability and ability to meet rising global demands for food, feed, and fibre. This two-fold problem is exemplified by the dilemma currently facing wheat production systems which must increase stagnating yields whilst also reducing their reliance on the environmentally costly agrochemical inputs that largely sustain them. The plant microbiome is increasingly understood as an unexploited resource with which to address these challenges (Qiu *et al*., 2019), especially belowground where resident microbes hold a significant influence over plant nutrition (Wang *et al*., 2022). Indeed, a plethora of microbial traits exist that may be incorporated within the 'extended genome' of the plant (Turner *et al*., 2013a), some with impacts on plant growth analogous to those of chemical fertilisers – hence the term biofertilisation used to describe their action (Adesemoye and Kloepper, 2009). However, the factors effecting the diversity of agricultural microbiomes and the relevance of routine managements decisions in determining crop associated microbial community structures are not well defined. Whilst reductionist approaches outlining the ecological functions and mechanisms of action of potentially PGP microbes are central to defining their potential utility (Liu *et al*., 2019), an advancement of our understanding of extant PGP interactions is necessary for such globally important cropping systems (Kavamura *et al*., 2021). Moreover, in terms of bioprospecting there is substantial merit in using such existing agricultural microbiomes as the starting point for the development of PGP microbial solutions, rather than culminating in sink-or-swim tests of their competence in these field settings.

The research presented in this thesis aimed to assess the relevance of a renowned long-term field experiment as a resource for understanding agriculturally relevant PGP microbial associations *in situ* and extracting microbes with the potential to address the

dilemmas outlined above. Such microbial interactions with plants are likely under strong selection in agronomic field experiments such as Broadbalk, where by definition crops are subject to various growth-limiting pressures under which they may benefit from relevant microbially-mediated activities. Indeed, the main focus of the experimental design at Broadbalk is the application of mineral fertilisers supplying the elemental macronutrients N, P, K, and Mg central to plant growth and ultimately wheat yields. The overarching hypothesis of this work was that under such well-defined treatments, reinforced by continuous cropping through the years, any enrichments of microbes able to alleviate said nutrient limitations would be evidenced. This was investigated in the three Chapters of this thesis using complementary culture-dependent and -independent methodologies, contextualised by comprehensive soil chemical analyses.

It is in the intersections of these three key pillars of the project that the novelty of this approach becomes apparent. Indeed, the insights afforded by the characterisations of pools of nutrients variously available to plants in Chapter 2 offered potential explanations for the initially confounding patterns of enrichments of putatively PGP microbes cultured in Chapter 4. Only with the context provided by comprehensive characterisations of soil nutrients not just in forms directly available for uptake by plant roots but more importantly in pools not directly available to the plant but that may be made available by microbes did we start to make sense of these findings. I thus reasoned that the recalcitrant pools of nutrients able to be liberated by PGP microbes had been depleted by repeated selections for such microbes in plots in which those particular nutrients are withheld and thus further selection for these traits was precluded.

The insights afforded by bringing these data together helps build a more holistic understanding of the ecological functions of arable soils consistent with current conceptualisations of soil health in terms of its application in sustainable agriculture. Indeed, of the physical, chemical, and biological components typically considered in such assessments, the comprehensive characterisations of soil chemistry and relevant microbiological functions presented here address two of these areas. Whilst physical variables such as soil texture and porosity were not considered, increasingly these can be predicted by more easily measured soil chemical metrics such as the Mehlich-III extractable elemental concentrations measured here, at least at a regional scale (Drescher *et al*., 2024). Furthermore, using appropriate sampling design with suitable reference libraries calibrated using the appropriate 'wet' chemical analyses (Breure *et al*., 2022), low-cost and high throughput 'dry' spectral analyses may be used to assess both chemical (O'Rourke *et al*., 2016) and physical (Thomas *et al*., 2021) properties in future investigations. Moreover, the interconnectedness of the physical and biological components of soils is increasingly understood (Neal *et al*., 2020), as is their influence over various aspects of sustainable agricultural soil functioning such as nitrogenous gaseous emissions (Neal *et al*., 2023), investigations of which may be linked to our data in future. The integration of such diverse datasets will only improve our interpretations of each of these measures individually and may ultimately aid the development of reliable indicators of soil health as such.

### *FUTURE INSIGHTS*

Potentially the richest source of novel insights is however likely to be found in the integration of the culture-dependent and independent strands of this work. Indeed, the potential to relate the functional insights gained in Chapter 4 to the broader microbial

communities studied in Chapter 3 is highly attractive. This has however been largely precluded thus far by the function-first approach taken towards the culture work here which in itself enabled the testing of hypotheses relating to the soil chemical analyses described above. Future, taxonomic identification of microbes isolated here will allow findings regarding the beneficial microbial traits screened for to be related to the wider microbial community. Indeed, the subset of isolates cultured by Reid *et al.* (2021) that were identified by individual Sanger-based 16S rRNA gene sequencing enabled determinations of the relevance of functional screening data in the wider microbial community via integration with culture-independent bacterial microbiome data. Future identifications of the large number of isolates in culture collections generated using the high-throughput approaches developed here could adopt streamlined, well-barcoded multiplex amplicon sequencing approaches as per Armanhi *et al.* (2016) and Zhang *et al.* (2021), increasingly allowing high resolution interpretations. Indeed, using their mixed-culture isolation approach Armanhi *et al.* (2018) were able to use such a technique to prioritise cultured microbes highly abundant in culture-independent surveys for the construction of an effective PGP multi-strain inoculant.

Moreover, further analysis of the substantial bacterial amplicon sequencing dataset generated here will provide additional avenues with which to determine the relevance of microbes cultured, or indeed identify targets for future isolations. The large number of sequenced rhizosphere samples associated with a single wheat genotype in a single field location should allow the determination of the membership of a robust ‘core’ microbiota comprising taxa common to all Broadbalk plots. Such ‘cores’ may be variously defined (Risely, 2020), but are always hypothesized to reflect underlying functional relationships with the host (Shade and Stopnisek, 2019) that may be exploited (Toju *et al.*, 2018; Kavamura *et al*., 2021). Indeed, the filtering out of transient

associations should allow the prioritisation of any microbes with replicable interactions with wheat crops under the various treatments at Broadbalk.

Furthermore, the targeting of keystone-like ‘hub taxa’ that are disproportionately important in shaping the microbiome community structure of plants may also be possible based on inferences from microbial co-occurrence networks (Agler *et al*., 2016; Hassani *et al*., 2018). Such taxa exhibit similarly replicable interactions with the host and appear to mediate the influence of host genetics over the wider microbial community in a manner that is robust to environmental variation (Brachi *et al.*, 2022). Prioritisations of such robust plant-microbe associations are key to development of microbial solutions for agriculture (Toju *et al*., 2018) which have often been marred by inconsistent field implementation (Bacilio *et al*., 2017). It should be stressed however, that such microbial keystone species classified using network theory alone consider all possible microbe-microbe interactions a given strain has the potential to mediate. Establishing causality in such relationships and defining the true ecological significance of designated ‘hub taxa’ demands rigorous hypothesis-driven investigations of microbial community dynamics *ex situ* (Faust and Raes, 2012; Vorholt *et al.*, 2017). This provides further justification for the complementary culture-dependent approach taken here, which may provide the basis for such experimentation in addition to the functional profiling of individual isolates conducted thus far. Indeed, ‘hub taxa’ that may not themselves promote plant growth but play important roles in the predictable assembly of beneficial microbiome types are increasingly seen as the most important targets for robust PGP solutions (Toju *et al*., 2018; Qiu *et al*., 2019). On the other hand, microbes specific to particular experimental treatments, and those that increase in abundance under their associated stresses coined DefenseBiomes by Liu *et al.* (2020) are also of particular interest. Indeed,

potentially more so for their functionality which may be consistent with answering plants ‘cries-for-help’ (Rolfe *et al*., 2019) common to plots sharing treatments. If any such microbes have been cultured thus far, these should be prioritised for further investigations of their stress-ameliorating effects.

***FURTHER DIRECTED BIOPROSPECTING***

Moreover, and potentially more likely, if such microbes of interest are not yet represented in our culture collections these analyses may provide targets for future culturing efforts. The streamlined microbial isolation pipeline developed here is in fact particularly amenable to parallelised cultivations using different growth media and incubation conditions targeting particular taxa. The merit of this approach was explored by Reid *et al.* (2021) who demonstrated the increased retrieval of taxa detected by culture-independent methodologies in wheat-associated culture collections utilising additional, albeit fairly similar simple media types previously utilised for the establishment of extensive culture collections from *Arabidopsis* by Bai *et al.* (2015). The wider trend towards ‘culturomics’ described in Chapter 4 should also be pursued, including the use of plant extracts in media imitating the root-associated niches targeted here (Sarhan *et al*., 2019). Moreover, predictive tools now exist for pairing appropriate culture media with bacterial taxa including those based solely on 16S rRNA gene amplicon sequences (Oberhardt *et al*., 2015) that may be utilised here as such.

Additional diversity may also be targeted by focusing on more selective physical niches such as the wheat root endosphere as previously investigated at Broadbalk (Robinson *et al*., 2016), and which represents an additional pool of characterised microbes. Similarly, various aspects of root system architecture are increasingly understood as important drivers of the rhizosphere microbiome (Galindo-Castañeda

*et al*., 2024), with certain root types exerting greater selective pressures (King *et al*., 2021). Indeed, the magnitude of differences in microbial diversity at the tips of wheat roots compared with their bases are comparable with the variation in root microbial diversity between different plant species grown in the same soil (Kawasaki *et al*., 2021). Focusing on more selective and potentially more intimately plant-associated micro-environments could be a promising option for future microbial cultivation campaigns as well as increasing the credibility of any inferred microbe-microbe interactions from culture-independent investigations. Moreover, these selection factors should be combined in a sampling regime that leverages sampling intensity to maximise the efficient recovery of diverse isolates (Park *et al*., 2013) especially for targeted bioprospecting proper, for which the requirements necessary for the robust comparisons between plots here are removed. Such a sampling strategy may largely preclude the introduction of taxonomic and indeed functional redundancy to resultant culture collections associated with the repeated isolation of dominant strains. Indeed, the microbial culturing carried out here chiefly serves as a proof-of-principal for the highly adaptable isolation pipeline developed here and was not aimed at bioprospecting as such.

Furthermore, the functional aspects of the rhizosphere microbiota characterised here have only begun to scratch the surface of traits relevant to agricultural sustainability that may be investigated as such. Indeed, even considering the nutrient mobilisation traits that have been the primary focus of this project under the banner of biofertilisation (Figure 1.1), the mechanisms considered by our *in vitro* screens have been far from exhaustive in terms of the liberation of the nutrients in question (Bashan *et al*., 2013). Further functional characterisations of this and future isolate libraries should also incorporate screens more specifically targeting biotic and abiotic stress

mitigation via phytohormone-modulation and biocontrol-type traits (Figure 1.1) such as ACC deaminase production (Penrose and Glick, 2003) and *in vitro* pathogen inhibition (Pacheco-Moreno *et al*., 2021) assays. Finally, the extent to which these putatively PGP functions may be recaptured *in planta* should also be determined using the washed compost system developed in our group (Masters-Clark *et al*., 2020). For example, by providing similarly recalcitrant sources of the elements whose solubilisation was screened for *in vitro as* the sole sources of these nutrients, the effects on plant growth of the inoculation of microbes positively responding *in vitro* may be determined under highly controlled settings.

Dissection of the genetic basis of the traits described here may also be explored through comparative genomic assessments of isolates of interest selected for individual genome sequencing. The use of integrative multi-omics tools such as Anvi'o (Eren *et al*., 2015) will facilitate the linking of specific genes to biological function via comparative genomics and consequently improve our understanding of the impacts of agronomy on belowground microbiological functions. Further exploitation of these valuable sequences may additionally co-opt existing pipelines for the bioinformatic-led discovery of secondary/specialised metabolite (SM) biosynthetic gene clusters (BGCs) including many with potential utility as microbial alternatives to pesticides and herbicides (Blin *et al*., 2023; Terlouw *et al*., 2023). Moreover, the utility of taking this approach with cultured isolates such that the results of various *in vitro* functional assessments may be integrated with sequence data has recently been realised within our group, allowing the linking of *Zymoseptoria tritici* suppression phenotypes to novel genes in several *Pseudomonas* spp. (personal communications: Lund, G., 2023). Such loci may themselves be the real targets of future bioprospecting efforts as these traits may now be directly incorporated into more efficient plant-interacting chassis

(Geddes *et al*., 2015) such as those utilising synthetic host-specific plant-to-bacteria signalling as recently developed from rhizobia-legume symbioses by Geddes *et al*. (2019), further enabling crop-exclusivity of such PGP interactions in the field (Haskett *et al*., 2021). These approaches avoid many of the limitations of 'first generation' microbiome management strategies (French *at al*., 2021) that have curtailed the success of many microbial inoculations in the field when compared with endosymbionts leveraging the specificity of such interactions with plants (O'Callaghan *et al*., 2022).

***TRANSLATING MICROBIAL PLANT GROWTH PROMOTION INTO THE FIELD***

Another large part of the reasoning for the poor translation of PGP microbial interactions outside of the lab (Bacilio *et al*., 2017) may be the more numerous metabolic constraints acting in the field as opposed to in the lab when using simplified growth media. Our understanding of the requirements for microbial solutions designed to survive and thrive across a range of agriculturally relevant conditions may therefore be improved by investigation of the metabolic capabilities of cultured microbes possessing putatively PGP functions isolated from various Broadbalk treatments. Moreover, such investigations of microbes enriched under the action of particular plant stressors may to a certain extent reflect the chemical profile of the root-exuded compounds that may act as cries-for-help implicated in the recruitment of such functional microbes to alleviate these stresses (Rolfe *et al*., 2019). Whilst culture-independent approaches involving stable isotope probing exist to identify microbes actively consuming plant-derived carbon (Fan *et al*., 2022) for example, more traditional microbiological phenotyping microarrays (e.g. BIOLOG™ plates) may be more appropriate for bioprospecting. Indeed, such assessments target cultured microbes thus more amenable to development into microbial solutions to the problems

faced by modern agricultural systems. Moreover, screening as such revealed that the utilization of several substrates commonly found in root exudates was significantly positively correlated with the PGP ability of rhizobacterial isolates analysed by Shi *et al.* (2022) who advocate for their inclusion in primary screening of PGP based on the necessity of rhizosphere competence.

Moving beyond characterisations of individual microbial cultures in isolate libraries, similar assessments of microbial functional capabilities may also be undertaken for whole microbial communities. Indeed, BIOLOG™ EcoPlates have long been used in so called community-level physiological profiling approaches (Weber and Legge, 2010) assessing the utilisation of a range of individual substrates. Such methodologies based on growth-induced colour changes (Garland and Mills, 1991) as well as those measuring substrate-induced respiration (Degens and Harris, 1997) (i.e. MicroResp plates (Campbell *et al*., 2003)) may be used to compare and classify different communities based on their substrate utilization patterns. Alternative substrates including known root exuded compounds (Campbell *et al*., 1997), as well as total root exudates collected under different plant stressors have also been employed as such, yielding important insights into resultant ecosystem functioning under plants cries-for-help (de Vries *et al*., 2019).

However, the extent to which the precise metabolic response to these assays actually reflects the functional capabilities of the extant soil community has been disputed (Preston-Mafham *et al*., 2002). Indeed, particularly for BIOLOG™ methods based on *ex situ* microbial growth in individual wells during incubations, such methods are subject to many of the same biases as the isolation of microbial cultures. As such the various wells of such arrays essentially represent a variety of enrichment cultures established using multiple defined selective pressures. The integration of culture-

independent analyses to standardised enrichment procedures by Flynn *et al.* (2017) did however reveal a strong effect of the inoculum source despite the abrupt divergences of enriched cultures from them in terms of 16S rRNA gene amplicon alpha and beta diversity metrics. Such reductions in complexity have in fact previously been leveraged to great effect in the recovery of novel soil microbial functions (Jacquiod *et al*., 2013), as well as the successful reassembly of rare microbial genomes (Delmont *et al*., 2015) using metagenomic sequencing of DNA from such microbially diverse systems. These controlled, replicable reductions in complexity have proved to be attractive methods with which to achieve higher resolution investigations of functional microbes and their interactions using culture-independent methods (Bell, 2019; Ossowicki *et al*., 2021).

Indeed, despite substantial stochasticity in terms of species representation between replicate cultures (Flynn *et al*., 2017), both the community-level function and the coarse-grained taxonomy of communities enriched by simple defined media have been demonstrated to be highly predictable and governed by availability of nutrients (Goldford *et al*., 2018). Such 'functional modules' of species coexisting under environmentally relevant stressors were probed by Naylor *et al.* (2020), providing a more comprehensive understanding of the biochemical potential of the soil microbiome than would otherwise be possible using a more holistic analysis. A similar approach may be taken here using substrates such as recalcitrant sources of plant nutrients designed to reflect putatively PGP functionality in order to increase the resolution with which microbes carrying out such functions may be interrogated.

### *MICROBIAL CONSORTIA FOR PLANT GROWTH PROMOTION*

Such self-assembled microbial communities may in fact also be utilised as robust consortia conferring PGP functionality to targeted agricultural production systems

(Gutierrez *et al*., 2020). Various methods of artificial selection may be used to derive these functional communities including the use of the host plant itself in so called host-mediated microbiome engineering approaches employing repeated growth cycles (Mueller and Sachs, 2015). Coincidentally such approaches resemble the Broadbalk experimental system and our reasoning for utilising it here, and promising results have demonstrated improved tolerance to drought stresses by utilising the wheat rhizosphere environment (Jochum *et al*., 2019). The application of such directed evolution strategies *in vitro* may however utilise more well-defined perturbations and propagations (Ossowicki *et al*., 2021; Sánchez *et al*., 2021) and have recently been successfully implemented to recapture highly relevant traits such as phosphate solubilisation (Faller *et al*., 2024). Moreover, this presents a better starting point for any future implementations based on their potential for upscaling production of such microbial solutions to the problems faced by modern agricultural systems (Gutierrez *et al*., 2020).

This top-down approach to deriving robust functional consortia leverages microbe-microbe interactions (Hassani *et al*., 2018) in a similar way to the construction of synthetic communities (SynComs) designed from the bottom-up (Lawson *et al*., 2019). Such SynCom approaches permit the inclusion of isolates of our choosing such as the top-performing isolates from *in vitro* screening of culture collections as described here, as opposed to whatever functions can be artificially selected for as part of a self-assembled community. Indeed, several promising approaches towards designing complex microbial consortia have recently been demonstrated that may incorporate multiple known cultures of interest. These may aim to introduce functional diversity, recently implicated in the enhanced root colonization and protection from the phytopathogen *Ralstonia solanacearum* by several highly clonal but phenotypically

dissimilar mutants (Li *et al*., 2023b) whilst also providing additive PGP effects. Indeed, multifunctionality may be more easily achieved using a SynCom strategy rather than the top-down approach which would require multiple simultaneous selection factors (Gutierrez *et al*., 2020). Alternatively, in order to improve the environmental resilience of the inoculum, the niche breadth of the SynCom may be expanded by introducing elements of functional redundancy.

However, in certain cases it has been observed that increasing the number of *Pseudomonas* spp. strains in microbial communities is accompanied by an escalation of antagonistic interactions, causing the loss of the biocontrol of crop pathogens affording plant protection (Becker *et al*., 2012; Mehrabi *et al*., 2016). This further underlines the importance of considering the incorporation of more diverse, even trans-kingdom microbe-microbe interactions which may be central to microbial communities maintaining plant growth (Durán *et al*., 2018). Such multispecies inocula can be used to exploit positive microbe–microbe interactions. For instance, consortia incorporating taxa that assume highly interconnected keystone-like ‘hub’ roles under certain conditions upon deployment are more effective growth-promoters (Li *et al*., 2023c). The identification of such microbes may be garnered from the integration of further analyses of culture-independent work presented in this thesis as suggested above with taxonomically resolved culture collections.

Moreover, the incompatibility of the *Pseudomonas* spp. in the experimental consortia mentioned above also highlights the bottleneck on the complexity of inocula presented by the intricacies of higher-order interactions between microbes (Sanchez-Gorostiaga *et al*., 2019). Several strategies for the rational design of compatible SynComs have however been developed that address this hurdle, using predictive binary-associations (Herrera Paredes *et al*., 2018) and massively-parallel screening using micro-droplet

technology (Kehe *et al*., 2019) for example. Indeed, by systematically studying the many factors that affect the success of SynComs we may gain more mechanistic insights into PGP interactions than may be afforded by using the top-down approach, but which may be used to improve the artificial selection strategies utilised as such. Regardless, by utilising Broadbalk as a resource for bioprospecting, both top-down and bottom-up strategies (Lawson *et al*., 2019) may embrace the relevance of microbes native to, and targeted for, specific agricultural environments as well as these potential benefits of microbial consortia over individual isolates.

### *INTEGRATING PGP MICROBES INTO AGRICULTURAL PRACTICE*

Moving towards the implementation of the outputs of bioprospecting, the modes of application and formulation of microbial bioproducts require significant optimisation (Mitter *et al*., 2021; O'Callaghan *et al*., 2022). Indeed, such considerations of the integration of inoculated microbes into complex native microbiomes present another key factor contributing to the limited translation into the field of PGP interactions studied in the lab (Bacilio *et al*., 2017; Parnell *et al*., 2016). Fortunately, the early stages of plant microbiome assembly most amenable to manipulation are quite easily accessed in most crop species due to their annual lifecycles, enabling efforts to promote the integration of PGP microbes and the establishment of beneficial microbiome types at the start of every cropping cycle (Toju *et al*., 2018). Indeed, the accessibility of the seeds of annual crops such as wheat for treatment prior to sowing presents significant opportunities for exploitation (O'Callaghan, 2016). An early innovative example of this contemporary approach towards the incorporation of PGP microbes in crop production systems successfully targeted the microbiota transmitted within elite crop seed embryos by introducing microbes to the flowers of the parent plants (Mitter *et al*., 2017), resulting in the modulation of various growth traits in field

grown wheat progeny. Moreover, interactions with the plant may be similarly enhanced by the development of 'biofilmed biofertilisers' (Mitter *et al*., 2021) integrating isolates such as those with the exopolysaccharide production trait screened for here such that multispecies biofilms physically protecting and co-locating functional consortia may be formed directly on root surfaces.

However, the typically poor integration of inocula in plant-associated microbiomes such as the rhizosphere may in fact mostly be a product of the plant genotype (Fadiji *et al*., 2023) which is demonstrated to confer reduced abilities to interact with PGP rhizobacteria in modern varieties of wheat (Valente *et al*., 2019). Several studies of this phenomenon have implicated key transitionary processes in the domestication and subsequent breeding of elite varieties with heightened responses to fertilisation with increasing amounts of inorganic inputs post-Green Revolution (Pingali, 2012). Indeed, the impact on the microbiome of ploidy, and domestication (Wipf and Coleman-Derr, 2021) have been investigated revealing more prominent roles of neutral assembly processes in domesticated wheat (Hassani *et al*., 2020), indicative of their reduced selective abilities. Furthermore, the reduced height (*Rht*) genes conferring gibberellin insensitivity-mediated yield increases that characterised the Green-Revolution are also implicated in the reduced connectedness of root-associated microbial communities in modern semi-dwarf varieties (Kavamura *et al*., 2020). Indeed, a return to the less intensively-bred genotypes previously cultivated at Broadbalk before the development of modern elite-germplasm may for example enable further extraction of growth-limiting nutrients from recalcitrant compounds in these soils than the subsequent succession of increasingly yield-optimised contemporary winter wheat cultivars (Figure 1.2) have been able to over the course of the experiment. If modern cultivars really are less equipped to recruit functional

microbes their associated microbiome, any recalcitrant nutrients still ‘left over’ from when the older varieties ended their tenure on Broadbalk may still be utilised as such if they were reintroduced.

Such a proposition is obviously not a viable alternative for sustainable agricultural production systems given the yield implications of returning to unimproved varieties. However, several proposed strategies exist by which we may reinstate beneficial plant-microbe interactions fostered by ancestral crop progenitors (Raaijmakers and Kiers, 2022). Indeed, investigation of the genetic basis for specific mechanisms by which such species may recruit functionally relevant microbes such as root exudation (Ianucci *et al*., 2017; Pacheco-Moreno *et al*., 2024) may ultimately lead to their introgression in otherwise modern, high-yielding cultivars. Moreover, de novo domestication (Fernie and Yan, 2019) of wild-relatives of crop species may be a more viable approach given that it is challenging to identify what exactly has been lost due to the huge amount of genetic diversity loss associated with so called ‘domestication syndrome’. However, the extent to which ancestral varieties, or genotypes carrying their traits of interest may in fact pick up where they left off in terms of microbially-mediated PGP interactions at Broadbalk may depend on changes to the reservoir of microbial diversity in the bulk soil. Indeed, whether a tipping point has been reached in terms of the structure of these communities, beyond which these beneficial interactions cannot reestablish is not known. Moreover, the effects of the different agronomic treatments at Broadbalk may well be expected to influence any such processes, as would the changes made to these treatments in order to reflect contemporary agricultural practise (Poulton *et al*., 2024a) that accompanied the succession of varieties planted. The necessity to conduct any such experimental cultivations in Broadbalk soils *ex situ* as per Kavamura *et al.* (2018) in order to

preserve the integrity and relevance of this living experiment may in fact represent the optimal trade-off for resolving extant plant-microbes interactions as well as any interventions developed given the increased level of microbial community variation explained when using this approach (Kavamura *et al*., 2018) and the generally diminishing observations of microbial plant growth promotion from lab to field (Bacilio *et al*., 2017).

It is important that any such future work should similarly consider all plant and microbial aspects of the holobiont (Sánchez-Cañizares *et al.*, 2017; Fadiji *et al*., 2023) as well as the importance of management factors that are at the heart of design of the renowned Broadbalk experiment and make up a third integral pillar of future microbiome-facilitated production systems (French *et al*., 2021; Kavamura *et al*., 2021). Moreover, any future exploitation of the rhizosphere microbiome will undoubtably be more successful when both plant and microbial partners are considered in tandem (Dessaux *et al.*, 2016; Haskett *et al*., 2021; French *et al*., 2021; Fadiji *et al*., 2023), a principle noted right at the dawn of efforts to manipulate the rhizosphere, the relevance of which continues to this day.

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To conclude, the work in this thesis demonstrates the utility of the Broadbalk long-term experiment to advance our understanding of the nature of selection for PGP microbial associations under agriculturally relevant variables. This thesis presented a multifaceted approach to bioprospecting such plant-microbe interactions, incorporating bacterial 16S rRNA gene amplicon sequencing in tandem with robust soil chemical analyses across the diversity of Broadbalk treatments. These data enabled a deeper understanding of the snapshot of functional diversity revealed by *in vitro* screening of microbial isolates with reference to the somewhat idiosyncratic

effects of such lengthy treatment histories, potentially a result of thorough geochemical depletions through repeated cropping. Moreover, this work provides the basis for future integrations of the observed functional data with the broader view of the factors affecting microbial community structure and diversity afforded by the complementary amplicon sequencing survey. This holistic approach towards understanding the nature of selection for PGP microbial associations holds significant promise and the data and biological resources generated should be further leveraged to develop microbial solutions to problems faced by contemporary agricultural systems. Finally, the partially automated, high-throughput microbial isolation pipeline developed here represents a valuable tool with which future targeted bioprospecting efforts may be undertaken based on such interpretations.

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***APPENDIX***

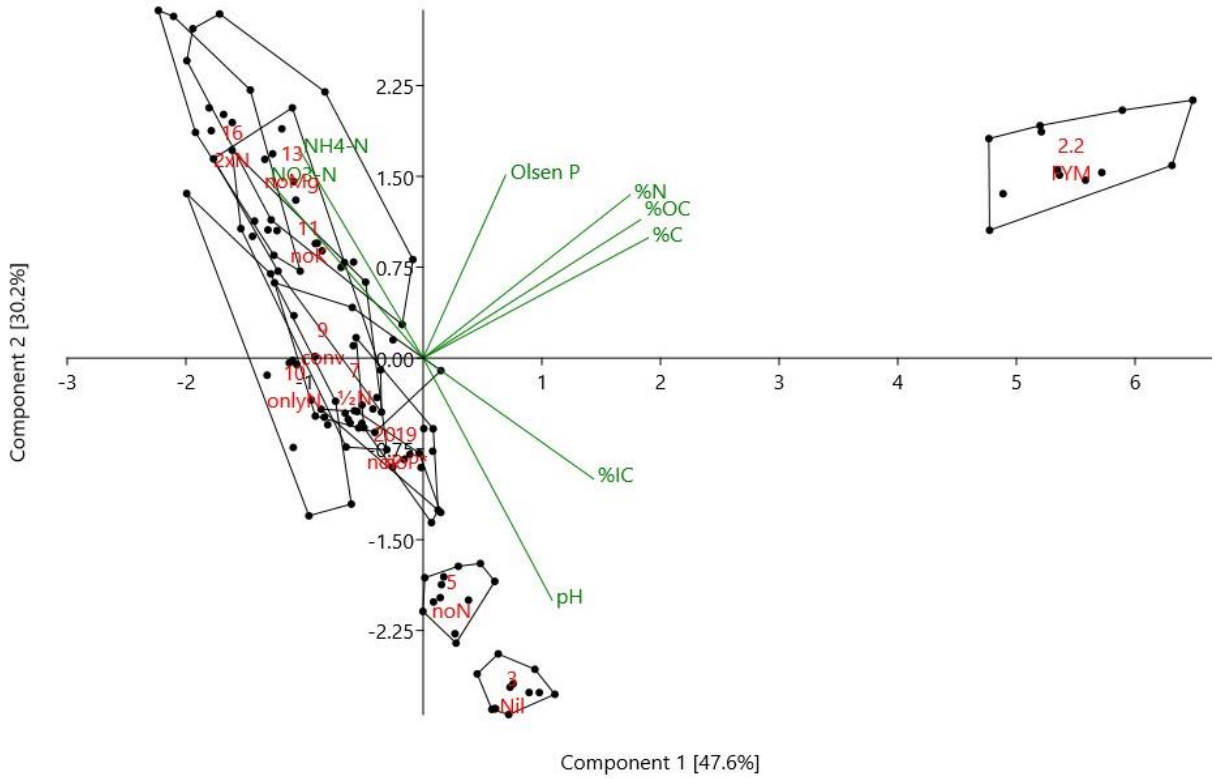


**Figure S1 – Principal components analysis (PCA) of all analysed soil chemical properties for Broadbalk soil samples across 11 nutrient amendment strips in 3 sections.** The main measurements responsible for observed variability are included in green.

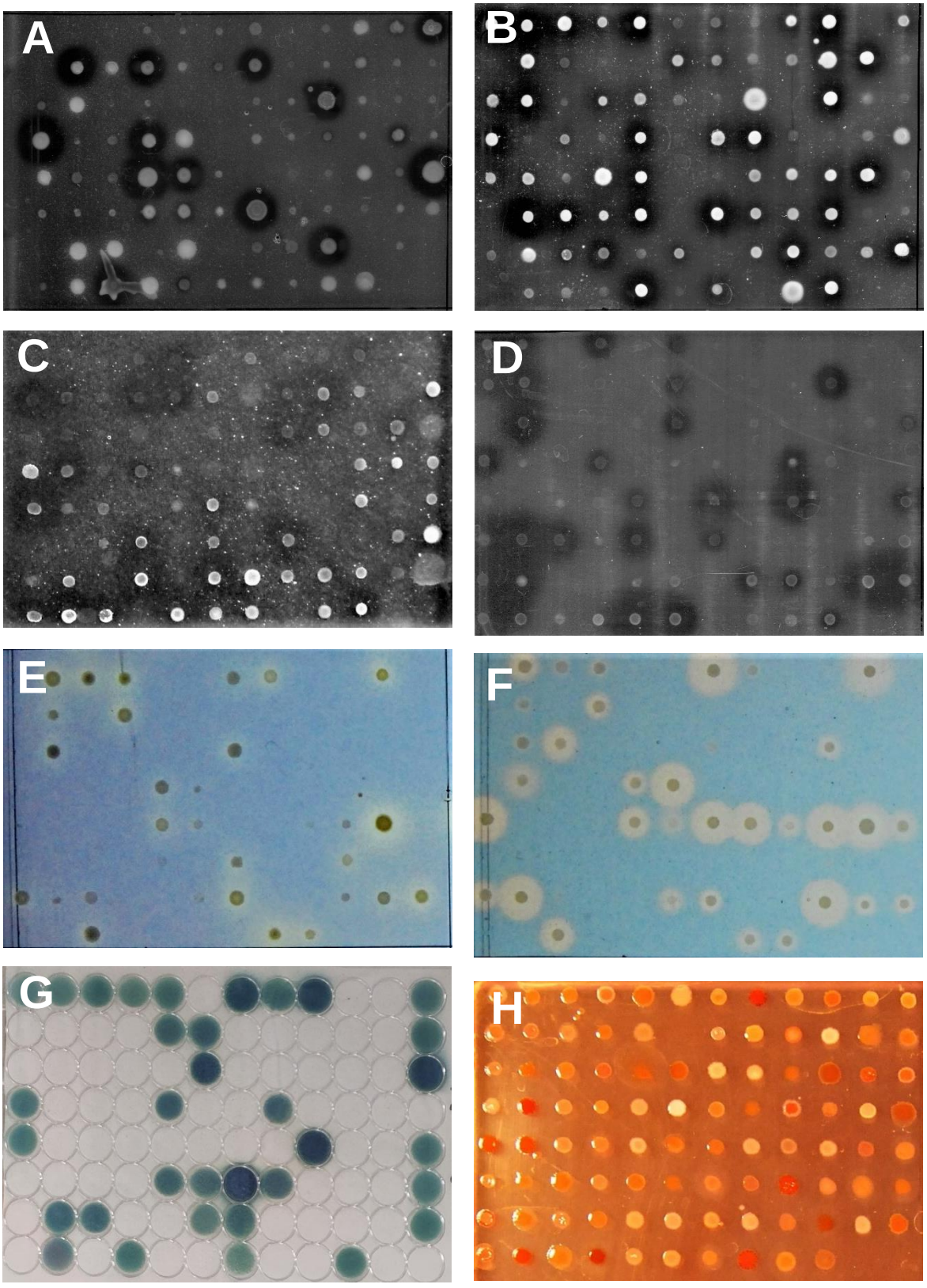


**Figure S2 – Exemplar *in vitro* phenotypes obtained by 8 bioassays** screening for (A) Casein hydrolysation; (B) Phosphate solubilisation; (C) Potassium solubilisation; (D) Magnesium solubilisation; (E) Extracellular acidification; (F) Siderophore production; (G) HCN production; (H) Congo Red-binding; all prepared as per Chapter 4 Materials and Methods under the subheading 'Bioassays'.