

Disentangling plant-microbe interactions under
environmental change

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Summary

The application of nitrogen fertiliser to maintain agricultural productivity is one of the key aspects of modern farming. These activities help provide for a growing population under climate change pressures with higher intensity droughts occurring more frequently. However, such manipulations alter soil health, lowering carbon: nitrogen (C:N) ratios and shifting plant-microbial interactions, on which plants are largely dependent. This thesis addresses the potential impacts on plant-microbial interactions under different C:N fertiliser regimes and water-availability by analysing the overall and N-cycling rhizosphere microbial communities and plant performance of a modern wheat variety, *Triticum aestivum* cv Mulika. In this thesis we have analysed the effects of concurrent drought and C:N fertiliser application on *T. aestivum* cv Mulika and its associated rhizosphere communities. We observed significant differences ($P < 0.05$) in plant A_{sat} , size, and grain weight between well-watered and droughted conditions. Lower C:N performed better than higher C:N treated plants, but N-only treatments were significantly higher ($P < 0.05$) in plant size, grain weight, and A_{sat} . Despite analysing root exudates, we did not find many significant differences as previously noted in the literature. This is likely due to the limited scope and analysis of root exudates in this thesis, with previous research performing broader analyses of compounds being exported from roots. However, due to an inability to analyse poor quality MiSeq reads we find that drought application alongside C:N fertilisation has little effect on plant-microbial interactions for *T. aestivum*. In conclusion, high C:N treatments move mineralised N away from plant use and toward plant-host specific microbial communities, with low C:N and N-only treatments preferentially favouring plant growth. Whilst significant differences were seen between droughted and well-watered plants, little effect was observed for the microbial communities.

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Abbreviations

A	Plant CO ₂ assimilation rate
ABA	Abscisic acid
ABC	ATP-binding cassette
ABC _t	ATP-binding cassette transporter
<i>A_{sat}</i>	Saturated assimilation rate
<i>A_{max}</i>	Maximal assimilation rate, assuming optimal light and [CO ₂]
ACC	1-aminocyclopropane-1-carboxylate
ACC _d	1-aminocyclopropane-1-carboxylate deaminase
AMF	Arbuscular mycorrhizal fungi
AMO	Ammonia monooxygenase
ANOVA	Analysis of variance
AOA	Ammonia oxidising archaea
AOB	Ammonia oxidising bacteria
ARE	Artificial root exudates
ATP	Adenosine triphosphate
BLAST	Basic local alignment search tool
C _i	Internal leaf CO ₂ concentration
[CO ₂]	CO ₂ concentration (or concentration of enclosed compound)
CTAB	Cetyltrimethylammonium bromide
DCM	Dichloromethane
DES	De-epoxidation
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
DON	Dissolved organic nitrogen
DNRA	Dissimilatory nitrate reduction to ammonia
dNTP	Deoxyribonucleotide triphosphate
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
EF	Ectomycorrhizal fungi
ESM	Earth system model
FMS	Fluorescence monitoring system

$(F_m/F_m' - 1)$	An estimate for total energy other than fluorescence and qP
F_q'/F_m'	An estimate of PSII operating efficiency in light-adapted leaves
F_q'/F_v' or qP	Photochemical quenching
F_v/F_m	An estimate of PSII maximum efficiency (dark-adapted leaves)
F_v'/F_m'	An estimate for PSII maximum efficiency (light-adapted leaves)
FW	Fresh weight
GC/MS	Gas chromatography / Mass spectroscopy
GHG	Greenhouse gas
GS	Growth stage
G_s	Stomatal conductance
HMWC	High molecular-weight compound
(RP) - HPLC	(Reverse phase) - High performance liquid chromatography
HSR	Hypersensitive response
IRGA	Infrared gas analysis
ITS	Internal transcribed spacer
LMWC	Low molecular-weight compound
LMWON	Low molecular-weight organic nitrogen
MATE	Multidrug and toxic compound exudation
MDS	Multidimensional scaling
MRCA	Most recent common ancestor
MDR	Multidrug resistant protein
MRP	Multidrug resistance-associated protein
MV	Modern varieties
NEP	Net ecosystem production
NOB	Nitrite oxidising bacteria
NPP	Net primary production
NPQ	Non-photochemical quenching (see $F_m/F_m' - 1$)
NUE	Nitrogen use efficiency
NVA	Nitrate vulnerable area
OTU	Operational taxonomic unit
PAR	Photosynthetically active radiation
PARi	Internal leaf photosynthetically active radiation

PCA	Principle component analysis
PCB	Polychlorinated biphenyls
PCR	Polymerase chain reaction
PDR	Pleiotropic drug resistance protein
PEG	Polyethylene glycol
PERMANOVA	Permutational analysis of variance
PGPR	Plant growth promoting rhizobacteria
PMP	Peroxisomal membrane protein
P_n	Net photosynthesis
PPFD	Photosynthetic photon flux density
PSbS	Photosystem II subunit S
PSI	Photosystem I
PSII	Photosystem II
ϕ PSII	Quantum yield of PSII
qE	Energy-dependent quenching
QIIME	Quantitative Insights In Microbial Ecology
Q-PCR	Quantitative-PCR
qZ	Zeaxanthin quenching
RISA	Ribosomal intergenic spacer analysis
RFU	Relative fluorescence unit
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RWC	Relative water content
RuBP	Ribulose bisphosphate
SAR	Systemic acquired resistance
SLA	Specific leaf area
SOC	Soil organic carbon
SOM	Soil organic matter
SON	Soil organic nitrogen
TAE	Tris-acetate-EDTA

TC	Total carbon
TE	Tris-EDTA
TIC	Total inorganic carbon
TIN	Total inorganic nitrogen
TN	Total nitrogen
TOC	Total organic carbon
TW	Turgid weight
UAA	Utilised agricultural area
UV	Ultraviolet
WUE	Water use efficiency

Chapter 1: Introduction

1.1 Agriculture and plant-microbe interactions

1.1.1 Historical contextualisation of agriculture

Homo sapiens have been impacted greatly by agriculture since its conception in the Neolithic Revolution c.10,600-10,000 years ago (Zohary & Hopf, 2000). Most notable are the impacts on global population rising from pre-agricultural estimates of 4 million to 7,349 million in 2015 (Melorose *et al.*, 2015; Tilman *et al.*, 2001). The global population continues to grow at a rate of 1.18%, or approximately 83 million people annually (Melorose *et al.*, 2015). Although the new growth rate is an improvement from the previous rate of 1.24% it still places an unprecedented amount of pressure on agriculture to provide caloric needs of people, with moderate future estimates suggesting a global population of 9.7 billion and pessimistic estimations of 10.1 billion by 2050 (Melorose *et al.*, 2015).

To meet the caloric demands of our growing population the world summit set a goal in 2009 to increase food production by 70% in preparation for 2050 (FAO, 2009). To achieve this goal, a sustained increase of 44 million metric tonnes on average for crop production - 38% above historical increases in yield - is necessary (Tester & Langridge, 2010). Such increases would be difficult to attain under ideal conditions and almost impossible to achieve with the barrage of environmental changes including, temperature increases, chaotic weather events, shifting climates and degrading soil quality antagonising yield increases needed from superior crops by an estimated 2% yield loss per decade for wheat due to climate change (IPCC 2014).

The UK itself dedicates c. 70% (17.3 million hectares) of land to utilised agricultural area (UAA), which includes around c. 4.5 million hectares of arable cropland, of which wheat is c. 1.8 million hectares ~ 40% of total cropland (Defra, 2017). With the third highest population in Europe of 66.4 million (Office for National Statistics, 2018), 80% of the population lives in urban developed areas that comprise just under 7% of total land (Watson & Albon, 2011), increasing concerns for population and environment health creating competition for land between housing, conservation and agriculture. One factor for global leaps in agricultural production has been land expansion, yet land competition greatly restricts potential agricultural expansion for most countries (Barbier, 2004). Except for sub-Saharan Africa, South America and parts of Asia, most places are unable to agriculturally expand (Barbier, 2004). Even if land could be utilised for agricultural expansion, the detrimental biodiversity effects associated with farmland conversion undermine the progress towards conservation (Phalan *et al.*, 2013). Considering this, we are restricted to increasing future yields through maintaining 'ideal' crops under peak physiological conditions. Agriculture has centred around three crops, Rice, Wheat, and Corn.

Wheat and Barely yields can make drastic differences in agricultural – food market. Such increases in yields have historically been made through the breeding and maintaining of plants under their ideal conditions, yet over a third of global crop growing areas for rice (35%) and wheat (37%) have yield stagnation (Ray *et al.*, 2012). For example, the global leaders in wheat production, China and India, saw large stagnations in wheat yield of 55.5% and 69.9% respectively (Ray *et al.*, 2012). Hitherto agronomic and breeding improvements have pushed previous global cereal production up from 877 million metric tons in 1961 to 2,351 million metric tons in 2007 (Tester & Langridge, 2010), with an

expected rise to 3,056 million metric tons in 2050 (Alexandratos & Bruinsma, 2012). Although we have thus far managed to sustain an increasing trend in global cereal production these gains may be superseded according to some global climate models with predicted future yield decreases between -5.45% to -13.40% and -3.74% to -18% globally for irrigated and rain fed wheat respectively (Nelson *et al.*, 2010). Combining these factors with declines UK wheat area (-1.9% Wheat area from 2016-2017), make it imperative to protect and increase yields per hectare without raising the levels of chemical application (Defra, 2017).

1.1.2 The Green Revolution and modern agriculture

The existing techniques to increase yields include selective breeding, genetic modification and nutrient supplementation, do not normally address belowground microbial processes. Yet the observation of bolstered plant growth being highly linked to soil nutrient levels (Marschner, 1995) resulted in copious mineral supplementation to soils, particularly nitrogen based fertilisers. A period starting in the mid-1960s known as the 'Green Revolution' (Evenson & Gollin, 2003; Pingali, 2012; Tilman *et al.*, 2001) utilised a combination of recently bred 'Modern varieties' (MVs), soil control through irrigation and fertiliser use, pest control via pesticide and herbicide application, and an increase in land use that doubled global cereal crop production over 40 years (Evenson & Gollin, 2003; Tilman, 1999; Tilman *et al.*, 2001, 2002).

Inputs during the green revolution saw a 6.87-fold, 3.87-fold, and 1.68-fold increase in nitrogen, phosphorus and irrigation respectively (Tilman, 1999), consequently damaging

geological structures and contaminating surrounding land, which can have long-term and unforeseen consequences (Nikolic & Nikolic, 2012; Rogers *et al.*, 2014). For example N-fertilisation caused ammonia pollution, observed globally for agricultural practices totalling 18.5 Tg Nyr⁻¹ of the total 54 Tg Nyr⁻¹, which was only dwarfed by domesticated animal emissions (Bouwman *et al.*, 1997). These figures were found to be most intense for Europe, China and subcontinental India, reflecting use intensity of synthetic fertiliser (Bouwman *et al.*, 1997). Modern agronomic practices look to minimise the impacts of pollution, ecological and geological damage associated with agriculture through the incorporation of no-burning and no-clearing techniques, that look to return carbon to the field by laying the mulch down on top (Lal & Kimble, 1997). Common practices are to monitor soil nutrients before fertiliser application, to ensure minimal nutrients leach into unwanted areas, and crop rotations, that look to conserve the use of fertiliser, benefitting the farmer economically and the need to extract such minerals from the ground (Rigby *et al.*, 2001). These practices work alongside policies to restrict the impact of agriculture on the environment around us.

1.1.3 Plant and microbial responses to N-fertilisation

Nitrogen (N) is an essential component for life and is highly sought after by both plants and micro-organisms (Moreau *et al.*, 2019), normally being a limiting nutrient for the former's growth (LeBauer & Treseder, 2008). Such manipulations of soils by mineralised-N fertilisation, whereby there is an artificial increase in nitrate (NO₃⁻) and ammonium (NH₄⁺), has many indirect effects. Firstly, increased nitrate concentrations may stimulate denitrification processes which may lead to an increase in greenhouse gas productions (NO_x), whereas NH₄⁺ increase facilitates nitrification, the systematic oxidation of NH₄⁺ to

NO_3^- . These processes constitute a large portion of N-cycling, and alteration of them can lead to N-acquisition or loss from soil. Besides microbial cycling of nitrogen within soils a direct effect is seen on plant uptake of nitrogen when adding fertiliser. After the maximal uptake rate for NO_3^- for the crop is reached the plant actively reduces NO_3^- to NO_2^- and ultimately NO^- , increasing gaseous-nitrogen lost from stomata (Stöhr *et al.*, 2002). Such alterations in $\text{NH}_4^+:\text{NO}_3^-:\text{NO}_2^-$ ratios alter wheat root uptake rates, with ammonium being the preferred molecule (Criddle *et al.* 1988), therefore affecting the abundance of substrates for N-cycling organisms. Not knowing the end results of these actions greatly affects the accuracy of global ecosystem models and impacts N and carbon (C) cycling (Galloway *et al.*, 2008; Pacala *et al.*, 2001), a system that has already been altered beyond recognition.

Alternatively, some plants maintain highly intimate relationships with soil microbes in order to gain N through symbiotic N-fixation (Requena *et al.*, 1997). Other plant-microbe interactions exist which depending on the species can provide physiological benefits such as resistance to drought (Marasco *et al.*, 2012) or detrimental infections through pathogenic attack, changing gene expression (Bi *et al.* 2011). An evolutionary trade-off between exploitative (fast-growing) and conservative (slow-growing) growth strategies has been recognised (Chapin & Stuart, 1980; Grime, 1977; Moreau *et al.*, 2019; Wright *et al.*, 2004), and are now used to better categorise plant distributions in relation to these trait-based strategies (Cornwell & Ackerly, 2009; Moor *et al.*, 2017; Moreau *et al.*, 2019; Ordoñez *et al.*, 2009), the focus of which has now shifted to below-ground root traits as opposed to above-ground leaf traits (Bardgett, 2017; Laliberté, 2017).

Plants may require these relationships in order to remain competitively viable and therefore present in vegetation (Bever, 2003). Pine has utilised such established obligatory plant-microbe interactions with ectomycorrhizal fungi to become successful invasive species in Costa Rica (Bever, 2003). Plants can typically benefit from such positive microbial influences and although negative interactions (inhibition of competing plants) is more ecologically relevant (Bever, 2003), positive relationships are of interest for agricultural crops as the use of agrochemicals and mono/bi-cultures renders the effect of negative relationships mute. Therefore, plants surrounded by non-beneficial pathogenic microbes and/or unfavourable chemical conditions would be physiologically disadvantaged compared to roots surrounded by beneficial PGPB (plant growth promoting bacteria) and/or chemically optimal conditions when facing inevitable environmental fluctuations, e.g. drought or soil nitrogen status (Rolli *et al.*, 2015). In fact, plant-microbial interactions date back at least 400 million years from fossil evidence (Lemanceau *et al.*, 2017; Redecker *et al.*, 2000) and so these interactions have been fine-tuned over many millennia.

These interactions reflect that soil N availability is largely dependent on the presence and activity of guilds transforming N₂ into easily assimilable N compounds (Moreau *et al.*, 2019). Of which, plants can alter the activity and composition of N guild communities (Berendsen *et al.*, 2012; Moreau *et al.*, 2019; Philippot *et al.*, 2013), and are likely to have multiple mechanisms with which to manipulate such communities to obtain mineralised N (Moreau *et al.*, 2019). Similarly, plants and micro-organisms are in direct competition for NO₃⁻, NH₄⁺, and organic N forms (Courty *et al.*, 2015). Organic forms of N are the less energy intensive molecules for plant uptake as they bypass the assimilation process (Jones *et al.*, 2005; Näsholm *et al.*, 1998), as a result a large range of plants have been

found to utilise L- and D- enantiomers of intact proteins, short peptides, and amino acids (Hill & Jones, 2019; Paungfoo-Lonhienne *et al.*, 2010; Warren, 2014). Additionally, uptake of organic N molecules has largely been shown to be taken up by microbes for their C-content as opposed to the present N, reducing N-competition between plants and microbes when these mineralised N molecules are released into the soil environment (Farrell *et al.*, 2014; Geisseler *et al.*, 2009; Treseder, 2008). Whilst, on the other hand, it is thought that soil amino acids are an unlikely significant source of C to terrestrial plants (Hill *et al.*, 2011; Paungfoo-Lonhienne *et al.*, 2012; Warren, 2012). However, NO_3^- is energetically costly for plants as it requires the active use of ATP to import across root membranes (Moreau *et al.*, 2019), yet NO_3^- , due to its negative charge, does not interact with the soil cation complex and therefore has more mobility than NH_4^+ in soils (Courty *et al.*, 2015). As a result, NO_3^- is generally more available than NH_4^+ for plant uptake (Courty *et al.*, 2015) and is a key compound of N competition between plants and micro-organisms.

In the absence of mineralised N fertiliser, organic matter N sources play a dominant and significant role in soil N-content, which can comprise up to 95% of soil N being bound by organic matter (Bingham & Cotrufo, 2016; Knicker, 2011; Nannipieri & Eldor, 2009; Rillig *et al.*, 2007). Therefore, in N-limited ecosystems, plants with mechanisms to assimilate earlier components in the protein breakdown process may have a competitive advantage (Chapin *et al.*, 1993; Hill *et al.*, 2011; Hill & Jones, 2019; Näsholm *et al.*, 1998; Weigelt *et al.*, 2005). Furthermore, competition between plants and microbes for N-sources are not only unequal in energetic favourability but also in the residency time in the soil. For example, for *Triticum aestivum* and soil, competition for L-alanine was found to be complete within a few minutes in soil, whilst NO_3^- was available for over 24 h (Hill & Jones,

2019), with 40% of alanine being converted to NH_4^+ within 5 minutes. Such residency times can be indicative of the complex interactions present in soil when applying a compound. Alanine, for example, can undergo both microbial and plant uptake, as well as being extracellularly deaminated, allowing a portion of alanine to enter both microbes and plants as pyruvate (Hill & Jones, 2019). The pathways each molecule takes is dependent on the competition between the present plants and microbes in each soil dependent on current abiotic factors, affecting the breakdown of proteins within soils. As a result, soil history has an important role when regarding how the competition will be affected by these processes as it sets many of the abiotic conditions. Therefore, previous land history can have major implications on where competition will lead, and ultimately determining the composition of end products when adding external compounds to soils containing plants.

The extent of past land alterations has led to a substantial change in global cycling of nitrogen and carbon with c. 36% of the earth's non-frozen land being utilised for agriculture (Food and Agriculture Organization (FAO), 2002). When comparing native grasslands, which consume CH_4 and produce $1.9 \mu\text{g m}^{-2} \text{hr}^{-1}$ of N_2O , with local croplands, both decrease in CH_4 consumption and up to 50% higher N_2O emissions are observed (Mosier *et al.*, 1997), overall increasing the concentration of greenhouse gases in the atmosphere, contributing to climate change. Furthermore agricultural practices such as ploughing exacerbate N_2O emissions 8-fold for an 18-month period (Mosier *et al.*, 1997), which for a molecule with a lifespan c. 160y and an estimated 300 times the global warming potential is devastating (Del Grosso *et al.*, 2010). Unfortunately, the alterations we have made to our planet are long-term and sometimes irreversible. A field last cropped in 1939 reverted back to natural N_2O emissions levels (Mosier *et al.*, 1997).

However, this is a long and slow process, a wheat field in 1987 still maintained cropland N₂O emission rates after c. 10 years of re-naturalisation (Mosier *et al.*, 1997) and typical values for croplands able to re-naturalise reside >50 years (Hyder, 1975; Reichhardt, 1982). Focus on either the above or belowground sections of this two-part system (Figure 1.1) would not result in achieving maximum yield potentials. As the condition of soil and thus plant health are highly linked to the status of soil microbes present below (Marasco *et al.* 2012; Glick 2014; Lakshmanan *et al.* 2014) considerations for how fertiliser applications affect these communities should be taken into account.

1.1.4 Plant influence on microbial communities

Plants exhibit great influence over the general surrounding soil microbiota by providing variable carbon sources in the form of root exudates (Klironomos, 2002), driving soil food webs (Bever, 2003). Such exudates are intricately reliant on the physiological state of the plant i.e. rate of CO₂ assimilation (Farrar & Jones, 2008), which in turn selects microbiota based on the affinity for compounds produced (Klironomos, 2002). As a result, competition is altered both between functionally distinct groups and within them, such as denitrifiers. Communities will therefore be shaped in and around an area known as the rhizosphere (Figure 1.2), defined in three main parts: the endorhizosphere (within the plant root), the rhizoplane (the surface of the plant root), and the ectorhizosphere (the area of soil influenced by the plant root). It is mainly the ectorhizosphere we are concerned with in this thesis, defined here as the soil directly adhering to and immediately surrounding the root. Despite our focus on the ectorhizosphere, interactions throughout the entire rhizosphere are of vital importance to plant-microbial interactions.

Microbial communities form in the rhizosphere through optimal niche occupation via competition for resource acquisition. Within these communities are populations that have physiologically different rates of acquiring resources (in this case exudates) and varying rates of metabolic processes and waste-product formation as demonstrated by a suite of traditional experiments creating the Lotka-Volterra, Michaelis-Menten and Monod equations.

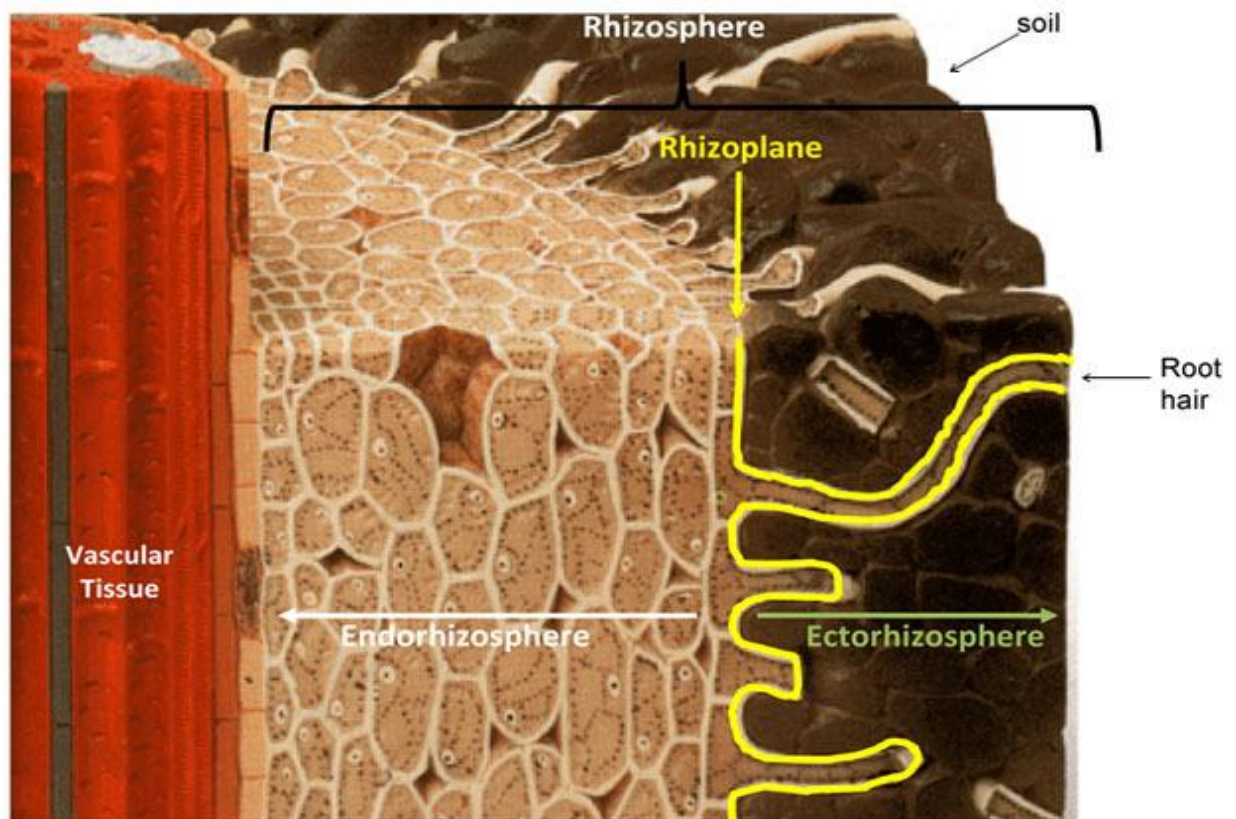


Figure 1.2: A cross-section of the Rhizosphere broken down into its three components as seen in McNear and David 2013. **1) Endorhizosphere:** sections of the cortex and endodermis that can be inhabited by microorganisms, **2) Rhizoplane:** the zone directly adjacent to and including the epidermis and surrounding mucilage, **3) Ectorhizosphere:** The zone stretching out from the surrounding soil to the bulk soil.

The profound effect plants exert on soil microbes, is illustrated by rhizospheric soils being main active sites for microbial growth (Lynch & Audus, 2008). Additionally, a 4-fold increase in activity is seen in rhizospheric soils compared to bulk (Bartholomew & Clark,

1950). However, these systems prove difficult to disentangle as root movement through soil simultaneously alters pH, aeration, nutrient availability and bacterial communities (Micallef *et al.*, 2009) whilst simultaneously chemically mediating microbiota abundances (Walker *et al.*, 2003a). Furthermore soils are complex heterogeneous environments with chemical gradients and non-simple stoichiometry, which should be considered when undertaking any research in this area (Micallef *et al.*, 2009; Sposito, 1989). These plant-soil feedbacks are bidirectional relationships with microbes influencing root exudation, rhizodeposition, yield and both abiotic and biotic resistances (Wardle 2002, Kardol *et al.* 2006), in return microbiota activity and assemblages are altered based on the carbon sources exuded (Cowan, 1999; Levine *et al.*, 2006; van der Putten *et al.*, 2013) and the affinity of organisms for those compounds.

By preventing diseases such as stem rust, induced by *Puccinia graminis* and/or fortifying resistance against drought, yields can be maximised from crops using beneficial bacterial, archaeal and fungal communities. It is therefore evident that to meet mounting population demands, a significant improvement of belowground activity (in addition to ongoing aboveground work) is necessary. To achieve this a necessary foundation of knowledge on how such belowground systems behave and their response to root exudates and changing environmental factors is essential. Hitherto, to our knowledge, nobody has investigated the role of simultaneously applied labile C (glucose) and mineralised N ($\text{NH}_4^+\text{NO}_3^-$) for the effects on plant-microbe interactions with regards to community structure and plant physiology.

Similarly, plants affect soil processes such as denitrification, with *Arabidopsis thaliana* and *T. aestivum* rhizospheres having a positive effect on nirK gene expression and

denitrification, whilst *Brassica napus* showed a suppression of both denitrification and nirK expression in the rhizosphere (Achouak *et al.*, 2019). This is likely a result of root exudates supporting both denitrifying bacteria growth and diversity in the former plants, as diversity loss has been associated with lower denitrification rates (Achouak *et al.*, 2019; Philippot *et al.*, 2013) whilst inhibition of denitrifiers for the latter *B. napus*.

This review looks to provide an overview, in the context of plant-microbe, microbe-microbe and microbe-chemical interactions, the current knowledge of: nitrogen cycling in soils, plant-root chemicals produced through environmental factors (*e.g.* drought, N-fertilisation), NO⁻ influence on plant physiology and chemical influence on denitrification (C/N ratio), chemical reactions within soil. Additionally, throughout the review links between N/C-cycling in soil (focused on denitrification), atmospheric changes, and plant physiology (chemical production thereof) will be highlighted. We will also be mentioning carbon metabolism where necessary, but a comprehensive review of both the carbon cycle and other topics listed earlier is beyond the scope of this review.

1.2 The terrestrial nitrogen-cycle and its influence

The potential for microbes to cycle N in soil ecosystems is in constant flux due to changes in pH, carbon sources, O₂ concentration, water retention and soil compaction altering microhabitats, particularly denitrification fluxes around these factors (Sgouridis *et al.*, 2015). These conditions shift the within microhabitat abundance and activity of key organisms regulating the nitrogen cycle, which are: nitrifiers (e.g. *Nitrosomonas* sp. and *Nitrobacter* sp.), split into ammonia oxidisers and nitrite oxidisers; nitrate reducers are divided metabolically into assimilatory and dissimilatory (denitrification), and nitrogen fixers (e.g. *Azotobacter* sp.) which are needed to fix N₂ before any other process can take place in the nitrogen cycle (Figure 1.3).

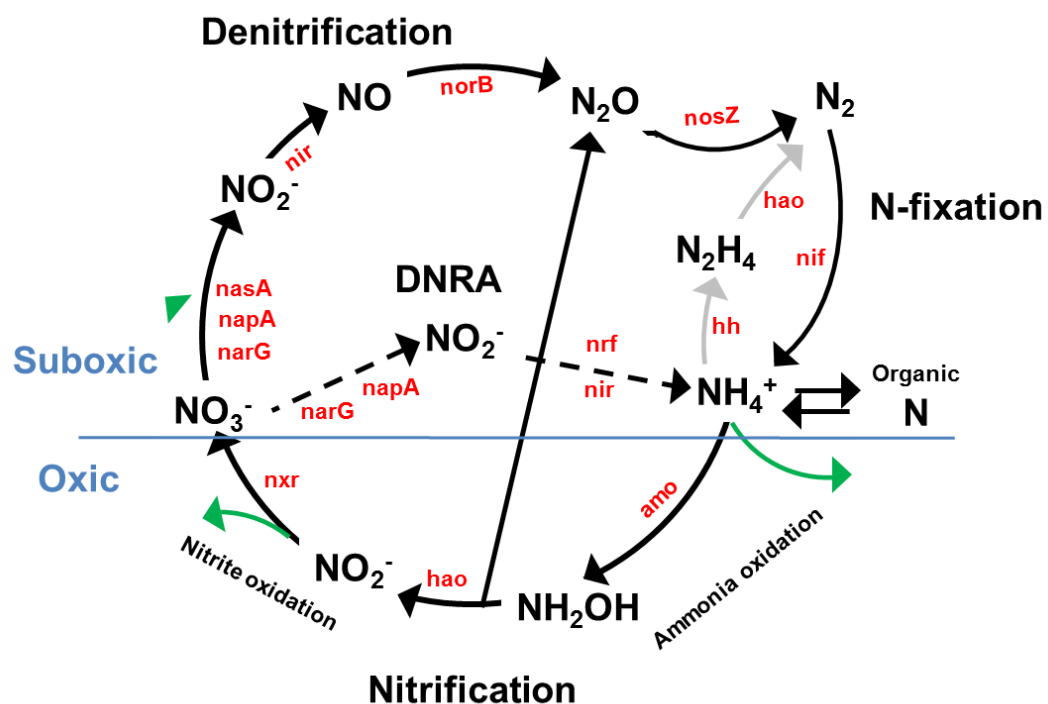


Figure 1.3: Representation of terrestrial nitrogen cycle with respective genes for each process. | = discussed N-cycling steps | = additional N-cycling steps | = plant uptake points. Information on cycling adapted from Canfield 2010

All of these processes are crucial to the maintenance and turnover rates of the nitrogen compounds NO_3^- and NH_4^+ (Figure 1.3), which are essential for plant growth (Criddle *et al.*, 1988) and root development (Forde & Lorenzo, 2001). Nitrate in particular has been suggested to function as an inducer molecule for root development, due to mutated plants *nia* gene defects (functional nitrate reductase encoding genes in plants) showing the same reactions as wild-type to high NO_3^- (Forde & Lorenzo, 2001; Zhang & Forde, 2000). Therefore, it is vital to understand the requirements of growth and competition for each of these functional groups in relation to plant products due to their vast influence over rhizospheric interactions.

1.2.1 Nitrogen-fixation

Nitrogen needs to be fixed before any other process in the nitrogen cycle can take place. The majority of terrestrial N_2 -fixation generally takes place in symbiotic relationships with plants (Cleveland *et al.*, 1999). Rhizobia bacteria induce plant root organogenesis and form nodules by releasing nod factors, which facilitate expression of plant nodulin (Horvath *et al.*, 1993). Once infection has occurred the host plant provides carbohydrates in the form of fructose and glucose in exchange for close proximity to high levels of nitrogen containing compounds (Graham & Abbott, 2000). However, not all plants form this symbiotic relationship but alternatively use fungi performing the same function (Graham & Abbott, 2000). Additionally there are free-living N-fixing bacteria in soil that exist independently of plants and contribute to maintaining N levels within soils (Van Der Heijden *et al.*, 2008).

1.2.2 Nitrification

1.2.2.1 Ammonia-oxidisers

The ammonia-oxidising bacteria (AOB) and archaea (AOA) are chemolitho-autotrophs and were considered obligate aerobes tolerant of sub-oxic or anoxic conditions (Kowalchuk & Stephen, 2001) utilising NH_3 , not NH_4^+ as their electron (e^-) donor (Suzuki *et al.*, 1974). Since then this obligate aerobe view has been debunked due to the discovery of “lithotrophs missing from nature”, micro-organisms able to perform anaerobic ammonia oxidation (Anammox), originally identified in a *Planctomycete* bacterium (Strous *et al.*, 1999). Anammox organisms utilise ammonia or H for e^- donation (Bock *et al.*, 1995) yet both aerobic and anaerobic ammonia oxidisers can utilise N_2O_4 for e^- donation (Schmidt *et al.*, 2002). This was thought of as a multistep and multi-organism until the discovery of complete ammonia oxidation “comammox” in one organism (Daims *et al.*, 2015).

This unique metabolic ability of AOB/AOA to utilise NH_3 as the sole energy source opened up a global distribution which has been maintained in various environments such as: freshwater habitats including soda lakes at pH of 10 (Sorokin *et al.*, 1998, 2001), soils including Antarctic (Arrigo *et al.*, 1995) and pH 4 acidic soils (Burton & Prosser, 2001; De Boer & Kowalchuk, 2001), within masonry stones (Bothe *et al.*, 2000; Mansch & Bock, 1998), and at high temperatures (Egorova & Loginova, 1975), despite the optimal pH range for cell growth being 7.6-7.8 (Schmidt *et al.*, 2002). It is generally thought that for most environmental systems ammonia oxidation is the rate-limiting step due to the lack of nitrite accumulation in nature (De Boer *et al.*, 1990, 1992; El - Demerdash & Ottow,

1983; Prosser, 1989). Nitrification can greatly influence N-retention in systems by oxidising the volatile ammonia to less volatile molecules, therefore retaining that which could be lost through gaseous diffusion (Kowalchuk & Stephen, 2001).

1.2.2.2 Denitrification, Dissimilatory Nitrate Reduction to Ammonia

Denitrification is the systematic reduction from NO_3^- to $\text{N}_2\text{O}/\text{N}_2$ (Figure 1.3) constituting one of the main processes of nitrogen conversion in soil (Šimek & Kalčík, 1998). Nitrate concentrations limit denitrification and this has been demonstrated through nitrate supplementation experiments (King & Nedwell, 1985), which showed that increasing NO_3^- produces higher concentrations of N_2O , a by-product of denitrification (Trogler, 1999). Support also exists from supplemented salt marsh cores with 1M/1M acetate/ KNO_3 - and noted increases in N_2O and N_2 production (Cartaxana & Lloyd, 1999). The inverse of higher C: N ratios inhibiting denitrification has also been observed (Kelso *et al.*, 1997). This inhibition exists due to competition for NO_3^- substrate with dissimilatory nitrate reduction to ammonia (DNRA) (Kelso *et al.*, 1997), decreasing denitrification production of $\text{N}_2\text{O}/\text{N}_2$, key atmospheric gases.

N_2O production has been rising since mid-1700s (Albritton *et al.*, 2001), constitutes 8% of greenhouse gases (Harter *et al.*, 2014) and is highly damaging to the ozone and our health (Crutzen, 1981; Crutzen & Ehhalt, 1977). With an estimated 300 times the global warming effect than CO_2 reducing N_2O emissions is a top priority (Del Grosso *et al.*, 2010). Major crop agricultural sites have been estimated to emit 201Gg N yr^{-1} yet this is highly uncertain with a 95% CI between 133-304 Gg N yr^{-1} (Del Grosso *et al.*, 2010). Still agricultural sites remain the primary source of N_2O (Mosier & Kroeze, 2000) and should

be the focus of N₂O research. The gaseous loss of nitrogen from terrestrial biospheres results in an altered C:N ratio, increasingly polluted atmosphere and reduced vegetative productivity. Altered rates of denitrification and DNRA subsequently alters NH₄⁺, NO₃⁻ and NO₂⁻ ratios, altering plant N uptake (Criddle *et al.*, 1988). As the originator substrate NO₃⁻ concentration limits denitrification rates (Cartaxana & Lloyd, 1999). It has been suggested and shown that carbon substrate type and concentrations affect nitrate reduction (Kelso *et al.*, 1997, 1999; Sears *et al.*, 1997).

Previous work on determining topsoil carbon (Bradley *et al.* 2005, Figure 1.4), and regional distributions of N/P (Earl *et al.* 2014, Figure 1.5) will allow us to approximate C:N ratios of the UK. With lower C:N ratios associated with higher denitrification and lower DNRA rates (Cartaxana & Lloyd, 1999; Kelso *et al.*, 1997). Evident the UK contains a patchy distribution (Figure 1.6), these figures provide a visual guide to possible C:N ratios but are not calculated representations. The UK's agricultural scene is dominated by wheat, its high production (>16,000 tonnes for the past two years), and its large distribution (Figure 1.7) causes wheat to be the foremost crop in the UK influencing microbiota and C:N ratio, making it the key crop to investigate.

Topsoil Carbon Stocks for England and Wales

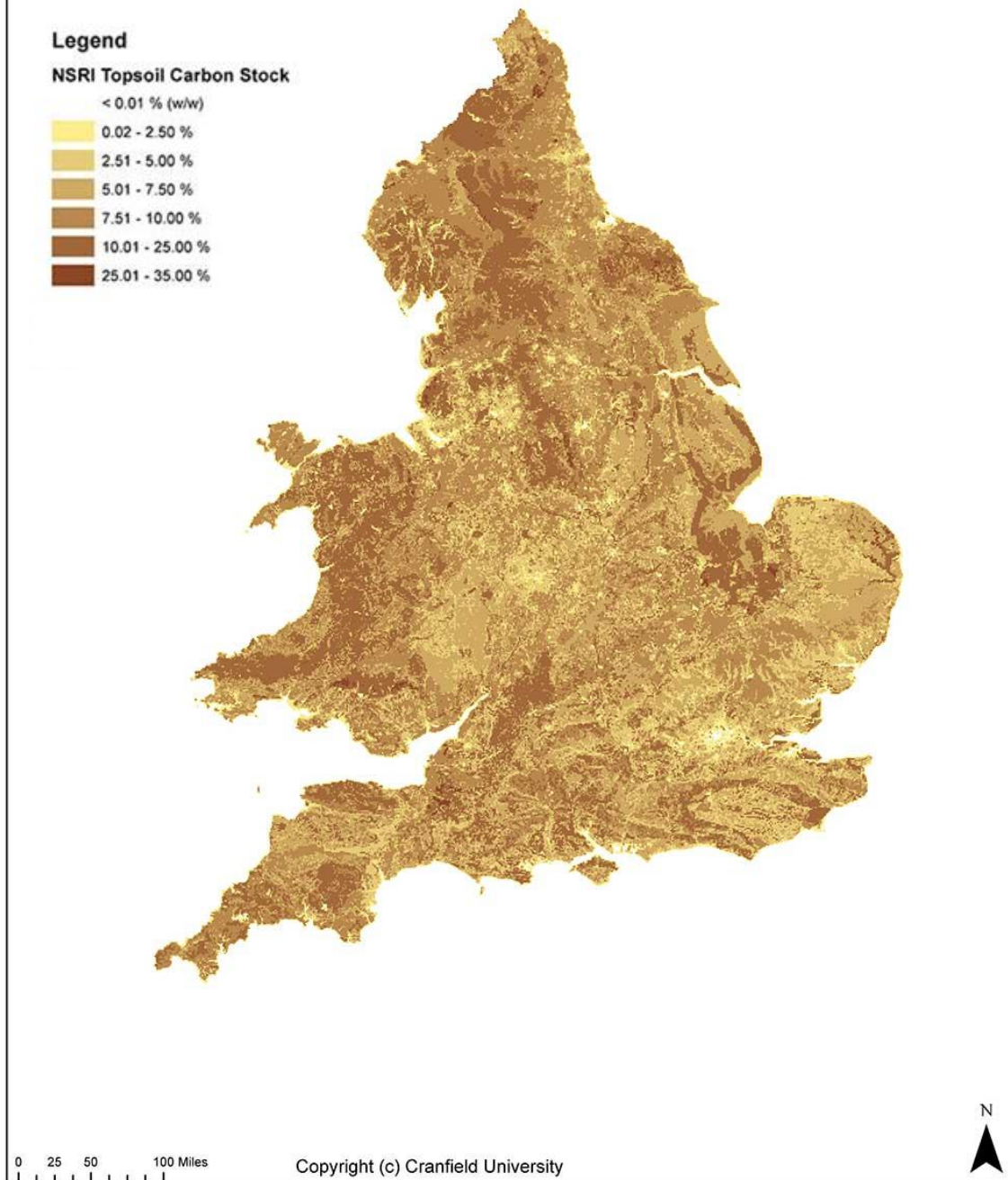


Figure 1.4: Topsoil distributions for the England and Wales from Bradley *et al*/2005

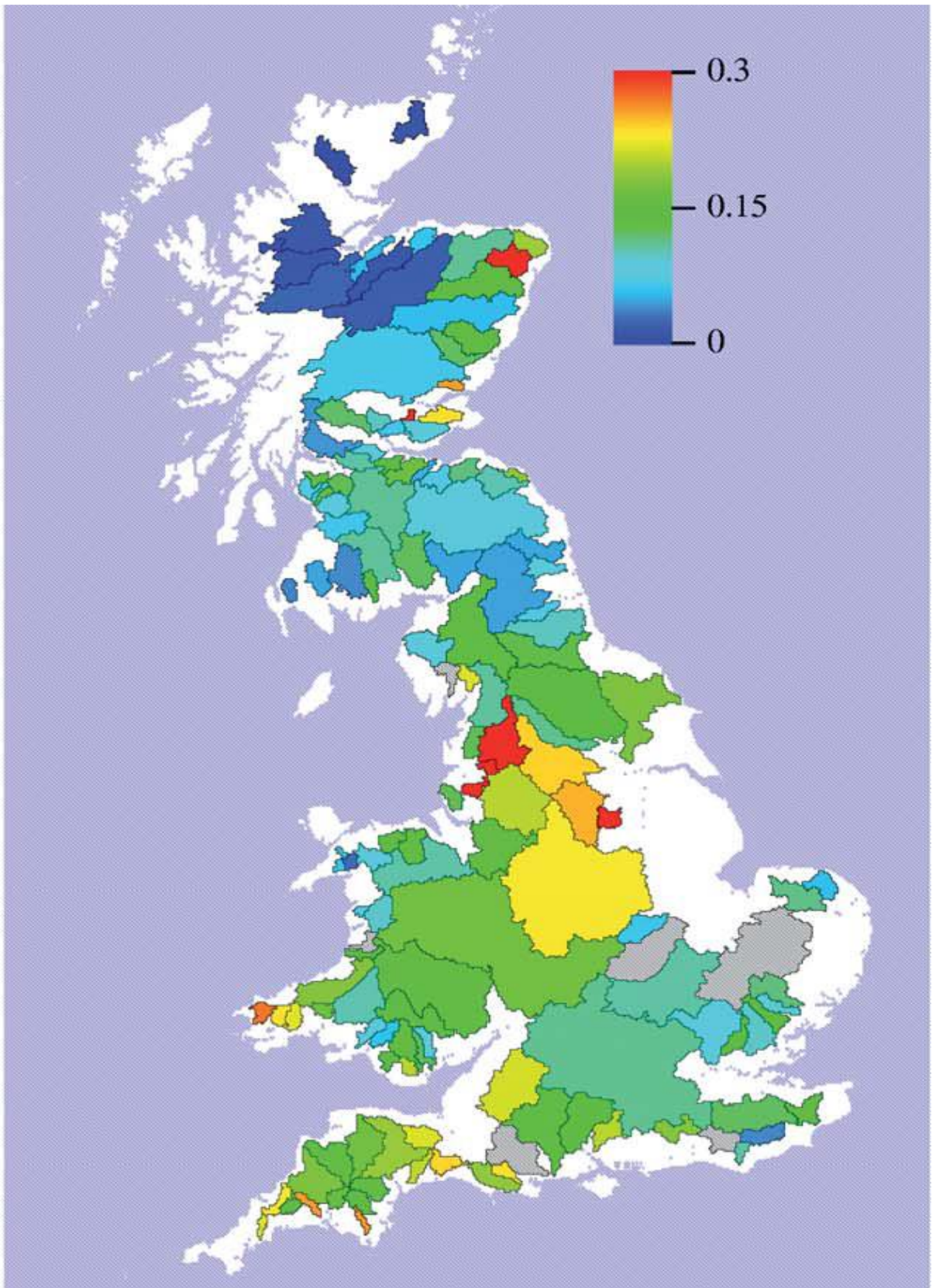


Figure 1.5: Modelled annual nitrate loads for the United Kingdom from Earl *et al*/2014

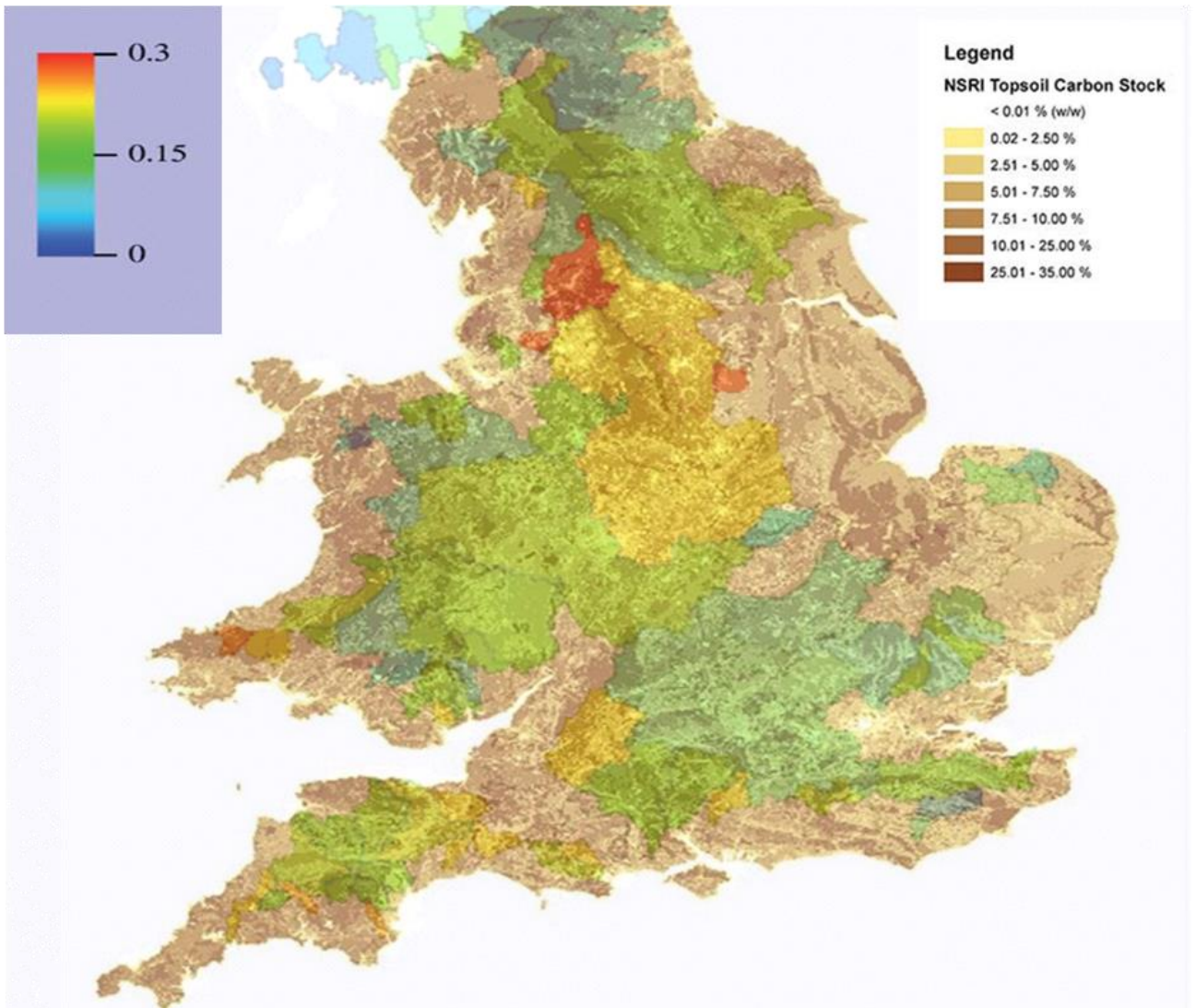


Figure 1.6: Visual representation of C: Nitrate ratios across England and Wales adapted from Bradley *et al* 2005 and Earl *et al* 2014

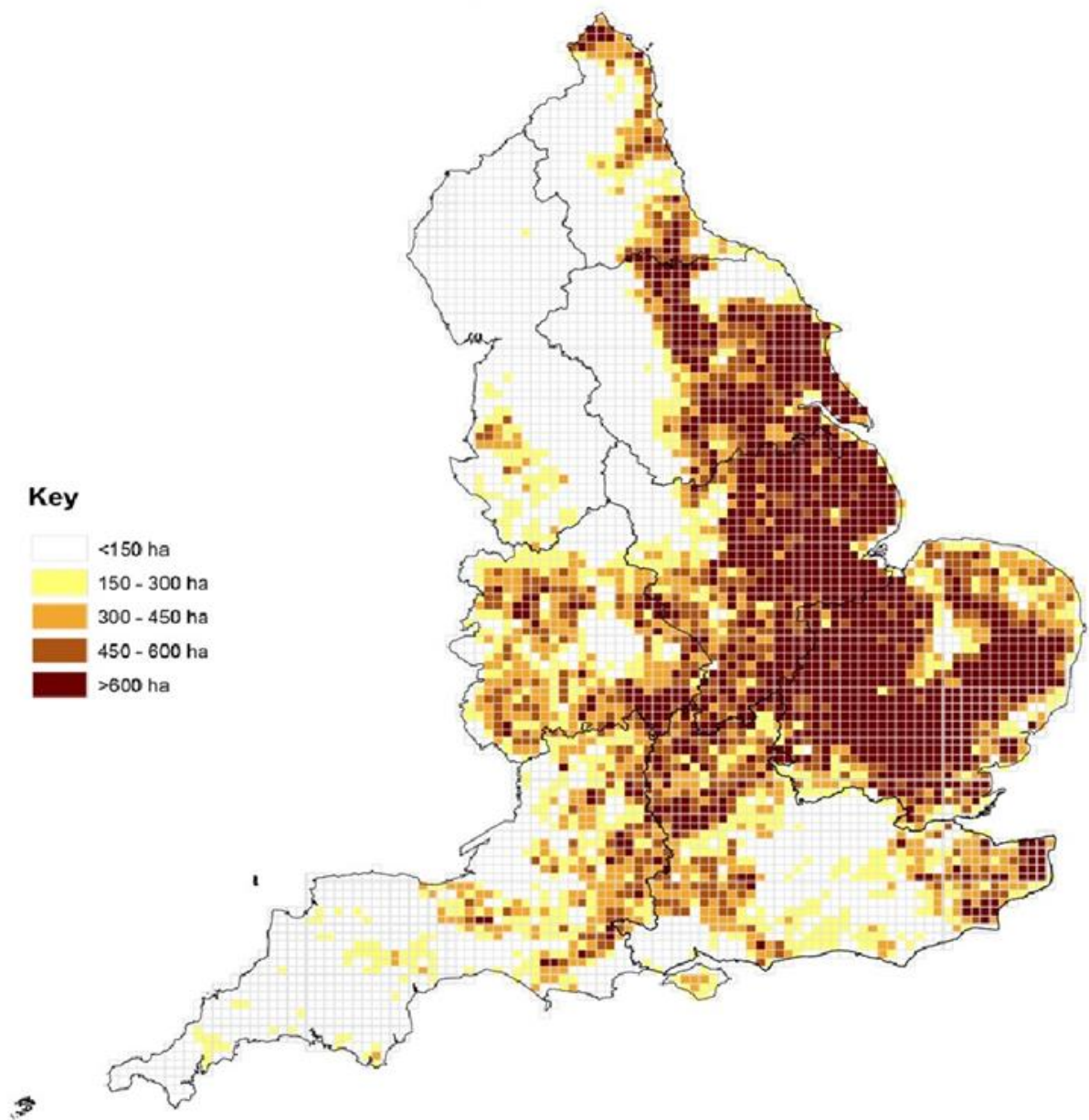


Figure 1.7: Distribution of wheat in 2010 for England as represented by DEFRA, "Maps of crop areas in 2000 and 2010 across England", 2011

1.2.2.3 Nitric oxide (NO⁻)

Nitric oxide (NO⁻), a component of the nitrogen assimilation pathway in plants, is an important signalling molecule and constitutes one of the smallest known signalling molecules (Fang, 1997). First identified as a nervous and immune system regulator in mammals (Wink & Mitchell, 1998) in which it has mainly been studied, however it is also produced by bacteria (Goretski *et al.*, 1988) and plants (Stöhr *et al.*, 2002). As an apolar molecule NO⁻ easily diffuses across plasma membranes, resulting in secondary cytosolic reactions, altering internal cellular metabolic concentrations (Kröncke *et al.*, 1997; Lessig *et al.*, 1998). These cytosolic reactions are particularly important in the production of root exudates due to NO⁻ roles in root system architecture (RSA) and metabolite production (Corpas & Barroso, 2015; Zhu *et al.*, 2017).

A growing body of evidence demonstrates the roles NO⁻ plays in plant growth (Beligni & Lamattina, 2000; Ya'acov & Pinchasov, 2000), regulating plant defences for both biotic (Klessig *et al.*, 2000; Wendehenne *et al.*, 2004) and abiotic (Beligni & Lamattina, 1999; H-Mackerness S John CF Jordan B, 2001) factors including regulation of disease-resistance genes and related factors (Parani *et al.*, 2004). NO⁻ in plants is a systemic elicitor signalling molecule in relation to defence responses (Li *et al.*, 2002; Stöhr *et al.*, 2002) and can manipulate the rate of secondary metabolite production (Zhao *et al.*, 2005) via interfering with plant hormones salicylic and jasmonic acid (Grün *et al.*, 2006). Such stimulation has been observed in several plants including soybean (*Glycine max*), Madagascar periwinkle (*Catharanthus roseus*) and potato tuber (*Solanum tuberosum* L.) (Modolo *et al.*, 2002; Noritake *et al.*, 1996; Xu *et al.*, 2005). Furthermore NO⁻ is required

for ABA-induced stomatal closure, an important physiological requirement for conserving water in plants (Desikan *et al.*, 2002).

These effects combined with the influence NO^- has on morphogenesis (Beligni & Lamattina, 2000; Ya'acov & Pinchasov, 2000), delaying leaf senescence (Leshem & Haramaty, 1996) and facilitating/inhibiting root tissue expansion at low/high concentrations (Gouvea *et al.*, 1997) leads to altered quality (composition) and quantity (concentration) of root exudates by inducing the production various organic compound families (Badri *et al.*, 2008). The diversity of possible induced compounds with varying chemical properties exhibit eclectic effects on soil chemistry (see sections 1.4 and 1.5) and the micro-organisms therein.

The importance of NO^- signalling to plant physiology implies the regulation is as, if not more, important than the presence of NO^- itself. However, NO^- production in and around plants is highly complex and influenced by many factors both external and internal to plant cells (Stöhr *et al.*, 2002). Nitrite is the main precursor for non-enzymatic chemical production of NO^- in plants, which favours acidic conditions due to nitrite forming NO^- or nitrate through dismutasing (Stöhr *et al.*, 2002). Yet, between pH 3 – 6 NO^- formation is due to ascorbate reduction of nitrite (Henry *et al.*, 1997). It is possible for carotenoids to reduce NO_2^- to NO^- in plants (Cooney *et al.*, 1994), but these processes require unnaturally high concentrations of nitrite (Stöhr *et al.*, 2002) and therefore unlikely to be the main source of NO^- production in plants.

As a result, it is suggested that the NO^- pathways are a response to external nitrate concentrations by preventing excess nitrate nutrition and interactions with plant hormones to regulate root morphogenesis (Stöhr *et al.*, 2002). Furthermore to evidence NO^- activity in bacteria the identification and purification of a NO^- reductase from *Paracoccus denitrificans* (Hoglen & Hollocher, 1989) has reinforced the NO^- reducing activities previously found (Grant *et al.*, 1984; Mancinelli *et al.*, 1986; Payne *et al.*, 1971; Zumft & Frunzke, 1982). Although the influence denitrifiers have over NO^- signalling within in plants is questionable due to low amounts observed (Bleakley & Tiedje, 1982), the removal of NO_3^- alters internal plant NO^- production (Stöhr *et al.*, 2002), affecting stomatal closure (Desikan *et al.*, 2002).

1.3 The interconnectivity of terrestrial C and N cycles

1.3.1 Terrestrial biosphere and the cycles

Biogeochemical cycling of N is highly interconnected with the cycling of C (Lloyd, 1999; Zaehle *et al.*, 2010). N regulates net primary production (NPP) and therefore controls CO_2 assimilation from vegetation (Lloyd & Farquhar, 1996; Magnani *et al.*, 2007; Vitousek & Howarth, 1991). The terrestrial biosphere plays an integral part in coupling biogeochemical interactions between land surface to the atmosphere's radiative balance (Foley *et al.*, 2003; Gerber *et al.*, 2010). Nitrogen is widely regarded as the constraining element (Vitousek & Howarth, 1991) both in short and long-term terrestrial responses (De Graaff *et al.*, 2006; Finzi *et al.*, 2007; Luo *et al.*, 2004), demonstrated by enhanced vegetative growth when supplementing N-containing compounds (Aber *et al.*, 1998; Matson *et al.*, 2002; Pregitzer *et al.*, 2008). It has been suggested that elevated temperatures due to global warming may increase soil decomposition, N mineralisation

and N availability (Fisher *et al.*, 2010; Melillo *et al.*, 1993; Pastor & Post, 1988; Peterjohn *et al.*, 1994). Yet the converse of this has been proposed with theories of N being 'locked' up in vegetative biomass (Luo *et al.*, 2004).

Through fertiliser creation, burning fossil fuels and terraforming humans have greatly impacted the cycling of N and C (Galloway *et al.*, 2004; Pacala *et al.*, 2001). This creates a necessity to understand and predict the implications of such effects (Gerber *et al.*, 2010) as the uncertainties of feedback quantifications have important consequences (Cox *et al.*, 2000; Friedlingstein *et al.*, 2006; Gerber *et al.*, 2010). So far many earth system models (ESMs), which look to predict fluxes of compounds within these cycles, have failed to capture the vast complexity of C - N cycling and the hydrological cycle due to parametrising on specific locations (Gerber *et al.*, 2010). Nonetheless, many models focus on integrating the two cycles, IBIS (Kucharik *et al.*, 2000), GFDL LM3V (Gerber *et al.* 2010); accounting for N fixation in resource optimisation (Rastetter *et al.*, 2001) and DON losses (Gerber *et al.*, 2010; Rastetter *et al.*, 2005) with some even taking into consideration C-N interactions in order to maintain specific C: N ratios, MEL (Rastetter *et al.*, 1997). Full analyses of such models are beyond the scope of this review, yet it is necessary to highlight that these models rely on plant-community abundance and composition, below ground microbiota and therefore on the activity of such microbes (Bever, 2003; Del Grosso *et al.*, 2010).

Denitrification remains the most uncertain and elusive part of the nitrogen cycle, but has been said to contribute up to 50% of N-removal from terrestrial systems relative to N-deposition in semi-natural ecosystems (Sgouridis *et al.*, 2015). Influenced by, moisture,

pH, bulk density and C: N ratios (Sgouridis *et al.*, 2015), denitrification interlinks the carbon and nitrogen cycles. Previous C-cycling models have predicted that an increase in atmospheric CO₂ will be offset by an increase in photosynthetic carbon assimilation subsequently stored in the terrestrial biosphere sink (Cramer *et al.*, 2001; Gerber *et al.*, 2004). However such models often overlook the nutritional requirements for terrestrial environments as C sinks (Gerber *et al.*, 2004; Hungate *et al.*, 2003; Oren *et al.*, 2001). These assumptions can cause models to become wildly inaccurate on the effects of “CO₂ fertilisation”. Particularly important is the effect such an increase in soil C storage would have on C: N ratios and denitrification (Cramer *et al.*, 2001). Models that include the effects of climate change as well as increased CO₂ predict that terrestrial environments will slow as C-sinks beyond 2030 due to diminishing returns from NEP (Net ecosystem production) declines as vegetation communities’ shift and respiration rates increase (Cao & Woodward, 1998; Cramer *et al.*, 2001; King *et al.*, 1997). However soil C will continue to rise until c. 2200 according to these models (Cramer *et al.*, 2001). This higher C: N ratio is countered by reductions in denitrification increases in DNRA, resulting in N being retained in soil.

It has previously been shown soil C content and fertility alter peptide uptake by soil microbial communities with higher C and low fertility soil significantly inhibiting uptake (Farrell *et al.*, 2014). It was concluded that the uptake of LMWON (low molecular weight organic nitrogen) was mainly for the C content within and not N (Farrell *et al.*, 2014). This study has major implications for the perception of field management and how C and N is perceived in the environment. This study also demonstrates that we have difficulty interpreting total soil C:N as opposed to C:N of compounds for which the microbial community will be competing.

However, soil respiration in aggregates form anoxic environments, where denitrification can then take place. Unlike previously thought denitrification can occur in overall aerobic soils given the anoxic conditions are met on a microcosmic scale. Mainly O₂ diffusivity governs anoxia, which is reduced in aggregates (Sexstone *et al.*, 1985), this sustained decrease in O₂ diffusion and increased respiration within aggregate formations can form anoxic environments (Dowdell & Smith, 1974; Flühler *et al.*, 1976; Parkin, 1987; Tiedje *et al.*, 1984). The direct measurements of O₂ within such aggregates have led to postulation of anaerobic microsites (Greenwood & Goodman, 1967; Sexstone *et al.*, 1985). Humic substances released by plants are crucial aggregate formation chemicals for these anaerobic areas (Sposito, 1989) and therefore control to a greater or lesser extent denitrification rates. However, recent research has shed light on the paucity of evidence supporting the existence of humic substances in the environment, chalking up their role in the literature to an artefactual effect from alkaline soil extraction processes (Kleber & Lehmann, 2019).

ESMs tend to overlook the activity of such micro-organisms, although over 200 proposed models predict biogeochemical transformations of N/C (Manzoni & Porporato, 2009; Pansu *et al.*, 2010), only a handful consider the implications of Lotka-volterra, Michaelis-Menten, and Monod equations for resource acquisition (Manzoni & Porporato, 2007; Wutzler & Reichstein, 2008). A few previously hypothesised climate-induced die-off triggers, that affect soil microbiota diversity through changes in vegetation abundance, affecting litter decomposition and C-cycling (Barba *et al.*, 2016), have demonstrated the cyclic effect decomposition rates can have. As a result, these models may open dramatic

advances in accurately predicting local/global plant nutrient status, carbon storage and pollution if the interrelationship between microbial cycling potential and plant root exudates is disentangled and incorporated.

1.4 Plant root exudates

As noted to earlier, plants interact in various ways with the organisms around them through chemical communication (Badri & Vivanco, 2009; Canarini *et al.*, 2019). Whilst root exudates mainly consist of organic matter it also includes oxygen, inorganic acids, protons, and water (Badri *et al.*, 2010, 2013; Badri & Vivanco, 2009). Extreme variety exists in these exuded secondary metabolites from plant roots including phytohormones, carbohydrates, amino acids, organic acids, mucilage, proteins, and fatty acids (Badri & Vivanco, 2009; Bais *et al.*, 2006; Canarini *et al.*, 2019). Although a large array of information is available on these secondary metabolites their exportation mechanisms are not fully understood (Badri & Vivanco, 2009; Canarini *et al.*, 2019).

1.4.1 Mechanisms of root secretion / exudation

Methods of compound transportation can be divided by whether they export primary or secondary metabolites, with two main routes for compounds leaving plant roots, exudation and secretion (Bertin *et al.*, 2003; Canarini *et al.*, 2019; Neumann *et al.*, 2000). Root exudation is the active transportation of a compound against a concentration gradient, utilising a proton(H^+)-pump coupled with ATP, such as the MATE (Multidrug and toxic compound exudation) transporters or directly using ATP as in the ATP-binding

cassette transporters (ABCts) (Canarini *et al.*, 2019). On the other hand, root secretion is the passive diffusion of compounds across the membrane down a concentration gradient either diffusing due to small size or utilising channels such as the UMAMIT and SWEET transporters which assist in amino acid and sugar transportation respectively (Canarini *et al.*, 2019). A suite of transporters has been characterised and recognised for metabolite transportation: glutamine dumper proteins (GDU), CAT8, ABC, MATE, UMAMIT, SWEET, ALMT, CDF, MDR, PMP, and MRP to name a few (Badri & Vivanco, 2009; Canarini *et al.*, 2019; Dinkeloo *et al.*, 2018; Pratelli *et al.*, 2010; Sánchez-Fernández *et al.*, 2001; Williams *et al.*, 2000; Yang *et al.*, 2010). Here we first address root secretion and then move on to focus on root exudation.

1.4.1.1 Secretion

This mainly passive diffusion process utilises common ion-channels and vesicle transportation to co-export compounds across the root membrane (Bertin *et al.*, 2003; Neumann & Romheld, 2000). Small polar and uncharged molecules can diffuse across the lipid membrane without the assistance of ion-channels or vesicles (Bertin *et al.*, 2003; Neumann & Romheld, 2000). Whilst this anion-transporter transportation is common, there are specific, less-promiscuous transporters that selectively target and export compounds. For example, the transporter knock-out mutant maize YS1 (*Zea mays* L. cv Yellow stripe) secretes mugineic acid into surrounding soil to make up for its inability to release and uptake the siderophore 2[prime]-deoxymugineic acid (DMA) (Roberts *et al.*, 2004; von Wiren *et al.*, 1994). The release of mugineic acid into soils forms Fe(III)-MA, which can be taken up by YS1. Previously thought to solely be an iron siderophore transporter, YS1 has been seen to transport other metals such as Copper (Roberts *et al.*,

2004). Similarly, *A. thaliana* releases malate instead of mugineic acid through *ALMT1* to handle Fe-acquisition, which is seen as critical for root development under low phosphate (Mora-Macías *et al.*, 2017). This highlights the multitude of ways each plant species responds to cope with the same environmental pressures, utilising different transporters to achieve the same goal. Golgi-mediated transportation has been demonstrated for mucilage and exopolysaccharide exportation across the root cap for larger molecules (Neumann & Romheld, 2000).

1.4.1.2 Exudation (ATP-binding cassette transporters)

In opposition to secretion, exudation utilises ATP to translocate a variety of compounds against a concentration gradient (Badri & Vivanco, 2009; Canarini *et al.*, 2019; Walker *et al.*, 2003a, 2014). One of the largest set of transporters involved in this translocation is ATP-binding cassette (ABC) transporters (Sánchez-Fernández *et al.*, 2001), which is the largest known protein superfamily (Henikoff *et al.* 1997). Not all ABCTs are membrane-bound and involved in transportation, but most are (Sanchez-Fernandez *et al.* 2001). These ABCTs are diverse in number, with each transporter being specific to a handful of compounds (Sánchez-Fernández *et al.*, 2001). There have been around 133 identified ABCTs in *A. thaliana*, with ~103 thought to be involved in transmembrane transport, which is more than found in *Homo sapiens* (48), *Caenorhaditis elegans* (58), *Drosophilla melanogaster* (51) or *Saccharomyces cerevisiae* (29) (Sánchez-Fernández *et al.*, 2001). These ABCTs are involved in a variety of functions including the excretion of toxins, salts, nutrient transportation, and disease resistance (Sánchez-Fernández *et al.*, 2001). ABCTs have been split into more than 12 subfamilies based on molecule size (full or half), orientation (forward or reversed), the presence or absence of idiotypic

transmembrane/linker domains, and sequence similarity (Sanchez-Fernandez et al 2001). Although a full review of ABCTs are beyond the scope of this introduction, the most relevant ABCTs here are the multidrug resistance-associated protein (MRP) – transports a large range of compounds (Badri & Vivanco, 2009; Canarini *et al.*, 2019; Walker *et al.*, 2003a, 2014) -, peroxisomal membrane protein (PMP) – transports long chain acyl-CoAs, and multidrug resistance protein (MDR) – involved in phospholipid translocation and exportation of hormone factors (Badri & Vivanco, 2009; Canarini *et al.*, 2019; Walker *et al.*, 2003a, 2014). The functions of ABCTs could help reveal a full picture of plant-microbial interactions as monitoring these compound gateways would allow a high-level understanding on compound trafficking across root membranes. However, the mechanisms for secretion and exudation we have addressed are not uniform along the root (Badri & Vivanco, 2009; Canarini *et al.*, 2019; Walker *et al.*, 2003a, 2014), with root structure and regional activity playing large roles in the compounds exported.

1.4.2 Root system architecture (RSA)

Root system architecture (RSA) is extremely important to plant survival as it is the essence of nutrient and water uptake through the roots. Two main root systems are found, a taproot system with smaller lateral roots, mainly found in dicot plants, and an adventitious root system with primary roots of comparable size to secondary and lateral roots, mostly found in monocots (Bellini *et al.*, 2014; de Klerk *et al.*, 1999). Although our focus here will be on adventitious roots, both systems' growth patterns are dictated by abiotic and biotic factors.

Many factors are known to affect RSA including abiotic environmental factors: nitrogen, light, iron, water and phosphorus, abiotic plant induced factors: auxins, cytokinins, gibberellins, ABA, brassinosteroids and ethylene, and biotic factors such as pathogens and PGPR. It is well established that RSA changes with various levels of phosphate and nitrate (Ho *et al.*, 2005; Paterson *et al.*, 2007), with shallower-rooted acquisition strategies being used in nutrient-deplete situations. This strategy increases the amount of lateral root development to seek out new 'pockets' of nitrogen sources. Similarly, biotic factors also affect RSA as seen in the rhizobacterium *Bacillus megaterium*, which has been shown to promote root growth in *A. thaliana* and *Phaseolus vulgaris* through an ethylene-independent and ABA-independent cytokinin signalling mechanism (Castro *et al.*, 2007; López-Bucio *et al.*, 2007).

The effects of RSA are bidirectional, with both aboveground and belowground factors affecting a cyclic relationship between plant physiology, plant phenotype, and the surrounding chemical environment. For example, phosphorus starved plants accumulate starch and sugars in the leaves and root systems (Cakmak, 1994; Qiu & Israel, 1992). This accumulation increases the sucrose load in the phloem and alters root-shoot carbon allocation towards larger root systems (Cakmak, 1994; Jordan-Meille & Pellerin, 2008; Marschner *et al.*, 1996). Through activating sugar signalling cascades, these changes lead to RSA changes, with root size increasing relative to shoot size (Hammond & White, 2008), which in turn affects the phosphorus load received by the plant. In general, this is a brief root-shoot imbalance not an issue and typically regulates itself, with larger root systems acquiring more nutrients and relieving the aboveground deficit. Yet it should be noted that optimal plant health and performance requires a delicate balance of aboveground and belowground portions (Mokany *et al.*, 2006).

1.4.2.1 Root region-specific compound exportation

As mentioned earlier, roots are not equally active in compound exportation along their entire length. In addition to RSA responses to biotic and abiotic factors different cells and regions of the root respond in various ways (Badri & Vivanco, 2009; Canarini *et al.*, 2019; Walker *et al.*, 2003a, 2014). In general, root secretion has been found to be higher at the apical root meristem and elongation zone than in other areas (Badri & Vivanco, 2009; Canarini *et al.*, 2019; Walker *et al.*, 2003a, 2014), typically releasing Asparagine and threonine (Badri & Vivanco, 2009; Canarini *et al.*, 2019; Walker *et al.*, 2003a, 2014). The root hair zone typically releases glutamic acid, valine, leucine, and phenylalanine, whilst the whole root releases aspartic acid (Badri & Vivanco, 2009). It was found that diffusible material can be found in the whole root, whilst materials that cannot diffuse are usually found at the apical tips of both primary and lateral roots (Badri & Vivanco, 2009).

1.4.2.2 Border cells

A crucial structural aspect of root apical meristems is the presence of a group of specialised border cells that form the root cap (Badri & Vivanco, 2009). These border cells slough off whilst the root moves through soil easing movement through the soil by reducing resisting pressure (Badri & Vivanco, 2009). As these cells cover the cap, they were aptly named 'border cells' and they fulfil several important functions for roots. Border cells reduce frictional resistance at the root tip, decrease the frequency of pathogenic attack, increase PGPR interaction, and negate heavy metal toxicity (Barceló & Poschenrieder, 2002; Hamamoto *et al.*, 2006; Hawes *et al.*, 2000, 2002, 2003). Whilst

border cells are of significance for both physical functioning of the root and chemical exportation, they are also a source of chemical noise in the rhizosphere.

1.4.2.3 Root hairs and other cells

Apart from border cells the next most important sections of the roots are root hairs, which are an extensively integral part to roots and can account for up to 77% of the total root area for some crop species (Badri & Vivanco, 2009). Root hairs are formed from protrusions of single epidermal cells, which can sometimes be an important source for root exudates. For example, *Sorghum* sp. Was noted to exude sorgoleone, an important soil-active phytotoxic lipophilic compound (Dayan *et al.*, 2009), exclusively from root hairs (Czarnota *et al.*, 2003). Therefore, root hairs can be an important and exclusive source for many root exudates that cannot be overlooked in importance when considering their roles in rhizosphere microbial dynamics (Badri & Vivanco, 2009). In addition to border cells and hair cells, stellar and cortex cells are involved in root compound exudation. For example, stellar and cortex cells have been noted to be involved in citrate exudation for *Zea mays* under Al toxicity (Pineros *et al.*, 2002).

As we have seen, root activity varies along its length, with the younger root apical meristems typically being the most active, especially regarding HMWCs (Badri & Vivanco, 2009; Canarini *et al.*, 2019; Walker *et al.*, 2003a, 2014). Although higher exudation rates have been reported in the upper roots (Lugtenberg *et al.*, 2001), it is likely these results are biased towards the compounds investigated, as different patterns of compound exportation are expected to differ along the root length (Badri & Vivanco, 2009; Canarini *et al.*, 2019; Walker *et al.*, 2003a, 2014).

1.4.3 Root compounds

Whilst most plants rely on interactions between soil microbiota for nutrient mineralisation, some do not require assistance. The *Proteaceae* family increase compound acquisition through a specialised exportation of root exudates (Groom & Lamont, 2010; Lambers *et al.*, 2011; Shane *et al.*, 2004). *Proteacea* species, such as *Hakea prostrata*, create proteoid root formations under nutrient limiting conditions (Groom & Lamont, 2010; Lambers *et al.*, 2011; Shane *et al.*, 2004). These proteoid roots are small, dense clusters 2 – 5 cm thick that release carboxylates, water, and phenolics to increase the solubility of inorganic nutrients in the soil, thus facilitating their uptake (Lamont, 2003).

Most exudation/secretion are released from intact and healthy root cells, but many cells experience apoptosis releasing a larger range of metabolic products into the soil. In addition to affecting metabolism, community structure and activity of soil microbiota (Cowan, 1999; Walker *et al.*, 2003a) these compounds exert large influence of complex soil chemistry. Generally organic acids are upregulated due to drought stress (Marasco *et al.*, 2012). A previous detailed analysis quantifying wheat exudates found fatty acids along with their alkanes to be the major components both in classes and abundance (Monreal & Schnitzer, 2013) yet bioassays need to be performed in order to truly diagnose bioactive compounds.

As with RSA, there are many abiotic factors that affect exudation. Elicitor signals have a large effect on root exudation profiles, salicylic acid, methyl jasmonate, jasmonic acid, and nitric oxide (Badri & Vivanco, 2009; Canarini *et al.*, 2019; Walker *et al.*, 2003a, 2014).

Additionally, light, nutrient availability, soil moisture, hypoxia, and temperature all affect root exudation. For example, *Vicia faba* reduces root exudation of tannins and phenolics at low temperatures (Badri & Vivanco, 2009; Canarini *et al.*, 2019; Walker *et al.*, 2003a, 2014). Furthermore, to avoid phytotoxic effects of lactic acid, ethanol, and alanine accumulation, roots actively exude these compounds into the rhizosphere (Badri & Vivanco, 2009; Canarini *et al.*, 2019; Walker *et al.*, 2003a, 2014). This has implications for interpreting internal root metabolite concentrations, as it is likely that beyond a certain threshold most compounds are exuded if they were not previously exuded.

The biotic factors affecting root exudate profiles are: Neighbouring plants, communication between plants are likely to have detoxifying mechanisms (carbohydrate conjugation, sequestration and oxidation) to cope with toxins produced by neighbouring plants (Duke, 2003; Inderjit *et al.*, 2005), and plant maturation, root exudates are produced least by seedlings, increasing until flowering and then decreasing again at maturity (Aulakh *et al.*, 2001). Additionally, as is expected due to the different metabolic processes undertaken, different species produce distinct exudate profiles (Cieslinski *et al.*, 1997; Iannucci *et al.*, 2017), with the distinction being greater the less phylogenetically related they are (Iannucci *et al.*, 2017).

Previously, many studies have focused on the use of artificial root exudates (ARE), which are a select set of compounds found to be released by plants – usually through hydroponic studies – used to mimic active plant exudation in plant absence to disentangle chemical effects from simultaneous biological and chemical effects that exist in the presence of active plant material. For example, Baudoin *et al.* 2003 applied a mixture of

glucose, fructose, saccharose, citric acid, lactic acid, alanine, serine and glutamic acid previously identified in Maize exudates (Krafczyk *et al.*, 1984) with varying C/N ratios (20.5 and 40.1 respectively) and applied them at a rate of 100 µg C g⁻¹day⁻¹ to columns of topsoil (0-20 cm from surface) to investigate the effect of root exudates on the microbial communities. They found that both solutions equally increased culturable heterotrophic bacteria compared to dH₂O but formed different community composition from RISA (ribosomal intergenic spacer analysis) results (Baudoin *et al.*, 2003).

Although hydroponic experiments are useful in determining exuded compounds from living plants, they are limited in their analysis through the very methods used as artificial environments created for ease of study do not reflect compound exudation under natural conditions. This results in a myriad of clear compound identification with little knowledge on whether those compounds would be exuded at such concentrations within normal (*e.g.* soil) environments, if at all. Many hydroponics experiments utilise an aerated liquid media without mechanical impedance (Bengough *et al.*, 2011; Boeuf-Tremblay *et al.*, 1995; Groleau-Renaud *et al.*, 1998; Horst *et al.*, 1990) - physical resistance to root growth usually provided by soil – which limits these studies for root exudate analysis as mechanical impedance plays important roles in both root morphology and functioning (Groleau-Renaud *et al.*, 1998). Chemical interactions of compounds need to be kept in mind when investigating potentially bioactive compounds, as artefactual results may appear through indirect effects on soil chemistry. This is particularly true in acidic soils where many free protons interact with exuded compounds to form by-products. However, for most plants an important group of characterised defence compounds exist, known as phytoalexins.

1.4.3.1 Phytoalexins

Phytoalexins are stressed-induced low-molecular-weight metabolites found throughout many chemical families, e.g. terpenoids, polyacetylenes, isoflavonoids and dihydrophenanthrenes (Grisebach & Ebel, 1978) and are upregulated by NO⁻ (Noritake *et al.*, 1996). These chemicals are important for plant defence (Ebel, 1986; Hain *et al.*, 1993; Ingham, 1972) with extensive antimicrobial properties (Kurosaki & Nishi, 1983) and are produced by many plants through biotic stressors, such as pathogenic attack (Jeandet *et al.*, 1995), and abiotic stressors through low temperatures (repressing production) and UV exposure (Ensminger, 1993; Sanchez-Ballesta *et al.*, 2006).

One phytoalexin in particular, a simple stilbene, *Trans-resveratrol* (3, 4', 5-trihydroxy stilbene) has been widely observed upregulated in grapevines (*Vitis vinifera*) in response to UV radiation exposure or pathogenic attack (Langcake & Pryce, 1976) producing concentrations up to 400 µg/g in stressed leaves and 700 µg/g in lignified stem tissue (Langcake & Pryce, 1976), which in turn decreases susceptibility to pathogen *Botrytis cinerea* (Langcake & McCarthy, 1979). Phytoalexin transgenic crops have been generated for tobacco (*Nicotiana tabacum*) and cereal crops: wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) (Leckband & Lörz, 1998) by inserting the *vstI* gene found in *V. vinifera* which upon expression has led to an increased resistance to fungal pathogen infection in barley and tobacco (Leckband & Lörz, 1998).

Although phytoalexins are important in many species for abiotic and biotic resistances other species such as wheat have a lack of phytoalexins, with only one identified compound, 1,4-Benzoxazinone, having phytoalexin potential in wheat (Bücker &

Grambow, 1990). Such plants rely on lignification, systemic acquired resistance (SAR) and the hypersensitive response (HSR) as an alternative defence against pathogenic infection. The HSR; a rapid-rate necrosis of cells in the locality of infection in order to reduce the risk of systemic infections (Heath, 2000), is linked to lignification for determining HSR as inhibition of lignification disrupts HSR (Moerschbacher *et al.*, 1990). The lignification process is affected by N-availability through morphological shortening and widening of xylem fibres (Pitre *et al.*, 2007). As N-availability is crucial to lignification, and therefore HSR, it is vital to understand N-availability for plants that rely on HSR for pathogenic defence, such as wheat. As a result, it is important to understand plant-microbial interactions in the N-fertilisation context as denitrification and DNRA rates have profound implications on soil N-availability.

1.4.3.2 Micronutrient mineralisation

Metal chelators are used by plants to enhance bioavailability of numerous micronutrients such as iron, manganese, copper and zinc, graminoid phytosiderophores are the best known for this. Although Fe is present in soil it is often present as Fe (III) and forms precipitates in soils with neutral or high pH. Yet the graminoid-secreted phytosiderophores form a Fe(III)-phytosiderophore complex that greatly increases the rate at which Fe can be taken up. It has been shown that graminoids can generally uptake the phytosiderophores-chelated compounds of other species, suggesting that these compounds increase Fe availability across the board for graminoid sp. Although organic acids also chelate these micronutrients, they are thought to play a more important role in phosphorus limitation. Phosphorus is normally found abundantly in calcium, ferrous and aluminium-phosphates. Organic acids (such as citric, malic and oxalic) form complexes

with these metal-phosphate pairs and increase bioavailability. It is also thought that organic acids also increase phosphorus availability through blocking soil adsorption sites for phosphorus or by interacting with cations. Linked with the proteoid roots above it seems that Lupines are also *Protaceae*, they decrease organic acid metabolism whilst increasing the exudation in these proteoid roots. It has yet been undetermined whether this exudation increases phosphate for other species other than Lupines.

1.4.4 Root compounds in rhizoengineering

Rhizoengineering has focused on roots due to them being the active sites for microbial growth in soil. A large population of metabolically active microbes is necessary for the success of agriculture or bioremediation (Metting, 1993). Rhizoengineering requires that nutrient-limitation (which is prevalent in rhizospheres) is present to starve natural rhizobacterial populations (Normander *et al.*, 1999). In this case, transgenic plants created to produce opines selectively alter their rhizobacterial community by providing a metabolic advantage to opine degrading bacteria over those unable to metabolically utilise this carbon source, nutritionally biasing the rhizosphere (Lugtenberg *et al.*, 2001).

By the reasoning above it was thought by Narasimhan *et al.* 2003 that naturally occurring secondary metabolites may be exuded in high enough concentrations to produce a nutritional bias without the need for transgenic plants. They used an *Arabidopsis thaliana*-*Pseudomonas putida* system, where they produced a phenylpropanoid auxotrophic strain of the wildtype *Pseudomonas* that was initially able to synthesize phenylpropanoid. The *Pseudomonas* also had the crucial capability in degrading PCBs, which at the time of research were only second to Benzene as global organic pollutants

and 6th in the list of hazardous substances (Narasimhan *et al.*, 2003). They found the phenylpropanoid utilising bacteria, Pp-wt, successfully colonised the rhizosphere of all flavonoid producing *A. thaliana* lines over the null mutant which didn't (Narasimhan *et al.*, 2003). Furthermore the authors demonstrate that the Pp-wt utilising strain consistently had an advantage in colonising the roots of *A. thaliana* mutants, this increase lasted 21-days in wt *A. thaliana* and 28-days in the flavonoid producing mutants, and did not exist in the flavonoid null mutant, this effect persisted even in mixed inoculations (with Pp-wt out-colonising, Pp-mut).

After colonisation, both strains (Pp-wt & Pp-mut) were shown to decrease 2Cl- and 4Cl- PCB levels by c. 30 and c. 90% respectively. Sand was then used to determine a gnotobiotic effect of the phenylpropanoid advantage and PCB degradation as PCBs have been shown to bind to soil particles, decreasing bioavailability (Narasimhan *et al.*, 2003). Bacterial populations were consistently found to be higher near the root than away from it (as expected) and PCB degradation was pronounced in all the inoculated samples. PCB degradation between the null mutant (tt4) and the wild type showed remaining levels of 2Cl- and 4Cl- PCBs c. 55% and 40% for bulk soil and c. 30%, for both, in adhering soil (tt4) and c. 60% and c. 30% for bulk with 40% and 10% remaining in adhering for (wt) respectively.

Overall, the differences between the two *P. putida* strains was the ability to utilise phenylpropanoids (aurones, lignins, flavonoids, coumarins, sinapates and anthocyanins) which *A. thaliana* naturally produced. Narasimhan *et al.* 2003 demonstrated an immediate practical use of understanding plant-microbial interactions in the

bioremediation field, and they laid out three key criteria to replicate such rhizoengineering. Firstly, the compound must be exuded by all plants so the microbe can colonise several root systems, despite competition. Secondly, the microbe must be one commonly abundant in the rhizosphere, so that it is generally robust and will not be outcompeted. Thirdly, the compound of choice must have a complicated structure and be considered 'unusual' so not all rhizobacteria can successfully metabolise it (Narasimhan *et al.*, 2003).

1.4.5 Root compound summary

In all, through ABCTs, ion channels, anion transporters and diffusion, plants release a wide array of compounds, differing in concentration and composition along the root length, plant age, abiotic factors, and biotic interactions. A need has arisen to make sense of this large array of compounds and disentangle how the aboveground section links to the belowground section and the surrounding soil microbiota. In order to achieve this, it is necessary to first carefully quantify and identify all compounds involved in an upregulation under certain conditions, then it is necessary to determine how these compounds are being exuded and the effect they're having on the physiology of the plant. It has been stated in this review that several avenues of research interest should be pursued in order to benefit agriculture: 1. MATE channels should be investigated, and their substances involved in secretion in phytohormones 2. Efforts should focus on decoding the dialogue between organisms in the rhizosphere 3. The link between how root chemicals influence the aboveground and belowground interactions needs to be deepened and explored to gain a full understanding, and 4. The responses to these interactions under changing environmental factors needs to be characterised.

1.5 Soil chemistry overview

Around 90% of the approximate 50-66% of solid matter in soil is organic, with the exception of muck and peat soils, which have much higher organic matter contents (Sposito, 1989). This abundance in organic matter exists from a range of molecules including humic substances, organic acids, fatty acids, carbohydrates and carbonates to name a few (Sposito, 1989). These abundances may change the rate of respiration within soils and therefore alter the typical aerated soil O_2 (200 ml/L) and CO_2 (~15 ml/L) concentrations, which can drop and rise to 20 ml/L and 100 ml/L respectively in microenvironments (Sposito, 1989). CO_2 and organic matter (organic acids, humic substances etc) play large roles in determining the acidity, redox potentials and aggregate formation in soils (Sposito, 1989), which ultimately determine the cohort of microbes present. Furthermore, inorganic stoichiometry in soils is highly complex due to the presence of metastable states (Sposito, 1989).

The complexity of native soil chemistry (without any plant presence) makes the interpretation of plant-microbial results difficult as indirect effects through co-variables may be present. Although the soil chemistry remains key and central to plant-microbe interactions and their interpretations it is too large to cover within this literature review.

1.6 Conclusion

In summary, plants experiencing differing conditions will alter their physiology in response. This alteration causes a systematic change in root C-acquisition and thereby shifts the composition and concentration of root metabolites exuded, which subsequently

alters the C: N ratio and thereby the N-cycling processes, nitrification, denitrification and DNRA. When N-cycling rates alter so do ratios between the substrate chemicals ($\text{NO}_3^-:\text{NO}_2^-:\text{NH}_4^+$) which in turn affect plant N-uptake via roots, affecting plant physiology which needs to be maximised to meet anthropogenic demands. The 'recruited' microbiota favouring such conditions may provide beneficial adaptations for plants experiencing the current stressor which, coupled with ongoing crop breeding, would assist with maximising yield/NPP. To make use of these systems a firm foundation needs to be created addressing how, when and why these relationships respond to external conditions such as drought and C:N ratios. The changing climate and C:N ratios of agricultural soils make these poignant systems to investigate to verify whether benefits of increased crop yields, more accurate and robust ESMs, and the ability to manipulate ecological interactions to maximise terrestrial C-sinks and minimise NO_x emissions can be gained from these systems. Such benefits would help us reduce the impacts of agriculture on global scales, whilst simultaneously improving crop yields and reducing climate change, alleviating two problems through one solution.

Here we look to address how the application of a labile C, which largely stimulates microbial growth, and a mineralised N, a limiting factor for plant growth, affects both the plants and microbial communities, and the interactions thereof. It cannot be overstated that the simplification and use of one simple labile C compound, as opposed to the diverse array naturally available in soil, limits the interpretations and inferences that can be had from the data. Particularly, the use of glucose, whilst common amongst the literature, is limited in real world inference due to not reflecting the complexity of both labile and recalcitrant C sources usually present, providing a heavy bias towards r-selected, copiotrophic micro-organisms.

The aims and hypotheses of this PhD are to disentangle plant-microbe interactions under environmental change by:

1- Assessing the effect C:N-fertilisation has on plant-microbial interactions

H₁ – Higher C:N-supplementation will have negative implications for plant photosynthetic rates and decreased biomass due to higher N-competition with soil microbiota

H₂ – Lower C:N-supplementation will increase plant photosynthetic rates and biomass, which will produce more distinct deterministic rhizosphere communities as a by-product of this relatively higher metabolic rate

2- Discover how root exudate profiles change in response to simultaneous drought and C:N-supplementation

H₃ – Root exudates will differ significantly between well-watered and droughted plants, with droughted plants producing a smaller range of compounds but with increased quantities of those compounds, forming deterministic communities

H₄ – C:N-supplemented plants will produce more exudates than water-only plants and will experience more N-competition with soil microbiota and will show have more stochastic communities than droughted soils

3- Determine how alterations in root exudate composition and concentration affect soil microbial communities

H₅ – Up regulated root exudates will significantly alter microbial communities compared to water-only controls, which will form deterministic microbial communities

H₆ – C:N-supplemented plants will have a more stochastic and less deterministic microbial community compared to water only controls due to the external influence of adding labile C

Chapter 2: General Methods

2.1 Plant measurements

2.1.1 Plant growth conditions

All plants (with exception of experiments performed in Chapter 3 –) were grown in 4 L pots containing a sand-loam (23% sand, 46% silt, 31% clay) agricultural topsoil at 1.2 g cm⁻³, obtained from Suffolk (Deans' Nursery, Colchester, UK). The soil complied with BS3882:2015 standard for topsoil as set by British Standards Institute (BSI). This ensured the topsoil was free from contamination (including pesticides, herbicides and metalloid contamination), was fertile to support plant growth, and the soil structure was not altered in transit and handling (which can inhibit topsoil functions).

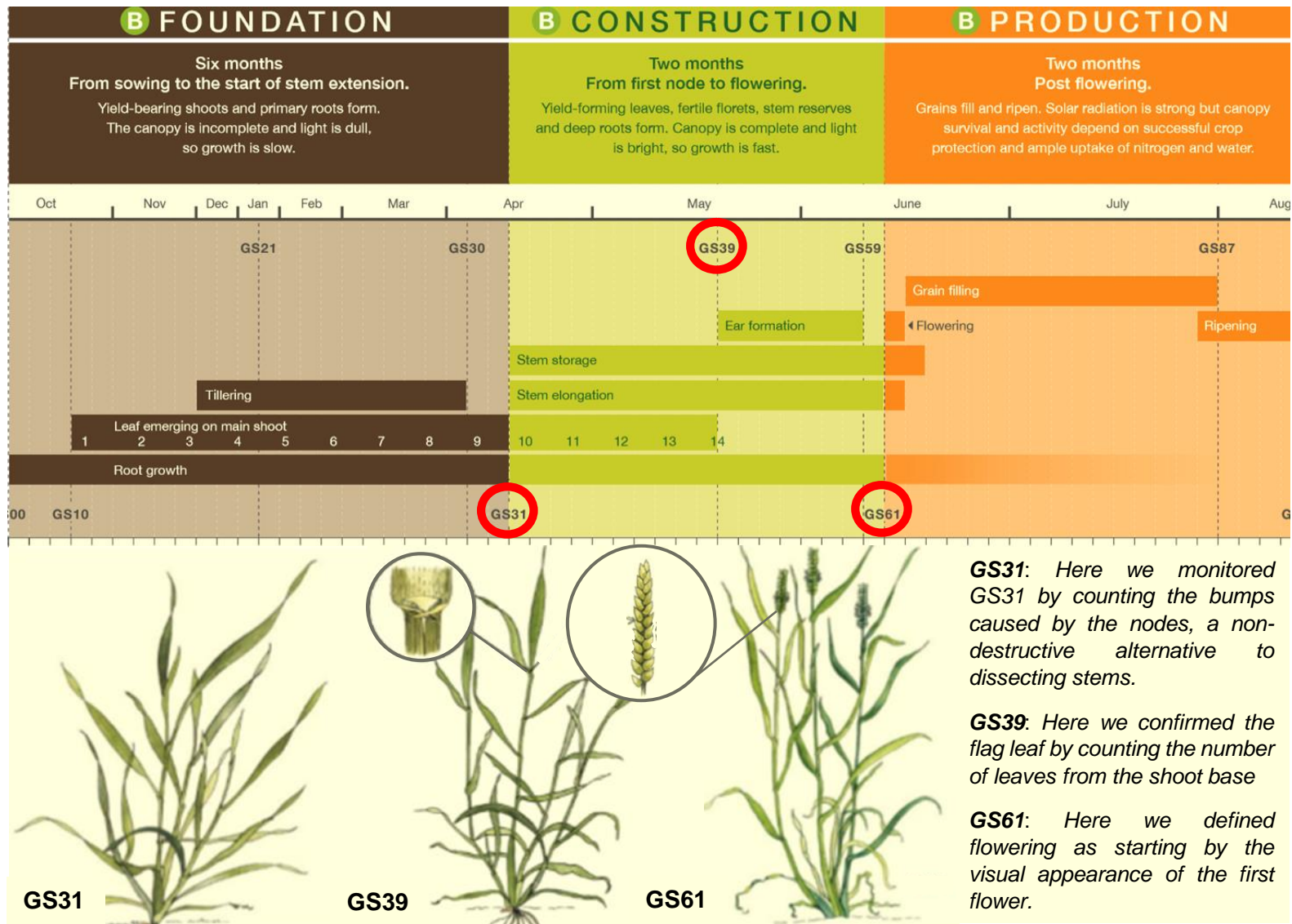


Figure 2.1: Visual display of the growth stages and fertilisation timings for the experiments in Chapter 4 and 5. This figure has been adapted from the information available in the AHDB wheat growth guide 2018.

○ = fertilisation

Below the fertilisation chart we have displayed the key construction growth stages that were the focus of chapters 4 and 5.

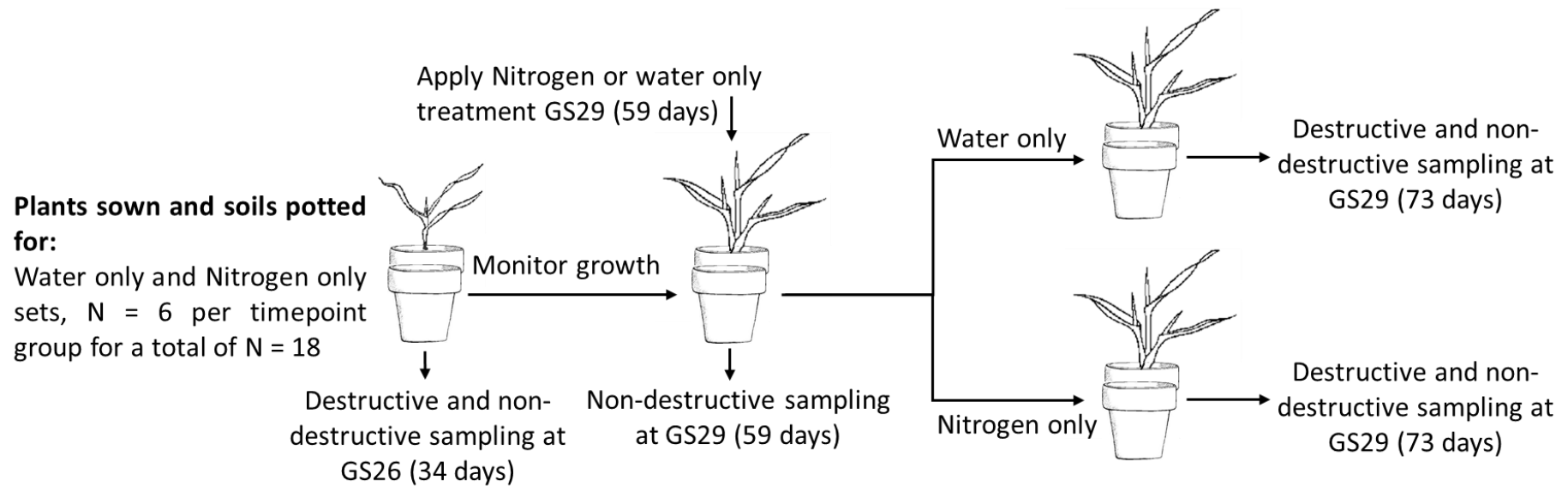


Figure 2.2: Overall experimental plan for chapter 3 for the three species used: Barley (*Hordeum vulgare* L.) cultivar Cassata, Einkorn (*Triticum monococcum*) Wheat, Emmer (*T. turgidum* subsp. *dicoccoides*), and soil controls.

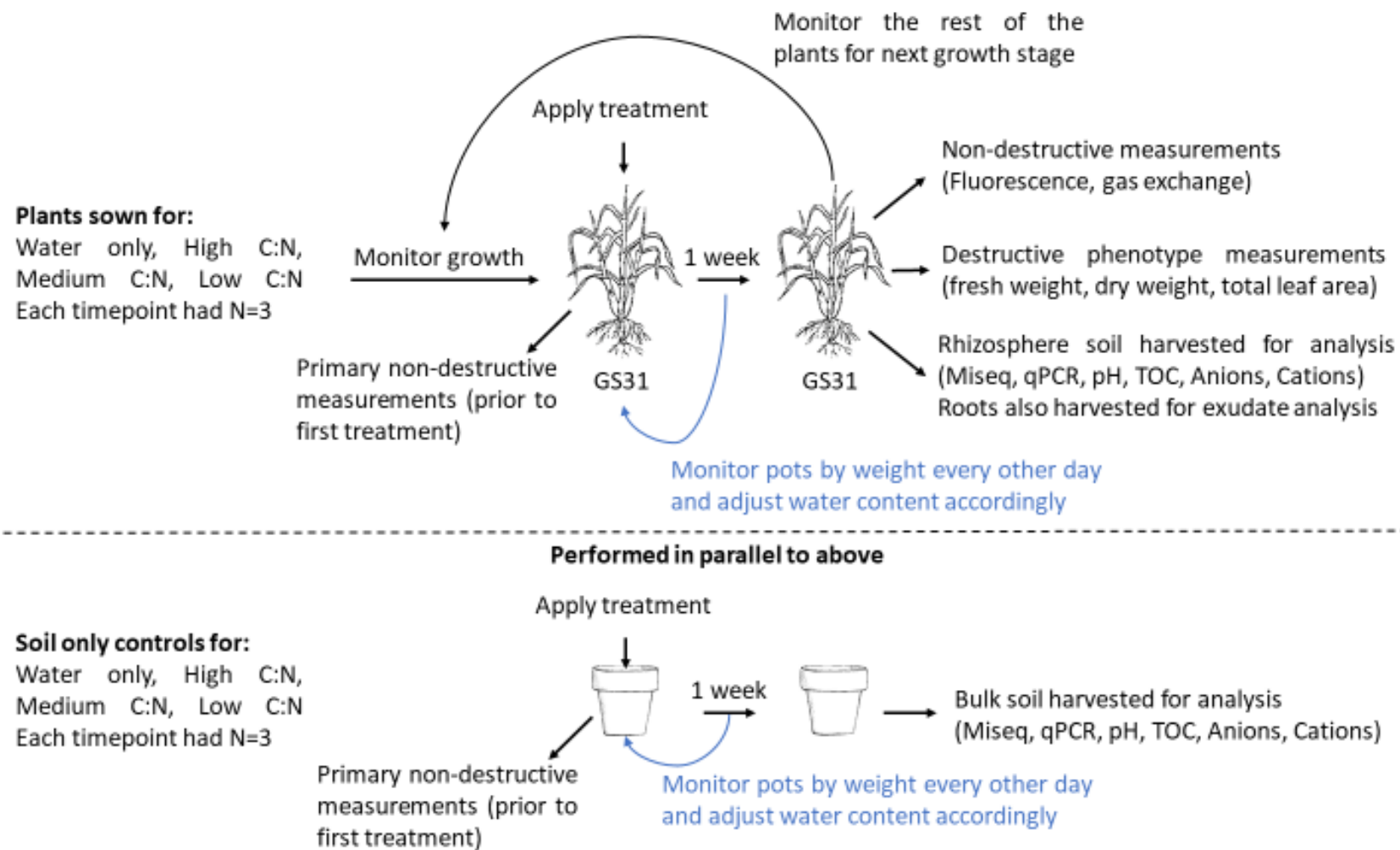


Figure 2.3: Experimental plan diagram summarising the steps taken in the experiment for chapter 4

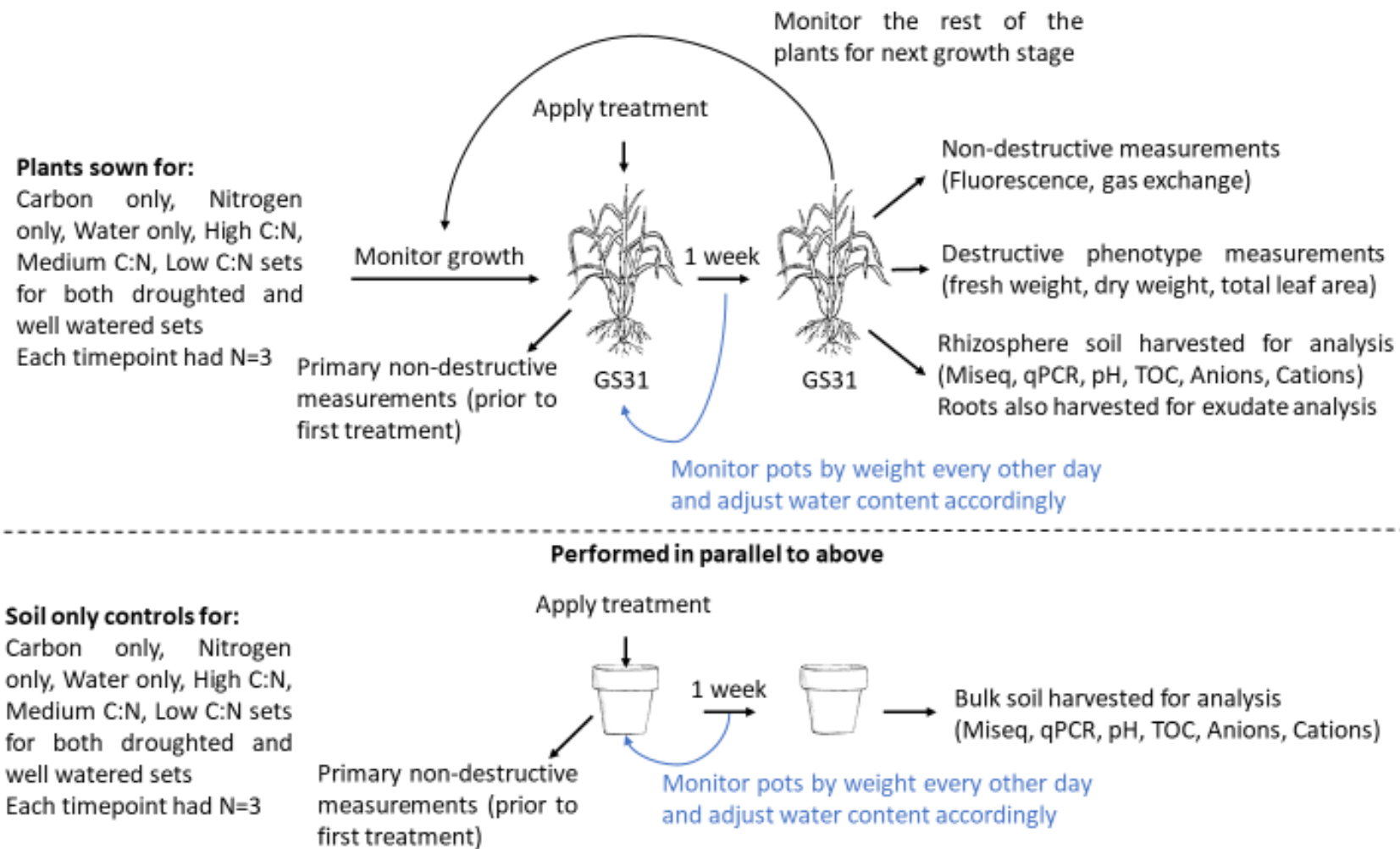
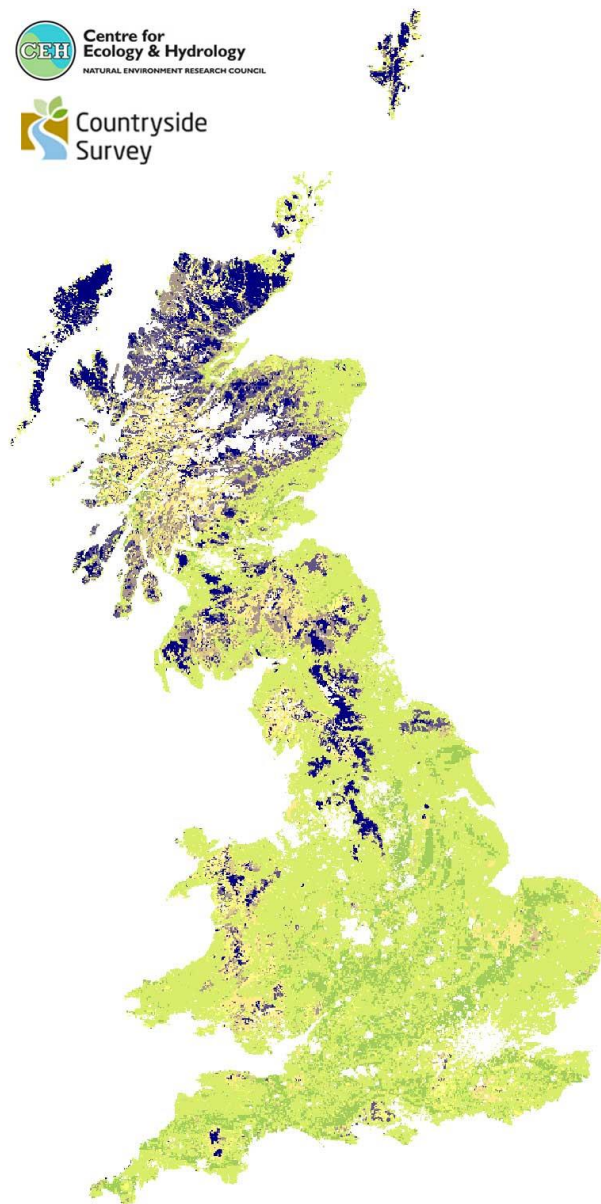


Figure 2.4: Experimental plan diagram summarising the steps taken in the experiment for chapter 5

2.1.2 C:N fertiliser treatments

Three C:N (D-glucose:NH₄⁺NO₃⁻) fertiliser treatments (32.58:1, 17.22:1, 9.37:1) were applied to *Triticum aestivum* cv Mulika wheat during three different growth stages (GS31, GS39, and GS61). The fertiliser treatments were selected based on mineralised N concentrations applied to UK tillage crops, 140 kg N ha⁻¹ (ONS, 2013), arriving at the different ratios by varying the amount of glucose added. The C:N ratios were calculated using UK Soil Observatory UKSO data from across the United Kingdom and selecting the extremes and average of that dataset to represent the high, low and medium C:N ratios used respectively (Figure 2.5). At each plant growth stage (GS31, GS39 and GS61) soils and plants were sampled for plant physiological measurements, gas exchange and soil microbial community analysis.



Map Key

CS Topsoil Nutrients – C:N ratio Carbon:Nitrogen Ratio










	< 11
	11 - 13
	13 - 15
	15 - 17
	17 - 19
	19 - 21
	21 - 23
	23 - 25
	>25

Figure 2.5: Soil C:N ratios adapted from the CEH (Centre for Ecology and Hydrology) website, (Henry *et al.*, 2012).

2.1.2 Anatomical analysis

Biomass calculations – Fresh (FW), dry (DW) and turgid (TW) weights of total aboveground biomass and single leaves (for specific leaf area) were used to determine the relative water content (RWC) through the formula:

$$RWC = \frac{(FW-DW)}{(TW-DW)} \times 100$$

With fresh biomass being the weight recorded immediately after harvest, turgid being recorded after 24hrs of the biomass sitting in wet tissue paper at 4°C and dry biomass being the stable low value when drying out mass in an oven at 60°C.

2.1.2.1 Total leaf area

For Chapters 4-8 total leaf area (cm²) used a calibrated LI-COR 3100C Area Meter (LI-COR, Lincoln, Nebraska, USA) to measure total leaf area. Individual leaves were detached from the plant, separated and systematically funnelled through the machine immediately after harvest. The machine was calibrated using a 10 mm² disc 10 times and adjusting the output accordingly. For Chapter 3 total leaf area (cm²) was analysed by image analysis of pictures taken from fully separated leaves for each plant (ensuring no overlap of the leaves). Images were processed on ImageJ using a 99.75 mm length object as an internal standard to relate pixel length with physical length.

2.1.2.2 Specific leaf area

Measured by image analysis of a leaf (via ImageJ, described above) with the addition of RWC (calculated as described above).

2.1.3 Leaf chlorophyll fluorescence analysis

2.1.3.1 Chapter 3 Fluorescence measurements

Chlorophyll fluorescence measurements were performed weekly on whole plants from GS14 to GS29 plants using a CF Fluorolmager imaging system (Technologica Ltd., Colchester, UK). When too large to stand upright in the Fluorolmager the plants were placed on a metal grid with nylon wiring to keep them within the focal plane. The maximum quantum efficiency of PSII photochemistry (F_v/F_m) was determined following a 30-min period of dark adaptation followed by a saturating pulse of $5,200 \mu\text{mol m}^{-2}\text{s}^{-1}$ for 800 ms. Plants were then maintained under actinic light ($500 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 20 min with light adapted measurements F_q'/F_m' (PSII operating efficiency, the efficiency of absorbed light being associated with stable photochemistry), F_v'/F_m' (a measure that provides the maximum operating efficiency in light adapted leaves, when some photosystems are closed due to photochemical activity) and $F_m - F_m'/F_m$ (a measure of NPQ, non-photochemical quenching) being taken at a saturating pulses ($5,200 \mu\text{mol m}^{-2}\text{s}^{-1}$ for 800 ms) applied 10 times at 2-min intervals. Following this a stepwise change in light from $1500-0 \mu\text{mol m}^{-2}\text{s}^{-1}$ in 11 steps (1500, 1250, 1000, 750, 500, 400, 300, 200, 150, 100, 50, 0) was applied to plants with light adapted measurements being determined by saturating pulses ($5,200 \mu\text{mol m}^{-2}\text{s}^{-1}$ for 800ms at 2-min intervals). F_q' ($F_m' - F'$ – variable fluorescence in the light) was calculated from the difference between F' (steady state fluorescence of light adapted leaves) and F_m' (maximum fluorescence following a saturating pulse ($5,200 \mu\text{mol m}^{-2}\text{s}^{-1}$) of light). F_o' (minimum fluorescence signal when all centres open) was calculated through the following calculation:

$$F_o' = F_o / \left(\frac{F_v}{F_m} + \frac{F_o}{F_m'} \right)$$

F_v' (the variable fluorescence of light adapted leaves) was calculated at the difference between F_o' and F_m' , (operating efficiency of PSII, a calculated estimate of how much light is used in PSII photochemistry), Images were processed using the editing tools of the FluorImager computer programme (Technologica Ltd., Colchester, UK).

2.1.3.2 Chapter 4 and 5 Fluorescence measurements

A Fluorescence Monitoring System 2 (FMS2 – Hansatech Instruments Ltd, King's Lynn, Norfolk, PE32 1JL, United Kingdom) was used to take spot measurements on mature wheat. A clip was placed on a section of the leaf for 30 m, subsequently the dark-adapted F_v/F_m measurement was taken for that leaf.

2.1.4 Photosynthetic gas exchange

2.1.4.1 Light response curves

Gas exchange parameters (including A) was measured as a function of light (Q , to produce A/Q response curves) using LI-COR gas exchange systems (6400XT, LI-COR, Lincoln, Nebraska, USA). Cuvette conditions were kept at ~60% relative humidity, 23°C and ambient $[\text{CO}_2]$ of $400 \text{ mmolm}^{-2}\text{s}^{-1}$. Initially leaves were stabilised within the cuvette at a saturating irradiance of $2000 \text{ } \mu\text{molm}^{-2}\text{s}^{-1}$ for 20 minutes before measurements, then they were changed in a stepwise manner through 13 steps until reaching a PPFD of $0 \text{ } \mu\text{molm}^{-2}\text{s}^{-1}$ where the final measurement was taken. Measurements were recorded after A was found to reach steady state for 1-3 minutes determined through the variance in A detected within a 30s period.

2.1.4.2 A/C_i response curves

Gas exchange parameters were measured as a function of internal leaf $[CO_2] - C_i$ - (to produce A/C_i response curves) using the LI-COR gas exchange systems (6400XT, LI-COR, Lincoln, Nebraska, USA). Cuvette conditions were kept at ~60% relative humidity, 25°C and 2000 $\mu\text{molm}^{-2}\text{s}^{-1}$ of light. Measurements were taken after reaching a new steady state (1-3 minutes) at each point. After an initial 20-minute stabilisation period at 400 $\text{mmol}^{-2}\text{s}^{-1}$ CO_2 the concentration was decreased in a stepwise manner 6 times to 50 $\text{mmolm}^{-2}\text{s}^{-1}$, after which a re-stabilisation period at 400 $\text{mmolm}^{-2}\text{s}^{-1}$ for 3 min, followed by a stepwise increase in $[CO_2]$ 7 times until 1750 $\text{mmolm}^{-2}\text{s}^{-1}$ ambient CO_2 was reached where the final measurement was taken.

2.1.5 Root exudate extractions

Samples for root exudate analysis were taken from young, largely metabolically active roots (Stūrīte *et al.*, 2005), determined by a white-cream or light brown colouration rather than dark-brown or black colouration and thus potentially metabolically active (Stūrīte *et al.*, 2005). The Ectorrhizosphere defined here is any soil that actively adhering to metabolically active roots when the roots were taken from soil samples during harvesting. Roots previously harvested were removed from -80°C storage and root-adhering soil removed and weighed for exudate extractions whilst the remaining root tissue was processed for internal root compounds. Two extraction methods were used to obtain a range of polar and non-polar compounds: a) Monophasic water extraction, b) Biphasic $CaCl_2$ -dichloromethane (DCM) extraction. The samples were then processed on a HP5890 series II gas chromatograph with a Nukol column (30m length x 0.25mm ID x 0.25 μm film thickness. The oven program for the gas chromatographer was as follows:

from an initial temperature of 80°C a temperature of 150°C was achieved through an increase of 10°C/minute for 7m. The temperature was then increased to 200°C at a rate of 20°C/minute for 2 minutes and 30 seconds, where it was held at this temperature for a further 10 minutes for a total run time of 19 minutes and 30 seconds. These steps were performed at Essex University under the advice of Dr. Kelly Redeker from York University and then subsequently taken to York University and processed alongside Dr. Kelly Redeker.

2.1.5.1 Monophasic water extraction

Frozen root tissue was weighed out into reinforced bead-beating tubes with QIAGEN™ stainless steel 5mm beads (QIAGEN, Qiagen Str. 1, Hilden 40724, Germany) with 10 µl of ice-cold distilled water added per milligram of tissue. The tubes were then sealed and homogenised for 2 x 30s bursts at 6,500 rpm the homogenised mixture was removed into a clean 1.5 ml plastic tube and kept on ice for at least 5 minutes. These mixtures were then briefly put in a vortex and subsequently centrifuged at 15,000 rpm (21,885 rcf) at 4 °C for 10 minutes in a Biofuge rotor. An aliquot of the supernatant was removed, pushed through a 0.2 µm filter and immediately placed in an iced 1 ml glass GC vial, capped and stored at 4°C overnight. The samples were then analysed at York University with Kelly Redeker for volatile fatty acid (VFA) analysis using a HP5890 series II gas chromatograph (HP Inc. Palo Alto, CA 94304, United States).

2.1.5.2 Biphasic CaCl₂-DCM extraction

Weighed frozen samples were homogenised for 2 x 30s bursts at 6,500 rpm, then added to a 1:10 extraction ratio (W/V) of 1.0M CaCl₂ at 25°C horizontally shaken at 150 rpm for

30 minutes. The samples were then centrifuged at 816 x g for 10 minutes. A 1.5 ml supernatant aliquot was then removed and centrifuged at 12,240 x g for 15 minutes to remove all particles > 0.05 µm. Then 1 ml of this supernatant was added with 1 ml of DCM and stored at 4°C until VFA processing on the HP5890 Series II gas chromatograph.

2.2 Soil sampling

For bulk soil sterile universal tubes were used to sample soil cores measuring 3.1cm in diameter and 9.3 cm deep were used to obtain soil cores at the depth of 5 cm, these cores were visually inspected to ensure that the soil had no root contact. Conversely, rhizosphere soils were sampled by removing the plant from the pot, cutting a section of the root ~ 5 cm from the stem, giving a gentle tap to remove excess soil and then rapidly freezing it in liquid nitrogen. Once frozen these samples were processed later with only soil adhering to, or within 1 cm of, a metabolically active root (light brown/cream in colouration) was considered rhizospheric soil.

2.2.1 Soil physicochemical analysis

2.2.1.1 Anions and Cations analysis

Soil anion and cation concentrations were determined by adding 1g wet weight soil to 10 ml of sterile distilled water and sonicating for 30 minutes in a Fischer Scientific™ M Series Mechanical Ultrasonic bath. Samples were filtered through a 0.2 µm filter and the filtrate analysed using a Thermo Fisher™ Dionex™ ICS-3000 (Dionex™ ICS-3000, Dionex™, Sunnyvale, CA).

2.2.1.2 Soil pH, Total Organic Carbon (TOC) and Total Inorganic Carbon (TIC)

Soil pH was measured on a Mettler-Toledo™ FiveEasy™ (Mettler-Toledo Ltd, 64 Boston Road, Beaumont Leys, Leicester, LE4 1AW) F20 pH meter using a 1:5 soil:dH₂O mix previously described (Čuhel *et al.*, 2010). Total Organic Carbon (TOC) and Total Inorganic Carbon (TIC) were measured on a Skalar Formacs^{HT} (Skalar Analytical B.V., Tinstraat 12, 4823 AA Breda, The Netherlands) TOC Analyzer with the Primacs^{MCS} add-on for solid state samples.

2.2.2 Microbial community analysis

DNA was extracted from triplicate soil samples using CTAB (hexadecyltrimethylammonium) method previously described (Griffiths *et al.*, 2000). Q-PCR was performed in 10 µl amplifications using 1 µl DNA template, 5µl SensiFAST™ SYBR NO-ROX dye (Bioline Reagents Limited, United Kingdom) and 400 nl of 10 µM primer, run at 95°C for 3 min followed by 40 cycles of 95°C for 10 s and 55°C for 30 s and a final cycle of 95°C for 10 s followed by 65°C. All amplifications were performed on a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, UK) with primer sets purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd., The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT, United Kingdom), please see Table 2.1.

Table 2.1: Details of primer pairs used in the following studies

Primer Name	Gene target	Sequence 5'-3'	Annealing T (°C)	Reference
Bakt_341F	16S rRNA bacteria	CCTACGGGACGTGGCATGCAG	55	Herleman <i>et al.</i> , 2011
Bakt_805R	16S rRNA bacteria	GACTACATCGACGGGTATCAATCC	55	Herleman <i>et al.</i> , 2011
344F	16S rRNA archaea	ACGGGGCTGCAGCAGGCGCGA	54	Lane <i>et al.</i> , 1985
915R	16S rRNA archaea	GTGCTCCCCCGCCAATTCCT	54	Lane <i>et al.</i> , 1985
<i>nirS</i> -Cd3aF	<i>nirS</i>	AACGCTGCAAGGAAGACGCGG	57	Kandeler <i>et al.</i> , 2006
<i>nirS</i> -R3cd	<i>nirS</i>	GAGCTTCGGAGTGGCGTCTTGCACTGAA	57	Kandeler <i>et al.</i> , 2006
<i>amoA</i> -1F	Bacterial <i>amoA</i>	GGGGTTTCTACTGGTGGT	60	Rotthauwe <i>et al.</i> , 1997
<i>amoA</i> -2R	Bacterial <i>amoA</i>	CCCCTCGTGGCAAAGCCTTCTTC	60	Rotthauwe <i>et al.</i> , 1997
crenamoA23F	Archaeal <i>amoA</i>	ATGGTCTGGCTWAGACG	53	Tourna <i>et al.</i> , 2008
crenamoA616R	Archaeal <i>amoA</i>	GCCATCCATCTGTATGTCCA	53	Tourna <i>et al.</i> , 2008
nosZ1F	<i>nosZ</i>	WCSYTGTTCMTCGACAGCCAG	62	Henry <i>et al.</i> , 2006
nosZ1R	<i>nosZ</i>	ATGTCGATCARCTGVKCRTTYTC	62	Henry <i>et al.</i> , 2006
ITS3F	Fungi ITS	GCATCGATGAAGAACGCAGC	60	White <i>et al.</i> , 1990
ITS4R	Fungi ITS	TCCTCCGCTTATTGATATGC	60	White <i>et al.</i> , 1990

Standards were made from extracted DNA samples amplified under PCR (polymerase chain reaction), using Appleton® AppTaq RedMix (Appleton Woods Limited, New Lindon House, Catesby Park, Kings Norton, Birmingham, B38 8SE, United Kingdom) and the PCR product was cleaned using QIAquick® purification kit following the manufacturer's instructions.

Standard curves were generated by serial 10-fold dilutions for each gene and using CFX Manager software (Bio-Rad Laboratories, United Kingdom) with default Cq values and baseline settings target genes were quantified. Limit of detections were determined at the lowest end of the standard curve, with samples appearing within 3 cycles of NTC cycles being discounted as no amplification. Melt curves were performed after all cycles had completed, heating to 65°C for 5 seconds and then increasing to 95°C by increments of 0.5°C, holding for 5 seconds once 95°C had been achieved.

After quantification conversion from ng/μl to copies was performed as follows:

$$\text{Copy Number} = \frac{6.023 \times 10^{23} \times [\text{DNA}] \text{ ng } \mu\text{l}^{-1}}{(\text{Length of target}) \times 660 \text{ g mol}^{-1} \times 1 \times 10^9}$$

To further define copy abundances per gram dry weight (DW) of soil formula output was then divided by the corresponding g DW soil to give gene copies g⁻¹ DW soil.

2.2.2.1 Next Generation Sequencing of phylogenetic and functional genes

We prepared amplicon sequencing libraries for 16S rRNA bacteria, 16S rRNA archaea, bacterial *amoA*, crenarchaeota *amoA*, *nirS*, and ITS genes according to Illumina workflow guidelines. Pipette tips were always changed to avoid cross-contamination of samples.

2.2.2.2 Initial PCR

Firstly, we performed a 25 μ l reaction amplicon PCR of each gene and sample according to reaction reagents and primer conditions highlighted above and in Table 2. A gel was then run to check for an amplicon product of the expected size, this step was repeated twice, the second time with dilution, as an unsatisfactory amount of PCR products did not amplify.

2.2.2.3 Amplicon PCR clean-up 1

After achieving an amplicon, we cleaned the amplicon product using 20 μ l AMPure XP beads at room temperature, 52.5 μ l of 10mM Tris buffer (pH 8.5), and 400 μ l of freshly prepared 80% ethanol per 25 μ l sample. After a 1-minute spin at 1,000 x g at room temperature we added 20 μ l of AMPure XP beads, which was then gently pipetted up and down at least 10 times, the mixture was then incubated for 5 minutes at room temperature. After incubation, we placed the solution on a magnetic stand and waited 2 minutes or until the supernatant had cleared. Once clear we removed the supernatant and discarded it. With the Amplicon PCR plate still on the magnetic stand we added 190 μ l of 80% ethanol, stood for 30 seconds on the magnetic stand, after which the

supernatant was carefully discarded. This step was repeated twice. After the two ethanol washes the magnetic beads were left for 10 minutes on the magnetic stand for excess ethanol to evaporate.

After ethanol evaporation, we removed the Amplicon plate from the magnetic stand and added 52.5 μ l of Tris buffer, gently mixing to fully resuspend the beads. The plate was then allowed to sit for 2 minutes at room temperature before placing the Amplicon plate back onto the magnetic stand and waiting a further 2 minutes or until the supernatant had cleared. After the supernatant cleared, we transferred 50 μ l to a clean 96-well plate.

2.2.2.4 Index PCR

We used boxes A, B, C, and D Nextera XT Index Primers from the Nextera XT Index kit to label each sample in the 96-well plates with a unique pair of primers. We done this by the dual indexing principle, we used unique N7XX primers horizontally across the plate (wells 1 to 12), and unique S5XX primers vertically down the plate (wells A to H), thus giving each sample a unique pairing of N7XX and S5XX indexes. Firstly, 5 μ l of sample from the last step was added to a new 96-well plate. We then added a master mix consisting of 10 μ l PCR grade water and 25 μ l of 2 x Appleton Woods RedTaq per sample, after which 5 μ l of each primer was added to their corresponding rows or columns, being sure to change tips in between pipetting to avoid cross-contamination. Whilst the last set of primers were added we gently mixed the wells for mixing. A seal was then added to the plate and it was centrifuged at 1,000 x g for 1 minute. After centrifugation we performed the following PCR on a thermal cycler: 95°C for 3 minutes, followed by 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, with a

final 72°C for 5 minutes after the 8 cycles had finished. Samples were then held at 4°C until promptly collected and stored in a -20°C freezer.

2.2.2.5 Amplicon PCR clean-up 2

Another clean-up was performed in the same manner as described above, except for different volumes of reagents. We used 56 µl of AMPure XP beads, and 27.5 µl Tris buffer (pH 8.5) for the respective steps in this clean-up. At the end of the clean-up, we transferred 25 µl to a new 96-well plate. After clean-up the products were quantified as below.

2.2.2.6 PicoGreen Assay quantification

We used the ThermoFisher PicoGreen kit to quantify the ds DNA indexed amplicon products. In brief, a 7-point standard curve was created from serial diluting a 100,000 ng/mL stock of lambda DNA provided in the kit with 1X TE buffer to: 1,000 ng/mL, 500 ng/mL, 250 ng/mL, 125 ng/mL, 62.5 ng/mL, 31.25 ng/mL, 15.5 ng/mL standards. PicoGreen dye provided by the kit was also diluted from the original 200X concentration to 1X using 1X TE buffer. Samples were thoroughly mixed with 10 µl of prepared dye to 10 µl of sample, giving 20 µl total. These samples were either run on a NanoDrop-2000, or a Microplate reader as follows:

NanoDrop-3300: The NanoDrop 3300 (Company details) was blanked with 1X TE buffer, each sample was run individually on the Nanodrop using the machine's ds DNA protocol. After each sample both pedestals were wiped with low lint laboratory wipes, cleaned with

1X TE and subsequently wiped again to ensure minimal contamination across samples. Technical replicates were as follows: Standards n=5, samples n=3, negative controls n=3, blanks n=3.

Microplate reader: Each plate contained all 7 standards in technical replicates of 5, samples in technical replicates of 3, negative controls of 10 μ l TE and 10 μ l dye in technical replicates of 3, and blank samples of 20 μ l TE in technical replicates of 3. The plate was then spun down briefly for 10 seconds before being run on the microplate reader as follows.

Each plate was set up on the microplate reader using the Mars software with the following settings: Excitation filter = 482-12, emission filter = EM520, optic = top. One selected the plate layout was created on the software. When ready to run, we initialised the gain at 90 % on the highest relative fluorescence unit (RFU) sample, the 1,000 ng/mL standard. Once the gain had been adjusted, we started the measurement on the machine. If samples were too high to be measured by the machine (or were outside of the standard curve) they were diluted, and the plate measurements were repeated.

2.2.2.7 Initial library pooling

Once each sample had a reading, we calculated the concentration of the sample based on its RFU to ng/ μ l DNA concentration. When converted to ng/ μ l DNA each sample was then converted into nM concentration of DNA using the following formula:

$$nM = \frac{\text{concentration in } \frac{ng}{\mu l}}{660 \frac{g}{mol} \times \text{average library size}} \times 10^6$$

This was done for each gene of each sample. After obtaining each sample concentration we calculated the volumes of each sample to add for an equimolar solution. We added 1 μ l of the most concentrated sample, and more of less concentrated samples. For example, if the next sample was half the concentration, we added 2 μ l instead of 1 μ l. This step was performed for each gene separately, producing one equimolar pool for each gene. These gene pools were then re-quantified by the NanoDrop 3300 step described above and then pooled into final library pools with twice the concentration of bacterial 16S rRNA to all other genes. Once these final pools had been created, they were quantified using the NEBNext Library Quant Kit.

2.2.2.8 NEBNext Library Quant Kit

Following the NEBNext Quant Kit instructions we diluted our libraries by 1:1,000, 1:10,000, and 1:100,000 using the NEBNext Library Quant Dilution Buffer (1X). We then prepared Q-PCR assays using 16 μ l NEBNext Library Quant Master Mix (with primers), and 4 μ l of sample, kit provided DNA standard (10 pM, 1 pM, 0.1 pM, or 0.01 pM), or library dilution buffer. These 20 μ l reactions were then run on a Bio-Rad CFX96 Touch Real-Time PCR Detection System set to detect SYBR in a 96-well plate under the following cycling conditions: 95°C for 1 minute, and 35 cycles of 95°C for 15 seconds followed by 63°C for 45 seconds.

The Cq results from the Q-PCR were then entered onto NEBNext Quant Kit online calculator for each standard and library sample. The results were then used to determine the final concentrations in the steps for MiSeq. However, we needed to concentrate our libraries to achieve the 4 nM concentration needed for MiSeq. This concentration step was achieved using an ethanol precipitation step as follows:

To each sample we added 1/10th 3 M Na-Acetate (pH 5.2) and 3X the volume of 100% ethanol and left at room temperature overnight. The next morning, we centrifuged the samples at 14,000 x g for 30 minutes. We carefully discarded the supernatant and rinsed the pellet with 70% ethanol. Another centrifugation was performed at 14,000 x g for 15 minutes. The supernatant was discarded, and the pellet dissolved in 1X TE. After this we re-quantified the libraries using the NEBNext Library Quant Kit as described above and received desirable concentrations.

2.2.2.9 Library Denaturation and MiSeq Sample Loading

Following the Illumina MiSeq protocol, we combined 5 µl of 4 nM pooled library to 5 µl of 0.2 M NaOH, which was pH checked using litmus paper to ensure high alkalinity (13-14). This was then briefly vortexed, centrifuged at 280 x g for 1 minute at room temperature, and left to incubate at room temperature for 5 minutes. We then added 990 µl of pre-chilled HT1 to the denatured pool to produce a 20 pM denatured library in 1 mM NaOH. Similarly, we combined 2 µl of 10 mM PhiX library to 3 µl of 10 mM Trish buffer (pH 8.5). We then added 5 µl of 0.2 M NaOH, briefly vortexed the solution, and allowed it to incubate at room temperature for 5 minutes. We then added 990 µl of HT1 to this solution to produce a 20 pM PhiX solution.

Both denatured library and PhiX tubes were diluted separately to 8 pM by adding 240 µl of sample to 360 µl of HT1 respectively. Once diluted they were thoroughly mixed. We then added 90 µl of PhiX to 510 µl of library to produce a 15% PhiX at 8 pM solution. This solution was then added to a pre-heated 96°C water bath for 2 minutes, after which it was inverted twice to mix and then immediately placed in an ice-water (3:1) bath for 5 minutes. After this 5-minute period the sample was loaded into an Illumina MiSeq cartridge and run on a MiSeq machine.

The outputs were then run bioinformatically by Dr. Alex J. Dumbrell using previously outlined guidelines for paired-read Illumina amplicon libraries (Dumbrell *et al.*, 2016). Briefly, next generation sequencing (NGS) quality control was performed following previous recommendations (Schirmer *et al.*, 2015). Using Sickle version 1.33 (Joshi & Fass, 2011), raw reads were trimmed only from the 3' end using paired-end mode Q20 quality threshold and reads with unknown bases were discarded. Using default settings for BayesHammer (Nikolenko *et al.*, 2013) in SPAdes (Nurk *et al.*, 2013) these reads were then error corrected. After aligning forward and reverse reads primers were removed in PANDAseq version 1.33 (Masella *et al.*, 2012) using the PEAR algorithm (Zhang *et al.*, 2013). A length filter of 200bp was used to remove short reads and paired reads were de-replicated, using VSEARCH (Rognes *et al.*, 2016) to remove chimeras. Using a 97% similarity threshold the remaining reads were clustered into operational taxonomic units (OTUs) and sorted by abundance. Additionally, due to the likelihood of non-biological origins, OTUs with less than 3 occurrences were removed (Flynn *et al.*, 2015).

Unfortunately, after this bioinformatic process very few reads of a suitable quality were present for each gene (< 1,000), with short fragments being amplified across the samples. These low-quality fragments (>Q40) failed to cluster into OTUs and could not be used for data analysis, rendering the two plates of 684 total samples across 2 plates unusable. As the checks (outlined above) showed expected results, it was thought that the oligos were synthesised incorrectly or that odd primers were used.

Table 2.1: Details of MiSeq read usability

Gene target	Average usable reads	Usable reads* (%)
16S rRNA bacteria	9.48	2.06
16S rRNA archaea	10.64	1.67
Bacterial <i>amoA</i>	9.33	4.67
Archaeal <i>amoA</i>	2.85	2.34
Fungi ITS	1.77	0.75

* Where 18,000,000 reads were used as a proxy for a successful run

2.3 Statistical Analyses

Data were initially visualised through histograms and normality Q-Q plots to assess homogeneity and normality. Then Levenes test for equality of variances were carried out to statistically check data homogeneity, and Shapiro-Wilk tests were used to test for normality. After checking data distribution, analysis was carried out according to normality. Analysis of Variance (ANOVA) tests were performed on data sets that

conformed to normality, with a Tukey HSD (Tukey honest significant differences) test being used as a post-hoc test after significance showed under ANOVA. Kruskal-Wallis tests or pairwise Mann-Whitney U tests where appropriate were used for non-parametric data sets, with Dunn tests being used as a post-hoc for the Kruskal-Wallis. Where possible, datasets were transformed to comply with parametricity and then corresponding parametric tests were applied. The principal component analyses (PCA) utilised the R-packages `missMDA`, `FactoMineR`, and `factoextra` (Josse & Husson, 2016; Kassambara & Mundt, 2017; Lê *et al.*, 2008). Whilst the package `dunn.test` was used for non-binomial distribution failure (Dinno, 2017), whilst `ggplot2` was used for some graphical visualisations (Wickham, 2009). All other statistics were performed using R v3.2.3 (R. Core Team, 2015). Throughout the thesis the probability value of $P = 0.05$ was used as a significance threshold.

Chapter 3: Rhizosphere community responses to N-treatment in *Triticeae* members

3.1 Introduction

Grasses have played a large role in the development of agriculture and they comprise 44.5% of modern calories (FAO, 2013a). Worldwide agriculture relies on grasses: Rice (*Oryza spp.*), Corn (*Zea spp.*), Wheat (*Triticum spp.*), and Barely (*Hordeum spp.*) to directly and indirectly provide caloric needs. Wheat is said to be the “universal” cereal of old-world agriculture and alongside Barely formed the cornerstone of Neolithic agriculture and its success in spreading (Zohary & Hopf, 2000). Through breeding efforts, we have moved away from smaller grain producing species such as Einkorn (*Triticum monococcum*) and ancient Barley to more modern varieties and species such as Emmer wheat (*Triticum turgidum*) and Bread Wheat (*Triticum aestivum*). With most of Earth’s surface covered by wheat than any other crop (FAO, 2013a), providing 18.3 % of total per capita kcal globally – only exceeded by rice, 18.8% - (FAO, 2013a) it is one of Earth’s biggest impacting crops. Wheat and Barely are also the most important grasses to UK agriculture with annual productions of c. 14 Mt and c. 7 Mt, from 29.2 % and 19.2 % of total UK croppable area respectively (Defra, 2012).

Wheat and Barely yield increases make drastic differences in agricultural food markets due to their dietary influence (FAO, 2013b). Historically, such yield increases have been reached through a combination of agricultural expansion, crop breeding and maintaining plants under ideal conditions (Barbier, 2004; Tester & Langridge, 2010). Hitherto, these agronomic and breeding practices have surged previous global cereal production up from

877 million metric tons in 1961 to 2,351 million metric tons in 2007 (Tester & Langridge, 2010), with an expected rise to 3,056 million metric tons in 2050 (Alexandratos & Bruinsma, 2012).

Recently however, over a third of global crop growing areas for rice (35%) and wheat (37%) have shown yield stagnation and see reduced yield improvement (Ray *et al.*, 2012). For example, the global leaders in wheat production, China and India, saw yield stagnation in wheat yield for 55.5% and 69.9% of their cropped wheat area respectively (Ray *et al.*, 2012). Furthermore, future yields may be compromised by weather patterns and bouts of drought according to some global climate models, predicting decreases between -5.45% to -13.40%, and -3.74% to -18%, globally for irrigated and rain fed wheat respectively (Nelson *et al.*, 2010). Total crop production for wheat is further exacerbated by decreasing potentials for agricultural expansion, for example, in the UK total agricultural holdings, although fluctuating, have largely remained the same (Figure 3.1). Despite still experiencing global yield increases in 61% of wheat harvested areas, yield stagnation is a disturbing and potentially catastrophic trend alongside increasing land use competition and a growing global human population which demands yield improvement per unit area whilst maintaining soil quality.

Future nitrogen fertilisation may be an issue due to the ecological and physical damages done to our environments when they are overused. With the UK implementing several NVA policies that look to limit the impact nitrogen fertilisation has upon the surrounding health, yet it is well known that over-farming land can lead to lower land productivity (Withers 2002). These low productive soils, or fallow periods, are a common occurrence

when essential nutrients are extracted from the soils without being replenished. This leads to a large decline in soil health and productivity as major factors such as soil structure, water retention, pH and aeration profiles change with such artificial manipulations.

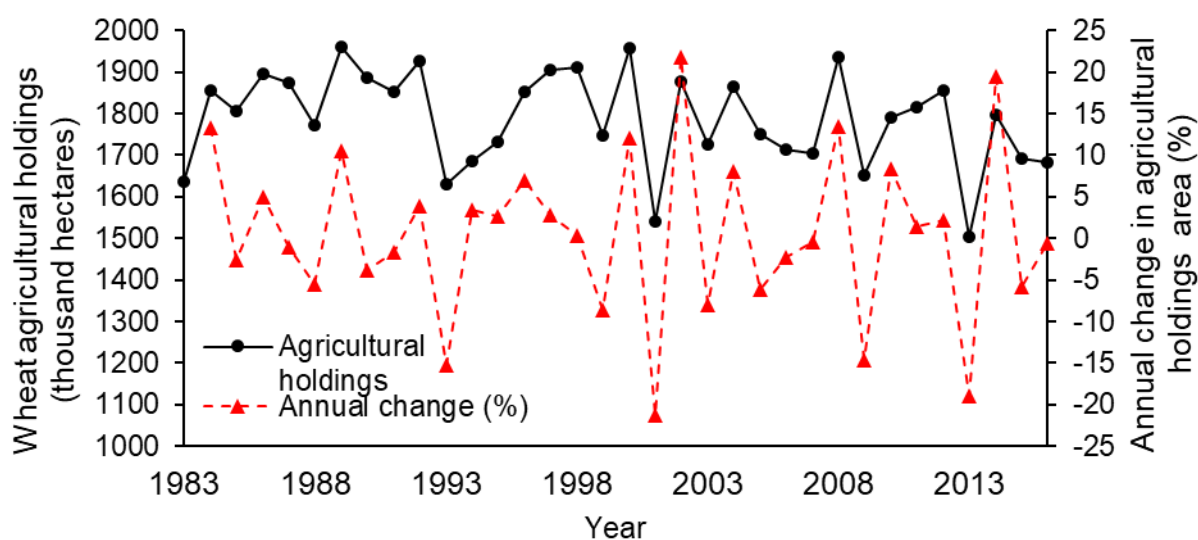


Figure 3.1: Total UK wheat agricultural holdings and annual percentage change from 1983 to 2016. Data was obtained through June Survey of Agriculture and Horticulture, England, DEFRA.

Plants are not only affected by agricultural inputs and manipulations but also greatly influenced by belowground microbiota: fungi, bacteria, archaea, nematodes, amongst others (Walker *et al.* 2003). These relationships (mutualism, parasitism and commensalism) are bidirectional with mutualistic relationships being at the forefront of research interests for their plant benefit from mineral mobilisation and phytoremediation (Pacwa-Płociniczak *et al.*, 2016; Sessitsch *et al.*, 2013; Vangronsveld *et al.*, 2009). Some examples have included bacteria containing the enzyme ACC_d (1-aminocyclopropane-1-carboxylate deaminase), which degrades 1-aminocyclopropane-1-carboxylate, the precursor of ethylene, a plant hormone that can reduce productivity if present in high concentrations (Glick, 2014). Plants containing these plant growth promoting

rhizobacteria (PGPR) in the rhizosphere have previously been shown to have increased tolerance and performance to a multitude of stressors such as heavy metals, flooding, drought, and pathogens (Glick, 2014). These bacteria alleviated detrimental effects compared with plants that did not have these bacteria or microbial communities and can have drastic implications for plant growth. For example, Zahir *et al.*, 2009 demonstrated that inoculation of wheat with *Pseudomonas putida* (N21) could increase grain yield up to 76% more than uninoculated controls under high salinity stress (Zahir *et al.*, 2009). However, most relationships are likely to be opportunistic commensalism, whereby microbiota benefit from passive C exuding from roots but have no direct effect on plant physiology.

Deterministic microbial assemblages in close association with plants play such an active role in plant health and performance they are sometimes referred to as the “Second genome” of plants (Berendsen *et al.*, 2012; Yan *et al.*, 2017). Such microbial assemblages are constantly altered based on the micro-organisms affinity for exuded carbon compounds (Cowan, 1999; Levine *et al.*, 2006; van der Putten *et al.*, 2013), in turn altering the microbial community composition surrounding the rhizosphere. The profound effect plants exert on soil microbes is illustrated by a 4-fold increase in activity seen in rhizospheric soils compared to bulk soils (Bartholomew & Clark, 1950) and their role as main active sites for microbial growth (Lynch & Audus, 2008). Therefore, it is clear that even (and perhaps especially) in agricultural environments these relationships need to be disentangled, particularly as there is potential for environmentally friendly agriculture strategies to form via improving NUE (Shaharoona & Naveed, 2008).

Increased C_{org} input into soil has been shown to influence rhizospheric communities by root exudates providing a convenient energy source for organisms with relevant metabolic abilities (Baudoin *et al.*, 2003; Marschner *et al.*, 2003). Such C inputs encourages the movement of these microbial assemblages from more stochastically driven communities - where microbial overturn is dominant community formation - to more deterministic ones dominated by competition and changing environmental conditions (Zhou & Ning, 2017). However, N-supplementation within agricultural environments shift the relationship between above and belowground processes in a complex manner (Donnell *et al.*, 2001). Nitrogen fertilisation generally increases photosynthetic rate due to upregulation of the key enzyme RuBisCO, a limiting factor at low CO_2 (Farquhar & Sharkey, 1982), and it can increase rates of protein, metal, and amino acid accumulation (Thomas & Thorne 1975; Evans 1983; Shi *et al.* 2010; Wieser and Seilmeier 1997; Gunthardt and McGinnis 1957). This in turn may affect the quality (composition) and increase quantity (concentration) of root exudates reaching the rhizosphere through passive diffusion gradients (Canarini *et al.*, 2019). These changes to rhizosphere resources can alter the surrounding microbial communities as the differences in exudate compound profiles may preferentially benefit particular microbial communities, which are highly species and variety specific (Fan *et al.*, 2001).

Previously, large variations in rhizosphere metabolites between tetraploid wheats have been observed (Iannucci *et al.*, 2017), with sugars changing depending on soil type and organic acids largely remaining similar. Furthermore, *T. aestivum* winter wheat cultivars are known to alter the microbial assemblage and were found to consistently alter 24 of the 962 bacterial OTUs found in 95% of rhizosphere samples (Mahoney *et al.*, 2017), which was found across all cultivars. In addition to the work done on Wheat, differing

microbial community structures were found in wild and cultivated barley accessions (Bulgarelli et al., 2015), where Comamonadaceae, Flavobacteriaceae and Rhizobiaceae bacterial families dominated the rhizosphere communities, and Eukaryotes accounted for a minor proportion (Bulgarelli et al., 2015). Additionally, root exudates of different grass genus' can have dramatically different effects, where barley and wheat root exudates had no effect on faba bean N₂ fixation, yet maize exudates did (Li et al., 2016).

Therefore, not only should differing microbial communities be present in the rhizosphere when compared to bulk soil, these communities should be distinguishable by species. As root exudation rates can largely be dependent on CO₂ assimilation rates, it would be intuitive to presume that higher assimilation rates lead to more distinct and unique rhizosphere communities through generating increased supply of organic compounds. As CO₂ assimilation is greatly affected by nitrogen fertilisation (Evans, 1989; Hikosaka, 2004; Seemann *et al.*, 1987; Sinclair & Horie, 1989), we would expect N fertilised plants to produce a more specific community at a younger stage.

To explore the relationships between below and aboveground processes alongside changing nitrogen application three crop plants, two Wheat species, a diploid species "Einkorn" (*T. monococcum*) and a tetraploid species "Emmer" (*T. turgidum subsp. dicoccoides*) (Figure 3.2) and a variety of Barley (*H. vulgare L. cv. Cassata*) "Cassata" were assessed in their response to a one-off application of mineral nitrogen at an agricultural concentration (16 mM NH₄⁺NO₃⁻). The aim of this chapter was to identify community structural differences between Emmer, Einkorn, and Barley (Cassata) for 34 (GS26) and 73 (GS29) day old plants with changing abiotic conditions and to assess the

suitability and variation of each species for future experiments. With key objectives being to establish a community fingerprint of each species' rhizosphere community, evaluate the physiological characteristics of each species, and to identify a suitable species with which to carry out future experiments.

We hypothesised that:

- 1) Einkorn would have a lower photosynthetic capacity and efficiency as compared with the more cultivated species
- 2) Einkorn would have a reduction in microbial community distinctiveness due to lower photosynthetic capacity and lower assimilation rate
- 3) Emmer, Einkorn and Barley microbial communities would be distinct from bulk soil and distinct from each other due to differences in unique species exudation profiles

3.2 Material and methods

3.2.1 Plant materials

T. monococcum and *T. turgidum* subsp. *dicoccoides* seeds were supplied by Prof. Debbie Sparkes from the University of Nottingham. Barley cv. “Cassata” seeds were obtained from Limagrain (Limagrain UK Ltd, Rothwell, England).

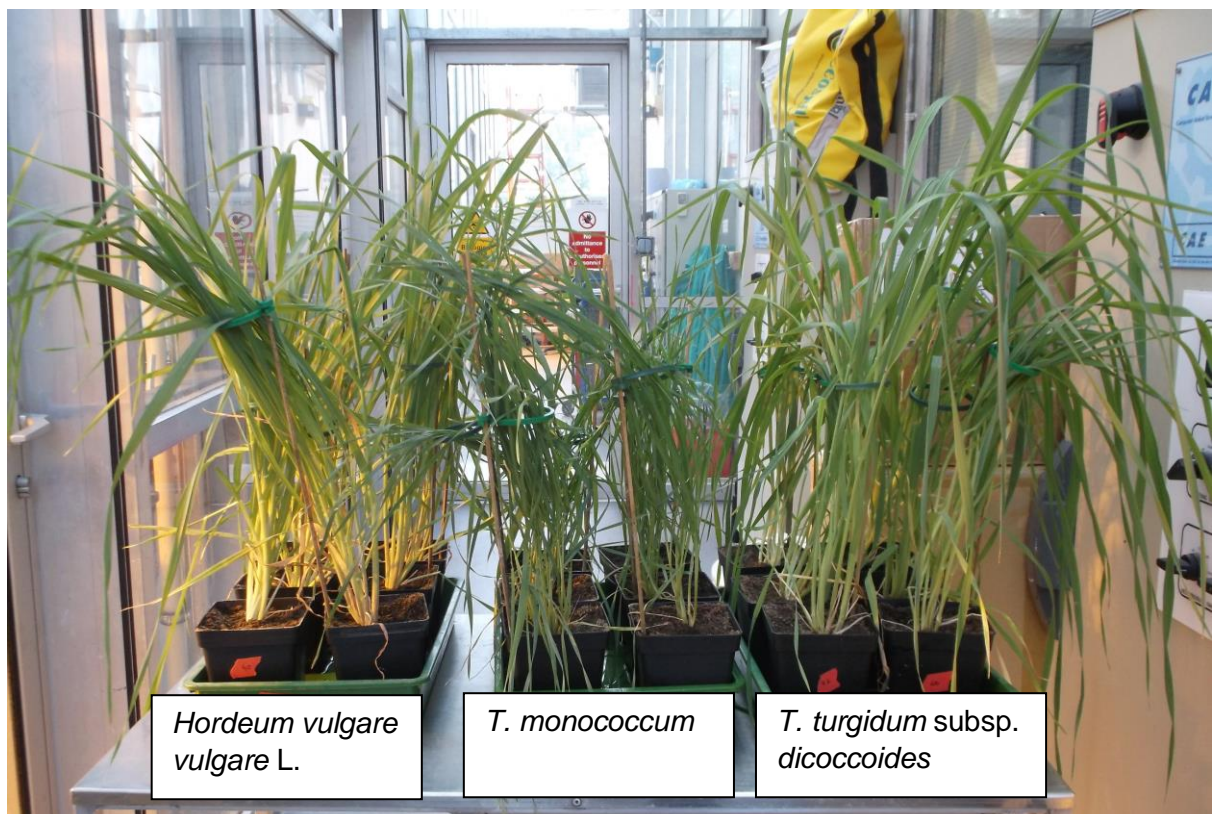


Figure 3.2: Representative image of the three used species Barley (*Hordeum vulgare* L.) cultivar Cassata (left), Einkorn (*Triticum monococcum*) Wheat (middle) and Emmer (*T. turgidum* subsp. *dicoccoides*) Wheat (right).

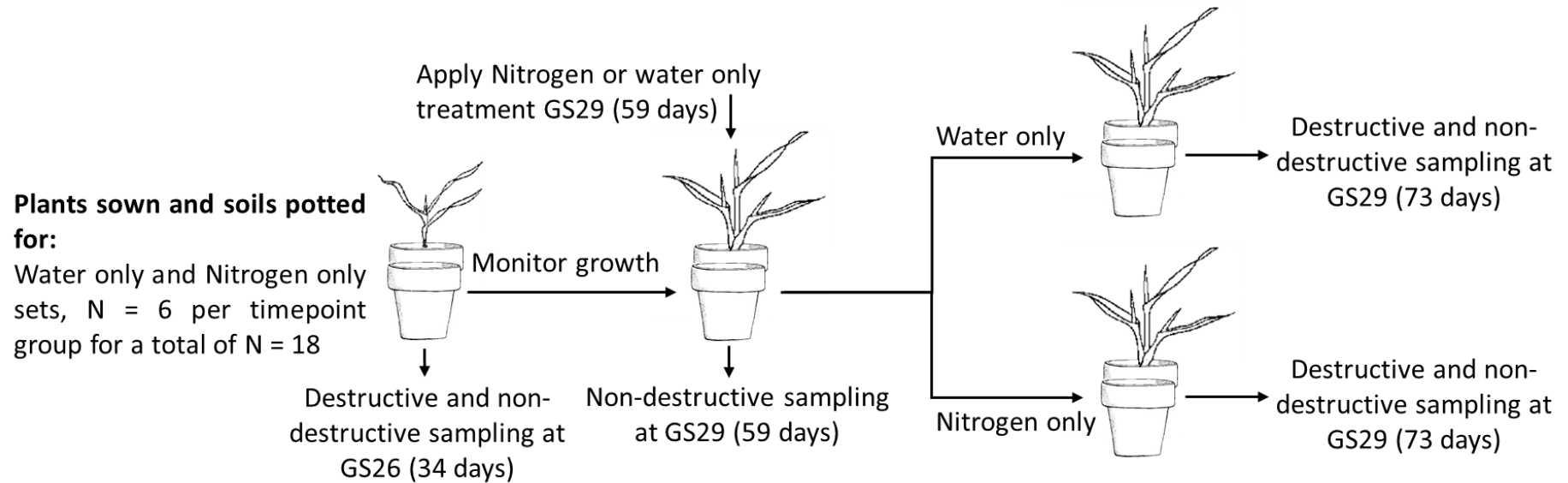


Figure 3.2: Overall experimental plan for the three used species Barley (*Hordeum vulgare* L.) cultivar Cassata, Einkorn (*Triticum monococcum*) Wheat, Emmer (*T. turgidum* subsp. *dicoccoides*), and soil controls.

3.2.2 Plant growth conditions

Plants were sown in Levington F2 + S peat loam-based compost (ICL Speciality Fertilizers, Waardenburg, Netherlands), which has pH range of 5.3-6.0, added nutrients: N – 144 mg L⁻¹, P (Phosphorus)– 73 mg L⁻¹, K (Potassium) – 239 mg L⁻¹. For vernalisation plants experienced a 3-week period of $100 \pm 34 \mu\text{mol m}^{-2} \text{s}^{-1}$ of light at 5 °C. Following vernalisation, the plants were moved to a growth room with $200 \pm 40 \mu\text{mol m}^{-2} \text{s}^{-1}$ of light and a temperature of 22 ± 1.7 °C. After 4-weeks at growth stage GS14 (defined as: no tiller present and 4 leaves in total), plants were re-potted into larger containers (still in Levington F2 + S) and moved to a glasshouse where light was $260 \pm 90 \mu\text{mol m}^{-2} \text{s}^{-1}$ and temperature 26.9 ± 2.3 °C, no measurements were taken for 48 hours after movement and re-potting. The youngest fully expanded leaves of plants were always measured, first at GS14 and then measured weekly for A/C_i and A/Q until the final measurements at GS29. All leaves of plants were measured through whole plant chlorophyll a fluorescence measurement from GS14 to GS29. All plants were well-watered throughout the experiment and maintained at an $80 \% \pm 10 \%$ soil water content. Nitrogen treatment, applied at GS29, consisted of applying a 16.16 mM solution to plant pots to provide 25.9 mg N per pot, equivalent to UK agricultural levels, 184.8 kgN ha⁻¹ (Defra, 2012).

3.2.3 Chlorophyll fluorescence imaging

Chlorophyll fluorescence measurements were performed weekly on whole plants from GS14 to GS29 plants using a CF FluorImager imaging system (Technologica Ltd., Colchester, UK). When too large to stand upright in the FluorImager plants were placed on a metal grid with nylon wiring to keep all leaves within the focal plane. Care was taken to minimise overlapping of leaves.

F_q' ($F_m' - F'$ – variable fluorescence in the light) was calculated from the difference between F' (steady state fluorescence of light adapted leaves) and F_m' (maximum fluorescence following a saturating pulse ($5,200 \mu\text{mol m}^{-2} \text{s}^{-1}$) of light). F_o' (minimum fluorescence signal when all centres open) was calculated through the following calculation (Oxborough and Baker 1997):

$$F_o' = \frac{F_o}{\frac{F_v}{F_m} + \frac{F_o}{F_m'}}$$

F_v' (the variable fluorescence of light adapted leaves) was calculated as the difference between F_o' and F_m' , (operating efficiency of PSII, a calculated estimate of how much light is used in PSII photochemistry), images were processed using the editing tools of the FluorImager computer programme (Technologica Ltd., Colchester, UK).

The maximum quantum yield efficiency of PSII photochemistry (F_v/F_m) was determined following a 30 min period of dark adaptation after which minimal fluorescence (F_o) was measured, followed by a saturating pulse of $5,200 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 800 ms to capture maximum fluorescence (F_m) and used to determine the maximum quantum efficiency of PSII photochemistry ($F_v/F_m = (F_m - F_o)/F_m$). After which actinic light was supplied at $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ and the plant/leaf allowed to adapt for 20 min. Following stabilization PSII operating efficiency (F_q'/F_m') was measured from $(F_m' - F)/F_m'$ in which F_m' is the maximum fluorescence in the light (measured after a saturating pulse of light as described above and F' steady state fluorescence in the light adapted state. F_q'/F_m' provides a measure of the efficiency of absorbed light being associated with stable photochemistry). Measure of the maximum operating efficiency in light adapted leaves

(F_v'/F_m') , which provided an indication of the dissipation of excess excitation energy by non-photochemical processes was determined from $(F_m' - F_o') / F_m'$, in which F_o' represents minimal fluorescence in the light adapted state and is calculated based on the equation by Oxborough & Baker (1997).

3.2.3.1 Light curve

A stepwise change in light from $1500 - 0 \mu\text{mol m}^{-2} \text{s}^{-1}$ in 11 steps was applied to plants with light adapted measurements being determined by saturating pulses ($5,200 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 800ms at 2-min intervals). A/Q curves were maintained at $400 \mu\text{mol mol}^{-1} \text{CO}_2$ for the duration of curves. Measurements of A were initially taken at $2,000 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD (Photosynthetic Photon Flux Density) and then decreased stepwise to $0 \mu\text{mol m}^{-2} \text{s}^{-1}$ with each curve consisting of at least 12 separate measurements. Measurements of assimilation rate in both the A/C_i and A/Q curves were made only during steady-state, which was observed for 30 seconds before measurements were taken with a minimum waiting period of 3 min and a maximum waiting period of 5 min before automatically taking measurements.

3.2.4 Gas exchange measurements

A/C_i and A/Q response curves were created using a portable gas exchange system (LI-COR 6400xt; LI-COR, Lincoln, NE). Leaf temperatures were maintained at 25°C and humidity of the chambers were kept $\sim 60\%$. Leaves were illuminated with a red-blue light source at $2,000 \mu\text{mol m}^{-2} \text{s}^{-1}$ for the duration of A/C_i curves. Measurements of photosynthetic assimilation (A) were first taken at $400 \mu\text{mol mol}^{-1} [\text{CO}_2]$, after which $[\text{CO}_2]$ was decreased in a step wise manner to $50 \mu\text{mol m}^{-2} \text{s}^{-1}$, and then returned to $400 \mu\text{mol m}^{-2} \text{s}^{-1}$, before being increased to $1400 \mu\text{mol m}^{-2} \text{s}^{-1}$ in a step wise approach.

Each complete curve consisted of at least 12 separate measurements.

3.2.5 Soil sampling

Triplicate soil cores were taken using sterile universal tubes which were then used to measure pH, nutrients, and soil water content. Then the plant was removed from the pot and a section alongside roots was selected for the rhizosphere soil sample. Here we included only metabolically active roots, which were assessed as being cream or white in colouration as opposed to dark brown or black roots deemed metabolically inactive.

3.2.7 DNA extraction and analysis by DGGE

3.2.7.1 DNA extraction

DNA was extracted from soils as previously described (Griffiths *et al.*, 2000), with the following modifications: centrifugation at 13,000 x *g* for 5 min at room temperature and addition of 40% (w/v) polyethylene glycol 6000. PCR amplification of the 16S rRNA gene was performed using the PCR primers and cycling conditions as previously described (Muyzer *et al.*, 1993).

3.2.7.2 DGGE analysis

DGGE analysis of the 16S rRNA PCR products was performed using an acrylamide gel with a 40 - 60% denaturant (v/v) gradient (with 100% denaturant defined as 40% (v/v) formamide and 7M urea). Gels were run for 14 hours at 60 V and 64 °C, then silver stained as previously described by (Nicol *et al.*, 2005).

3.2.8 Statistical analysis

All statistical analysis was performed in statistical programming R v.3.2.3 (R. Core Team, 2015). All data were checked for distribution through Shapiro-Wilk tests and visually through the production of histograms and whisker boxplots (not shown). Once checked the analysis continued using either ANOVA, t-test or Wards linkage hierarchical cluster analysis depending on the situation, with equivalent changes to non-parametric tests where necessary.

3.3 Results

3.3.1 Chlorophyll fluorescence

F_v/F_m , F_q'/F_m' , F_v'/F_m' , F_q'/F_v' and $F_m'/F_m'-1$ parameters for plants at growth stage GS14 are shown in Figure 3.3 GS24 (data not shown) and GS29 Figure 3.4. No significant differences in fluorescence parameters were found before GS29; we display GS24 data (Figure 3.3 A-D) as representative data before any significant differences were found post GS29 (Figure 3.4).

At GS29 (pre-treatment) both control and treated Einkorn maintained a F_v/F_m value of 0.822 (Figure 3.4A) whilst treated and control Cassata and Emmer had significantly lowered values of 0.805 and 0.792 respectively ($F_{(5,5)} = 7.710$, $P < 0.05$), with N-treatment for Cassata and Emmer closing the difference between Einkorn F_v/F_m but remaining significant $P < 0.05$ (Figure 3.4A). Conversely N-treatment decreased these values for Einkorn (Figure 3.4A) in a non-significant manner ($p = 0.82$).

F_q'/F_m' was significantly greater in control Einkorn compared to Cassata ($F_{(5,5)} = 7.710$, $P < 0.05$) for PPFD $> 500 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Figure 3.4A). Yet, these observed differences disappeared with N-treatment (Figure 3.4A). Similarly, F_v'/F_m' values in both treated and untreated Einkorn was significantly higher ($P < 0.05$) than Cassata and Emmer (Figure 3.4B), however N-treatment increased Cassata F_v'/F_m' making it insignificantly different from Einkorn ($P > 0.05$).

No significant differences were found in any of the F_q'/F_v' values (Figure 3.4C) however, NPQ in Emmer was significantly higher ($P < 0.05$) than both Cassata and Einkorn regardless of treatment (Figure 3.4D), although treatment did affect the significance between Einkorn and Cassata with N-treatment increasing NPQ in Einkorn from 0.988 to 1.126 at $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD, making it insignificant between Cassata's 1.193 and 1.187 for control and treated respectively at light levels $\geq 50 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD (Figure 3.4D).

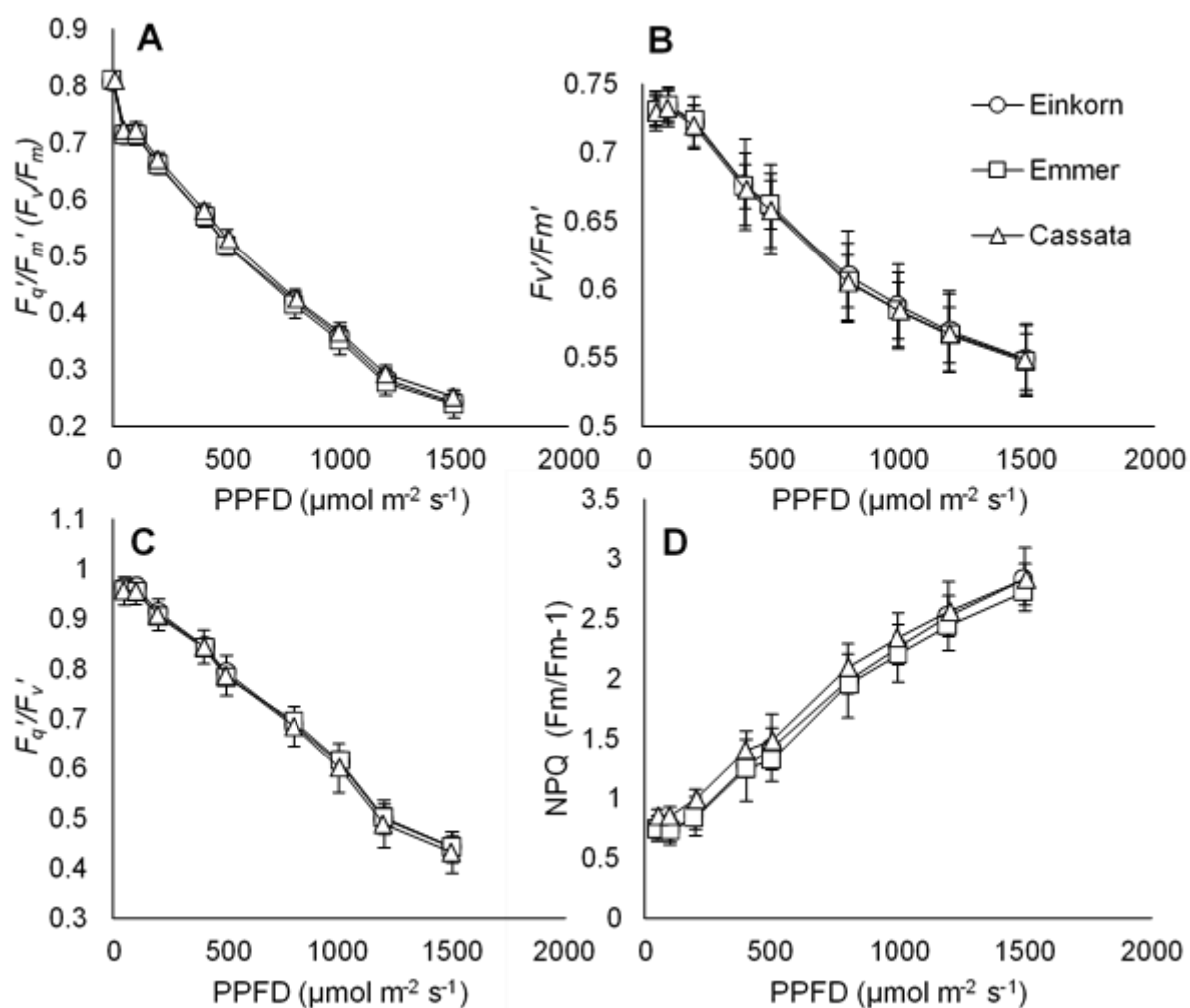


Figure 3.3: Whole plant **A)** PSII maximum efficiency (F_v/F_m) and PSII operating efficiency (F_q'/F_m') fluorescence values. The first values (F_v/F_m) were taken after a 30 m dark adaption period during which the plant received no actinic light. **B)** PSII maximum operating efficiency (F_v'/F_m') fluorescence values. **C)** qP or Photochemical quenching (F_q'/F_v') fluorescence values. **D)** NPQ or non-photochemical quenching ($F_m'/F_m'-1$) fluorescence values. All as functions of PPFD for pre-treatment Einkorn and Emmer Wheat and Barley cv. Cassata grown in environment-controlled growth room receiving $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD at GS24, 29 d. Error bars represent \pm SD, * denotes significance at $P < 0.05$, $n = 6$

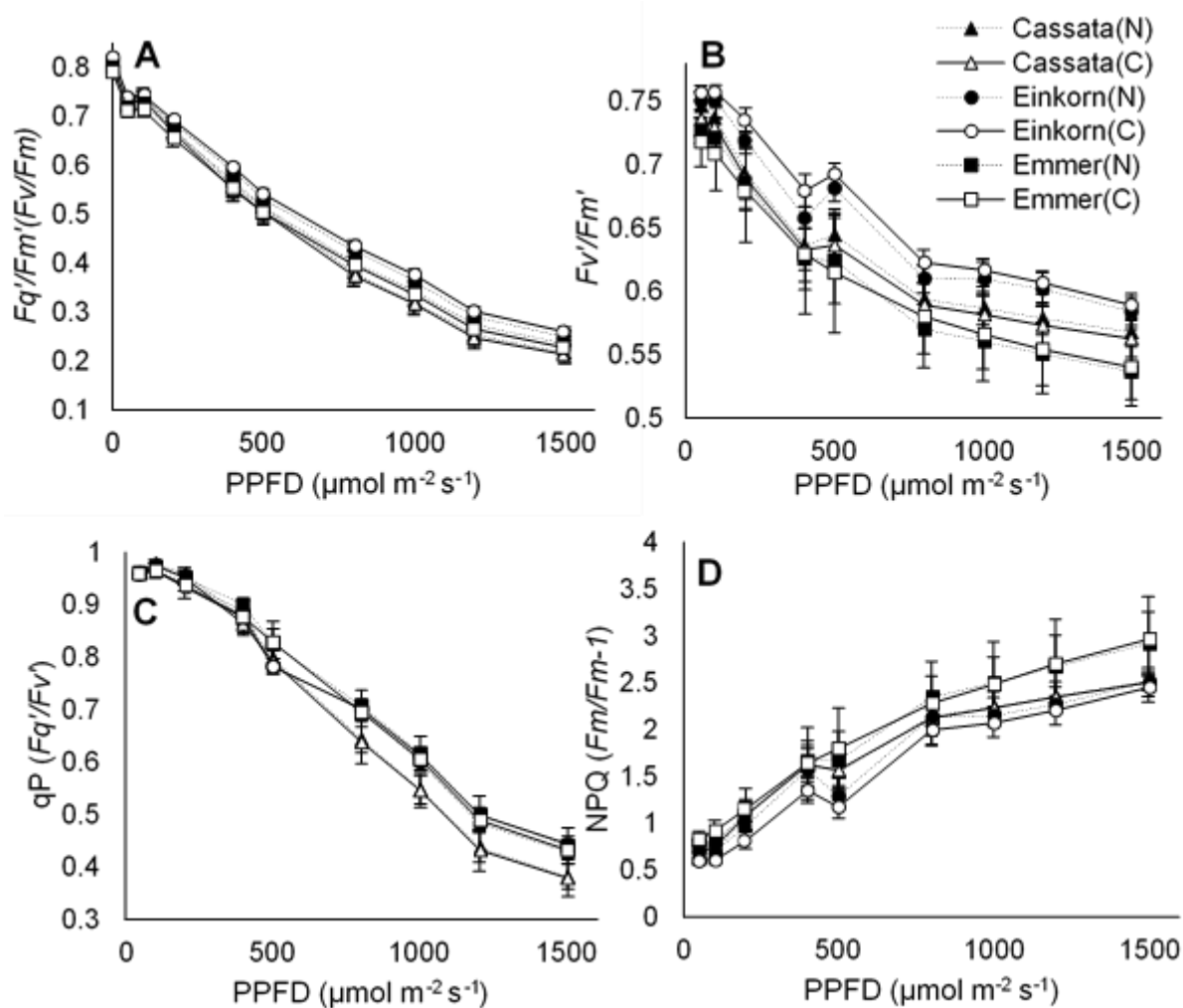


Figure 3.4: Whole plant **A)** PSII maximum efficiency (F_v/F_m) and PSII operating efficiency (F_q'/F_m') fluorescence values as a function of PPFD. **B)** PSII maximum efficiency (F_v'/F_m') fluorescence values. **C)** qP or photochemical quenching (F_q'/F_v') fluorescence values. **D)** NPQ or non-photochemical quenching ($F_m'/F_m'-1$) fluorescence values. All as a function of PPFD for control and N-treated Einkorn and Emmer Wheat and Barley cv. Cassata grown in environment-controlled greenhouse at 73 d old. Hollow markers are control plants (C), filled in markers are treated plants (N) with 16 mM of $\text{NH}_4^+\text{NO}_3^-$ applied at GS29 (59 d). The first values (F_v/F_m) were taken after a 30 m dark adaption period during which the plant received no actinic light. Error bars represent \pm SD, * denotes significance at $P < 0.05$, $n = 6$

3.3.2 Gas exchange (A/C_i and A/Q)

Photosynthetic CO_2 assimilation (**A**) as a function of PPFD (**Q**) and internal $[\text{CO}_2]$ (**C_i**) were used to determine photosynthetic capacity at GS14 (Figure 3.5), GS26 (Figure 3.6) and GS29 (Figure 3.7). A/C_i curves being assessed by determining J_{max} and V_{cmax} values in Figure 8. At GS14 the A/Q curves showed no significances ($P > 0.05$) for all A_{sat} between Emmer (mean \pm SD, $21.3\text{-}30.3 \pm 2.0\text{-}3.2$), Cassata ($19\text{-}24 \pm 0.98\text{-}0.73$), and Einkorn ($17.7\text{-}24.0 \pm 1.75\text{-}1.75$) (Figure 3.5A). Einkorn had significantly higher A at PPFD $> 200 \mu\text{mol m}^{-2} \text{s}^{-1}$ ($F_{(2,2)} = 51.16$, $P < 0.05$) than Emmer or Cassata which were statistically similar (Figure 3.6A).

In N-treated Einkorn A_{sat} became significantly higher ($P < 0.05$) than all treatments except from control Einkorn at PPFD $> 150 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Figure 3.7A). At PPFD $> 500 \mu\text{mol m}^{-2} \text{s}^{-1}$ N-treated Einkorn became statistically ($F_{(5,5)} = 5.86$, $P < 0.05$) distinct from control Einkorn (Figure 3.7A). And although N-treated Emmer had a higher A ($16.7 \mu\text{mol m}^{-2} \text{s}^{-1}$) than control Emmer ($13.2 \mu\text{mol m}^{-2} \text{s}^{-1}$) at light levels above $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD it was not significant ($P > 0.05$).

At GS14 (19 d) Einkorn had a significantly higher J_{max} than Cassata ($t(4) = -3.05$, $p = 0.03$). No significant increases in V_{cmax} were found at GS26 (34 d) yet at GS29 (73 d) significantly higher V_{cmax} was observed for N-treated Cassata ($t(4) = 4.12$, $p = 0.04$) and N-treated Emmer ($t(4) = 6.29$, $p = 0.02$) when compared to their controls, but this was not the case for Einkorn ($t(4) = 0.05$, $p = 0.96$) (Figure 3.8C).

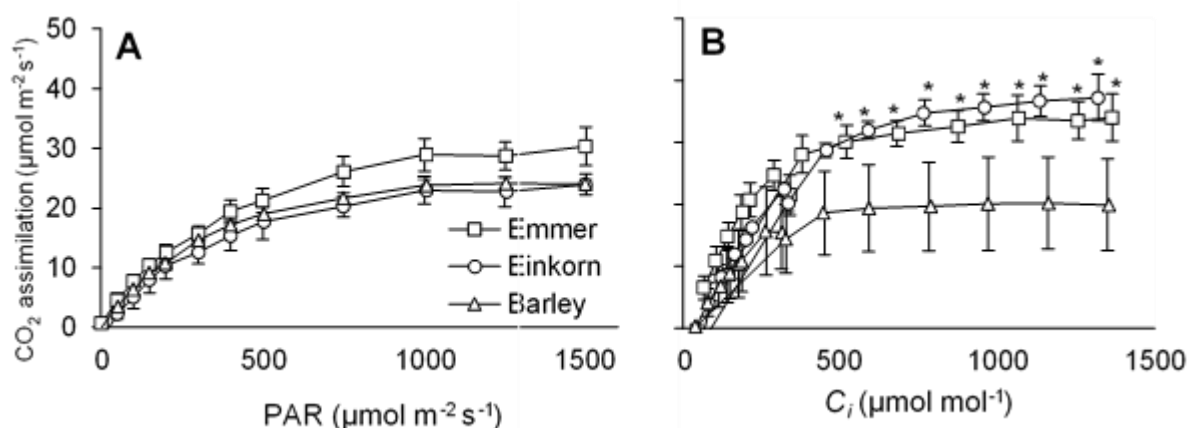


Figure 3.5: A) Photosynthetic carbon fixation as a function of increasing internal leaf PPFD (PAR) intensity at ambient CO_2 concentrations ($400 \text{ mmol m}^{-2} \text{ s}^{-1}$) and **B)** as a function of increasing CO_2 concentration at saturating light levels ($1500 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$) both for the youngest fully expanded leaves of pre-treatment GS14 stage (19d old) Emmer and Einkorn Wheat and Barley cv. Cassata plants grown in a controlled-environment growth room receiving $200 \pm 30 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ of light. Error bars represent \pm SD, * denotes significance, $P < 0.05$, $n = 3$

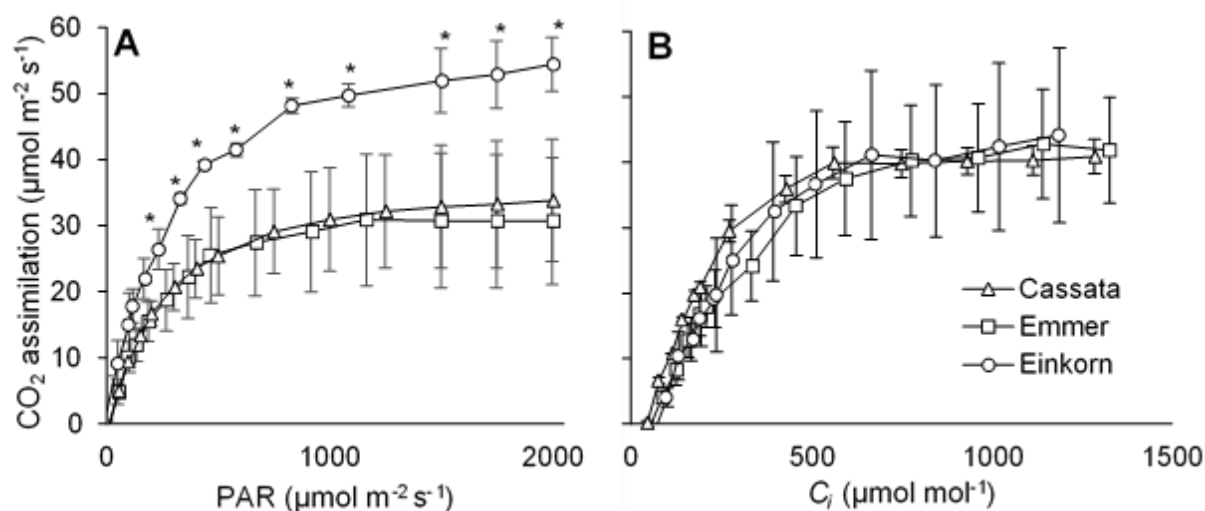


Figure 3.6: A) Photosynthetic carbon fixation as a function of increasing internal leaf PPFD (PAR_i) intensity at ambient CO_2 concentrations ($400 \text{ mmol m}^{-2} \text{ s}^{-1}$) and **B)** as a function of increasing CO_2 concentration at saturating light levels ($2000 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$) both for the youngest fully expanded leaves of pre-treatment GS26 stage (34 d old) Emmer and Einkorn Wheat and Barley cv. Cassata plants grown in a controlled-environment greenhouse receiving $290 \pm 70 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ of light. Error bars represent \pm SD, * denotes significance, $P < 0.05$, $n = 3$

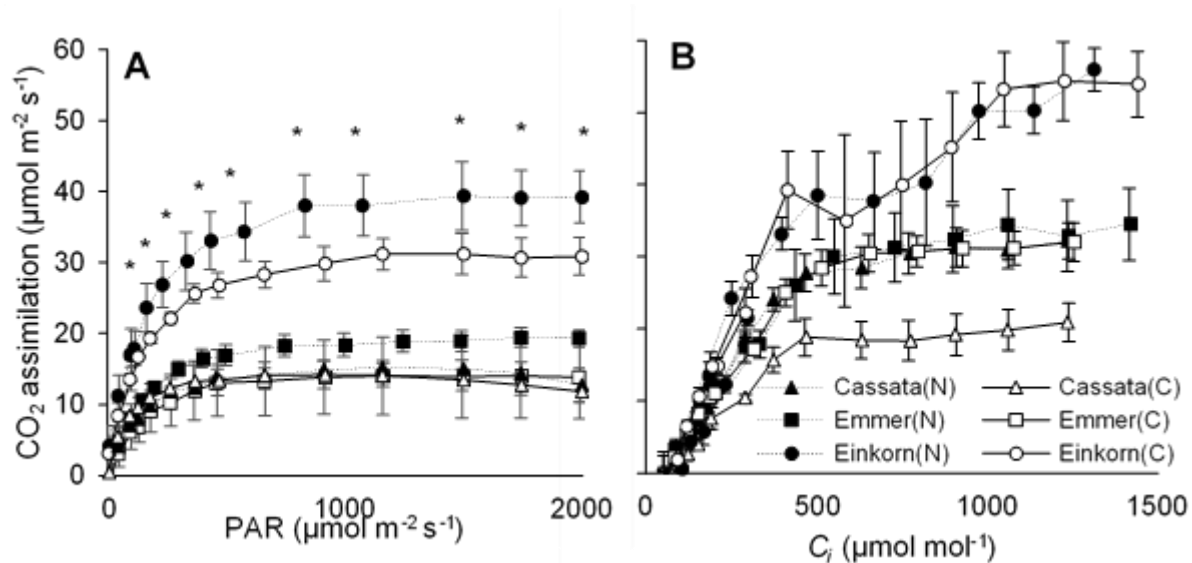


Figure 3.7: A) Photosynthetic carbon fixation as a function of increasing internal leaf PPFD (PAR_i) at ambient CO₂ concentrations (400 mmol m⁻² s⁻¹) and **B)** as a function of increasing CO₂ concentration at saturating light levels (2000 μmol m⁻² s⁻¹) both for the youngest fully expanded leaves on control and N-treated Einkorn and Emmer Wheat and Barley *cv.* Cassata grown in environment-controlled greenhouse at GS29, 73 d old. Hollow markers are control plants (C), filled in markers are treated plants (N) with 16 mM of NH₄⁺NO₃⁻ applied at GS29 (59 d). Error bars represent ± SE, * denotes significance at $P < 0.05$, $n = 6$

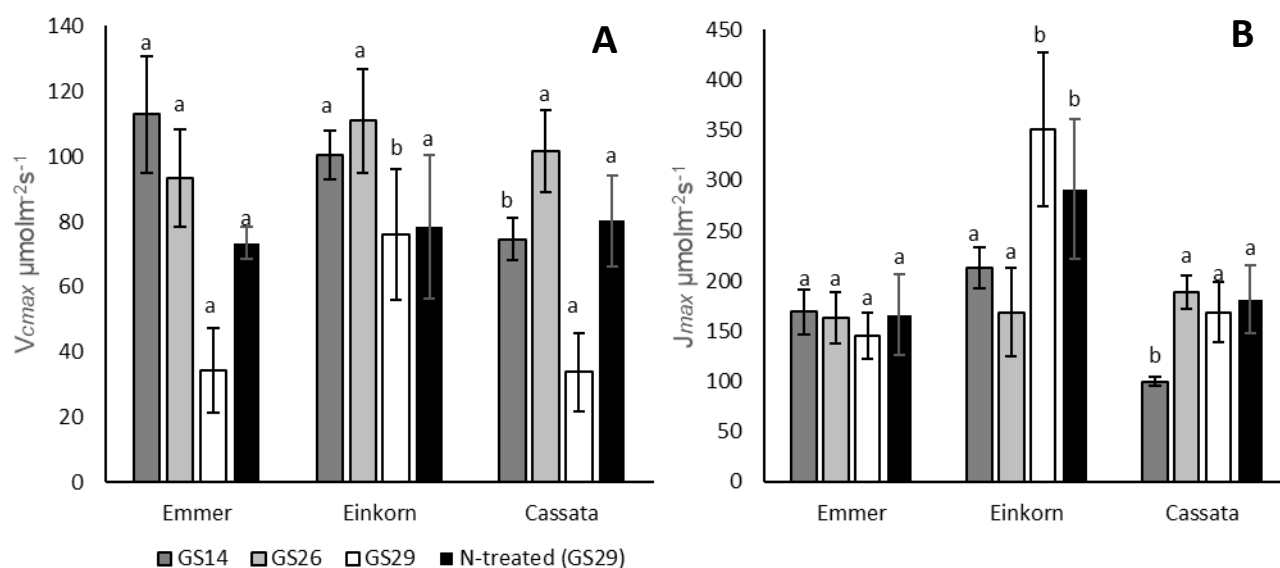


Figure 3.8: A) J_{max} and B) V_{cmax} for GS14 (19 d) GS26, (34 d), GS29 (73 d), and N-treated GS29 (73 d) for the youngest fully expanded leaves on control and N-treated Einkorn and Emmer Wheat and Barley cv. Cassata grown in an environment-controlled greenhouse. N-treated plants were treated with 16 mM of $\text{NH}_4^+\text{NO}_3^-$ applied at GS29 (59 d). Error bars represent \pm s.e.m, different lowercase numbers denote significant difference between species within each timepoint, $P < 0.05$, $n = 6$

3.3.3 Phenotypic measurements

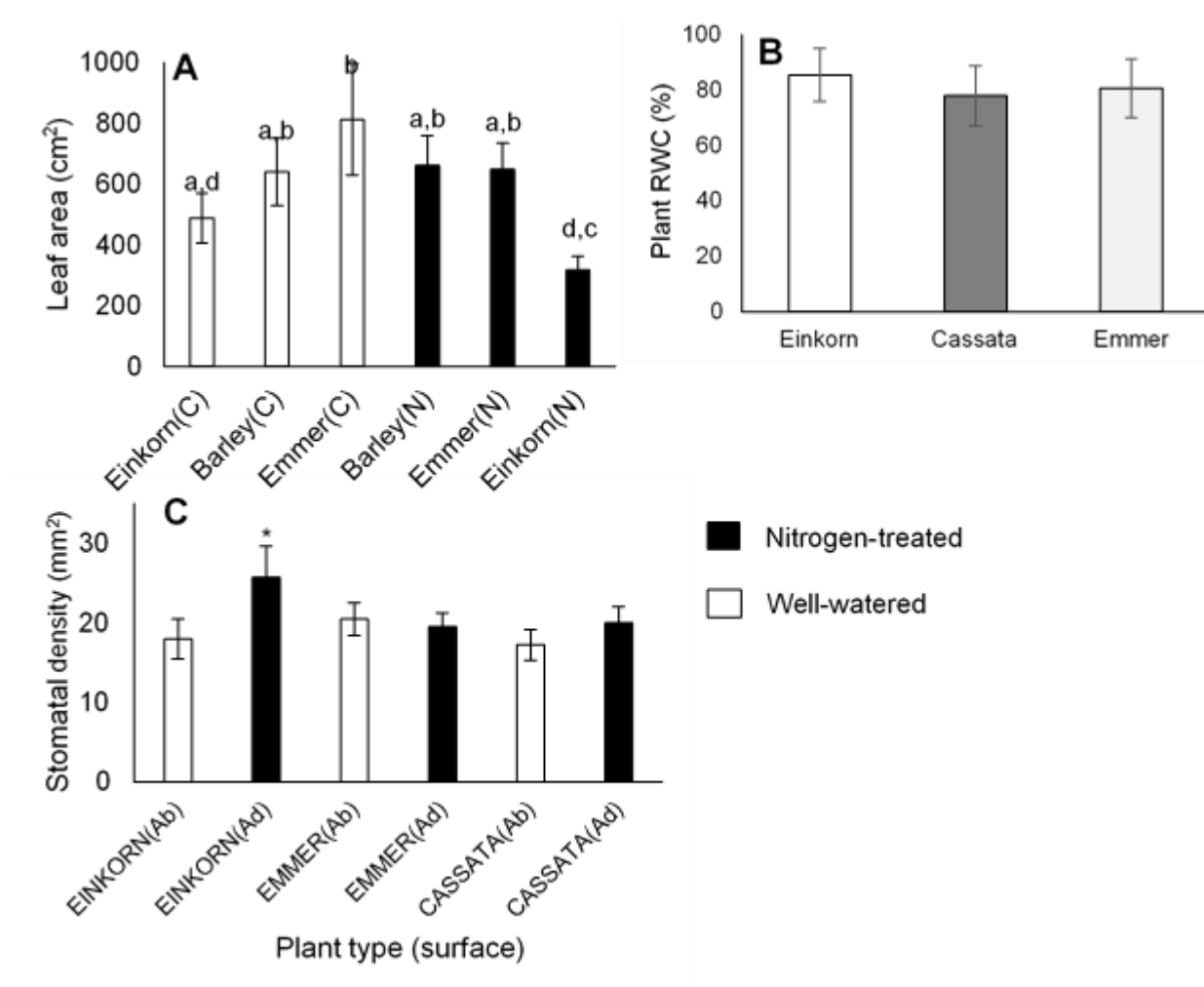


Figure 3.9: **A)** Leaf area **B)** Soil water content for GS26 (34 d) and **C)** Stomatal densities for the youngest fully expanded leaves on control and N-treated Einkorn and Emmer Wheat and Barley cv. Cassata grown in environment-controlled greenhouse at GS29 (73 d old). (Ad)/(Ab) = adaxial/abaxial. Hollow bars are control plants (C), filled in bars are treated plants (N) with 16 mM of $\text{NH}_4^+\text{NO}_3^-$ applied at GS29 (59 d). Error bars represent \pm SD, different lowercase numbers or * denote significance at $P < 0.05$, $n = 6$

Harvested aboveground biomass was used for area analysis, weighed for RWC and impressions were taken and counted for stomatal densities. Both control Einkorn had a smaller leaf area when compared to control Emmer ($F_{(5,5)} = 12.12$, $P < 0.05$) yet it remained insignificant from other plant groups (Figure 3.9A). However, the smallest area was found in N-treated Einkorn which was statistically distinct from all but control Einkorn plants ($F_{(5,5)} = 12.12$, $P < 0.05$). No significant differences were found between any of the

relative water content samples. Furthermore, only Einkorn showed a statistically higher stomatal density on the adaxial side of the plant leaves (Figure 3.9C).

3.3.3 Denaturing gradient gel electrophoresis results

Overall, three distinct groups can be seen in the multidimensional scaling (MDS) plot, with bulk soils, rhizospheric soils, and no plant control soils tightly clustering in the multidimensional scaling (Figure 3.10). In general, the bulk soil and control soil groups showed a much looser grouping than the rhizosphere soils (Figure 3.10), with the latter showing a high density of extremely similar community profiles, largely overlapping. Nitrogen seemed to have a grouping effect on rhizosphere samples, separate from the grouping present by being rhizosphere samples alone (Figure 3.10). Similarly, N-treated bulk soils showed closer similarity to rhizosphere and N-treated rhizosphere samples than they were to either bulk soils or soil controls, yet Cassata and Emmer samples were closer to each other than N-treated Einkorn bulk soils (Figure 3.10). Additionally, an effect of time can be seen with older 73 d old samples grouping more tightly than 34 d samples. Interestingly, Einkorn rhizosphere soils at 34 d were indistinguishable from bulk soils at similar (34 d) and older ages (73 d), whilst Emmer rhizosphere soils (34 d) showed a mediocre response, falling between the bulk and rhizosphere soil groups, and Cassata rhizosphere soils at 34 d were highly grouped to other 73d rhizosphere soils (Figure 3.10).

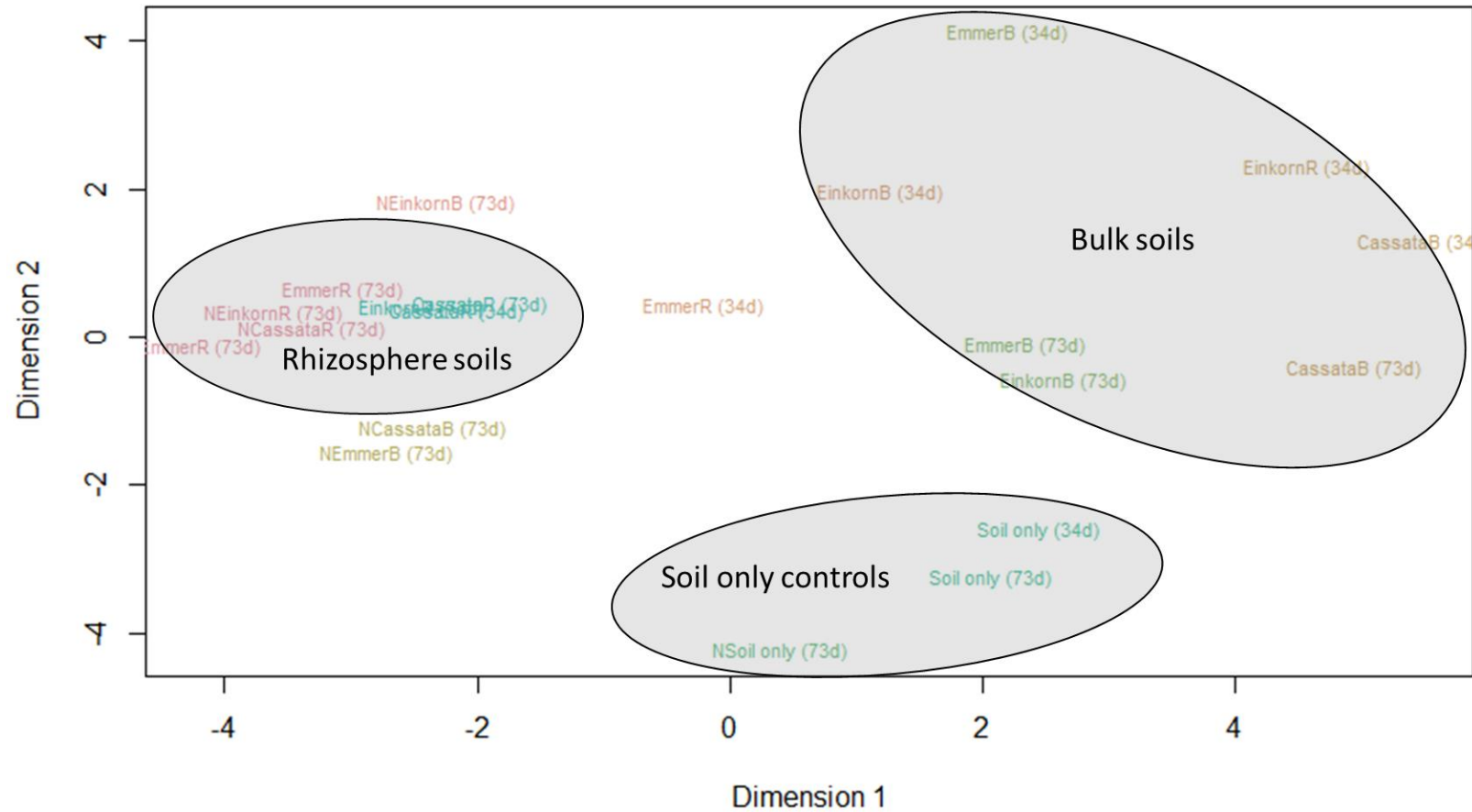


Figure 3.10: Multidimensional scaling plot illustrating the general grouping of rhizosphere (R), bulk (B) and soil only control soil community averages analysed via DGGE bands for of bacteria 16S rRNA amplified soil DNA samples (F2+S Levington) from both nitrogen-treated (N) and water-only control bulk (B) and rhizospheric (R) soils of Cassata, Emmer, Einkorn plants, and respective no plant controls at GS26 (34 d) and GS29 (73 d). Where ellipses represent general groupings.

An ANOSIM was performed and an R-value of 0.789 was observed ($p < 0.001$), suggesting the groups were quite dissimilar, supported by a PERMANOVA showing a $F_{(2,20)} = 6.7811$, $p < 0.001$. Most groups were statistically distinct from one another as determined by Dunn-tests through ANOVAs ($p < 0.05$), with only N-treated rhizosphere soils and untreated rhizosphere soils remaining indistinguishable from one another ($p > 0.05$), with little difference between 34 d and 73 d samples ($p > 0.05$) in each group (Figure 3.10). No overall effects were seen for plant type ($p > 0.05$), with exception to rhizosphere Einkorn 34 d, which showed significant difference ($p < 0.005$) from the other rhizosphere species.

3.4 Discussion

Plants have a highly conserved F_v/F_m value of 0.83 and only under extreme stresses do these values decrease (Bjorkman & Demmig, 1987). The partial alleviation from decreased GS29 F_v/F_m for Emmer and Cassata after N-application implies nitrogen limitation caused the observed F_v/F_m decreases. However, this cannot be fully concluded from our data. Previous studies have noted no difference in F_v/F_m with low and high N-treatments unless another stressor was applied simultaneously to low-N groups (Seemann *et al.*, 1987; Shangguan *et al.*, 2000). However, Huang *et al.*, 2004 withheld nitrogen in rice (*Oryza sativa* L. subsp. *indica* cv Zhengnong 952) and found differences in F_v/F_m from c. 0.83 to c. 0.70 and c. 0.64 for 15 and 20 days no nitrogen respectively (Huang *et al.*, 2004), which corroborates our F_v/F_m observations. Furthermore, it has been noted by several previous studies that low N experiencing plants

Increases A_{sat} in *T. monococcum* compared to Emmer and Cassata $> 200 \mu\text{mol m}^{-2} \text{s}^{-1}$ suggest that per unit area Einkorn is more photosynthetically efficient. However, given that Emmer and Barley are physically much larger than Einkorn, it is unlikely that a higher A rate materialises into an increased root exudation, as it has a diminished total assimilation compared to Emmer and Barley due smaller leaf surface areas. Previously, higher assimilations have been linked to higher leaf N content (Evans, 1983), with increases in leaf RuBP carboxylase, RuBisCO, and chlorophyll content being considered the main reasons (Seemann *et al.*, 1987). Amino acid and protein production elicit a higher energy cost than their soluble starch and carbohydrate counterparts (Munier - Jolain & Salon, 2005), which explain large declines in protein and carbohydrate levels at low N (Tamang *et al.*, 2017). Such declines in organics should lead to lower exudation rates and therefore a decreased community recruitment. This could explain why we did not observe distinct and specific community formation between the species.

Furthermore, all plants had a similar J_{max} (the ability to regenerate RuBP) values at saturating CO_2 where electron transport efficiency is limiting (Farquhar & Sharkey, 1982). J_{max} is strongly linked to V_{cmax} but not N, P, plant age, or specific leaf area (Sims *et al.*, 1998; Walker *et al.*, 2014). Therefore, as we were observing young plants experiencing N-addition, we did not expect to see differences in J_{max} values. Previous research agrees with our stunted V_{cmax} in non-N applied plants, such decreases in V_{cmax} limit CO_2 uptake at low concentrations and are strongly linked to leaf N content (Farquhar & Sharkey, 1982). These decreases then hinder potential C concentration in the rhizosphere, via limiting total CO_2 assimilation, the total accumulation of secondary metabolites within plant cells may decrease. The lowered secondary metabolite concentration then

automatically hinders the diffusion of smaller LMWCs across plant root membranes by stunting the concentration gradient available.

In the present study, whilst the groupings of bacterial communities tended to be specific between rhizospheric and bulk soil, they did not show a great plant specific response (Figure 3.10). This either highlights similarities in bacterial recruitment between the species or a lack of choice from a stunted microbial community through using Levington F2 + S. Whilst N-application had a separation effect between respective controls it was not a pronounced difference (Figure 3.10). The lack of separation between N-treated plants and respective controls suggests that N-application (and therefore increased A_{sat}) had little effect microbial community intensity or profile over 15 days. The indistinguishable communities between young plants and their bulk soil suggests: 1) small influence over the rhizosphere community, 2) little time to alter the community profile significantly, 3) insufficient influence range from the rhizoplane for differences to be detected, or 4) the sensitivity of techniques used (DGGE) were not able to detect such differences.

However, the similar microbial communities we observed could also be due to phylogenetic similarity between Einkorn, Emmer and Barely. Previous research has demonstrated larger differences in root exudation profiles with less phylogenetically similarity between species (Iannucci *et al.*, 2017) and it stands in direct contradiction to our lack of findings with regards to microbial community distinction by plant species of similar ages (Bulgarelli *et al.*, 2015; Mahoney *et al.*, 2017). This suggests the case that either Levington F2 + S likely stunts the plants ability to generate deterministic microbial

communities, due to its production process and heightened nutrient status compared to general soil. Or, as suggested above, the DGGE technique used in this study was insufficient in sensitivity for detecting the small, but significant, effect root exudates have over the bacterial assemblages (Mahoney et al., 2017).

Our results also suggest that below a certain plant-size threshold it is difficult to distinguish specific communities from bulk soil unless particular care is taken in sampling the fine space around the roots. Naturally, this sampling is more difficult to perform on small plants with fine root systems than it is to perform on larger plants with thicker and more distinguishable roots, thereby making it more difficult to find results for these finer root systems. As a result, we will move to a larger grass species spring wheat *Triticum aestivum* cv Mulika to allow for a larger detection window. Additionally, our plants may have experienced a N-depletion period due to rapid growth. To avoid this, future experiments should be performed in larger pots that can sustain plant root growth throughout the experiment.

However, more in-depth analysis of microbial communities would be required to distinguish differences in recruitment under N-limitation, with N-starvation treatment running for longer than 20 days to observe a large decrease in photosynthetic potential and performance (Huang *et al.*, 2004; Tamang *et al.*, 2017). Also, the response of microbial communities to root exudation would require quantification, along with analysis of soil total carbon, soil respiration and N₂O potential would be needed to quantify a functional community shift. Further studies in this area should focus on N-depletion and repletion for the three species in this study in addition to the closely related *T. urartu*, *A.*

squarrossa and *T. aestivum*. This study evaluated microbial community responses of member of the *Triticeae* family to N – application at early growth stages. To the best of our knowledge this is the first study that investigates the selectiveness of several *Triticeae* members' rhizosphere communities to $\text{NH}_4^+\text{NO}_3^-$ application.

3.4.1 Conclusion

Based on DGGE analysis, microbial communities present in the rhizosphere differed from those of bulk soil present in the same soil with Emmer and Barley, but not with Einkorn. These differences were likely due to the influence plant size has over root exudate concentration and therefore rhizosphere community influence overall. The observed changes in microbial communities were not species specific but rather time sensitive, with samples collected later in the development period showing greater distinctions between the rhizosphere and bulk soils. Whether the responses seen are due to direct effects of carbon accumulation in the rhizosphere, or differentiated carbon partitioning remains unclear, but these data demonstrate that community distinctions from bulk soil may arise within 73 days for grasses of the *Triticeae* tribe, with no species-specific variation being detected.

Chapter 4: Responses of spring wheat *Triticum aestivum* cv. Mulika and associated rhizosphere community to applications of C: N fertiliser

4.1 Introduction

In Chapter 3 DGGE bands of three N-fertilised *Triticeae* family members rhizosphere microbiotas were analysed to determine whether a significant difference was discovered within *Triticeae* rhizospheric communities. We found little evidence to suggest large differences between the three species we tested and so chose to proceed with a modern cultivar of spring wheat, *Triticum aestivum* cv Mulika.

It is typically thought that relationships between plants and microorganisms are formed through root exudate seepage into the rhizosphere, increasing local SOC (Soil Organic Carbon) (Badri & Vivanco, 2009). This exudation is largely dependent on the plant's metabolic condition (*i.e.* stressed or non-stressed), producing different quantities and qualities of root exudates depending on their metabolic state. This in turn affects the microbial community assemblage in the rhizosphere. However, one major component of competition between plants and micro-organisms is mineralised N (NO_3^- and NH_4^+) in the soil.

Major processes in the nitrogen cycle directly relate to soil N turnover and the ability for plants to utilise soil N. The denitrification process, for example, utilises NO_3^- and when complete produces gaseous dinitrogen (N_2), yet denitrification may only partially complete, producing N_2O , a detrimental gas to long-term global sustainability (Firestone & Davidson, 1989; Mosier *et al.*, 1998; Ravishankara *et al.*, 2009; Weier *et al.*, 1993). On the other hand, nitrification converts NH_4^+ to NO_3^- , both of which are used by plants

(Cramer & Lewis, 1993; Hachiya & Sakakibara, 2016; Haynes & Goh, 1978; Recous *et al.*, 1998). The rate of each process is determined by a myriad of factors such as C-content, pH, temperature, and O₂ content (Barnard *et al.*, 2005). Many of these parameters are used to predict field productivity over a growing season, particularly soil C-content and N-content.

Soil C:N ratios have previously been linked to soil quality and microbial activity (Bengtsson *et al.*, 2003). Due to low organic influx and turnover, low C:N ratio soils may be influenced a greater extent by supplied organics from plant roots than high C:N ratio soils. Decline in C:N across agricultural land due to exacerbated soil respiration (Lal *et al.*, 2011) may lead to future losses in yields, increase field contribution to GHGs, or both. As microbial interactions with plants can be vital to soil health and plant productivity it is crucial to gain an understanding on how microbial processes are influenced by C:N ratios and how plant-microbial competition for N is affected under altering C:N conditions if we are to predict how these relationships may change in the future.

Therefore, the aim of this chapter was to investigate how relationship between *Triticum aestivum* cv. Mulika and its associated rhizospheric microbiota change when subjected to differing C:N ratio treatments. Our approach involved applying three levels of C:N fertiliser at C:N ratios found across UK soils, 32.58, 17.22, 9.37, using the high C:N application to 'suppress' root exudates as a sole carbon source. We monitored plant physiology through gas exchange, biomass and fluorescence whilst analysing the soil microbiota through Q-PCR and MiSeq. We hypothesised that low C:N ratio treatments would benefit plants by providing a widely bioavailable N source that would otherwise

limit plant growth, with high C:N ratios causing competition for bioavailable N through stimulation of copiotrophic activity due to high levels of labile C (glucose). We also hypothesised that low C:N ratios would stimulate an increase in autotrophic organisms such as denitrifiers involved in the N-cycle compared to the high C:N treatments that would stimulate a relative increase in heterotrophic organisms that utilise carbon as their primary energy source.

4.1.1 Aims and Objectives

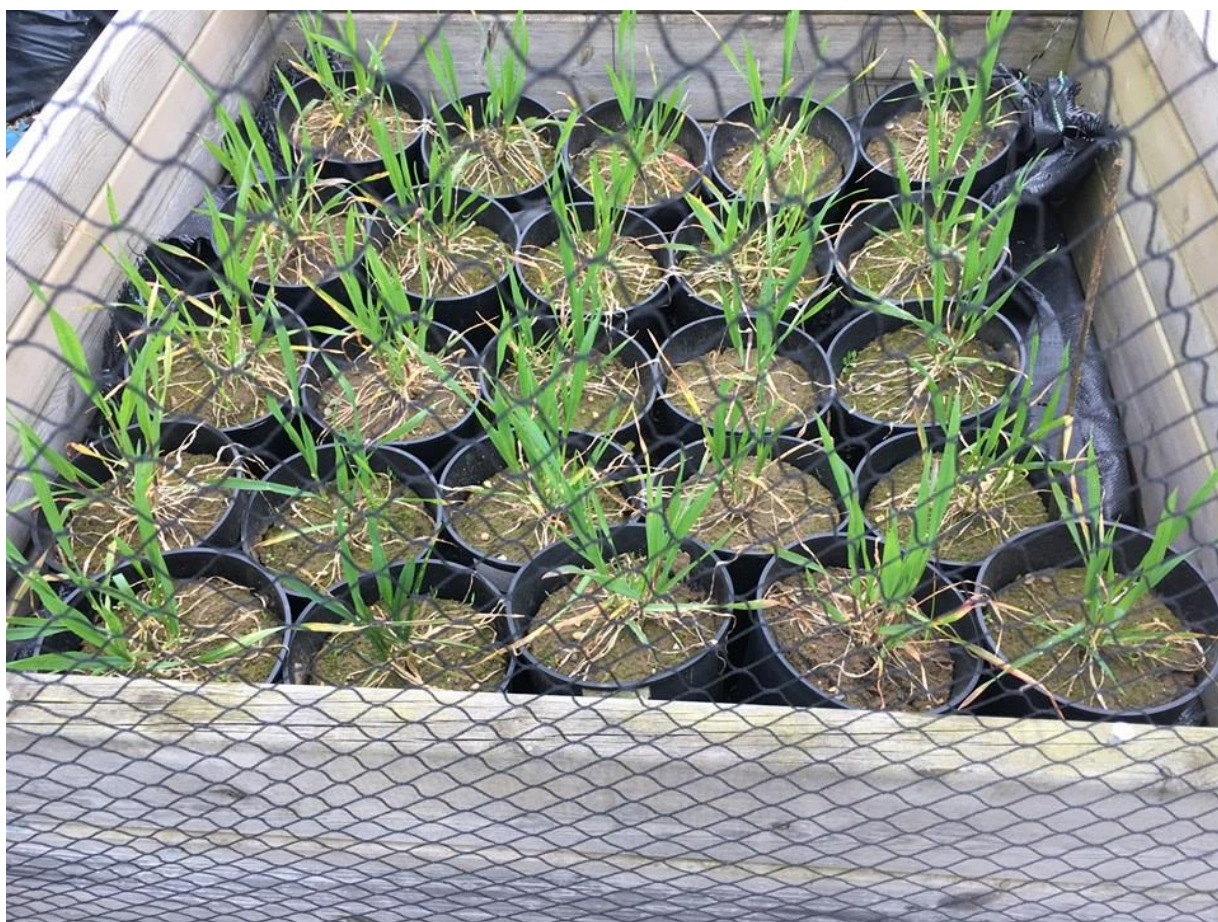
The main objective was to discover how wheat rhizosphere microbial communities change under differing C:N fertiliser applications. This was investigated using a modern agricultural spring wheat variety *Triticum aestivum* cv. Mulika, with the following specific aims:

1. Characterise the microbial community associated with the cultivar Mulika via qPCR and Miseq
2. Determine whether repeated applications of glucose-NH₄⁺NO₃⁻ (C:N) fertiliser reduce microbial community distinctiveness relative to bulk soil communities by qPCR and Miseq analysis
3. Establish the physiological effect on plant productivity from repeated applications of glucose-NH₄⁺NO₃⁻ (C:N) fertiliser via gas exchange and fluorescence measurements

4.2 Material and methods

Pots (4 litre) were packed with agricultural soil to a density of ~ 2.2 g cm⁻³ and randomised in three wooden quadrants (Figure 4.1) with a capacity of 25 pots to ensure each

treatment was well represented in each quadrant ($n = 6$). We avoided clumping of treatments by re-assigning a new position at random if multiple pots of the same treatment were allocated close-by to reduce the possibility of microenvironment biases. Similarly, we performed this step for the quadrant's edge to refrain from having one treatment proportionately placed along the edge of (or within) the quadrant to reduce the well-known "edge-effect" (different environmental conditions along the edge as



compared within the canopy) of this experiment design across treatments. All other methods are as described in Chapter 2. Small weeds that appeared were removed on a regular basis when they were noticed (Figure 4.2). Equivalent treatments for all the above were performed for soil-only controls.

Figure 4.1: An example quadrant from the experiment covered in a net to deter birds. 25 pots of *Triticum aestivum* cv Mulika can be seen with $n = 6$ of water-only, high C:N, medium C:N, or low C:N treatments, and one spare plant undergoing no treatment.

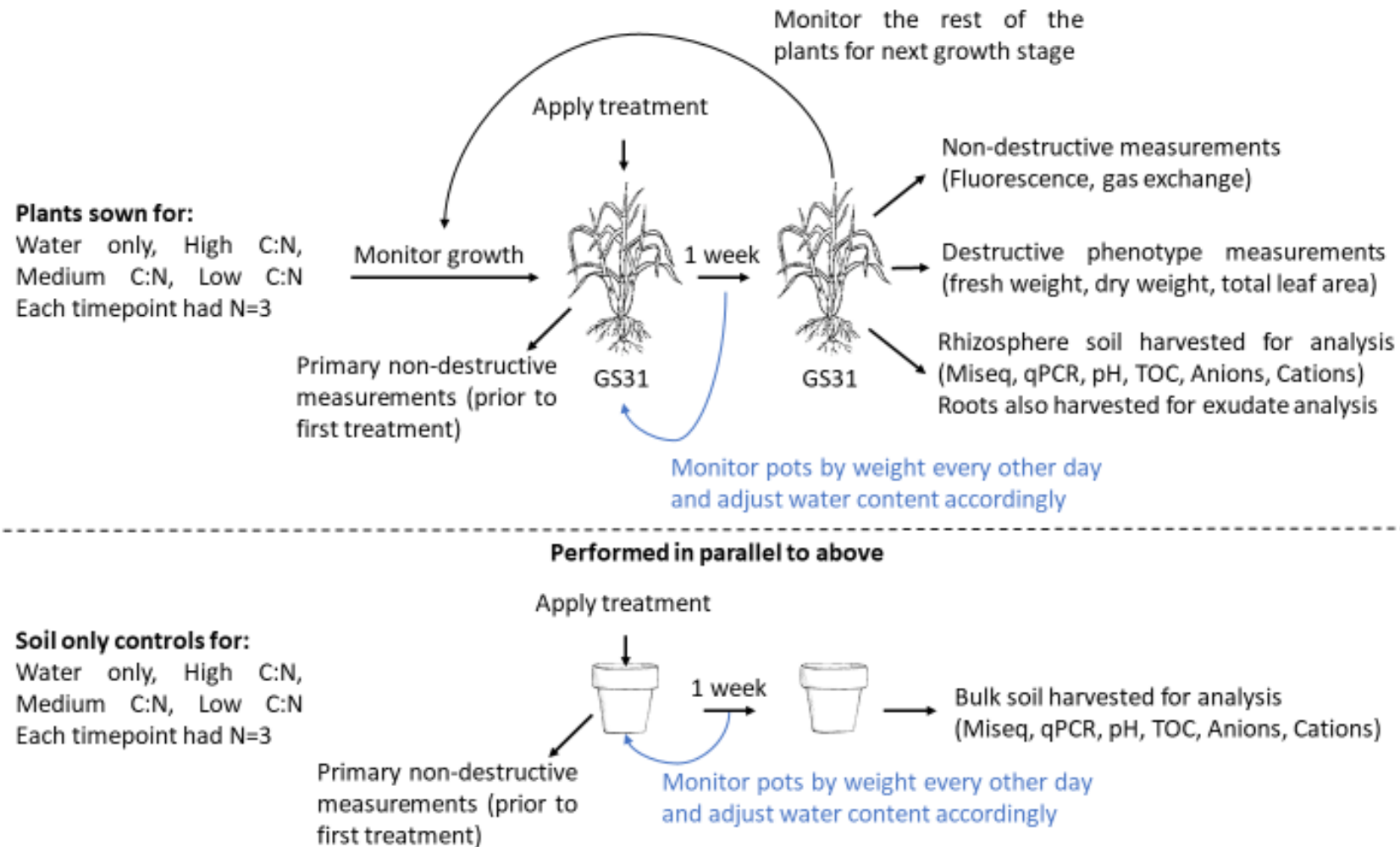


Figure 4.2: Experimental plan diagram summarising the steps taken in the experiment

4.2 Results

4.2.1 Plant physiology

Plants were measured at each growth stage (GS31, GS39 and GS61) for dark adapted fluorescence, F_v/F_m to assess the health of PSII, infrared gas exchange to assess the activities of photosynthesis under known conditions and biomass parameters (wet biomass, dry biomass, leaf area) to assess whether changes were realised in actual growth parameters. Below we show the results from the dark-adapted fluorescence values (F_v/F_m), gas exchange analysis (A/Q and A/C_i) and biomass harvesting.

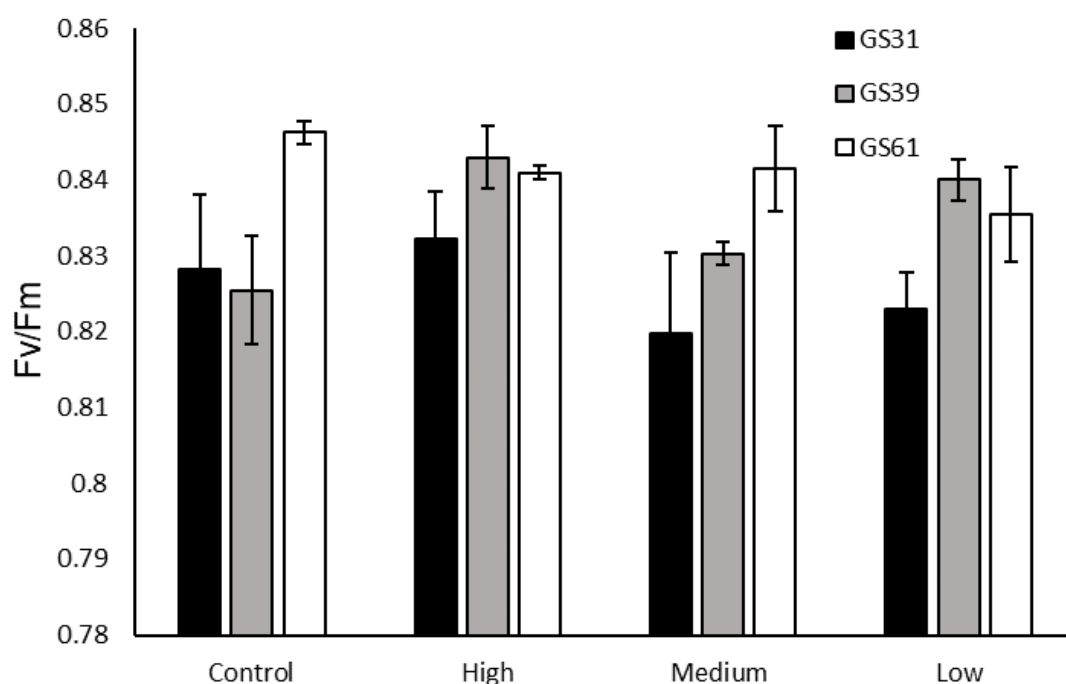


Figure 4.2: Dark adapted F_v/F_m values for *Triticum aestivum* cv Mulika for treatments over the three growth stages (GS31, GS39, GS61) either treated with C:N (glucose- $\text{NH}_4^+\text{NO}_3^-$) 32.58, 17.22 or 9.37 ratio fertilisers. Error bars represent \pm s.e.m, $n = 5$, and different lowercase letters denote significant differences.

For dark adapted F_v/F_m measurements no significant differences ($P > 0.05$) were found when comparing block groups for their respective weeks (Figure 4.2). Photosynthetic use of light by plants showed a positive trend of treatment application (Figure 4.3) with

treatments having a significantly ($P < 0.05$) higher P_{max} (the maximum use of photosynthetically active radiation (PAR)). Measurements at GS31 (Figure 4.3A) showed that the high C:N treatment was not different from control and at GS39 the high C:N had a higher P_{max} compared to other treatments ($P > 0.05$). High C:N treatment seemed to have maintained P_{max} whilst other treatments started dropping in photosynthesis (Figure 4.3B). At GS61 treatments were higher than controls with medium and high C:N being almost indistinguishable from one another at the top (Figure 4.3C).

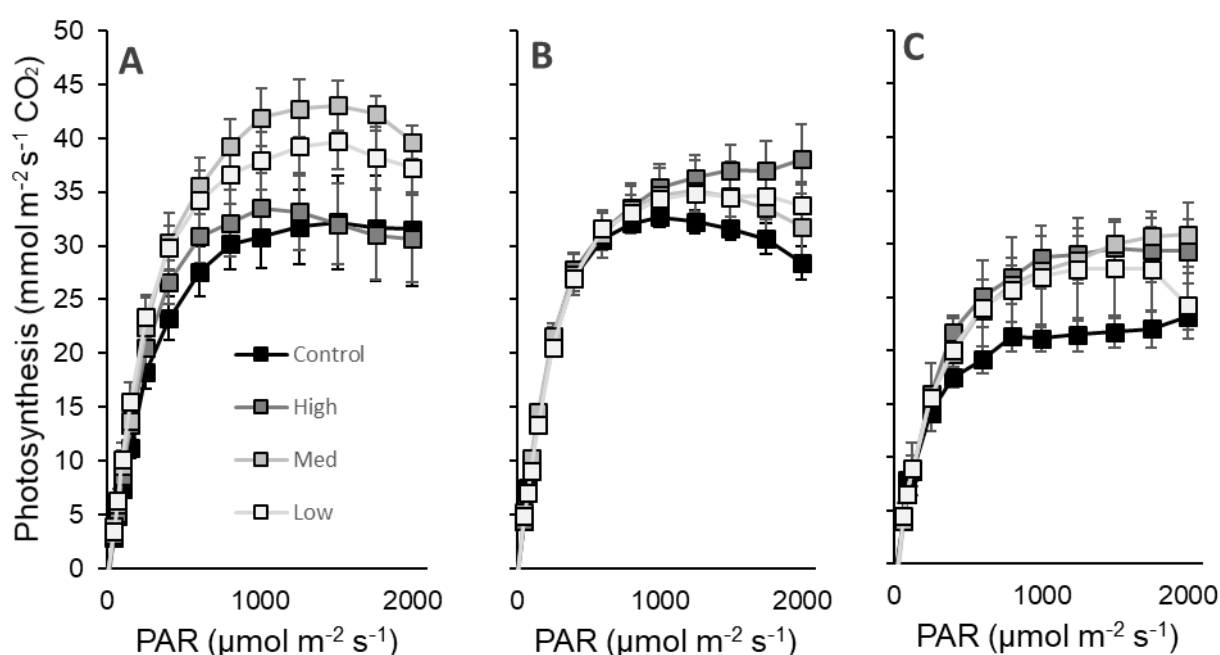


Figure 4.3: Assimilation against light intensity (PAR) for GS31 (A), GS39 (B) and GS61 (C) for *Triticum aestivum* cv Mulika when treated with high (32.58), medium (17.22) and low (9.37) C:N ratios in the form of glucose:NH₄⁺NO₃⁻. Error bars represent ± s.e.m, n = 3

At GS61 low C:N ratios numerically outperformed other treatments with CO₂ use and had an insignificantly ($P > 0.05$) A_{sat} (Figure 4.4), yet this was not observed for GS31 (Figure 4.4A) in which all treatments had equivocal curves but more pronounced at GS61 (Figure 4.4C). Although this trend was insignificant, the lower the C:N ratio applied the better

plants seemed to perform, with low C:N ratios residing at the top and high C:N ratios residing at the bottom along with H₂O controls (Figure 4.4).

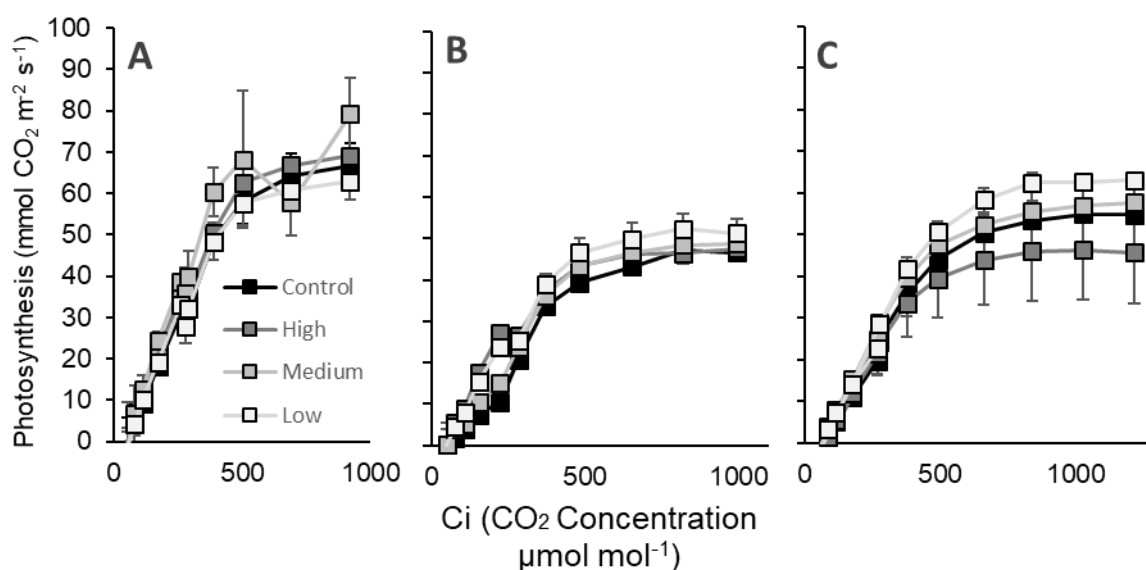


Figure 4.4: Assimilation against internal CO₂ concentration (C_i) for GS31 (A), GS39 (B) and GS61 (C) for *Triticum aestivum* cv Mulika when treated with high (32.58), medium (17.22) and low (9.37) C:N ratios in the form of glucose:NH₄⁺NO₃⁻. Error bars represent ± s.e.m, $n = 3$.

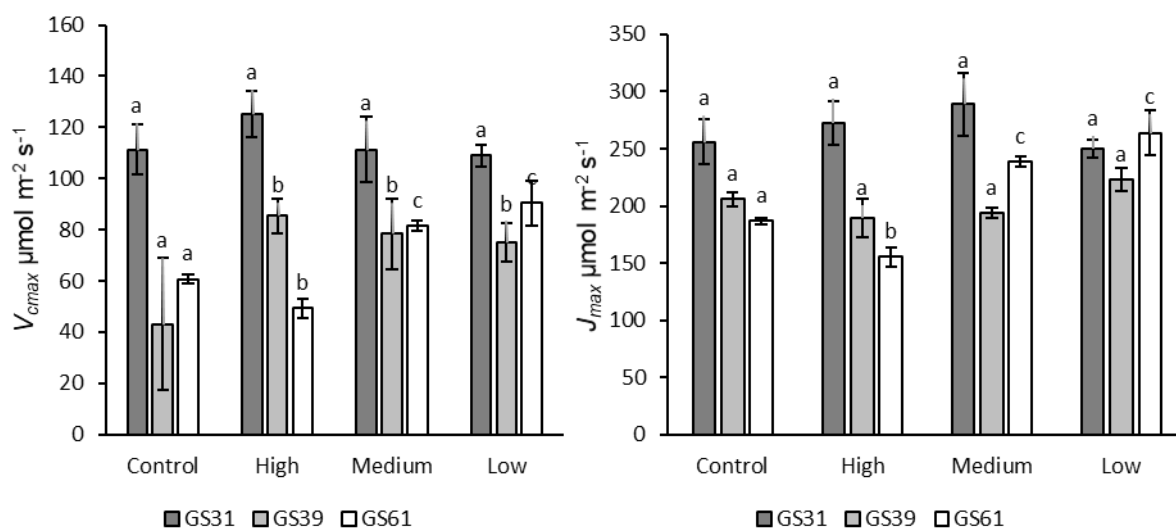


Figure 4.5: Gas exchange parameters V_{cmax} and J_{max} analysed from A/C_i curves using the 2016 edition of Sharkey's model (Sharkey 2016). C, H, M, and L represent control, high C:N, medium C:N and low C:N respectively. Error bars are ± s.d.m and different letters 3denote significant differences from the respective control, $n = 3$.

Model outputs from Sharkey 2016 indicate a similar pattern to the A/C_i results displayed in Figure 3. However, significant differences were found beyond GS31 at GS39 and GS61 respectively (Figure 4.5). One significant increase ($P < 0.05$) is seen in higher V_{cmax} over water controls for all C:N treatments, which are insignificant ($P > 0.05$) from one another (Figure 4.5A). Yet, when the plants have started to flower (GS61) high C:N shows an inhibitory effect on V_{cmax} compared to the water control, with low and medium C:N treatments significantly enhancing ($P < 0.05$) V_{cmax} values (Figure 4.5B). Similarly, these effects at GS61 are seen in J_{max} (Figure 4.5B), with high C:N treatment having significantly lower J_{max} values relative to water controls, whilst low and medium C:N show significantly higher ($P < 0.05$) J_{max} values.

Biomass and leaf area showed low and medium C:N ratios being significantly higher ($P < 0.05$) than high C:N and water controls (Figure 4.6). Low and medium C:N ratios showed a high total leaf area of *ca* 120 cm² at GS31 that declined to *ca* 75 cm² at GS61 (Figure 4.6A). However, high C:N and control treatments were stable at *ca* 75 cm² and 60 cm² respectively with insignificant ($P > 0.05$) declines being observed towards GS61 (Figure 4.6A). Water contents were insignificantly different ($P > 0.05$) throughout the experiment with a decline from 80% to 70% as plants aged from GS31 to GS61 (Figure 4.6C).

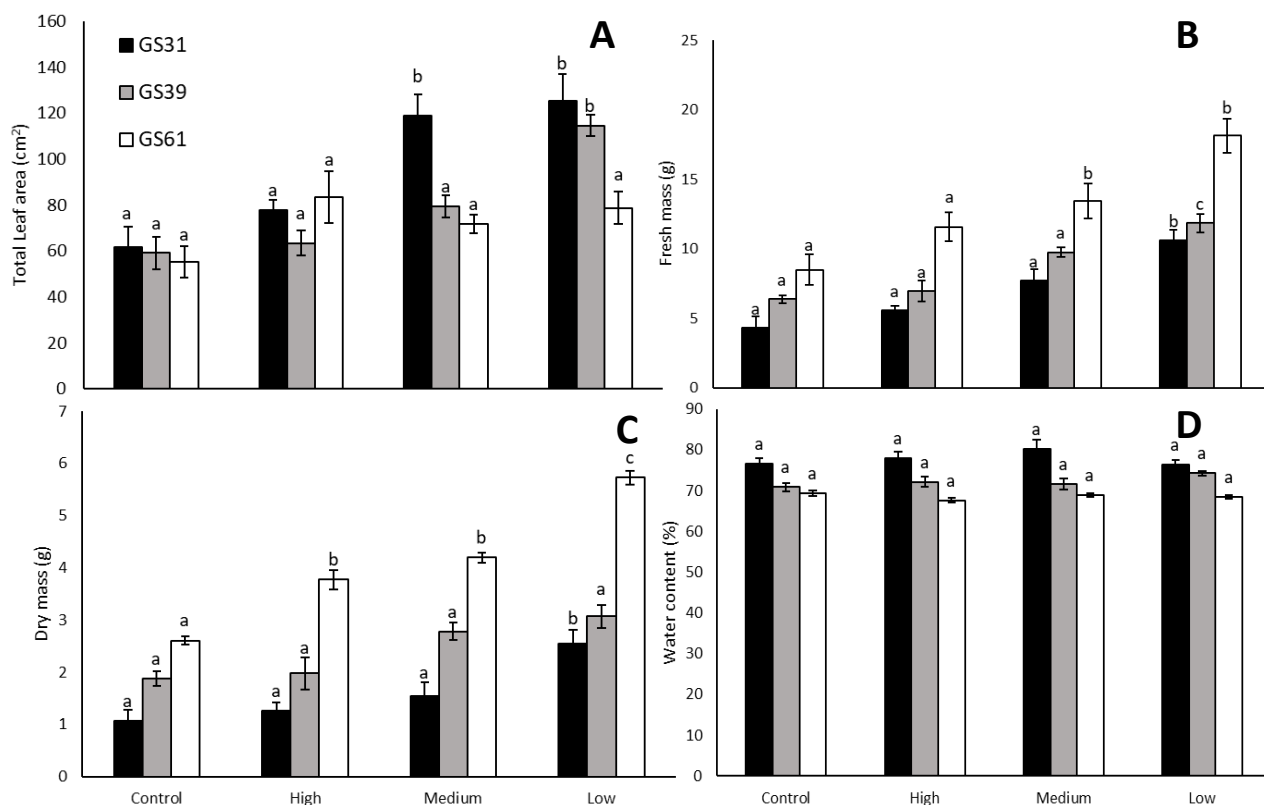


Figure 4.6: **A.** Leaf area analysis **B.** Fresh weight **C.** Dry weight and **D.** Water content, for *Triticum aestivum* cv Mulika when treated with high (32.58), medium (17.22) and low (9.37) C:N ratios in the form of Glucose: $\text{NH}_4^+\text{NO}_3^-$ for three timepoints GS31, GS39 and GS61 respectively shown for each treatment. Error bars represent \pm s.e.m, n = 3, and lowercase letters denote significant differences from the control compared to their respective week.

4.2.2 Soil microbial community and physicochemical properties

Here we move onto the belowground portion of the experiment, we will first address the microbial community from general bacterial, archaeal, and fungal abundance to N-cycling microorganisms. Then we will address the root exudate data and finish with the soil physicochemical factors.

Firstly, bacterial 16S rRNA gene abundance ranged between 8.19e^6 ($6.9 \log_{10}$) 9.47e^{12} (12.9) copies $\text{g}(\text{dw})^{-1}$ soil. In general, soil with plants present tended to have significantly higher ($P < 0.05$) abundance (Figure 4.7A) when compare to soil alone. Additionally,

these bacterial abundances tended to increase overtime between GS31 and GS61, with soil-only showing significant growth ($P < 0.05$) for low and medium C:N treatments, whilst planted soils showed significance in high C:N and water only treatments. Overall, bacterial abundance between treatments at each timepoint remained the same, the only significance between water-only and low C:N treatments at GS31 for soil-only pots (Figure 4.7A).

Although lower in abundance ($2.5e^4$ (2.4) to $3.3e^6$ (6.5)), archaeal 16S rRNA gene copies showed similar patterns to bacterial 16S rRNA (Figure 4.7B) with soil-only pots showing significant ($P < 0.05$) abundance increases between GS31 and GS61 for low and medium C:N treatments. However, archaeal abundance displayed a significant ($P < 0.05$) decrease in planted water-only treatments between GS31 and GS61 (Figure 4.7B). Conversely, very little difference was found in fungal ITS abundance, ranging from $7.5e^7$ (7.8) to $1.5e^9$ (9.2), only medium C:N treatments showed a significant ($P < 0.05$) increase in ITS abundance between GS31 and GS61 for soil-only (Figure 4.7C).

On the other hand, for the N-cycling genes, *nirS* ($2.2e^1$ to $9.2e^4$), *nosZ* ($5.9e^4$ to $1.1e^6$), crenarchaeota *amoA* (AOA, $1.8e^2$ to $4.4e^8$), and bacterial *amoA* (AOB, $2.9e^2$ to $3.4e^5$), significant increases ($P < 0.05$) in abundance were observed between GS31 and GS61 for all C:N treatments in soil-only pots (Figure 4.7D-G). Additionally, *nosZ*, AOA and AOB showed significant ($P < 0.05$) abundance increases in planted soils for high C:N treatments between GS31 and GS61 (Figure 4.7E-G), with *nosZ* and AOA showing significant ($P < 0.05$) decreases in water-only controls between these timepoints (Figure 4.7E,F).

Due to variation in control abundance levels (Figure 4.7A-G), it is difficult to interpret the raw abundances as differences. As a result, we compared plant-soil observations against their respective soil-only counterparts for each timepoint (Figure 4.8) to provide insight into how these abundances are changing with respect to no-plant counterparts. Distinct patterns are observable for each targeted gene when comparing planted: soil-only relative abundances. Bacteria showed no significant differences ($P > 0.05$) across treatments at GS31 (Figure 4.8A) with all treatments being between 3 to 4 the abundance levels of soil only. However, bacteria abundance showed a large increase relative to soil-only at GS61, with the abundance increasing significantly from low through high C:N treatments to water control respectively (Figure 4.8A). Archaea responded similarly but had varying levels of abundance to begin with (Figure 4.8B). Although relative archaeal abundance increased similarly to bacterial abundance, archaea had significantly less relative abundance at GS61 compared to GS31 in low and medium C:N treatments. Conversely, ITS showed no discernible pattern and displayed only one significant difference ($P < 0.05$) between medium C:N and water-only treatments at GS61 (Figure 4.8C), with all other treatments at all other timepoints remaining insignificant ($P > 0.05$).

The functional genes, *nirS*, *nosZ* and the *amoA* groups (AOA, and AOB) all demonstrated different responses to treatment over time in relative abundance. Apart from AOA and AOB under high C:N at GS61, every functional group investigated showed lower abundances in rhizospheric soils compared to soil-only at GS61 for all C:N treatments (Figure 4.8D-G). We observed a significant difference between GS31 and GS61 *nirS* abundances (Figure 4.8D), with *nirS* abundance decreasing from 1.22 times soil control for low C:N treatment in GS61 to 0.52 under high C:N treatment at GS61 (Figure 4.8D), significantly lower than the GS31 counterparts ($P < 0.05$), with *nirS* being significantly

higher in planted pots than soil-only for water-only controls at 20.5 times the abundance. Conversely, *nosZ* significantly increased ($P < 0.05$) with increasing C:N ratio, with only the high C:N treatment showing greater relative abundances at GS31 compared to GS61 (Figure 4.8E).

Both AOA and AOB showed significantly higher abundances ($P < 0.05$) from low C:N ratio to medium C:N ratio at GS31 (Figure 4.8F,G), with relative abundance dropping under high C:N treatments. Similarly, both displayed significantly higher ($P < 0.05$) abundances at GS61 under high C:N treatment compared to relative abundance at GS31 under the same treatment (Figure 4.8F,G).

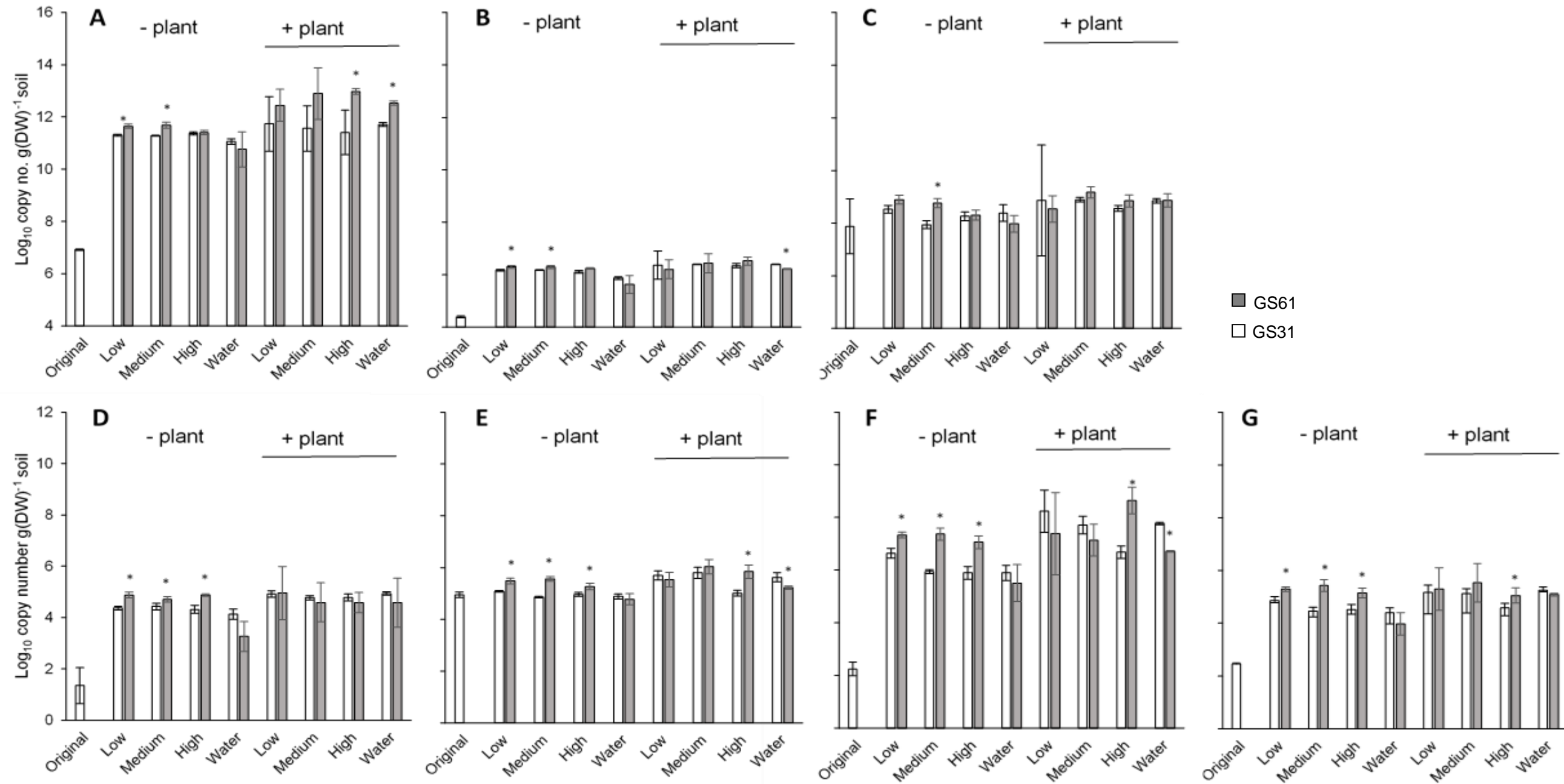


Figure 4.7: Log gene copy number for bacterial 16S rRNA (A), archaeal 16S rRNA (B), ITS (C), and functional genes *nirS* (D), *nosZ* (E), crenarchaeota *amoA* (F), and bacterial *amoA* (G) respectively per gram dry weight soil one-week post treatment for soils containing (+ plant) or not containing (- plant) *Triticum aestivum* cv Mulika wheat treated with either low (9.37), medium (17.22), or high (32.58) C:N treatments in the form of Glucose:NH₄⁺:NO₃⁻ sampled at plant growth stages, GS31 (white bar) and GS61 (filled bar) respectively. Error bars represent \pm s.e.m, $n=3$, and different letters where present represent significant difference between GS31 and GS61 treatments.

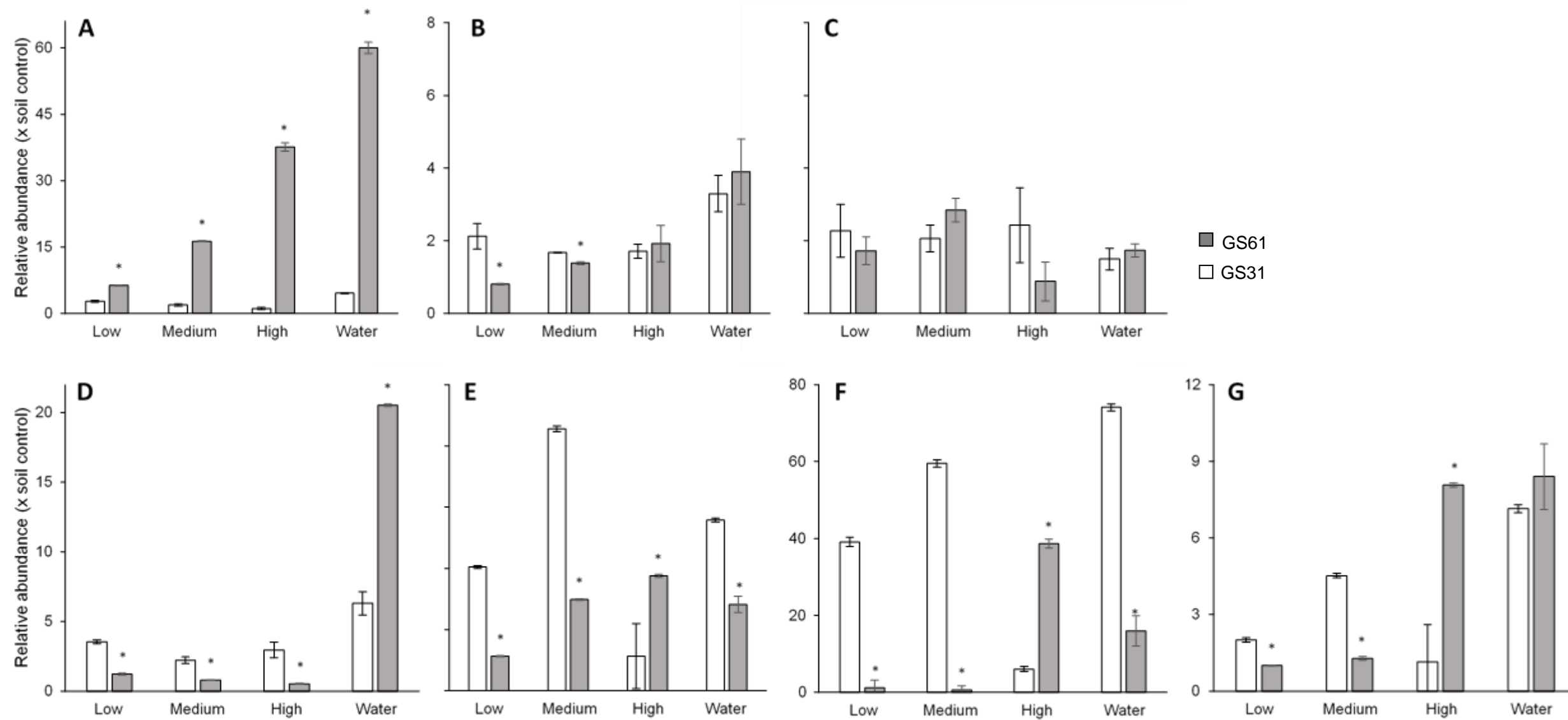


Figure 4.8: Relative abundance of gene copies relative to their respective soil controls for bacterial 16S rRNA (A), archaeal 16S rRNA (B), ITS (C), and functional genes *nirS* (D), *nosZ* (E), crenarchaeota *amoA* (F), and bacterial *amoA* (G) respectively per gram dry weight soil one-week post treatment for soils containing (+ plant) or not containing (- plant) *Triticum aestivum* cv Mulika wheat treated with either low (9.37), medium (17.22), or high (32.58) C:N treatments in the form of Glucose:NH₄⁺NO₃⁻ sampled at plant growth stages, GS31 (white bar) and GS61 (filled bar) respectively. Where the y-axis is the multiplication of soil control levels. Error bars represent ± s.e.m, n=3, and different letters where present represent significant difference between GS31 and GS61 treatments.

Throughout the experiment we observed the soil physicochemical parameters. We noted no significant ($P > 0.05$) difference in soil pH, which ranged from 6.4 (± 0.37 s.e.m.) to 6.7 (± 0.22), or in TOC and TIC. Furthermore, we observed significant increases ($P < 0.005$) in $[\text{NO}_3^-]$ and $[\text{NO}_2^-]$ throughout the experiment for all C:N fertilised pots when compared with water controls (Table 4.1). The same was seen for chloride concentration in low and medium C:N fertilisations ($P < 0.01$), however the high C:N fertilisation was statistically equivalent to water-only controls (Table 4.1).

We observed a similar response in cation concentration, with significantly higher $[\text{NH}_4^+]$ in C:N treatments compared to water controls (Table 4.2). Lithium was seen to be significantly higher in low and medium C:N fertilisations ($P < 0.01$) compared to water-only controls (Table 4.2). We observed that $[\text{Na}^+]$ and $[\text{Mg}_2^+]$ ($P < 0.01$) in high C:N treatments and $[\text{K}^+]$ ($P < 0.005$) in low C:N treatments were statistically higher than water-only controls throughout the experiment (Table 4.2). No significant differences were seen between ion concentrations across the three measured timepoints for the same treatments.

Whilst we were able to process and analyse the internal root exudates, when we processed the external root exudates, we noted extremely small concentrations, with no treatments having three detected replicates and many containing only one replicate measured above baseline fluctuation. Of the internal root exudates, we saw 57 unidentified metabolites appear inconsistently in the samples, many appearing only once. As a result, we have omitted those metabolites here to focus on the identified compounds

present in the internal standards as we were able to produce concentrations and therefore present them as a total of exudation.

Five main compounds were detected and analysed during the VFA protocol: acetic acid, propanoic acid, isobutyric acid, butyric acid, and glucose. On average, we observed that acetic acid comprised between 35% to 48% of total internal root exudation (Figure 4.9). The second most abundant compound was glucose (17% – 29%), followed by butyric (8% – 21%), propanoic (9% – 15%), and isobutyric (8% – 12%) respectively (Figure 4.9).

Water-only had statistically higher propanoic, butyric, and isobutyric acid abundance than other treatments ($P < 0.05$). However, glucose was statistically lower ($P < 0.05$) in low C:N treatments compared to high C:N and water-only treatments (Figure 4.9). Whilst acetic acid was although lower in low and medium C:N treatments, the levels did not differ significantly ($P > 0.05$).

Table 4.1: Bulk soil anion concentrations throughout the experiment for pots with and without plants, no significant differences were found between timepoints. Ion average \pm (s.e.m), n = 3. P < 0.05 (*), P < 0.01 (**), P < 0.005 (***) significances are relative to the relevant water control.

Treatment	Fluoride (ppm)	Acetate (ppm)	Chloride (ppm)	Nitrite (ppm)	Nitrate (ppm)	Sulphate (ppm)
Water	2.09 \pm (0.15)	6.91 \pm (4.8)	104.02 \pm (13.8)	4.52 \pm (0.33)	14.7 \pm (1.27)	180.1 \pm (11.2)
Low	2.04 \pm (0.14)	6.84 \pm (5.8)	135.4 \pm (9.3)**	6.47 \pm (0.2)***	29.81 \pm (1.54)***	176.1 \pm (10.3)
Medium	2.14 \pm (0.18)	7.17 \pm (3.5)	130.82 \pm (10.5)**	8.75 \pm (0.45)***	26.06 \pm (0.74)***	170.5 \pm (12.7)
High	2.13 \pm (0.21)	6.58 \pm (4.7)	89.81 \pm (13.4)	5.96 \pm (0.74)***	19.37 \pm (0.94)***	181.5 \pm (10.8)

Table 4.2: Bulk soil cation concentrations throughout the experiment for pots with and without plants, no significant differences were found between timepoints. Ion average \pm (s.e.m), n = 3. P < 0.05 (*), P < 0.01 (**), P < 0.005 (***) significances are relative to the relevant water control.

Treatment	Lithium (ppm)	Sodium (ppm)	Ammonium (ppm)	Potassium (ppm)	Magnesium (ppm)	Calcium (ppm)
Water	0.43 \pm (0.05)	1973 \pm (113)	2.06 \pm (0.28)	4.51 \pm (1.02)	156.9 \pm (9.7)	3377 \pm (105)
Low	0.84 \pm (0.11)**	1944 \pm (186)	5.99 \pm (0.14)***	7.05 \pm (0.87)***	157.9 \pm (11.3)	2913 \pm (292)
Medium	0.76 \pm (0.09)**	1937 \pm (128)	5.66 \pm (0.24)***	4.78 \pm (0.69)	156.6 \pm (10.3)	3130 \pm (150)
High	0.52 \pm (0.05)	1454 \pm (151)***	5.57 \pm (0.37)***	5.7 \pm (0.97)	119.8 \pm (10.6)**	3040 \pm (228)

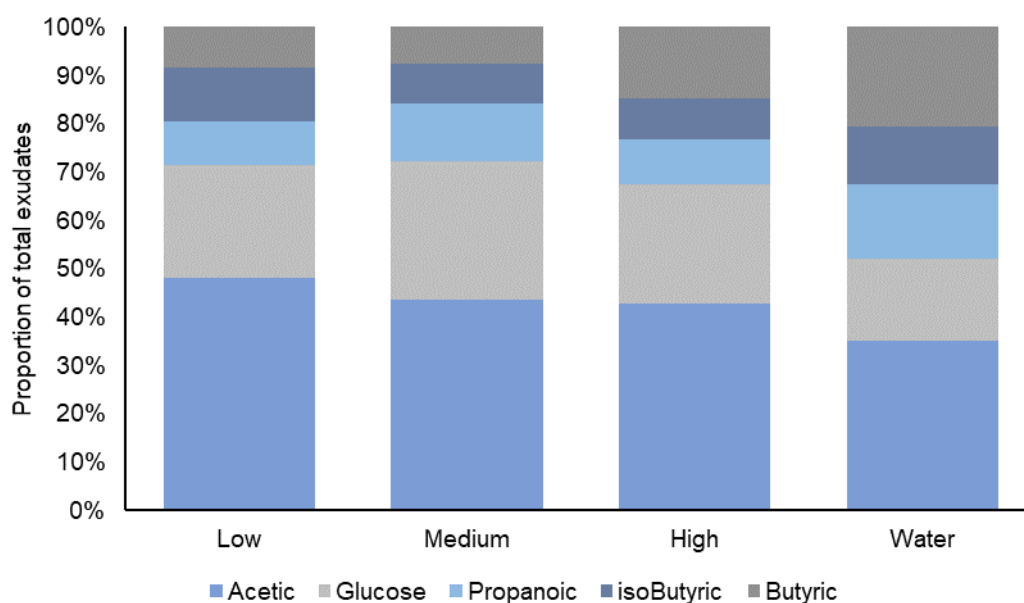


Figure 4.9: Internal root exudates of GS61 *Triticum aestivum* cv Mulika when subject to a well-watered or droughted watering regime and either nitrogen-only ($\text{NH}_4^+\text{NO}_3^-$), carbon-only (glucose), water-only or a high (32.58), medium (17.22), or low (9.37) C:N fertilisation treatment, n=3

PCA analysis showed large variation in the samples with nitrogen and high C:N treatments grouping the least (Figure 4.10). Clear separation existed between medium, low and water treatments, with medium and low treatments forming distinguishable clusters (Figure 4.10). Additionally, nitrogen and low C:N treatments were more closely related to each other than to medium C:N, high C:N, or water-only treatment groups (Figure 4.10). Despite the high C:N treatment showing a large variation, a general grouping pattern can be seen with high plant nitrogen bioavailability groups (low C:N and nitrogen only) forming clusters towards the bottom right, low plant nitrogen bioavailability (medium C:N) clustering to the top left, and water-only treatments clustering inbetween both macrogroups (Figure 4.10).

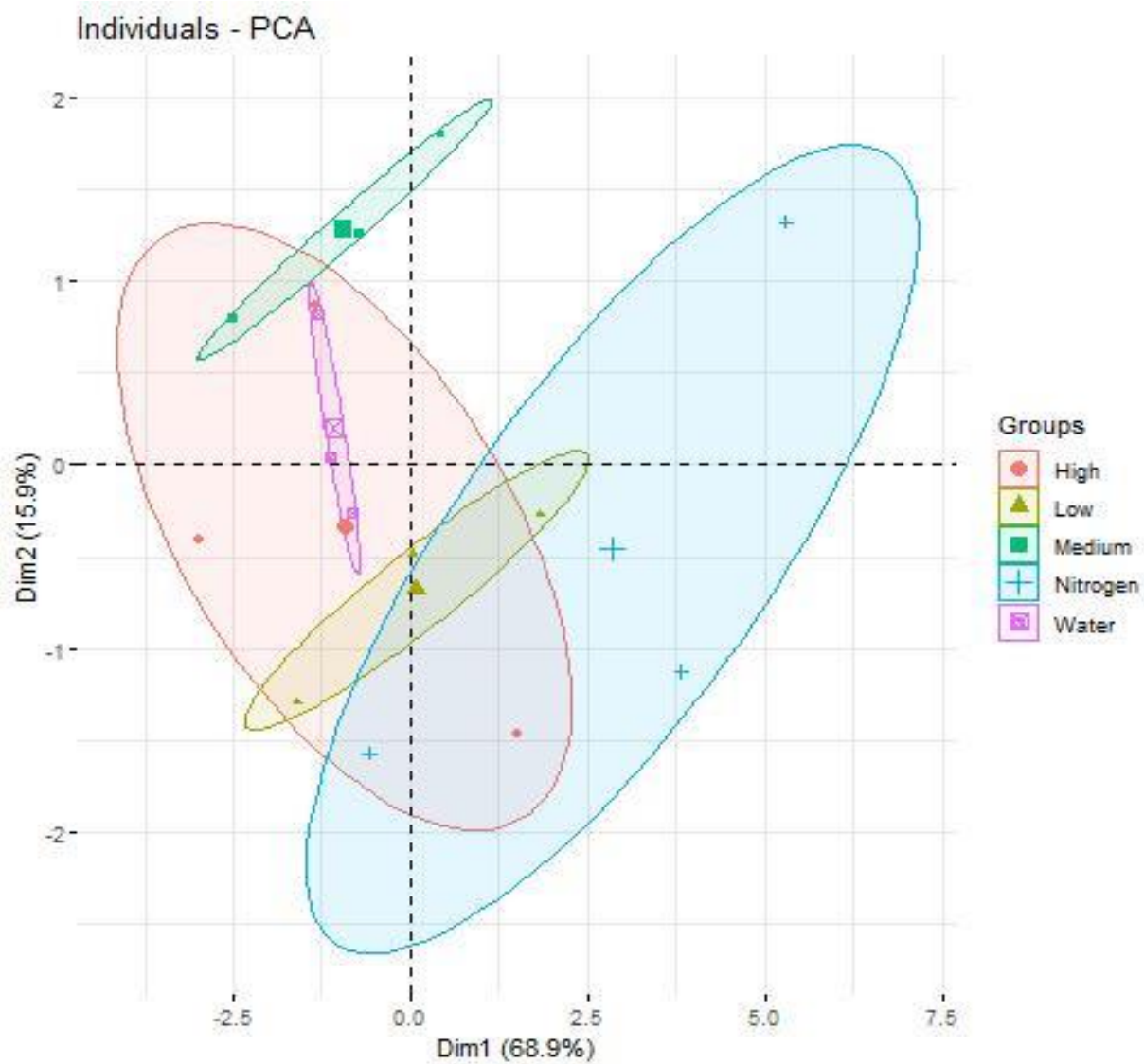


Figure 4.10: Principle component analysis of aboveground and belowground portions for GS61 *Triticum aestivum* cv Mulika when subject to either nitrogen-only ($\text{NH}_4^+\text{NO}_3^-$), water-only, or a high (32.58), medium (17.22), or low (9.37) C:N fertilisation treatment, ellipses represent 95% CI boundaries, $n = 3$

4.3 Discussion

P_{max} has been shown to increase with leaf N due to upregulation of RuBP carboxylase, increases in V_{cmax} , and chlorophyll content, which have drastic implications for photosynthetic performance for both young and old plants (Sims *et al.*, 1998; Walker *et al.*, 2014). For example, Sims *et al.*, 1998 demonstrated an increase in A for high N supplemented ($7.5 \text{ mol m}^{-3} \text{ NO}_3^-$) in comparison to low N supplemented plants ($0.75 \text{ mol m}^{-3} \text{ NO}_3^-$) in A/C_i analysis for older soybean (6-8 weeks) yet showed no difference when they were younger (Sims *et al.*, 1998). We find similar, but less pronounced results with *T. aestivum*, with older plants (GS61) showing a larger separation than younger stages. This could be due to N translocation and assimilation effects, younger plants may invest more into root systems and N assimilation than older plants, which may then start to translocate and utilise stored N. However, there is a latency effect, plants treated with N may not immediately show an increase in A until there is an upregulation of photosynthetic components. Therefore, bias sampling may be prevalent in younger plants as older plants experience several more weeks of treatment.

Our F_v/F_m data, which grouped around the optimal value 0.83, suggest no PSII damage (Bjorkman & Demmig, 1987; Johnson *et al.*, 1993) and that the PSII centres of all plants did not differ in potential light utilisation capacity. Therefore, treatments had no significant effect on plant stress as seen through PSII maximum potential and were not the cause for biomass differences seen in this experiment. It is likely that biomass differences were resulted from differences in A throughout the growing period. Although no significant differences were found between our A/Q or A/C_i curves these curves represent a small 20 to 30-minute picture of what is happening. Such small differences in trend, even if

insignificant, can amount to large and significant differences in biomass when considered throughout an entire growing season, as we have observed.

Generally, lower C:N ratios outperformed other treatments with CO₂ assimilation and had higher J_{max} (Figure 4.3), yet this was not observed for GS31 (Figure 4.4A) in which all treatments had equivocal curves but more pronounced at GS61 (Figure 4.4C). One possibility for this increase is a higher NH₄⁺NO₃⁻ concentration in low C:N treatments compared to higher C:N treatments. Total activity of RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase), which increases net assimilation of CO₂ (Mate *et al.*, 1996), has been shown to increase with elevated levels of N in the form of NH₄⁺NO₃⁻ up to a threshold (Manter *et al.*, 2005). These results support our observation of significantly higher leaf areas and biomass in the medium and low C:N treated plants (Figure 4.6) compared to other treatments. Leaf areas showed two distinct patterns, low and medium C:N ratios showed high total leaf areas of ca 120 cm² at GS31 that declined over time to around ca 75 cm² at GS61 (Figure 4.5A). However, high C:N and control treatments showed no discernible pattern holding at ca 75 cm² and 60 cm² respectively with insignificant ($P > 0.05$) declines being observed towards GS61 (Figure 4.6A).

Soil N-availability explains most of the plant physiological results. Plants compete with soil organisms for bioavailable N with bacteria being a main short-term competitor for mineralised N (Hodge *et al.*, 2000). Using ¹⁵N labelled NH₄⁺ and NO₃⁻ it was found that bacteria outcompeted plants within the first 4 hours of mineralised-N addition to soil (Inselbacher *et al.*, 2010), with NO₃⁻ being the preferred molecule. Within a day microbial N uptake substantially declined and plants then acquired more N (Inselbacher *et al.*,

2010). This 4-h period of microbial dominance decline after 24-h was suspected to be short due to soil C limitation (Inselsbacher *et al.*, 2010), it is therefore probable applying C alongside N application would lengthen this period of microbial dominance.

Labile C addition (in this case glucose), decreases plant N uptake due to microbial competition for N through the same immobilisation mechanism present in N applications (Dunn *et al.*, 2006; Schmidt *et al.*, 1997). This competition is particularly true for communities containing many copiotrophs as they prefer organic nitrogen and labile C (Treseder *et al.*, 2011). Yet, although immobilised N is often re-mineralised within a few days, our application at GS31 (a key growth stage for plant construction and N uptake) may have enhanced the effect of this temporary N immobilisation as uptake is critical at this stage. Thus, it is likely our high C:N treatments stimulated an uptake of N into microbial biomass, immobilising it in non-bioavailable forms for plants. This immobilisation caused a possible deficiency in plant N compared to lower C:N ratios where microbial uptake may be lower and where there was a relative abundance of mineralised N. Such competition for bioavailable N explains differences in our plant physiological data for A trends and total biomass.

Bacterial abundance increased between GS31 and GS61 under most treatments, with low and medium C:N being significant for no plants, whilst high C:N and water-only significant for rhizosphere soils. Other studies have shown an increase in bacterial abundance, the main utilisers of added glucose (Behera & Wagner, 1974), with LMWC (low molecular weight compound) supplementation (Chenu *et al.*, 2001). However, bacteria are known to utilise a large portion of added glucose within a 30h period

(Blagodatskaya *et al.*, 2007; Chenu *et al.*, 2001; Thiet *et al.*, 2006), with between 36 - 42% of glucose derived C being mineralised within 3-days (Mary *et al.*, 1993; Bremer and Kuikman, 1994) and 45 - 60% utilised by 10-days (Chander & Joergensen, 2001). This utilisation leads to an increase and subsequent decrease in biomass following a few days seen in many studies (Blagodatskaya *et al.*, 2007; Chander & Joergensen, 2001; Chenu *et al.*, 2001). This could explain why we saw no change in bacterial abundance sampling one-week post-treatment between treatments at the same timepoints.

Responses of microbial communities to LMWC are often associated with increases in community composition change, with specific assemblages forming depending on the added labile C (Cleveland *et al.*, 2007; Eilers *et al.*, 2010; Padmanabhan *et al.*, 2003), for example, additions of glucose to grassland soil did not change relative abundances of several bacteria phyla compared to water controls (Eilers *et al.*, 2010). However, the same additions of glucose to Hardwood and Coniferous forest soils significantly altered the abundances of bacterial phyla in varying ways, with additions decreasing Actinobacteria abundances in the Hardwood group but increasing them in the Coniferous group (Eilers *et al.*, 2010). This indicates a highly varied response to glucose addition is expected for bacterial communities in soil due to original community composition and the propensity for that community to respond to glucose. Therefore, although it is difficult to cross interpret from one study to another, we do not observe here the typical response of a general increase in abundance with higher additions of glucose. This could be due to our additions, whilst large in difference, may all be over a threshold of glucose addition and therefore all be producing a similar response in this manner.

Intuitively, adding labile C is known to elevate soil respiration (Bremer & Kuikman, 1994; Chenu *et al.*, 2001; Cleveland & Townsend, 2006; Thiet *et al.*, 2006), with strong positive relationships between labile C availability and respiration rates (Cleveland & Townsend, 2006). This relationship is closely linked to plant growth as the supply of organic residues increases with higher levels of assimilation, thus providing more substrate for soil decomposers, with the highest rates of CO₂ flux being in the tropics where plant growth is rapid and warm, moist temperatures are ideal for decomposer activity (Schlesinger & Andrews, 2000; Wardle *et al.*, 2004). Previously, when adding glucose between 10 - 25% was added to microbial biomass (Chander & Joergensen, 2001; Saggiar *et al.*, 1994), this effect was increased by 1.4 - 6.2% when adding N alongside glucose at a C:N of 15 compared to adding glucose alone (Chander & Joergensen, 2001). However, these spurts in microbial growth are often short-lived, with microbial biomass seen to peak on the second day after treatment and showing decreases below initial levels by day three (Chander & Joergensen, 2001), as we sampled 7 days post-treatment it is possible that sampling times over-looked a subsequent increase in bacterial biomass due to fertilisation.

These changes in both bacterial and archaeal microbial abundance do not support the hypothesis of higher C:N ratio treatments enhancing microbial growth by providing an abundance of C and N as we often saw no significant differences between treatments and water-only controls. Fungal abundance may give a better indication of N-competition, particularly as fungal growth is noted to increase on bacterial decline (Behera & Wagner, 1974) and contribute 78% of soil respiration (Anderson & Domsch, 1978), with one study noting fungi being involved more in N assimilation when glucose was added to soil (Mary *et al.*, 1993). Furthermore, elevated levels of C and N added to soil have adverse priming

effects for C-mineralisation and lower specific microbial growth rate, shifting from r-selected species to k-selected species due to preferential substrate utilisation (Blagodatskaya *et al.*, 2006). These factors affect the interpretation of our microbial data in drastic ways, which MiSeq could have eluded to. Unfortunately, as noted earlier the MiSeq run for this experiment produced poor quality and unusable reads. This would have given information into species identification, which coupled with the abundance data would have been useful in interpreting how the community may be responding.

As expected, bacterial *amoA* gene abundance was higher in low C:N ratios. It is likely at ratios below 17.22 a surplus of NH_4^+ for microbial growth was provided alongside glucose. This surplus of could have induced increases for *amoA* through higher $[\text{NH}_4^+]$, which has been previously observed (Di *et al.*, 2009; Jia & Conrad, 2009). The values of *amoA* abundance in this study are in correspondence with former studies in soil which range from 2.87×10^4 – 2.36×10^6 copies g^{-1} soil (Leininger *et al.*, 2006; Nicol *et al.*, 2008; Prosser & Nicol, 2012). It is also possible that under high C:N ratio treatments competition for NH_4^+ with heterotrophic organisms, leading to the observed decrease in *amoA* abundance, which corroborates other studies. However, soil *amoA* gene abundance is usually dominated by AOA (ammonia-oxidising archaea) rather than AOB (Leininger *et al.* 2006; He *et al.* 2007; Nicol *et al.* 2008), a relationship that increases toward AOA under lower pH (Nicol *et al.*, 2008), which glucose has been found to induce over a few days (Yan *et al.*, 1996).

As shown in Yan *et al* 1996 NH_4^+ or NO_3^- alongside glucose lead to small increases in soil pH, however, subsequent measurements over the 45d incubation period showed a

very stable pH. We did not find any significant increases in our soil pHs, finding them insignificantly different from each other at every timepoint. This could be due to variation induced in our outdoor roof experiment that Yan *et al.*, would not have experienced due to their controlled conditions (Yan *et al.*, 1996)., and therefore pH is unlikely to have affected the AOA:AOB relationship in this study. Although AOA and AOB growth are highly linked to ammonia concentration (Offre *et al.*, 2009), AOB growth, not AOA, is associated with nitrification after elevated levels of ammonium application (Di *et al.*, 2009; Jia & Conrad, 2009). This growth period for AOB could lead AOA: AOB ratios closer to 5 rather than the typical values > 15 (Prosser & Nicol, 2012), which we experienced in our study. Despite this dominance of AOA in numbers it has been shown that bacteria rather than archaea functionally dominate ammonia oxidation (Jia & Conrad, 2009).

Similarly, denitrification is a process known to increase with acidic soils, lowering the more alkaline a soil gets (Čuhel *et al.*, 2010). Our denitrification genes (*nirS* and *nosZ*) are generally in accordance with previously reported levels (Čuhel *et al.*, 2010; Lammel *et al.*, 2015). Whilst we saw significant increases in N-cycling gene abundances between many of our rhizosphere-bulk comparisons, we also noted a significant increase in *nosZ* abundance at GS31, which is in contradiction to ARE experiments by Henry *et al* 2008 who noted no increase in *nosZ* when applying ARE. However, this could be due to the extreme variation they experienced, with some samples having standard errors higher than the averages they represented (Henry *et al.*, 2008).

Here we observe the general response of increases in N-cycling gene and general microbial abundance in the rhizosphere as compared with non-planted controls. Yet, it

should be noted, while most of these differences were numerically true some were not significant (Figure 4.6), most of which was due to variation in the data. However, abundance does not necessarily translate into functional activity. Unfortunately, we did not measure N₂O emissions during this study, but previous studies have noted that additions of inorganic N lead to an increase in *amoA*, *nirS*, *nosZ*, *napA* and *narG* abundances and subsequent increase in denitrification as measured by N-loss in the form of N₂O and N₂ (Čuhel *et al.*, 2010; Lammel *et al.*, 2015). Additionally, in our research, we tried to capture the nitrogen-fixers through *nifH* amplification, yet we failed to obtain any *nifH* Q-PCR data.

4.3.1 Conclusion

In conclusion, our results illustrate that low and medium C:N outperform high C:N treated plants or those with no treatment due to N competition with the belowground microbiota. This has implications for farming practices with regards to mulching and adding labile carbon to agricultural soil, which may hinder performance if simultaneous additions of labile C and N are likely to hinder plant performance if the ratio exceeds 9.37. However, there are large limitations to this study which limit interpretations and applications of findings to other sectors. Namely, this study has used only glucose, a readily available organic compound that is widely used by microbiota. Secondly, we do not consider the possibility of N-containing organics such as amino acids and proteins, which constitute over 90% of soil N and are an integral part to soil ecosystems. Furthermore, long-term production and soil health are not addressed in this study, in which recalcitrant soil carbon and carbon turnover become increasingly important for maintaining and

enhancing agricultural productivity. Further studies should look to investigate a residency effect on these soils to determine whether subsequent generations of plants are affected.

Chapter 5: Spring wheat *Triticum aestivum* cv. Mulika responses to simultaneous drought and applications of C:N fertiliser

5.1 Introduction

In Chapter 4 responses of Mulika wheat and associated rhizosphere microbiota were analysed to assess the effects of fertiliser applications with differing C:N ratios (glucose:NH₄⁺NO₃⁻) regimes had on the relationship between plant and microbial communities. Yet, the experiments were performed under well-watered conditions with minimal changes in water availability. Most agricultural systems experience some amount of water availability fluctuation, making it necessary to assess how the responses between plants and microbes respond to droughts of low - or no - water availability. This global water shortage that affects more than 70% of arable land presents a huge obstacle for plant productivity (Timmusk *et al.*, 2014).

As a natural hazard, droughts are caused by global temperature cycles in the atmosphere and oceans, with higher temperature variations in a given region being associated with the temporary deficit or decrease in water availability (Amrit *et al.*, 2018; Cook & O'Grady, 2006; Grayson, 2013). Although these periodic droughts can be long or short-lived and of varying intensity, frequency and duration, they all have an impact on regional economies, with the agricultural sector being particularly prone (Kuwayama *et al.*, 2018). Climate change has vastly affected these temperature cycles, which has subsequently led to a visible increase in drought duration, intensity, and frequency (Ipcc, 2014; Kuwayama *et al.*, 2018). A few examples of recent notable droughts in the United States

are the long-lived drought in California from 2011-2017 and the intense 2012 Midwest drought.

The response of plants to drought is varied with modification of root architecture, leaf shape, and reproductive response that fall into three main categories of responding to drought: escape, resistance, resilience. Although the physiological and morphological responses to drought may differ between perennial and annual plants, and between cereal cultivars (Ranjan & Yadav, 2019), the closing of stomata through ABA induction is a common element in plant response to drought (Ding *et al.*, 2016; Kulkarni *et al.*, 2017). These physiological changes have been noted to impact metabolic processes within the aboveground and belowground portions of plants (Kulkarni *et al.*, 2017), and subsequent plant-microbe relationships have been seen to change under the environmental pressures of drought (Dai *et al.*, 2019; de Vries *et al.*, 2018; Hammerl *et al.*, 2019; Marasco *et al.*, 2013; Meisner *et al.*, 2018).

We already have a great understanding on how added N fertiliser affects plant physiology, yet underlying competition for N between plants and micro-organisms in these agricultural systems is less well understood, particularly under changing soil C:N. With the increasing occurrence of drought globally there is a necessity to understand how the effects of drought will also influence our agricultural systems alongside their already altering soil C:N ratios. As a result, here we investigate how microbial communities and wheat plants compete for N under drought with differing C:N fertiliser applications to better equip ourselves with knowledge for future events.

5.1.2 Aims and Objectives

The main objective for this chapter was to characterise *Triticum aestivum* cv Mulika and the associated rhizosphere microbiota in response to both C:N fertilisation and drought treatments concurrently. Where drought was defined by a soil water content of 40%, with the following specific aims:

1. Characterise the well-watered and droughted microbial communities associated with the cultivar Mulika via qPCR and Miseq
2. Determine whether repeated applications of glucose-NH₄⁺NO₃⁻ (C:N) fertiliser, an applied 40% water content drought, or the combination thereof, reduced microbial community distinctiveness relative to respective bulk soil samples by qPCR and Miseq analysis
3. Establish the physiological effect on plant productivity from repeated applications of glucose-NH₄⁺NO₃⁻ (C:N) fertiliser and/or the effect of a 40% water content drought, by fluorescence and gas exchange measurements

As in the previous chapters we will first approach the plant physiological side of the subject and then the belowground chemistry and microbiota.

5.2 Materials and methods

Pots (4L) were randomised in each quadrant and ensured each treatment was well represented in each quadrant ($n = 4$). Treatment grouping was avoided by re-assigning new positions at random if multiple same treatment pots were grouped, to reduce the possibility of microenvironment biases (Figure 5.1). Similarly, we performed this step for the quadrant's edge to refrain from having one treatment disproportionately placed along the edge of (or within) the quadrant to counter the well-known "edge-effect" (different environmental conditions along the edge as compared to within the canopy). All other methods are as previously described in chapters 2 and 4.

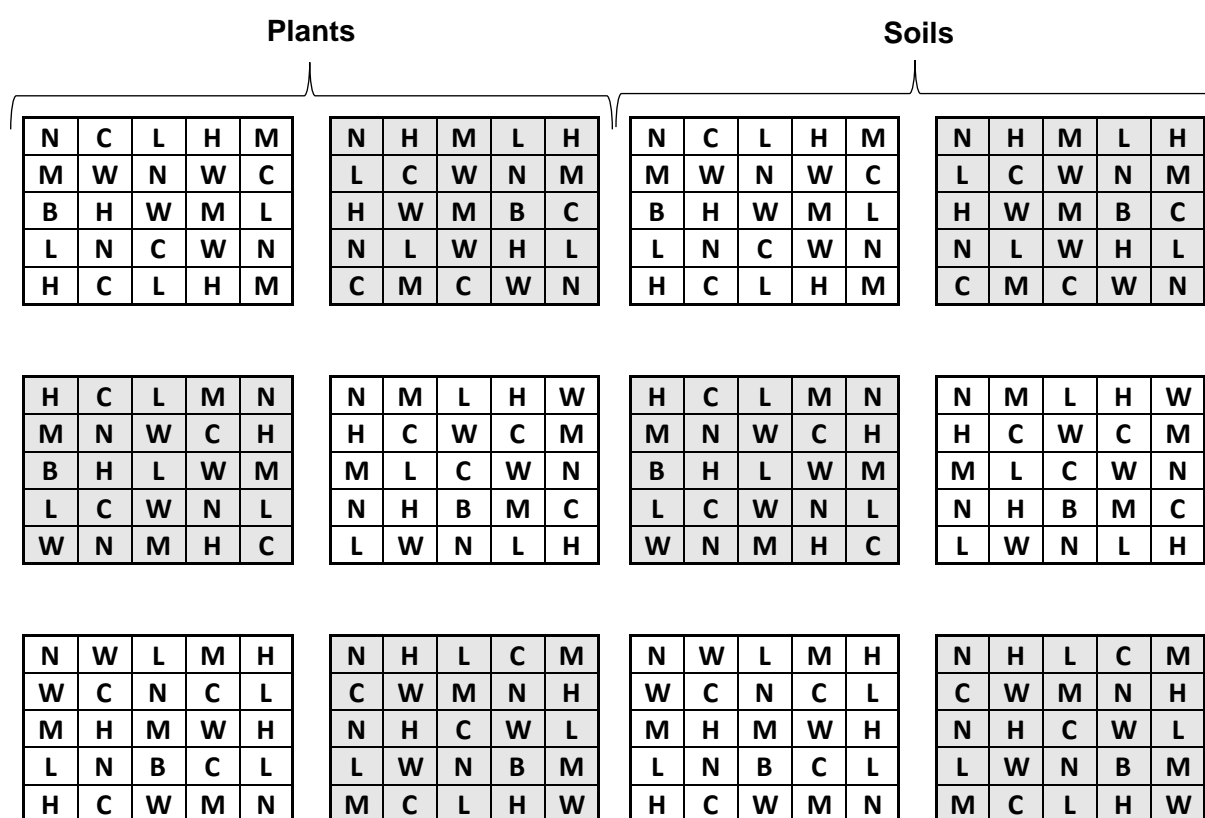


Figure 5.1: Experimental design, layout of wooden quadrants, grey is droughted whilst white is well-watered. Treatments are represented as follows: N = Nitrogen-only, C = C-only, H = High C:N (32.58), M = Medium C:N (17.22), L = Low C:N (9.37), W = Water-only, and B = Blank, spare and unused plant.

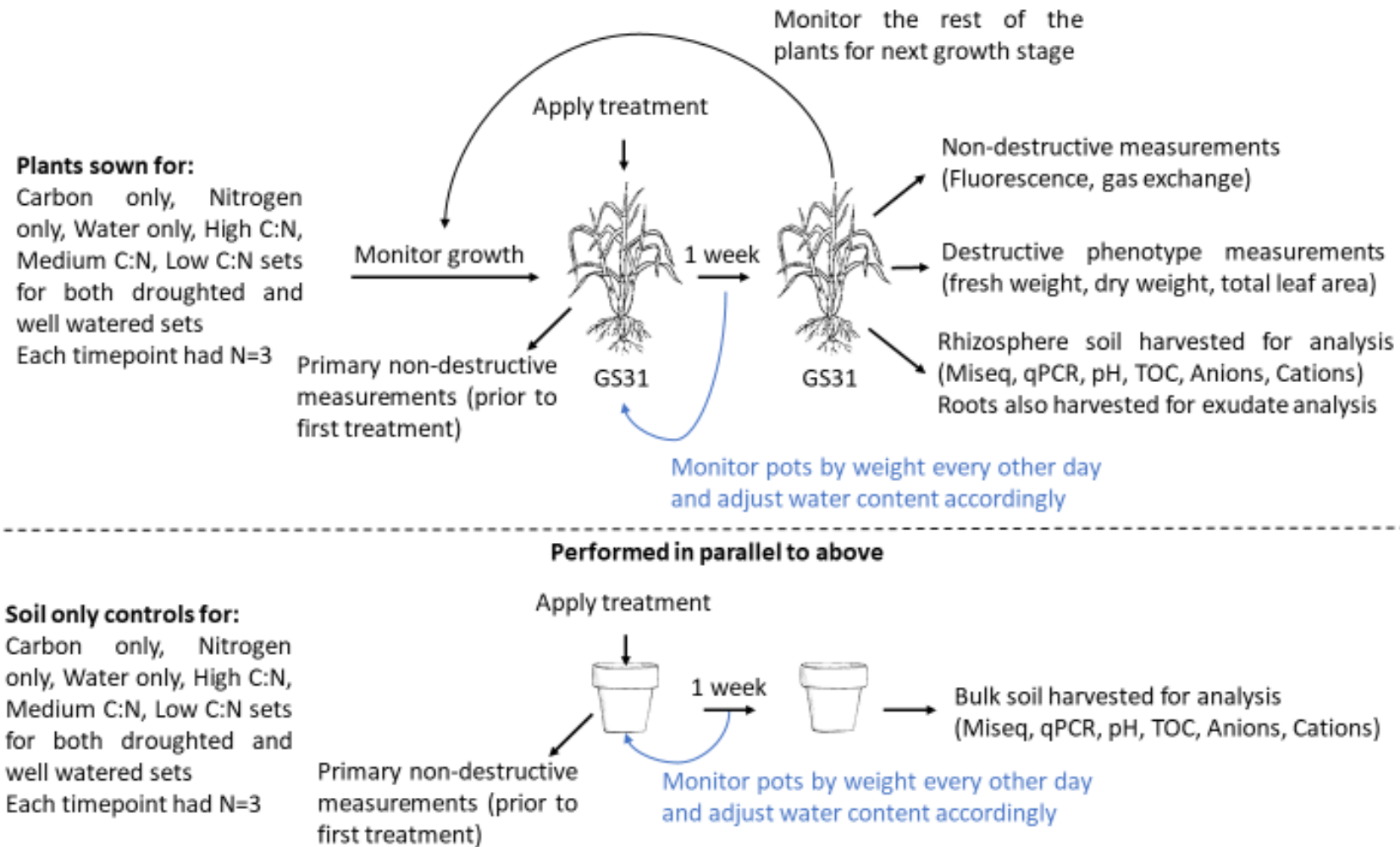


Figure 5.2: Experimental plan diagram summarising the steps taken in the experiment, where drought is considered 40% soil water content

5.3 Results

5.3.1 Plant physiology

Plants were measured at each growth stage (GS31, GS39 and GS61) for dark adapted fluorescence, F_v/F_m to assess PSII condition, infrared gas exchange to assess the activities of photosynthesis, and biomass parameters (wet biomass, dry biomass, leaf area) to evaluate whether treatment effects were realised in growth parameters. Below we show the results from the dark-adapted fluorescence values (F_v/F_m), gas exchange analysis (A/Q and A/C_i) and biomass harvesting.

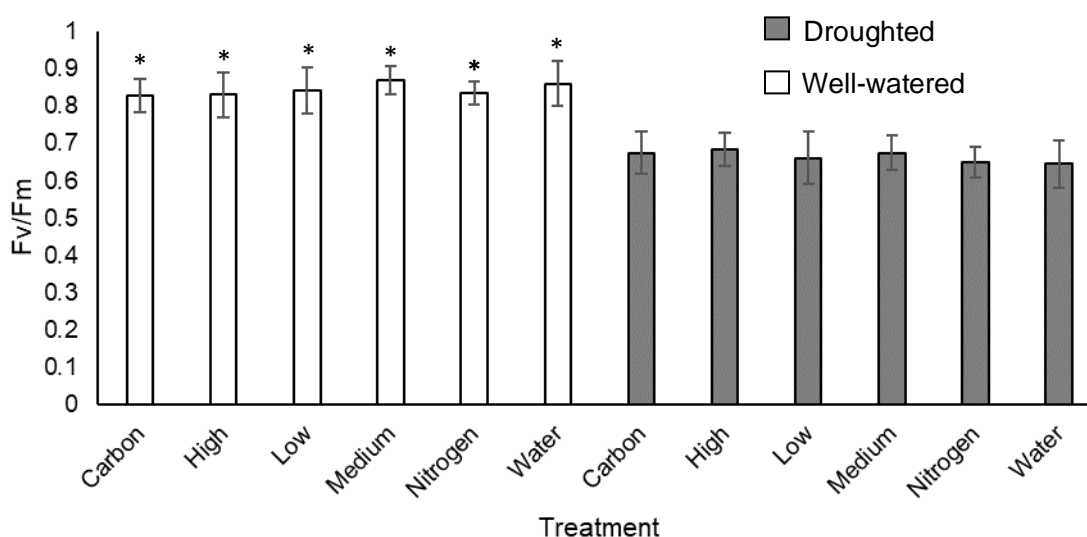


Figure 5.2: Average F_v/F_m measurements across the experiment for *Triticum aestivum* cv Mulika plants that have either experienced a high (32.58), medium (17.22), or low (9.37) C:N fertiliser treatment, or glucose, $\text{NH}_4^+\text{NO}_3^-$, or water only treatments under a well-watered or droughted condition. Error bars are \pm s.d n = 5, * denotes significant differences from the respective drought treatment.

Five plants were measured from each treatment at each growth stage (GS31, GS39, GS61) in both the drought and well-watered groups for dark adapted fluorescence (F_v/F_m). We found no significant F_v/F_m differences between treatments within the drought or well-watered groups when compared amongst themselves ($P > 0.05$). Similarly, we did not find any significant differences within the well-watered and droughted groups at each growth stage ($P > 0.05$). However, when comparing treatments across well-watered and droughted groups we found significant differences at each growth stage ($P < 0.05$), even when ignoring growth stage (Figure 5.2).

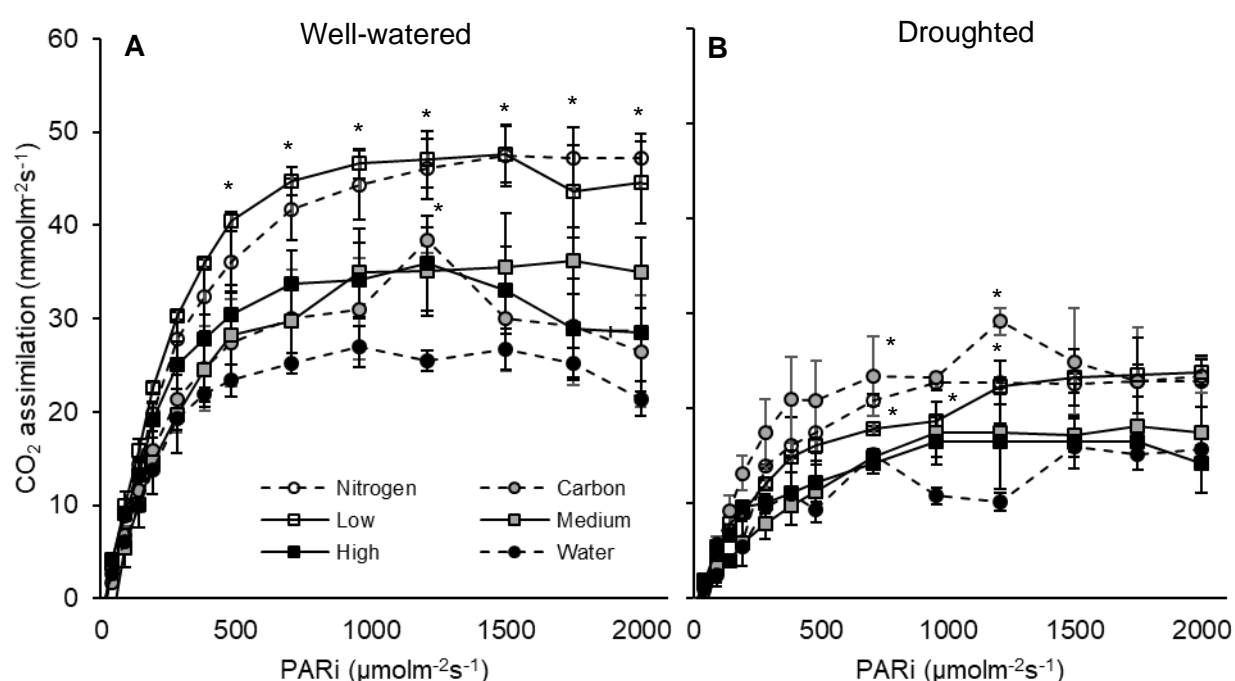


Figure 5.3: Assimilation against light intensity (PAR_i) GS61 *Triticum aestivum* cv Mulika when treated with high (32.58), medium (17.22) and low (9.37) C:N ratios in the form of glucose:NH₄⁺NO₃⁻, carbon only, or nitrogen only, for both droughted and well-watered conditions. Error bars represent ± s.e.m, n = 3

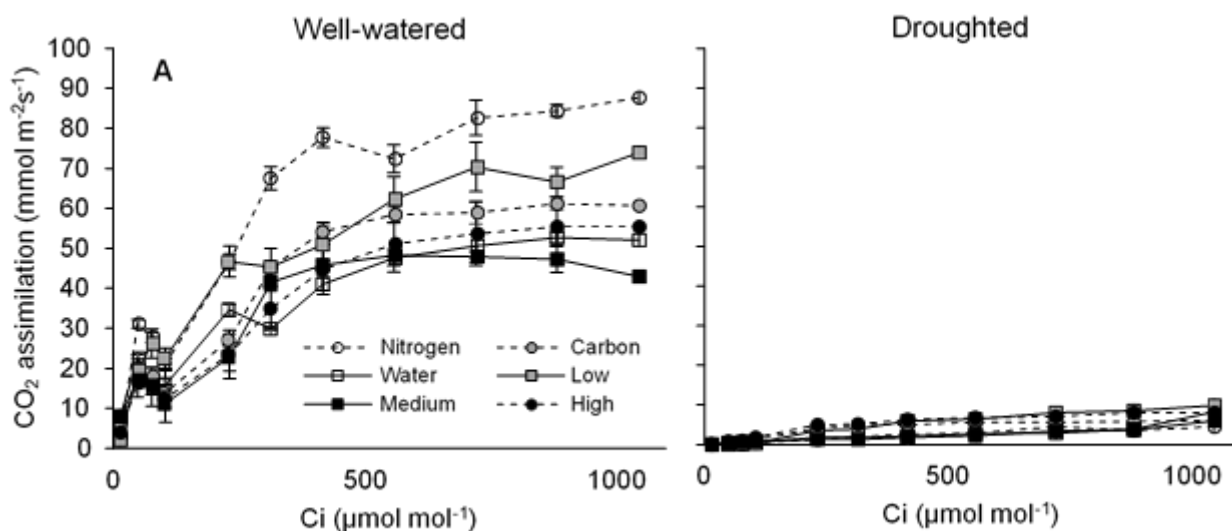


Figure 5.4: Assimilation against internal CO₂ concentration (Ci) G631 *Triticum aestivum* cv Mulika when treated with high (32.58), medium (17.22) and low (9.37) C:N ratios in the form of glucose:NH₄⁺NO₃⁻, carbon only, or nitrogen only, for both droughted and well-watered conditions. Error bars represent \pm s.e.m, n = 3

For the well-watered group, we observed significant CO₂ assimilation differences between nitrogen-only and low C:N treatments above 500 PAR_i (Figure 5.3A) when compared to water-only controls ($P < 0.05$). At 1250 PAR all treatments were significantly higher than water-only treatment ($P < 0.05$), but this significance was lost for all treatments aside from nitrogen-only and low C:N treatments (Figure 5.3). However, without significance the water-only treatment was consistently lower than all other treatments above 400 PAR_i (Figure 5.3A). The high C:N, medium C:N, and carbon-only treatments grouped together above water-only, and the low C:N and nitrogen-only treatments paired as the highest two (Figure 5.3A).

Large variation in values were seen within treatments for the droughted group (Figure 5.3B), which may explain the lack of significance observed. Most groups had significantly higher CO₂ assimilation at 1000 PAR and 1250 PAR ($P < 0.05$) when compared with

water-only, except for the high C:N treatment which displayed significant variation (Figure 5.3B). The droughted group responded similarly in assimilation to the well-watered group in order, with low C:N and nitrogen-only treatments having the highest assimilation. However, the carbon-only group joined the low C:N and nitrogen-only treatments with higher assimilation above 1250 PAR_i (Figure 5.3B). Additionally, the water-only treatment was indistinguishable in values from medium and high C:N treatments above 1500 PAR_i, which was not observed in the well-watered group (Figure 5.3).

We found significant differences ($P < 0.05$) when each well-watered treatment was compared to its droughted counterpart (Figure 5.3A,B). This was seen at every PAR_i except for PAR_i 2000, where water-only treatments were insignificantly different from each other (Figure 5.3).

The A/C_i curves for the well-watered group (Figure 5.4A) showed similar results to the A/Q curves (Figure 5.3A), with the nitrogen-only and low C:N treatments having the highest assimilation (Figure 5.4A). However, nitrogen-only displayed a significantly higher ($P < 0.05$) assimilation compared to all other groups at several C_i concentrations above 300 C_i (Figure 5.4A), which was not observed in the A/Q curves. Additionally, at C_i 1000 all groups showed significantly higher assimilation ($P < 0.05$) than the water-only group. Unfortunately, due to poor performance in the droughted group it was not possible to analyse the curves (Figure 5.4B). All assimilations for the droughted group were below 10 $\text{mmolm}^{-2}\text{s}^{-1}$ with no significant differences appearing ($P > 0.05$).

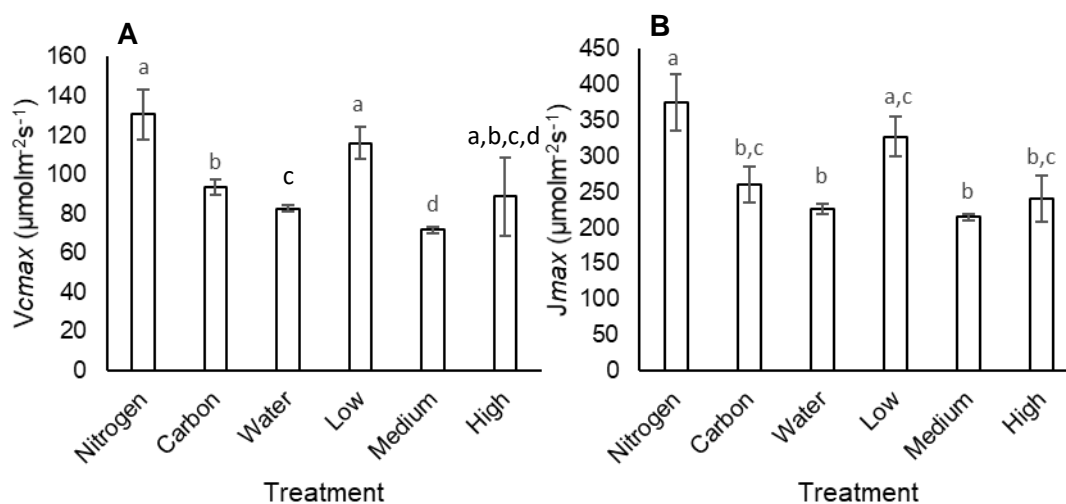


Figure 5.5: Gas exchange parameters V_{cmax} and J_{max} analysed from ACi curves using the 2016 edition of Sharkey's model (Sharkey 2016) for *Triticum aestivum* cv Mulika treated with glucose, $\text{NH}_4^+\text{NO}_3^-$, water, or C:N (glucose: $\text{NH}_4^+\text{NO}_3^-$) ratio fertilisers in high (32.58), medium (17.22), or low (9.37) treatments. Due to poor curve quality it was not possible to extract V_{cmax} and J_{max} values for droughted plants. Error bars are \pm s.e.m., different lowercase letters denote significant differences, $n = 3$.

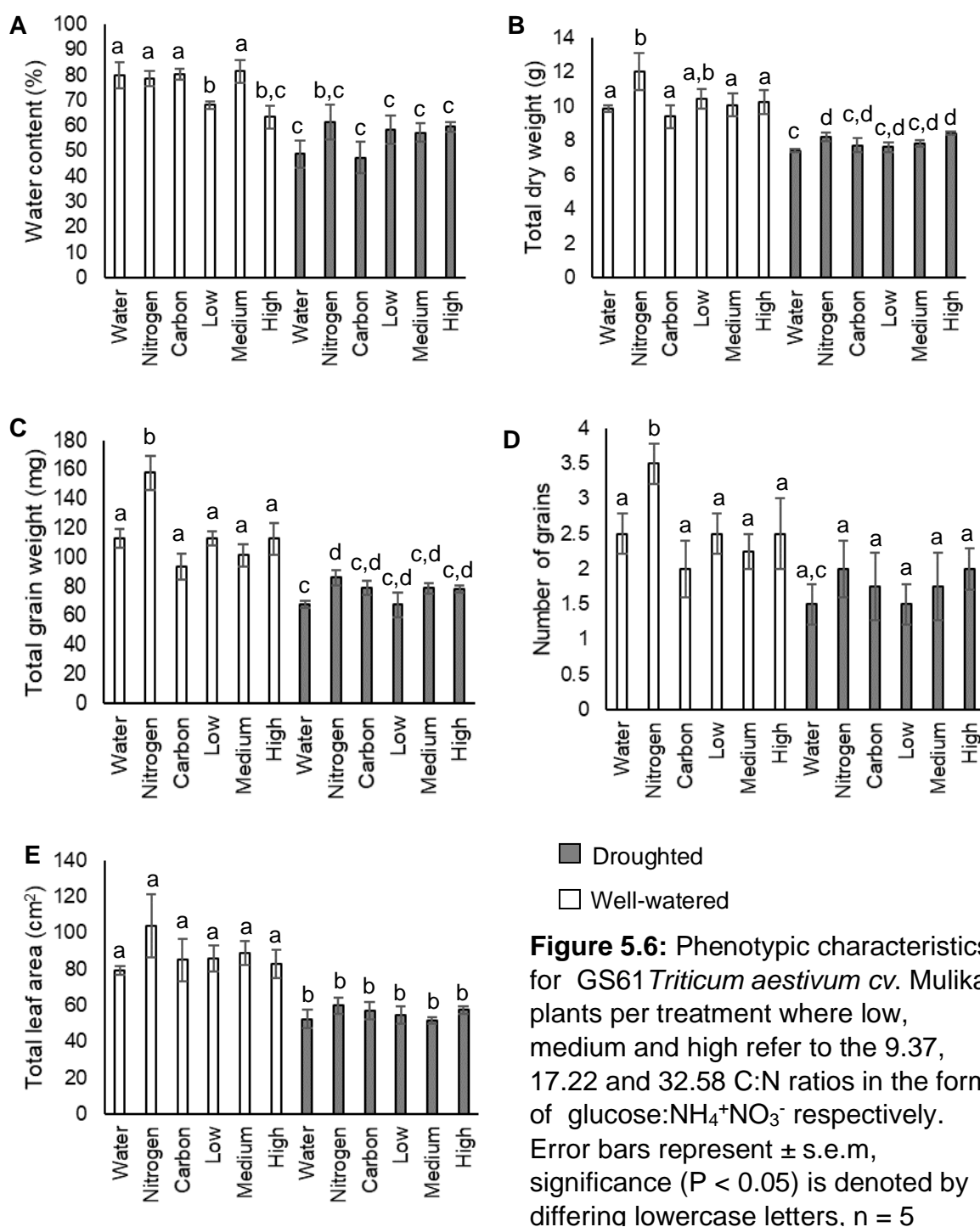
Nitrogen-only and low C:N treatments had significantly ($P < 0.05$) higher V_{cmax} values than all other treatments (Figure 5.5A) apart from high C:N which had a large variation and was insignificant from all other treatments ($P > 0.05$). The medium C:N treatment had significantly lower V_{cmax} values than other treatments ($P < 0.05$), with water-only being significantly higher than medium C:N, and carbon-only being significantly higher than water-only (Figure 5.5A).

Water-only and medium C:N treatments had significantly lower ($P < 0.05$) J_{max} than nitrogen-only and low C:N treatments, which had the highest and second highest J_{max} values respectively (Figure 5.5B). The carbon-only and high C:N treatments had lower J_{max} than nitrogen-only and low C:N, but higher J_{max} values than water-only and medium C:N treatments (Figure 5.5B). However, these differences were only significantly lower than the nitrogen-only treatment, with all other treatments insignificant from carbon-only

and high C:N treatment J_{max} (Figure 5.5B). As the Sharkey model requires full curves to estimate V_{cmax} and J_{max} values it was not possible estimate V_{cmax} and J_{max} for the droughted group due to poor curve performance (Figure 5.5B).

In general, we observed that water content was significantly higher ($P < 0.05$) for the well-watered group compared to the droughted group (Figure 5.6A). However, significant differences ($P > 0.05$) were lost between the well-watered low C:N treatment group and droughted nitrogen-only group (Figure 5.6A). Additionally, the well-watered high C:N treatment did not significantly differ ($P > 0.05$) from the droughted group (Figure 5.6A).

Similarly, we observed significantly higher ($P < 0.05$) total dry weights for the well-watered group when compared to the droughted group (Figure 5.6B). We found the well-watered nitrogen-only treatment was the only treatment to display significantly higher dry weight ($P < 0.05$). In the droughted group the water-only group had significantly lower ($P < 0.05$) dry weight than all other treatments (Figure 5.6B). These statistical patterns were repeated for the total grain weight, yet in the droughted group nitrogen-only was also significantly higher than the water-only treatment (Figure 5.6C). We also observed the same pattern for number of grains, with well-watered nitrogen-only treatment displaying significantly higher ($P < 0.05$) number of grains (Figure 5.6D).



The well-watered group had significantly higher total leaf area ($P < 0.05$) than the droughted group, but no significance ($P > 0.05$) was observed between the treatments (Figure 5.6E).

5.3.2 Soil microbial community and physicochemical properties

Similar to chapter 4 we will now consider the belowground microbiota, followed by the root exudate analysis and then soil physicochemical data. Due to the volume of data we present only the rhizosphere:soil abundances previously eluded to in chapter 4, complete counts of abundances will be provided in an appendix table format for further detail. We finish with a principle component analysis (PCA) of all variables during this experiment to help interpret our data and provide some potential insight into future projects.

Similar to chapter 4, we observed the same value ranges for each gene abundance. However, observed a difference in rhizosphere:bulk soil relative abundance comparisons. In general, relative abundances were lower in GS31 than in GS61 for both droughted and well-watered groups (Figure 5.7). The droughted group tended to have significantly ($P < 0.05$) higher relative abundance at GS61 than well-watered equivalents (Figure 5.7).

Bacterial 16S rRNA showed little difference at GS31 (Figure 5.7A), with only low and medium C:N treatments showing significant differences ($P < 0.05$) between droughted and well-watered counterparts. However, by GS61 we saw that every treatment had significantly higher relative abundance compared to well-watered counterparts (Figure 5.7A). This pattern of higher relative abundance under drought at GS61 compared to well-watered GS61 was seen for most genes with the exception of archaeal 16S rRNA gene relative abundances (Figure 5.7A-G) which showed significantly higher ($P < 0.05$) relative abundances for carbon and water-only well-watered treatments at GS61 than droughted equivalents at GS61 (Figure 5.7B).

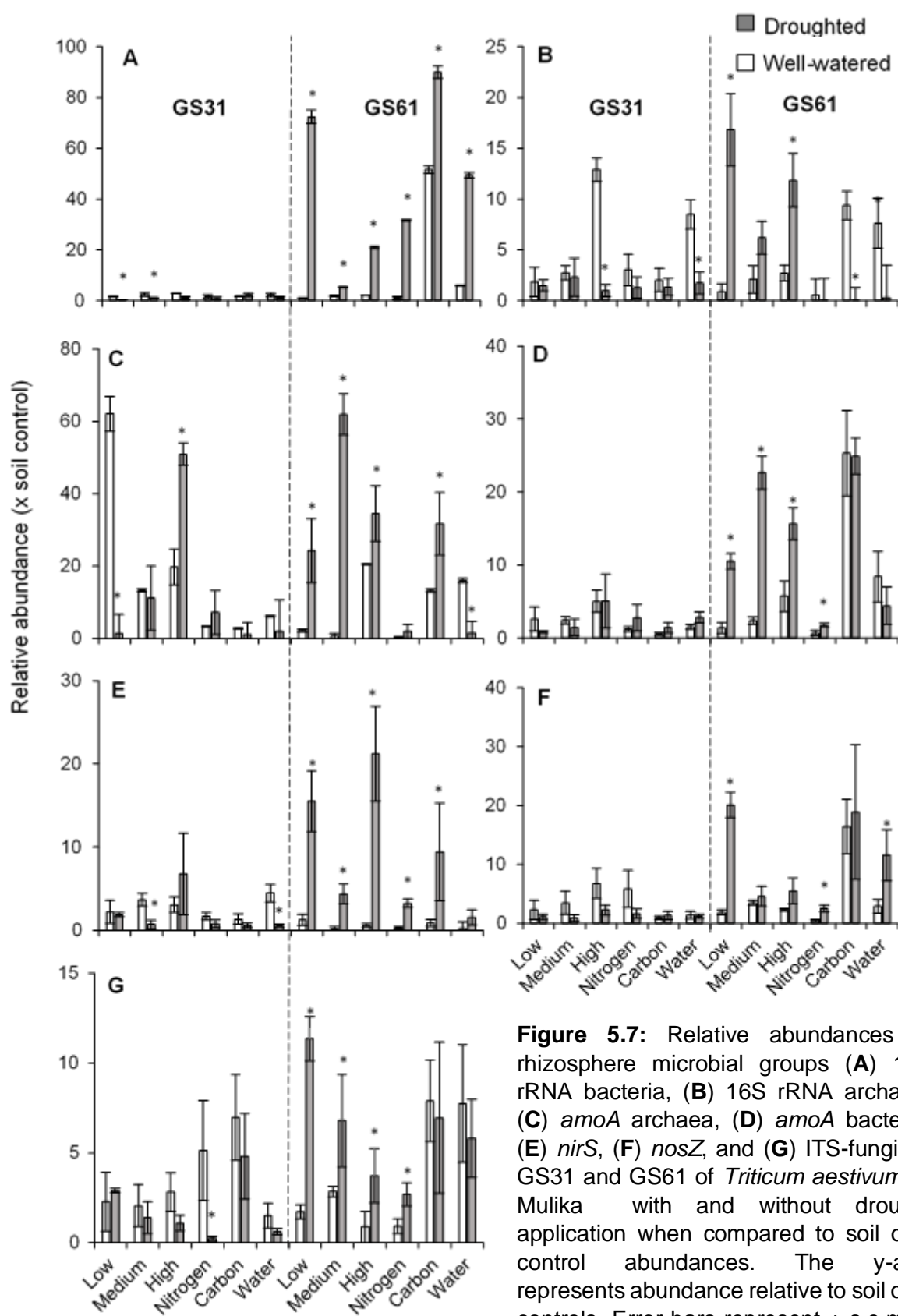


Figure 5.7: Relative abundances of rhizosphere microbial groups (A) 16S rRNA bacteria, (B) 16S rRNA archaea, (C) *amoA* archaea, (D) *amoA* bacteria, (E) *nirS*, (F) *nosZ*, and (G) ITS-fungi for GS31 and GS61 of *Triticum aestivum* cv Mulika with and without drought application when compared to soil only control abundances. The y-axis represents abundance relative to soil only controls. Error bars represent \pm s.e.m, n = 3, * denotes significance between watered equivalents

AOA and AOB had showed similar patterns of change, but AOA was more pronounced in the degree of change (Figure 5.7C). We observed AOA to have significantly higher relative abundance ($P < 0.05$) in all C:N treatments and carbon-only treatments experiencing drought when compared to well-watered equivalents at GS61 (Figure 5.7C), with water-only controls showing significantly lower relative abundances at GS61. At GS31 AOA demonstrated significantly ($P < 0.05$) higher relative abundances when well-watered and treated with low C:N fertiliser and significantly higher ($P < 0.05$) relative abundances under drought when high C:N treatment was received (Figure 5.7C). On the other hand, AOB had no significant differences ($P > 0.05$) at GS31 regardless of nutrient treatment or watering status (Figure 5.7D). However, N-only and all C:N treatments had significantly higher ($P < 0.05$) relative abundances for AOB at GS61 (Figure 5.7D).

Apart from well-watered medium C:N treated having significantly ($P < 0.05$) higher relative *nirS* abundance than droughted equivalents (Figure 5.7E), *nirS* had no significant differences at GS31. By GS61, *nirS* had significantly greater ($P < 0.05$) relative abundance in all droughted treatments (Figure 5.7E) with exception to water-only treatment which showed no difference ($P > 0.05$). Whilst *nosZ* on the other hand showed no significant differences ($P > 0.05$) at GS31 between well-watered and droughted groups (Figure 5.7F). Yet, at GS61, there were significantly higher ($P < 0.05$) *nosZ* relative abundances in droughted low C:N, N-only, and water only treatments (Figure 5.7E).

ITS showed significantly lower ($P < 0.05$) relative abundances at GS31 in the droughted group (Figure 5.7F). Whilst at GS61 ITS displayed significantly higher ($P < 0.05$) relative abundances for all C:N treatments and N-only under drought (Figure 5.7F).

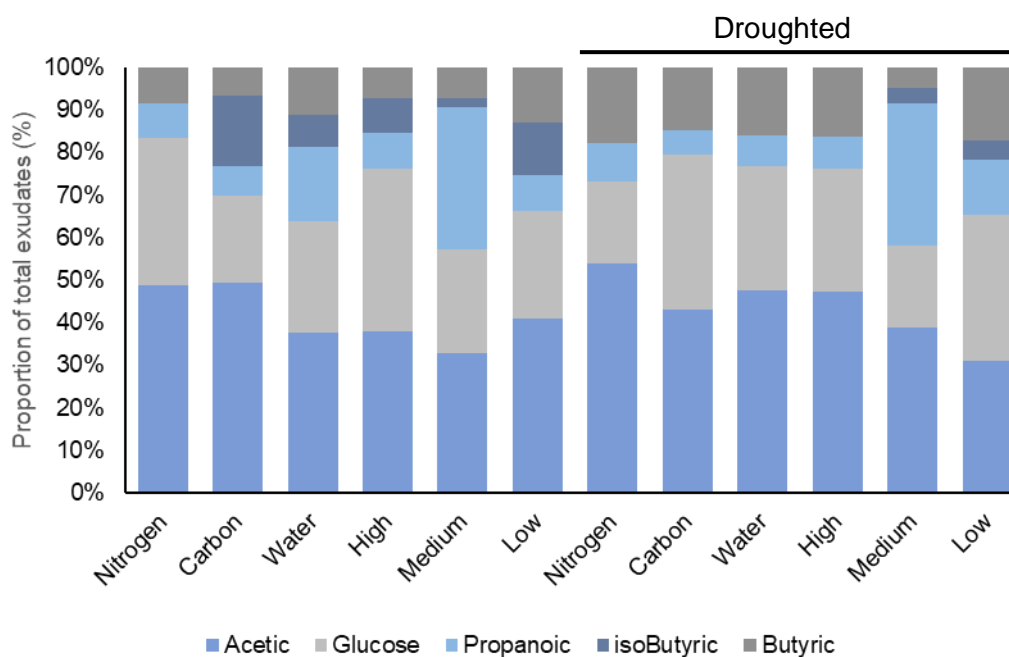


Figure 5.8: Internal root exudates of GS61 *Triticum aestivum* cv Mulika when subject to a well-watered or droughted watering regime and either nitrogen-only ($\text{NH}_4^+\text{NO}_3^-$), carbon-only (glucose), water-only or a high (32.58), medium (17.22), or low (9.37) C:N fertilisation treatment, $n=3$

As in chapter 4, we have omitted inconsistently appearing unidentified metabolites due to troubles quantifying and analysing them. Here we again focus on the five compounds detected and analysed during the VFA protocol: acetic acid, propanoic acid, isobutyric acid, butyric acid, and glucose present, pin the internal standards as we were able to produce concentrations and therefore present them as a total of exudation.

On average, we observed that acetic acid was the most abundant compound and comprised between 6% to 49% of total internal root exudation (Figure 5.8). The second most concentrated compound was glucose (15% – 38%), followed by butyric (4%)– 18%), propanoic (8% – 33%), and isobutyric (2% –17%) respectively (Figure 4.8).

When comparing droughted with well-watered we found that well-watered low C:N plants had significantly ($P < 0.05$) higher proportion of acetic acid at GS61 for internal root exudates. Conversely, water-only and high C:N droughted treatments had higher acetic acid, although the difference was insignificant ($P > 0.05$). Well-watered nitrogen and droughted carbon treatments had significantly higher glucose ($P < 0.05$) proportions than their counterparts (Figure 5.8). The only other significantly higher proportions we observed for internal root exudates were propanoic acid in well-watered treatments ($P < 0.05$) and isoButyric acid in carbon-only well-watered treatments ($P < 0.05$).

When observing the soil physicochemical properties, we observed few significant differences for the well-watered group, with more significances being found in the droughted group when comparing treatments to their respective water-only controls (Table 5.1, Table 5.2). TOC, TIC, and pH showed no significant differences ($P > 0.05$), with pH ranging from 6.6 (± 0.42) – 6.8 (± 0.18). We shall, as in chapter 4 start with anions and then move onto cations.

We observed significant increases ($P < 0.05$) in $[\text{NO}_2^-]$ for every treatment in the well-watered group except for N-only when compared to water-only (Table 5.1). These results were similar for $[\text{NO}_3^-]$, yet N-only was significantly higher ($P < 0.005$) and C-only was

not (Table 5.1). These findings were similar for the droughted group, with only medium and high C:N $[\text{NO}_2^-]$ being insignificant from water-only ($P > 0.05$), whilst $[\text{NO}_3^-]$ showed the same pattern as the well-watered group (Table 5.1). All other anions demonstrated no pattern in the well-watered group, with significant decreases ($P < 0.05$) found for acetate in N-only and $[\text{Cl}^-]$ for C-only and medium C:N (Table 5.1). Whilst N-only and C-only well-watered treatments had significantly higher ($P < 0.05$) $[\text{SO}_4^-]$ (Table 5.1) and showing significantly lower ($P < 0.05$) levels in all C:N treatments for the droughted group (Table 5.1). However, $[\text{F}^-]$ was significantly lower ($P < 0.05$) in all droughted treatments when compared to the water-only control (Table 5.1).

Cations showed much greater increases in concentration for the droughted group (Table 5.2). Every cation had significantly higher ($P < 0.05$) concentrations in all droughted treatments when compared with water-only (Table 5.2) except for $[\text{Ca}_2^+]$ that was found only to be significantly higher ($P < 0.05$) in medium C:N treatments. For the well-watered group, we observed no significant differences ($P > 0.05$) were for $[\text{Ca}_2^+]$, $[\text{Mg}_2^+]$, $[\text{K}^+]$, or $[\text{Na}^+]$ cations (Table 5.2). However, significant increases ($P < 0.01$) were found for $[\text{NH}_4^+]$ for all treatments except for C-only ($P > 0.05$) and $[\text{Li}^+]$ for all but N-only and medium C:N treatments (Table 5.2).

Table 5.1: Bulk soil anion concentrations throughout the experiment for pots with plants, no significant differences were found between timepoints, drought-experiencing plants are shaded grey. Ion average \pm (s.e.m), $n = 3$. $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.005$ (***) significances are relative to the relevant water control.

Treatment	Fluoride (ppm)	Acetate (ppm)	Formate (ppm)	Chloride (ppm)	Nitrite (ppm)	Nitrate (ppm)	Sulphate (ppm)
Water	2.02 \pm (0.21)	77.4 \pm (2.3)	n.a.	132.0 \pm (6.6)	9.53 \pm (0.53)	17.99 \pm (0.69)	174.2 \pm (12.1)
Nitrogen	1.91 \pm (0.17)	64.2 \pm (3.2)*	n.a.	109.5 \pm (10.9)	8.42 \pm (0.64)	29.82 \pm (1.58)***	196.3 \pm (7.5)**
Carbon	1.98 \pm (0.14)	68.4 \pm (6.1)	0.49 \pm (0.25)	93.9 \pm (17.1)*	3.93 \pm (0.28)***	11.59 \pm (1.23)	134.9 \pm (4.8)**
Low	1.97 \pm (0.20)	74.9 \pm (3.7)	0.49 \pm (0.31)	166.3 \pm (14.9)	7.32 \pm (0.37)*	30.74 \pm (2.27)***	172.7 \pm (12.7)
Medium	2.15 \pm (0.17)	69.2 \pm (5.5)	0.33 \pm (0.17)	97.6 \pm (5.2)*	7.35 \pm (0.47)**	24.38 \pm (0.93)**	162.4 \pm (12.1)
High	1.79 \pm (0.13)	64.6 \pm (4.8)	n.a.	153.0 \pm (10.7)	5.17 \pm (0.29)***	29.82 \pm (1.56)***	183.1 \pm (10.2)
Water	3.85 \pm (0.27)	65.6 \pm (4.6)	0.41 \pm (0.11)	182.7 \pm (9.1)	20.94 \pm (1.1)	15.56 \pm (0.92)	235.8 \pm (9.1)
Nitrogen	1.77 \pm (0.18)***	66.2 \pm (2.6)	n.a.	204.5 \pm (7.0)	1.59 \pm (0.12)***	27.71 \pm (1.76)***	209.7 \pm (12.4)
Carbon	1.12 \pm (0.63)***	69.0 \pm (2.1)	0.36 \pm (0.23)	166.1 \pm (6.6)	8.79 \pm (0.84)***	14.28 \pm (1.17)	240.3 \pm (21.7)
Low	2.27 \pm (0.20)***	70.9 \pm (4.9)	n.a.	165.4 \pm (7.3)	8.82 \pm (0.81)***	36.1 \pm (1.85)***	167.4 \pm (6.8)**
Medium	1.71 \pm (0.17)***	61.7 \pm (6.1)	n.a.	160.7 \pm (9.1)	18.71 \pm (1.63)	20.87 \pm (0.64)***	181.1 \pm (18.5)**
High	2.03 \pm (0.08)***	65.0 \pm (5.2)	0.27 \pm (0.12)	175.5 \pm (13.5)	20.94 \pm (1.21)	38.81 \pm (1.65)***	163.6 \pm (4.4)***

Table 5.2: Bulk soil cation concentrations throughout the experiment for pots with plants, no significant differences were found between timepoints, drought-experiencing plants are shaded grey. Ion average \pm (s.e.m), $n = 3$. $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.005$ (***) significances are relative to the relevant water control.

Treatment	<i>Lithium (ppm)</i>	<i>Sodium (ppm)</i>	<i>Ammonium (ppm)</i>	<i>Potassium (ppm)</i>	<i>Magnesium (ppm)</i>	<i>Calcium (ppm)</i>
Water	0.39 \pm (0.04)	1702 \pm (112)	2.89 \pm (0.09)	7.84 \pm (0.24)	151.8 \pm (13.6)	2582 \pm (180)
Nitrogen	0.27 \pm (0.05)	1812 \pm (181)	6.81 \pm (0.11)***	7.02 \pm (0.54)	170.2 \pm (15.3)	2835 \pm (113)
Carbon	0.49 \pm (0.04)**	1993 \pm (159)	3.16 \pm (0.19)	7.52 \pm (0.38)	147.5 \pm (10.3)	2544 \pm (178)
Low	0.12 \pm (0.05)***	1933 \pm (96)	5.53 \pm (0.38)***	9.97 \pm (1.03)	191.6 \pm (7.6)	3337 \pm (200)
Medium	0.44 \pm (0.03)	1256 \pm (113)	6.62 \pm (0.23)***	7.79 \pm (0.11)	118.2 \pm (11.8)	2126 \pm (106)
High	0.22 \pm (0.03)***	2056 \pm (205)	5.93 \pm (0.36)***	7.05 \pm (0.54)	180.1 \pm (9.6)	3008 \pm (240)
Water	0.36 \pm (0.02)	1159 \pm (194)	3.18 \pm (0.32)	1.29 \pm (0.05)	223.6 \pm (6.7)	3040 \pm (91)
Nitrogen	0.52 \pm (0.01)***	2296 \pm (90)***	9.66 \pm (0.46)***	8.97 \pm (0.95)***	270.6 \pm (27.1)*	3391 \pm (231)
Carbon	0.55 \pm (0.04)***	3156 \pm (157)***	2.61 \pm (0.18)*	6.83 \pm (0.61)***	619.6 \pm (55.7)***	3236 \pm (226)
Low	0.83 \pm (0.01)***	2762 \pm (105)***	11.65 \pm (0.14)***	4.69 \pm (0.28)***	544.7 \pm (13.0)***	3398 \pm (143)
Medium	0.61 \pm (0.03)***	2465 \pm (221)***	7.58 \pm (0.39)***	8.88 \pm (0.89)***	609.6 \pm (6.2)***	3695 \pm (221)**
High	0.69 \pm (0.02)***	2702 \pm (51)***	9.23 \pm (0.37)***	8.15 \pm (0.41)***	729.4 \pm (9.0)***	3301 \pm (207)

We performed a PCA on all the variables present in this study for each group at GS61. Overall, there is quite a lot of variation as seen by the 95% confidence ellipses being so large (Figure 5.9). However, in general, we can see a separation between droughted and well-watered on dimension 1, with the droughted group being tightly separated to the left from the well-watered treatments on the right, explaining 79.7% of the variance. The well-watered treatments show quite a bit of variation in range and show little separation between the treatments (Figure 5.9), whilst drought shows almost no separation between treatments.

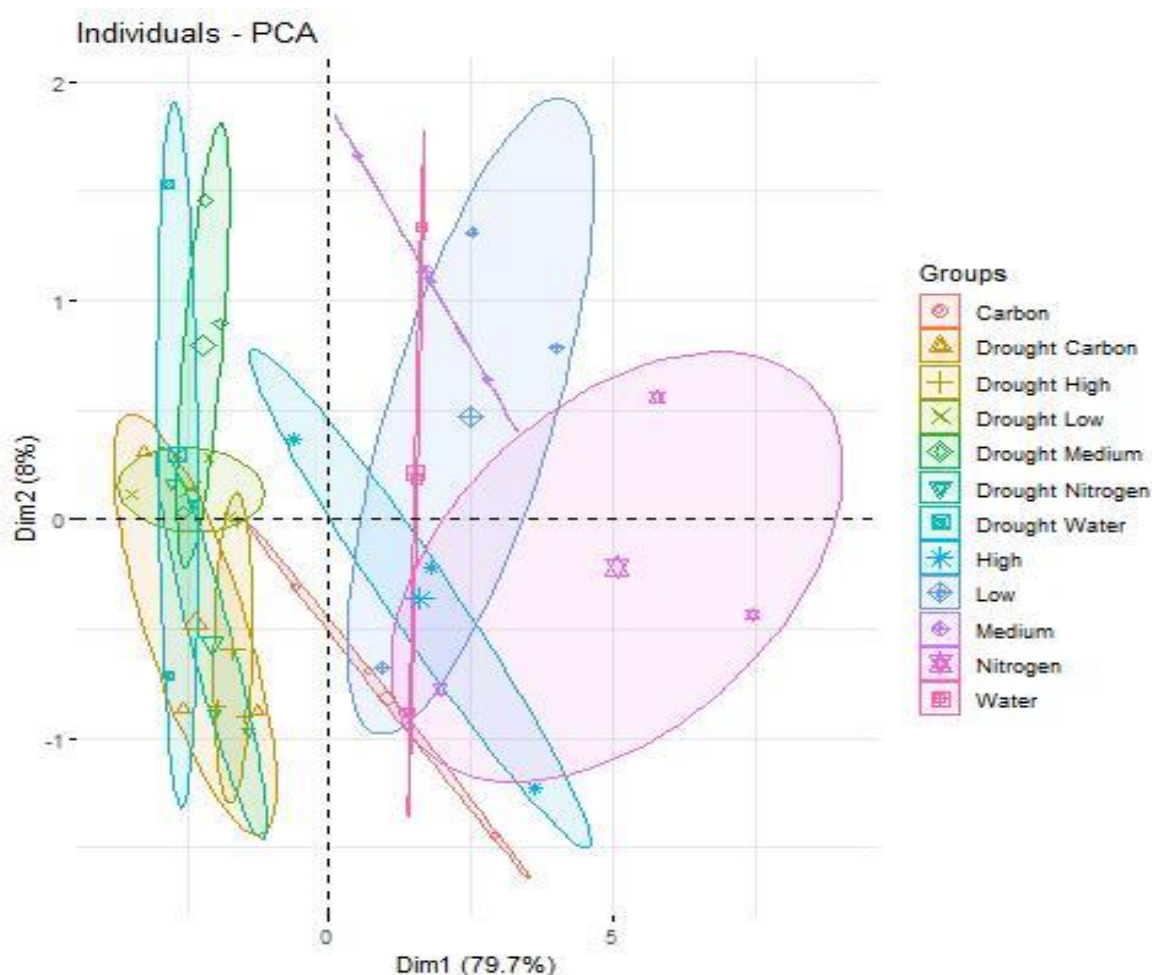


Figure 5.9: Principle component analysis of aboveground and belowground portions for GS61 *Triticum aestivum* cv Mulika when subject to a well-watered or droughted watering regime and either nitrogen-only ($\text{NH}_4^+\text{NO}_3^-$), carbon-only (glucose), water-only or a high (32.58), medium (17.22), or low (9.37) C:N fertilisation treatment, ellipses represent a 95% CI boundary

Additional MiSeq analysis on these samples unfortunately came out at a low read quality, with short fragments being amplified across the samples, rendering them unusable, with under 2% of total reads being considered usable quality (assuming ~18M total reads). 684 samples across all genes on two plates typically demonstrated low read numbers <1,000 per sample, with most reads being filtered out due to poor read quality. These libraries consequently failed to cluster into OTUs and appeared to be short fragments of random sequencing. As a result, we could not include them in the analysis. It was thought odd primers were used or that oligos were synthesised incorrectly.

5.4 Discussion

Here we have investigated the effects of both a C:N manipulation and drought-implementation on *Triticum aestivum* cv Mulika and its associated microbial communities. As we have previously addressed and discussed the C:N aspect in chapter 4 this discussion will centre around the droughted treatment. Whilst we will address the C:N effect it will mainly be in relation to the effect drought has had on the C:N. We ask you to please refer to the discussion in chapter 4 for detailed commentary on the C:N effects.

Two of the most essential environmental factors that affect plant health and growth are nitrogen and water with multiple prior studies investigating these effects in wheat (Allahverdiyev, 2016; Lopes & Araus, 2006; Wang *et al.*, 2016). Drought stress has many damaging impacts on plant physiology, and is known to alter fluorescence parameters, induce signalling pathways via gene upregulation, and alter metabolite concentrations (Farooq *et al.*, 2009; Fleury *et al.*, 2010; Reddy & Guerrero, 2004; Valliyodan & Nguyen, 2006; Zlatev, 2009). The aim is to achieve balance between preventing water loss,

preventing molecular damage via ROS generation, and maintaining a photosynthetic capacity (Farooq *et al.*, 2009).

We observed under droughted conditions a large decline in F_v/F_m , previously seen in many other studies (Wang *et al.*, 2016). This decrease in maximum photochemical efficiency has a knock-on effect by limiting the plants ability to utilise light, particularly high-intensity light, which would otherwise need to be dissipated via NPQ or risk photodamage (Ruban, 2015). This is resulting from a decreased of PSII quantum yield (Papageorgiou, 2011; Stirbet, 2011), which overtime results net photosynthesis (P_n) decreases (Tezara *et al.*, 2005; Wang *et al.*, 2016) and is demonstrated by our gas exchange data.

At GS61 we saw a significant decline in A/Q performance when drought was applied to plants, with a subsequent failure to perform in A/C_i curves. These results demonstrate a decrease in stomatal conductance (G_s) with a subsequent limitation of P_n . Although P_n is also known to be affected by many non-stomatal factors (Farooq *et al.*, 2009), it is quite likely ours is primarily stomatal as stomata close under drought stress to conserve water through improving water use efficiency (WUE), changing epidermal patterning factors for long-term responses (Dunn *et al.*, 2019; Wang *et al.*, 2016). Nitrogen application has been known to improve ϕ_{PSII} and somewhat reduce the negative effects of drought or nutrient stress (Wang *et al.*, 2016), which we have previously eluded to in chapter 3. However, our lower A/Q curves for the droughted group indicates the ineffective use of light by droughted plants, which is most likely due to a combination of reduced G_s and

photodamage to key proteins such as RuBisCO via ROS generation, limiting the plants' responses to N-application.

Generally, to assess A/C_i curves we utilise the two parameters introduced in chapter 3, J_{max} and V_{cmax} . These parameters give us an indication on electron regeneration via RuBisCO and the ability for RuBisCO to bind CO_2 (Sharkey, 2016). The observed J_{max} (the maximum rate of electron transport) values of N-only and low C:N treatments being significantly higher than other treatments implies N-fertilised plants are more efficient at utilising high light intensities. These results have been previously shown numerous times (Dunn *et al.*, 2019; Wang *et al.*, 2016), with multiple studies in wheat (Dunn *et al.*, 2019; Wang *et al.*, 2016). The maximum rate of electron transport rate has a positive feedback loop on preventing damage. If a plant has a low J_{max} they are slower at utilising incoming light, the rate of incoming energy (as light) is then more likely to be higher than the rate of utilisation (electron transport rate). This imbalance between incoming energy and energy utilisation creates an excess of energy that needs dissipation to prevent damage from occurring. To prevent this damage the plant would then need to invest stored energy (in the form of organics) in the xanthophyll cycle and NPQ systems so excess energy is dissipated. Whilst this prevents further damage to the plant, potentially avoiding photoinhibition, it also stops any investment in light-harvesting complexes and chloroplast formation, which leads to a lower J_{max} value, and thus exacerbates the cycle.

Our V_{cmax} (maximum rate of RuBisCO carboxylation) values display a similar story, with N-only and low C:N treatments being higher than other treatments. Again, there is a positive feedback loop, with damage preventing investment in RuBisCO formation.

However, this cycle is perhaps more dependent on N than J_{max} , with N-fertilisation previously being noted to increase V_{cmax} through RuBisCO upregulation (Hakeem *et al.*, 2012; Heidlebaugh *et al.*, 2008; McGuire *et al.*, 1995; Sims *et al.*, 1998). Unfortunately, due to the poor curve performance in droughted groups it was not possible to get J_{max} and V_{cmax} values. Yet, it should be noted that V_{cmax} is calculated from the steepness of the curve before the inflexion point, and J_{max} from that plateaued level of the typical sigmoidal curve seen in A/Q and A/C_i curves. It can be inferred from this knowledge that the droughted group would have had much lower J_{max} and V_{cmax} values, although it cannot be confirmed whether these values would have been significantly lower. We can however see from our A/Q results that the droughted group certainly have lower assimilation rates than the well-watered group, which is further supported by our phenotypic results.

Although these differences in assimilation can seem minor and insignificant to begin with, these changes in P_n can have drastic implications for plant growth and performance when tracked over a growing season. As our phenotype results show, well-watered counterparts tended to be bigger in mass, leaf area, and had larger grains. This is expected due to stomatal induced P_n inhibition through lower G_s . As previously noted, when drought is experienced plants close stomata to increase WUE (Dunn *et al.*, 2019), limiting assimilation (Wang *et al.*, 2016), and restricting growth. Even though we see large differences in assimilation even the small differences can be distinguished by phenotype as the assimilation difference builds up over time.

A reduction in P_n limits carbohydrate production, which reduces growth rates, limiting the total leaf area for P_n . This positive feedback loop limits grain productivity in the plant, with droughted or N-restricted plants producing fewer and smaller grains. The number of grains is also affected by the density of planting, with densely planted fields producing fewer grains per ha⁻¹ due to high intra-specific competition. Whilst we do not see any difference in grain number, we do notice a difference in grain weight which is likely linked to the higher P_n allowing for carbon allocation and partitioning to the grain (Emes *et al.*, 2003; Flood *et al.*, 1995; Gifford & Evans, 1981; Nicolas *et al.*, 1985; Yang *et al.*, 2000). However, it has been previously seen that droughted plants experience ABA-induced C redistribution to grains (Nagarajan *et al.*, 1999; Travaglia *et al.*, 2007; Yang *et al.*, 2001), which is likely a drought escape response.

The observed phenotypic changes would intuitively lead to a change in quality and composition of root exudates as previously seen (Canarini *et al.*, 2019; Henry *et al.*, 2007; Shone *et al.*, 1983; Song *et al.*, 2012; Svenningsson *et al.*, 1990). However, we note very few differences in root exudate composition, noting only significantly higher propionate levels in well-watered treatments and isobutyric levels in carbon-only well-watered treatments. In part, this could be due to the lack of comprehensiveness covered in our targeted metabolic analysis. Even though organic acids are key components in root exudates, particularly under droughted conditions (Canarini *et al.*, 2019; Marasco *et al.*, 2012; Naylor & Coleman-Derr, 2018), our methods do not detect many of the acids. Previous studies have noted large quantitative changes in root exudates with increases in organic acids, proline, mucilage, polar lipids, sterols, and water-soluble C (Canarini *et al.*, 2019; Henry *et al.*, 2007; Shone *et al.*, 1983; Song *et al.*, 2012; Svenningsson *et al.*,

1990). With organic acids being thought to increase plant drought tolerance without bacteria (Canarini *et al.*, 2019; Delhaize *et al.*, 1993; Ström *et al.*, 2002).

The microbial community has been seen to change significantly in response to drought, particularly for rhizosphere communities with large changes in microbial biomass, community diversity, and community compositions (Naylor & Coleman-Derr, 2018). It is thought that bacterial and archaeal abundances are affected by drought more than fungi, with fungal network responses minimal and more stable under drought (Barnard *et al.*, 2013; Bouasria *et al.*, 2012; de Vries *et al.*, 2018; Fuchslueger *et al.*, 2016; Naylor & Coleman-Derr, 2018; Yuste *et al.*, 2011). The typical response to drought is a decrease in bacterial biomass as a response to resource limitation (Alster *et al.*, 2013; Hueso *et al.*, 2012; Naylor & Coleman-Derr, 2018). However, it is thought that repeated exposure to drought increases resilience to drought as certain communities have been found to increase or maintain biomass under drought (Fuchslueger *et al.*, 2014; Hartmann *et al.*, 2017; Hueso *et al.*, 2012; Naylor & Coleman-Derr, 2018). Yet, these observed patterns require further elucidation due to methodological inconsistencies when measuring microbial biomass using either DNA-based quantification of phospholipid fatty acid (PLFA) methods (Fuchslueger *et al.*, 2014; Kassem *et al.*, 2008; Naylor & Coleman-Derr, 2018).

Bacterial community diversity is thought to be minimally impacted by drought (Acosta-Martinez *et al.*, 2014; Armstrong *et al.*, 2016; Bachar *et al.*, 2010; Naylor & Coleman-Derr, 2018; Tóth *et al.*, 2017), which allows soils to maintain functionality under water-scarce conditions (Nautiyal & Dion, 2008). However, community composition drastically changes under drought with shifts in relative abundance (Naylor & Coleman-Derr, 2018).

We observe these similar relative abundance shifts in our data, noting large changes in the droughted abundances compared to the well-watered relative abundances, which are often driven by a few phyla, with most remaining relatively unscathed by drought (Barnard *et al.*, 2013). It has previously been noted that gram-negative bacteria such as Bacteroidetes and Proteobacteria generally decrease under drought, whilst gram-positive bacteria such as Actinobacteria and Firmicutes typically increase (Barnard *et al.*, 2013; Bouskill *et al.*, 2013; Chodak *et al.*, 2015; Hartmann *et al.*, 2017; Naylor & Coleman-Derr, 2018). These changes are thought to largely be driven by copiotrophic versus oligotrophic competition, with the fast-growing (r-selected) copiotrophs preferring labile C under well-watered conditions whilst the slow-growing (k-selected) oligotrophs targeting specialist substrates maintain their populations under harsh conditions (Naylor & Coleman-Derr, 2018).

We expected to see an abundance of copiotrophs under our high C:N or carbon-only treatments in comparison to our low C:N or water-only treatments. Unfortunately, due to a poor quality MiSeq run, we were unable to identify changing microbes present. Therefore, whilst limited in interpretation, we must rely on our QPCR data and infer potential changes. Some such oligotrophs as mentioned earlier are the N-cycling microorganisms present in soil. Previous studies have found that nitrification is typically inhibited by desiccation, with 60% water-filled soil pores being reported as the optimum level for nitrification to take place (Hartmann *et al.*, 2013; Linn & Doran, 1984; Stark & Firestone, 1995). Although we did not measure nitrification, or nitrification potential, we did see a significant increase in $[\text{NH}_4^+]$ in droughted soils compared to well-watered counterparts. As previously mentioned by Hartman *et al.*, 2013 this could be a result of both an inhibition of nitrification, and a decrease in NH_4^+ uptake by the plants (Hartmann

et al., 2013). Denitrification genes were seen to be resilient to drought, with little to no change in abundance (Hartmann *et al.*, 2013; Keil *et al.*, 2015), we also observed this with well-watered treatments being similar in abundance to droughted treatments, yet we did not see *nosZ* decreases previously observed (Keil *et al.*, 2015).

Although it is thought that rhizosphere soils are acidic due to the H^+ abundance after nitrifiers oxidise NH_4^+ and plants exchange NH_4^+ for H^+ (Hartmann *et al.*, 2013), we did not measure rhizosphere pH due to small soil volumes. Furthermore, we did not see any decrease in general bulk soil pH as previously noted (Hartmann *et al.*, 2013), this could be due to methodological differences or due to the sampling distance of our bulk soil measurements being in the top 10 cm of soil. Furthermore, the significant increases we see in Na^+ , Li^+ , Ca_2^+ and Mg_2^+ in droughted treatments are likely due to accumulation over the treatment period, where in the well-watered treatments these micronutrients are likely to leach or utilised, instead accumulating in the droughted soils. The large changes in relative abundance between droughted and well-watered groups at GS61 compared to the smaller changes at GS31 suggests that rhizosphere microbial communities experiencing drought are continuing to grow whilst soil-only controls plateaued in abundance. This makes sense with the literature as the exudation of organics and mucilage from the plant roots allows a higher retention of soil moisture, and therefore preserving the microenvironment, protecting it from the effects of drought.

5.4.1 Conclusion

In this study we have analysed the effects of concurrent drought and C:N fertiliser application on *Triticum aestivum* cv Mulika and its associated rhizosphere communities.

Our findings are generally in accordance with the literature, with large differences in plant-performance between well-watered and droughted conditions. We did not find an overall difference in C:N treatments for plant physiology, with N-only treatments being the best performers all round. Despite analysing a few root exudates, we did not find many significant differences as previously noted in the literature. This is likely due to the limited scope and analysis of root exudates in this experiment, with previous research performing broader analyses of compounds being exported from roots. However, in general, we find that drought application alongside C:N fertilisation has little effect on plant-microbial interactions for *T. aestivum*, with rhizosphere communities being seemingly protected from drought in this experiment. In addition to drought, the author would recommend studying the additive effects of warming. Drought is rarely unaccompanied by an increase in warming, which is known to independently effect denitrification rates and N₂O production (Keil *et al.*, 2015). Studying the warming effect could conversely shed more light on the interactions between plant-microbe interactions under drought if it is accounted for. This is reinforced by previous models predicting that heat stress, not drought, will increase European wheat vulnerability (Semenov & Shewry, 2011).

In conclusion, drought adversely affected plant performance as expected, showing greater effect in unfertilised plants over fertilised plants. Similarly, drought had a powerful grouping effect, whereby most droughted treatments grouped tightly and seemed almost indistinguishable on the multivariate PCA analysis. Overall, the effect of drought masked the C:N-supplementation effects present in well-watered counterparts.

Chapter 6: General Discussion

This thesis has investigated the impact of changing environmental conditions (C:N fertilisation and drought) on plant-microbe interactions using *Triticeae* species, particularly *Triticum aestivum* cv Mulika. This area of research has mainly focused on interactions between plants and microbial communities in response to mineralised N, drought, or a combination of the two. These studies find that under N-fertilisation large changes in bacterial, archaeal, and fungal communities occur, whilst drought effects bacterial communities to a greater extent than fungal communities (Barnard *et al.*, 2013; Bouasria *et al.*, 2012; de Vries *et al.*, 2018; Fuchslueger *et al.*, 2016; Naylor & Coleman-Derr, 2018; Yuste *et al.*, 2011). However, to our knowledge, no study has addressed the effects of labile C (glucose) and mineralised nitrogen ($\text{NH}_4^+\text{NO}_3^-$) being concurrently added in differing C:N ratios (9.37, 17.22, 32.58) on plant-microbe interactions. The potted studies presented here are a step toward building an understanding on how C:N ratios and drought affect plant-microbial interactions in *T. aestivum*.

6.1 Do *Triticeae* species significantly affect rhizosphere microbiota?

Analysis of rhizosphere soils confirmed the presence of distinct community compositions between *Triticeae* members and bulk soil within 73 days (Chapter 3), which is in accordance with previous research (Bulgarelli *et al.*, 2015; Mahoney *et al.*, 2017). Using DGGE band separation of 16S rRNA amplified samples we found rhizosphere communities responded to both the present host plant species, and the addition of mineralised N (Chapter 3). Previous research has observed similar findings with the addition of mineralised N positively affecting fungal growth, whilst negatively affecting

bacterial growth (Rousk & Bååth, 2007). Furthermore, the current literature has a long-standing history of demonstrating that plants selectively recruit microbiota in a deterministic manner to form a rhizosphere community that is distinct from bulk soils (Bulgarelli *et al.*, 2015; Chen *et al.*, 2019; Mahoney *et al.*, 2017; Tkacz *et al.*, 2020), which we also find in our ability to distinguish between bulk soil and rhizospheric soil samples.

It has also been shown that rhizospheric community compositions are greatly affected by plant-seed microbial assemblage (Buchan *et al.*, 2010; Green *et al.*, 2006; Hardoim *et al.*, 2012; Johnston-Monje *et al.*, 2016; Shade *et al.*, 2017; Sultan *et al.*, 2017), with seed-surface proteins often being used to fingerprint the community. Such variations in original community assemblage could explain the large variation in community structures responses previously seen as it dictates the microbial recruitment pool. These spermosphere interactions usually bias toward copiotrophs as germinating seeds usually release many compounds such as organic/fatty/amino acids and carbohydrates (Lemanceau *et al.*, 2017; Nelson, 2004; Schiltz *et al.*, 2015).

It is perhaps because of these spermosphere communities that distinct communities are found between highly related species. For example, previous research has found distinct rhizosphere communities between cultivars of wheat (Yang *et al.*, 2018). In fact, utilising the Simpson's and Shannon's indices for bacterial and fungal amplification of rhizosphere soils Yang *et al.* 2018 found significant differences between six common wheat varieties in addition to other *Triticeae* members. They also observed variety clustering between varieties via an UPGMA dendrogram (Yang *et al.*, 2018). Whilst we did not analyse a range of wheat varieties our DGGE analysis results are did not manage

to differentiate between species, with no distinct community differences being found between *H. vulgare* L. Cassata, *T. monococcum*, and *T. dicoccum*.

In conclusion, the many other results of previous studies finding species-specific differences at the same plant ages (Bulgarelli *et al.*, 2015; Mahoney *et al.*, 2017) would suggest that DGGE, in this instance, was insufficient to distinguish microbial communities. However, overall, our results were in line with previous observations which demonstrate that communities in the rhizosphere are distinct from bulk soil communities within 73 days and we can confirm that *Triticeae* members shape these communities.

6.2 How do applications of C:N fertilisers affect wheat-microbiota relationships?

Previous research has noted significant increases in bacterial and fungal abundance with the addition of glucose to soil (Chenu *et al.*, 2001). However, it was observed that these increases were only on the surface of soil aggregates in clay soils, whilst sandy-loam soils had more bacteria and fungi both within and on the surface of these aggregates (Chenu *et al.*, 2001). This is interesting in relation to prior isotopic research which noted that clay soils had a greater capacity for preserving biomass, more efficient use of metabolic products and glucose, and retained decay products near surviving cells (Van Veen *et al.*, 1985).

Additionally, our research is in alignment with previous studies investigating the effects of plant residues with differing C:N ratios (Hadas *et al.*, 2004; Moritsuka *et al.*, 2004),

where higher C:N ratios result in net N immobilisation and low C:N ratios result in high N mineralisation (Truong & Marschner, 2018). Where our results support this through plant productivity, assuming net N immobilization hinders plant performance. Furthermore, a legacy effect is often seen in applying C:N residues, where N availability can differ after second treatment application depending on the type of C:N treatment (high or low) and amount of C:N treatment previously applied to the soil (Marschner *et al.*, 2015; Truong & Marschner, 2018; Zheng & Marschner, 2017). Similarly, N amendments have previously been associated with an increase in fungal abundance, whilst reducing bacterial growth rates (Rousk & Bååth, 2007). Our results support such findings, with our lower C:N treatments finding a higher abundance of fungi compared to higher C:N treatments.

C:N contents of soils usually refer to a macroscopic view with all present C being measured against total N present. This is useful on a larger scale when determining soil health but has a limited capacity within a microscopic environment as we then deal with individual compound C:N ratios. For example, the conclusions of Farrell *et al* 2014 that uptake of LMWON was mainly for the C, and not N, is pertinent as it highlights potential responses of micro-organisms to C:N. In this study, we have applied C:N ratios, but they are in two separate compounds, with one organism being able to take up either N or C entirely separately from the other. As a result, the responses seen here may be drastically different with adding LMWON compounds with C:N ratios of 9, 17, or 33. However, this study has major implications for the perception of field management and how C and N is perceived in the environment. The study in chapter 4 also demonstrates that we have difficulty interpreting total soil C:N as opposed to C:N of compounds for which the microbial community will be competing, as both views provide drastically different outlooks to the situational environment of both the micro-organisms and plants. For

example, two soils with the same overall C:N ratios can have different underlying compounds with a differing distribution of both N-containing and non-N containing organics, as well as different forms of mineralised N.

Yet, we have observed that lower C:N treatments are beneficial to the plant, with higher C:N ratios having negative implications for plant performance. This is likely due to mineralised N moving away from plants and towards the microbial community, which is observed in our increases in gene abundance, or alternatively lost to the atmosphere through denitrification, which our data cannot confirm.

6.3 How does drought impact wheat-microbiota interactions under different C:N fertiliser treatments?

Large changes in microbial diversity, biomass and compositions have been observed in response to drought community diversity, and community compositions, with bacterial communities being the most affected (Barnard *et al.*, 2013; Bouasria *et al.*, 2012; de Vries *et al.*, 2018; Fuchslueger *et al.*, 2016; Naylor & Coleman-Derr, 2018; Yuste *et al.*, 2011). Plant-microbe interactions in response to drought are complex due to direct and indirect effects of drought on microbial communities. In addition to the direct effects of drought on microbial communities is the plethora of physiological processes and genes activated by the plant when experiencing drought. These processes then set off a large cascade that ultimately effects plant root exudate composition and quantity (Bakker *et al.*, 2013). The rhizosphere community is then responding to both the direct effects of drought on the community and the changing resources exuded from the plant due to physiological shifts in C allocation (Palta & Gregory, 1997; Rampino *et al.*, 2006; Trillo & Fernandez,

2005). There is much interest in this area due to rhizoengineering or rhizosphere inoculation to prevent the dramatic yield losses when crop fields experience drought, an unacceptable phenomenon in the face of a growing population.

Whilst we observed large decreases in plant performance in droughted plants compared to well-watered plants, we could not confirm differences between microbial communities that had or had not experienced drought. Despite significant differences in the ratios of rhizosphere and bulk soil gene abundance between drought and well-watered pots, this seemed mainly a result of droughted soil-only abundances remaining stagnant whilst rhizosphere community abundances increased under drought.

6.4 Conclusions

In this thesis we been able to confirm that the *Triticeae* species *T. monococcum*, *T. dicoccum*, and *H. vulgare* recruit distinct communities from bulk soil within 73 days. We have also demonstrated that *T. aestivum* cv Mulika recruits distinct communities when compared to bulk soil. We also have demonstrated higher C:N ratios demonstrating a negative effect on plant performance, likely shifting mineralised N away from plant use and toward incorporation into rhizosphere microbial communities, or alternatively lost to the atmosphere by denitrification. Finally, we did not observe large effects on plant-microbe interactions under drought, despite the prior research pointing to this direction.

The conclusions of this thesis should be taken in context of the work limitations. These potted experiments operated under a simplified glucose: $\text{NH}_4^+\text{NO}_3^-$ treatment and/or the

withholding of water to produce a 40% soil water content. This is in opposition to real world environments whereby bioavailable fluctuations of both nutrients and water happen continuously in a complex environment with a large array of both organic C and N sources. As a result, the conclusions that can be drawn from these studies are confined in any interpretation to the bounds of the experiments. Any conclusions further extended into wider implications on soil management and plant-microbial interactions should be met with caution as to not over interpret the results, which are limited by the system they were performed in.

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