The effect of little and often fertiliser application on plant-microbe competition, nitrogen use efficiency, growth and crop yield of winter wheat.

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Masters by Research

University of York

Environment and Geography

June 2023

Abstract:

Nitrogen use efficiency of crops is an extremely important, topical issue. Even more so following current global events leading to rapid inflation of nitrogen (N) fertiliser prices. Because of this, there is an increased global interest in agricultural techniques which can maintain high crop yields by using less N fertiliser or to increase crop yield from the same fertiliser quantity. We investigated whether applying N fertiliser in smaller, more frequent doses, would lead to increased nitrogen use efficiency, crop biomass or crop yield in winter wheat. We also studied the effect of splitting N fertiliser applications into smaller more frequent doses on plant-microbe competition. We found that application of fertiliser had a positive effect on crop biomass and crop yield whilst reducing plant-microbe competition. We did not, however, find any significant results concerning the difference between the little and often fertiliser treatment and the regular fertiliser treatment. We suggest no significant differences between the treatments was observed due to timings of fertiliser application. We propose that under little and often fertiliser application, timings of application may not align with what is recommended for regular fertilisation applications. We recommend a late-stage application, around anthesis, to see improvement in crop biomass and yield following little and often fertiliser application.

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Acknowledgements:

I would like to take this opportunity to thank Dr Sylvia Toet and Dr Ben Keane for their support and kindness. Their continued efforts and patience towards me have been appreciated and I would not have been able to complete this degree if it were not for their support and understanding. I am extremely grateful to them both for being continually accommodating and gracious to me.

I would also like to issue a special thanks to the laboratory technicians at the University of York for allowing me to 'play' with their equipment! Thank you to Rebecca, Debs and Matt for all of your help and for going above and beyond to support me.

Finally, I would like to thank my dog Maisy for trying to bite my laptop as I am writing these words.

Authors declaration:

I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for a degree or other qualification at this University or elsewhere. All sources are acknowledged as references.

Chapter 1: Introduction

Plants and fertilisers:

The latest IPCC report predicts high risks to food security due to the impacts of climate change (IPCC 2022). We can combat this risk by working to increase the efficiency and yield of our agricultural practices. One of the key current mechanisms for increasing crop yield is the use of fertilisers. Fertilisers work by artificially enriching soils with nutrients so that crop growth is not limited by availability of these nutrients (Wang *et al*., 2011). Multiple studies have found the use of fertilisers to increase crop yield and nutrient use efficiency, regardless of the fertiliser used and the crop studied (Kaur, Kaur & Asthir 2017; Kenea *et al*., 2021; Li *et al*., 2021; Nkebiwe *et al*., 2016; Otie *et al*., 2016; Vaguseviciene & Juchneviciene 2015). Nitrogen (N) is the nutrient most required by plants, it is an essential macronutrient and is required to produce proteins (Aulakh & Malhi 2005). Nitrogen fertilisers are the most commonly used fertilisers in the UK (Fernández 2021; Kaur, Kaur & Asthir 2017).

Plants are able to take up both organic and inorganic forms of N, however, plants mostly assimilate inorganic N. Inorganic N is provided by fertilisers; there are numerous types of nitrogen fertiliser, depending on the crop, certain N-fertilisers can improve N use efficiency and crop yield more than others (Luo *et al*., 2018; Xing, Mi & Wang 2022). Crop N use efficiency describes how much available fertiliser crops are able to assimilate. Crops are able to access nitrogen provided by fertilisers through ammonification or direct assimilation depending on the form of N in the fertiliser (Zhang *et al*., 2019; Figure 1). However, microbes in soils are also responsible for 'stealing away' available nitrogen from plants; microbes uptake available N, reducing the amount available for plants (Inselsbacher *et al*., 2010). Nitrification is the process whereby ammonium compounds (produced by ammonification) are converted aerobically into nitrates and nitrites (Kumar *et al.,* 2020; Figure 1). Denitrification converts these nitrates and nitrites into nitrogen gas (N_2) (Figure 1). Nitrous oxide (N_2O) is one of the most damaging greenhouse gases (GHGs) to the environment and is produced in soils by these microbes during nitrification and denitrification (Kumar *et al.,* 2020; IPCC 2014). Nitrifiers can produce N_2O through

hydroxylamine oxidation and nitrifier denitrification (reduction of NO_2^-) (Kumar *et al.,* 2020). Denitrifiers produce N_2O by reducing NO_3^- or NO_2^-. N_2O is an intermediary in the process of denitrification, typically, the N_2O would be further reduced into N_2, completing the nitrogen cycle (Hénault *et al*., 2012). The final stage of denitrification (reduction of N_2O into N_2) is often halted by the diffusion of N_2O from soils before it can be reduced (Smith 2017; Figure 1). It is believed that denitrification is responsible for the majority of N_2O emissions in soil (Bateman & Baggs 2004; Hernandez-Ramirez, Ruser & Kim 2021). By enabling crops to uptake higher rates of applied N and increasing N use efficiency, we can reduce some of the N2O emissions associated with agricultural production.

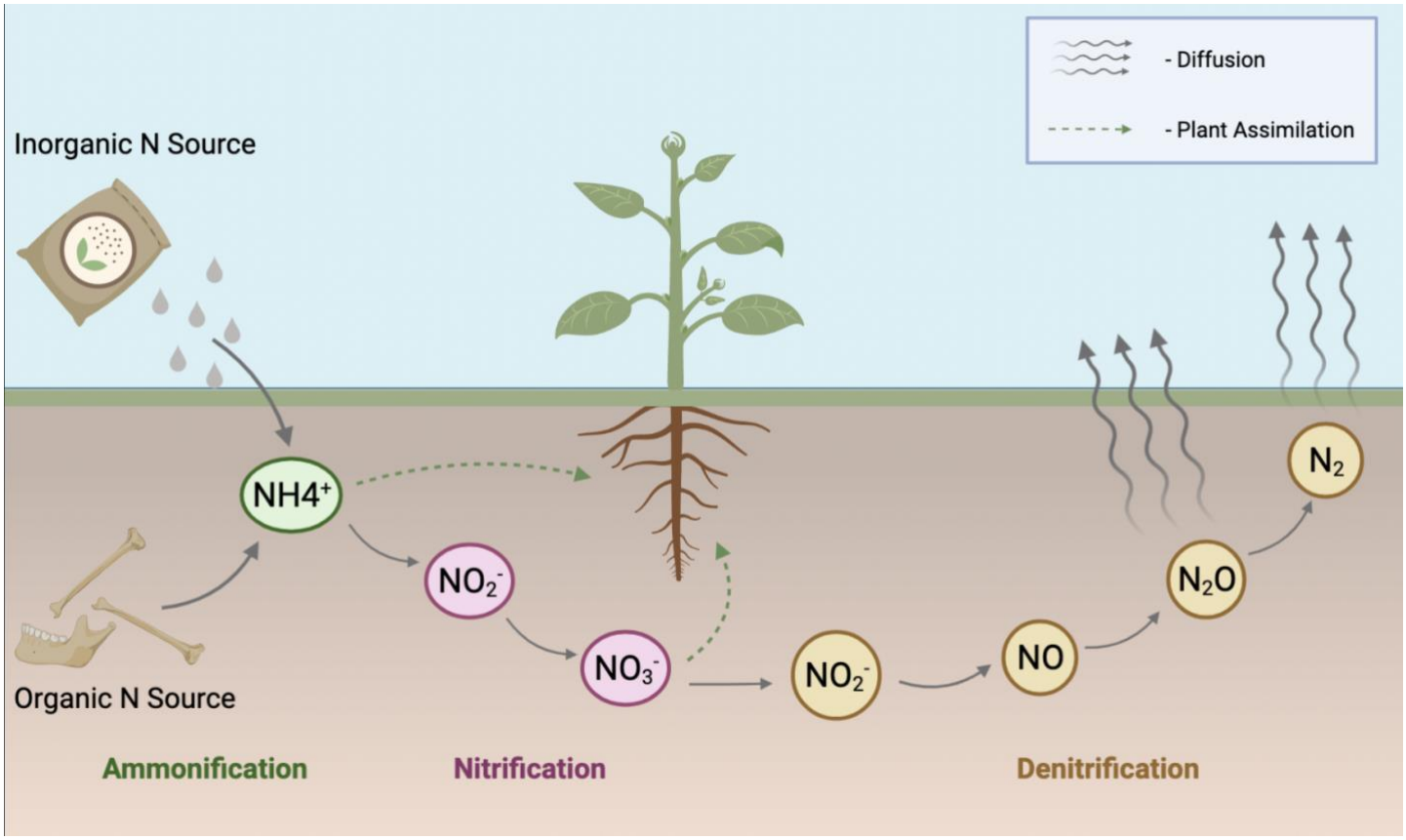

Figure 1 – The movement of nitrogen compounds through the nitrogen cycle.

Negative impacts of fertiliser use:

N_2O causes damage to the atmosphere by reacting with oxygen in the stratosphere to form NO which then enters the O_3 destructive cycle (Müller 2021). N_2O has an

atmospheric lifetime of 121 years and a 100-year global warming potential between 265-298, whereas the 100-year global warming potential of CO_2 is only 1 (Hénault *et al*., 2012; IPCC 2014). N_2O has been recognised as the most harmful GHG to ozone in the stratosphere (Hénault *et al*., 2012). Emissions of N_2O are increasing at a rate of 0.25% every year with agriculture being responsible for at least 70% of total N_2O emissions (Bateman & Baggs 2004; Kumar *et al.,* 2020). Soil microbial processes can mean soils act as a source or sink of N2O as N_2O is both produced and consumed in soils, current agricultural practices mean that globally, the effect of soils is as a source of N_2O (Hénault *et al.*, 2012).

Nitrogen-based fertilisers, despite increasing crop yield can have a detrimental effect on the environment. Soil microbial communities are affected by the use of N-fertilisers, specifically bacterial richness is negatively affected (Castellano-Hinojosa *et al*., 2020). The use of N-fertilisers leads to the leaching of nitrogen compounds from arable land by rainfall events, these compounds often build-up in other ecosystems where they can cause damage to wildlife (Jiménez *et al*., 2019; Huang & Uri 1995). The accumulation of nitrogen compounds in water systems leads to the excessive growth of algae on the surface of the body of water, which is impenetrable by light, preventing photosynthesis below this layer (Chislock *et al.,* 2013). Additionally, when the algae die due to microbial decomposition this leads to an elevation in pH and anoxic conditions which has knock-on effects for local biodiversity (Chislock *et al*., 2013). As well as this, the creation of synthetic N-fertilisers, the Haber-Bosch process, is an energy intensive process and has a high GHG output (Kaur, Kaur & Asthir 2017; Xing *et al*, 2019). The amount of synthetic N-fertiliser applied to arable land has increased significantly in recent years, increasing the rate of leaching and production of N_2O (Kaur, Kaur & Asthir 2017). Worldwide N deposition has increased from 12 Tg/year in 1977 to 104 Tg/year in 2017 and is set to continue as populations grow around the world and demand for food increases (Kaur, Kaur & Asthir 2017; Ritchie & Roser 2013). It is well understood that the use of N-fertiliser is directly related to N_2O emissions (Bell *et al.,* 2015; Cardenas *et al.,* 2019; Kumar *et al.,* 2020; Müller 2021; Stehfest & Bouwman 2006). Bell and colleagues (2015) found an exponential relationship between N-fertiliser application and N_2O emissions.

Mitigating negative impacts of fertiliser use:

Reducing N_2O emissions from fertilisation events not only will help to reduce global greenhouse gas output but also provides an economic benefit for farmers as less emissions means more nitrogen may be able to get into crops. In many cases, less than 50% of applied N-fertiliser ends up being taken up by crops (Adesemoye & Kloepper 2009). By working to increase N-use efficiency of crops, we can save costs for farmers and reduce emissions simultaneously. As part of a 10 year-long research study, millions of farmers in China were able to increase crop yield, on average, by 11% whilst reducing N-deposition by 1/6, this saved an equivalent of $12.2 billion USD (Cui *et al*., 2018). Controlled traffic farming (CTF) is a key method of reducing emissions which is widely practiced by farmers (Hernandez-Ramirez, Ruser & Kim 2021). CTF involves restricting use of farming equipment to particular areas within a field to reduce soil compaction. One meta-analysis found 82% of cases to show that higher soil compaction led to greater N_2O emissions and that CTF was an effective method of reducing emissions, generally halving emissions (Hernandez-Ramirez, Ruser & Kim 2021). Another common agricultural practice involves the timing of fertiliser application. Farmers will time fertilisation events depending on the growth stage of their crop in order to increase fertiliser uptake as much as possible (Efretuei *et al*., 2016). Farmers will also plan fertilisation events around weather to reduce the amount of fertiliser lost by leaching (Huang & Uri 1995; Thorman *et al*., 2013). There is debate among the literature concerning whether types of N-fertiliser can cause greater or lesser emissions, with some finding no effect of fertiliser type (Bell *et al.,* 2015) and others finding specific fertilisers, typically urea-based, to lead to higher emissions (Cardenas *et al.,* 2019; Castellano-Hinojosa *et al*., 2020). Another method of reducing N_2O emissions on cropland is the use of nitrification inhibitors (NIs) which have been found to successfully reduce emissions (Bell *et al*., 2015; Thorman *et al*., 2013).

There is difficulty with mitigating N_2O emissions from agriculture due to spatial and temporal variation in emissions (Smith 2017). Emissions are site specific and can be affected by a plethora of soil conditions including pH, water-filled pore space (WFPS) (Bateman & Baggs 2004; Smith 2017), temperature (Liu *et al*., 2011; Smith 2017), rainfall events (Bell *et al*., 2015; Jiménez *et al*., 2019; Kumar *et al*., 2020), soil aeration (Flessa *et al.,* 2002; Smith 2017) and soil type (Kumar *et al*., 2020). This means that any intervention to reduce

emissions needs to consider site-specific conditions and how emissions may be affected by these.

Little and often fertiliser regimes as a potential solution:

Little and often fertiliser regimes consist of applying the same volume of fertiliser but in more frequent, smaller doses. It is believed that these regimes can help to increase plant-fertiliser use efficiency, preventing loss of applied fertiliser, whilst reducing emissions. In theory, by applying fertiliser in more splits with smaller doses, less fertiliser is lost in the initial stage of application by leaching and microbial theft, meaning that the plants are able to take up a higher proportion of the applied fertiliser. Little and often fertiliser regimes have been found to reduce N_2O emissions (Bell *et al.,* 2015) and reduce leaching of fertiliser (Jiménez *et al.,*2019; Sitthaphanit *et al.,* 2009), as well as, increasing crop yield (Coventry *et al.,* 2011). Slow-release fertilisers, which work on a similar principle to little and often fertiliser regimes, have also been found to increase crop yield and N use efficiency (Li *et al*., 2021; Martin *et al.,* 2001; Xing, Mi & Wang 2022). Little and often fertiliser regimes have also been found to reduce loss of fertiliser by rainfall events, reducing detrimental environmental affects related to leaching and increasing availability of fertiliser to plants and microbes (Jiménez *et al*, 2019; Sitthaphanit *et al.,* 2009). Crops are limited by N and even if N becomes available they have to compete with microbes for it (Kaye & Hart 1997). If N is less limited, the effect of this competition is reduced. By increasing soil N availability, little and often regimes should reduce plant-microbe competition. Existing studies on little and often treatments have focused on fertiliser leaching and crop yield, our study will look at the specific role of plant-microbe competition in enabling plants to take up nitrogen. In the short term, it is well documented that microbes are better competitors for available N (Inselsbacher *et al*., 2010; Kuzyakov & Xingliang 2013; Ouyang *et al*., 2016). This can cause problems for crops, especially in unfertilised soils, however, in the long term, crops are able to outcompete microbes due to their longer lifespan. When microbes die, they release the N stored within them and this becomes available to crops (Inselbascher *et al*., 2010). In this way, microbes act as a store of N and can reduce leaching and losses of applied N. We will investigate whether or not we observe this effect in practice and the specific plant-microbe dynamics under a little and often fertiliser regime.

Our study aims to assess the effects of little and often fertiliser treatment on plant nitrogen-use efficiency and plant-microbe competition during the growing season of a winter wheat (*Triticum aestivum)*. The majority of existing work in this area has focused on the effects of splitting fertiliser application as opposed to applying in one dose. Research which has investigated splitting fertiliser into little and often doses has often overlooked plant-microbe competition. We aim to fill in some of these gaps in the literature by studying the impact of little and often fertilisation on soil and microbial N as well as plant N and the interaction between these variables. We expect that little and often fertiliser regimes will enable crops to take up more nitrogen by reducing plant-microbe competition, leading to less loss of applied fertiliser. We compared the impacts of three N fertiliser treatments: little and often, regular doses and a control where no fertiliser was applied. The effect of N treatment on crop N use efficiency, crop biomass, soil N, microbial N and plant N during the period of fertiliser applications in spring and at time of harvest was studied. The little and often treatment received the same total volume of fertiliser as the regular treatment (220 kg/ha), but it received it in six doses (each dose being a half of the regular dose).

The key hypothesis of this study are that the little and often treatment will reduce plant-microbe competition and will lead to increased plant N use efficiency, and therefore crop biomass and crop yield, compared to the regular (less frequent) N fertiliser application. In order to support this hypothesis, our subsidiary hypotheses are:

i. Little and Often Treatment will yield significantly lower soil N and microbial N and standing stocks then the regularly treated and untreated (control) soil.
ii. The Little and Often Treatment will yield significantly higher aboveground plant N and grain N standing stocks increasing plant nitrogen use efficiency and nitrogen uptake efficiency compared to the regular fertiliser and the unfertilised (control) treatment.
iii. Little and Often Treatment will yield significantly greater aboveground plant biomass standing stocks and greater grain standing stock than the regularly treated and untreated (control) crops.

Chapter 2: Materials and Methods

Field site and experimental treatments:

Our field experiment was conducted in a field in Skelton, York, UK (54°01'N, 1°07'W). Our study crop was Winter wheat (*Triticum aestivum*) cultivar Crusoe. The previous crop at the site was broad beans (*Vicia faba*). Any remaining bean volunteers were removed manually to prevent the influence of herbicides on the experiment. The site contains sandy loam topsoil with high drainage with a high clay content in the subsoil at 30 cm depth. Crops were treated with ammonium nitrate (NH_4NO_3) either regularly/conventionally (220 kg ha^{-1} fertiliser in 3-splits) or little and often (220 kg ha^{-1} fertiliser in 6-splits) or with no fertiliser at all which served as a control treatment (Figure 2). Twenty-four circular plots ("collars") were assigned and numbered across the crop rows a meter apart, for each treatment there were eight repeats i.e., eight collars of each treatment within each crop row (Image 1). Each collar was circular with a diameter of 40 cm and an area of 0.113 m^2. Little and often and regularly treated crops were randomly assigned from blocks based on soil bulk density as part of another study at the site measuring N_2O fluxes. A control treatment was added later and was therefore assigned to either end of the experimental plot. Crops received natural precipitation; it is of note that there was a fairly significant drought throughout the growth season of the crop during the spring and summer of 2022 in North Yorkshire.

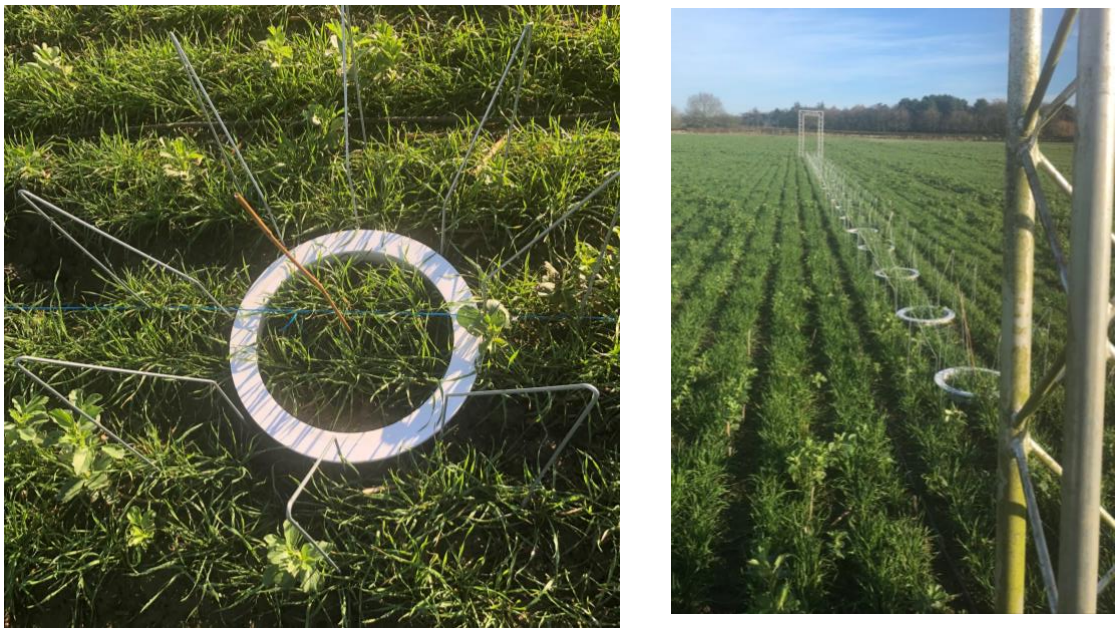

Image 1 – An example of one of the collars and an image of the transect showing all 24 collars along a crop row.

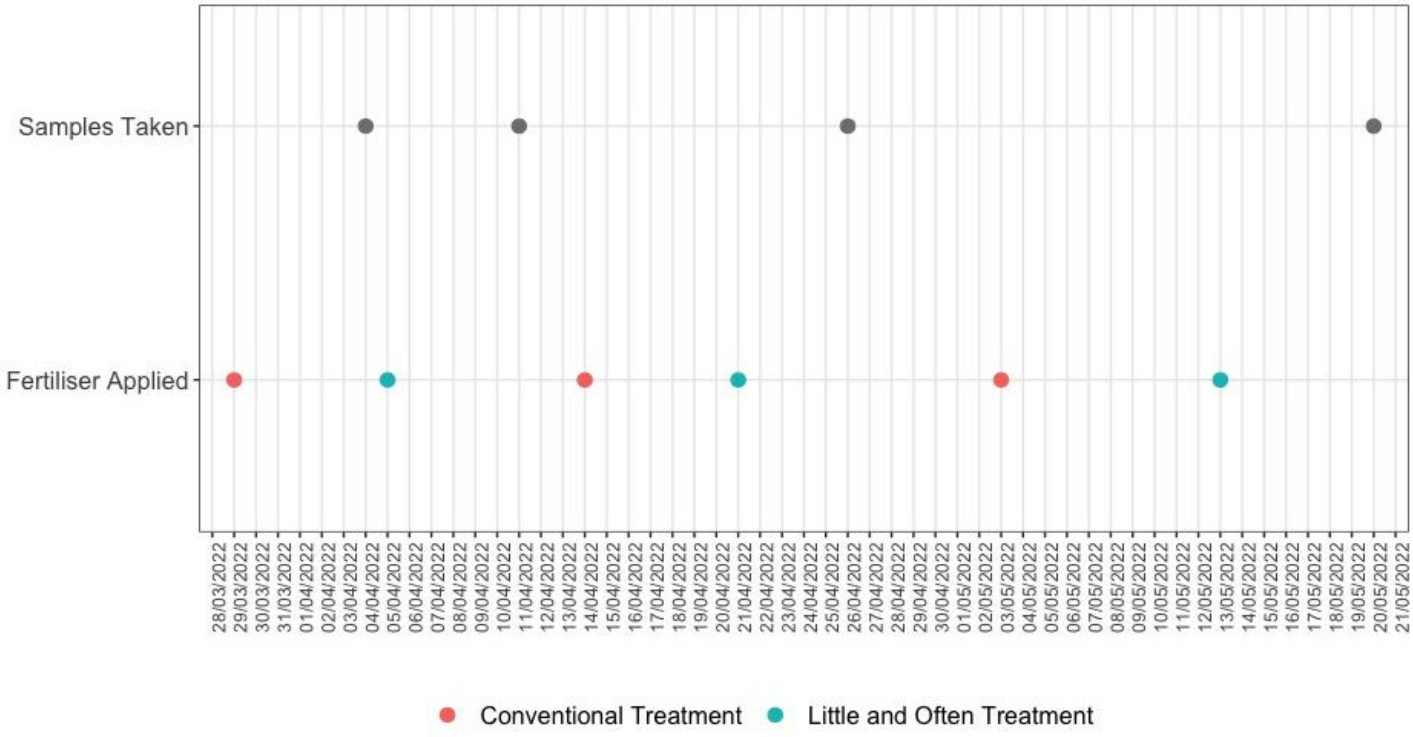

Figure 2 – Dates of sampling and fertilisation events during the growing season, the harvest sampling event occurred on 29/07/2022. Little and often treated crops were also fertilised when the regular fertilisation treatments occurred.

Sampling methods:

At each sampling event, a crop row was chosen at random and samples collected from that row. Soil and plant samples were taken four times throughout the growing season (on the 4th, 11th and 26th of April and 20th of May) and again just before the harvest of the crop on the 27th of July (Figure 2). In most instances, sampling occurred roughly a week after fertiliser application and in line with our trail plan (Figure 2; Image 2). At each collar along the selected crop row, total aboveground biomass was collected as well as two soil cores to 10 cm depth. The corer used had a diameter of 2 cm, except for the first sampling event where only one core was taken using a corer with a diameter of 5 cm. Additionally, for each plot, at each sampling event, plant height (cm), tiller density and ear density (when applicable) in a permanent 40 cm diameter collar were determined. Plant height was inferred using a measuring pole and a soft flat surface which rested on the top of the crop in

the middle of the collar and from which crop height could be measured. From these samples, for each plot, we determined: soil moisture content, bulk density, soil N (TNb, ammonium, nitrate and total N), soil C (DOC), microbial biomass N, microbial biomass C, root (belowground) biomass (g), total leaf area (cm^2), aboveground biomass (g), aboveground plant N and aboveground plant C. Total leaf area (cm^2) was determined using ImageJ software (version 1.53t).

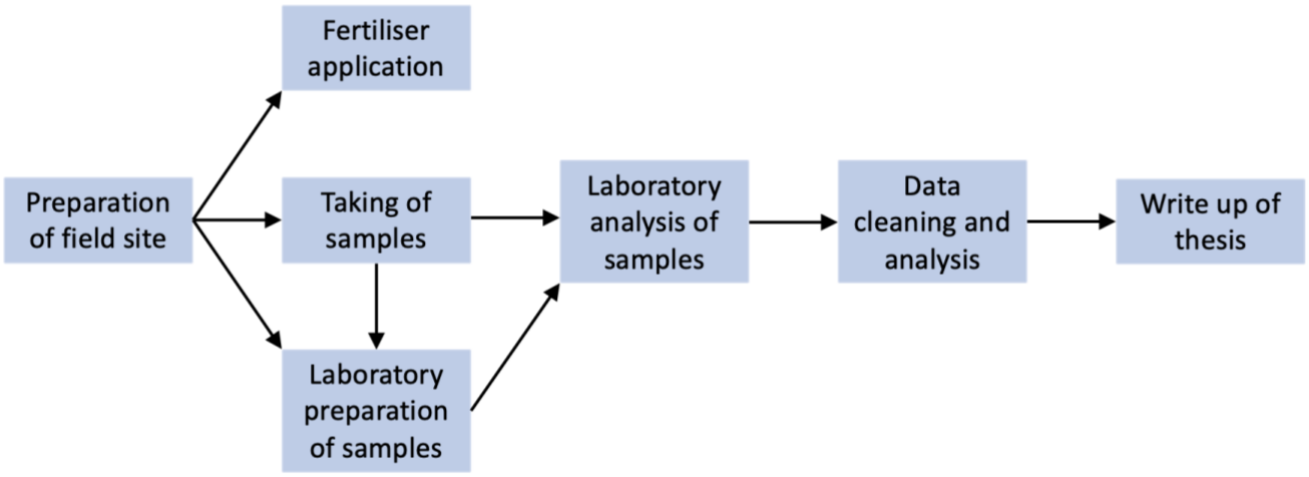

Image 2 – A trial plan outlining the process of this research project.

Plant sampling:

On each sampling date, from the assigned row, all aboveground plant biomass was taken from each plot. In each plot two soil cores to 10cm depth were taken, from which, root (belowground plant) biomass was determined. Measurements of tiller count, plant height and ear count (when applicable) were also taken at each sampling date. Plant material was separated into stems, green leaves, dead (brown) leaves and ears (when applicable) for each sampling date. For all plant tissue, sample dry biomass was taken by weighing samples after being placed in an oven set to 70 °C for approximately 48 hours. Once the crop had matured to a stage where ears were present, ears were also separated from the rest of the plant, ears were categorised as 'visible' or 'invisible' based on whether or not the ear had begun to separate itself from the flag leaf (Image 3). At harvest, ears were categorised as either 'fully formed' or 'non-fully formed' based on size; dwarfed ears were classed as 'non-fully formed'. Additionally, at harvest, ten ears were randomly selected

for each sample. From these ten 'representative' ears, group rachi dry weight, group chaff dry weight, individual seed count and individual seed dry weight were determined. For all sampling dates, except at harvest, green leaf surface area was calculated using imageJ software. Biomass data was reported as standing stock (SS) by dividing biomass within a collar to the area of the collar and in units per tiller or per ear where applicable.

Image 3 – An example of an ear classed as 'visible' on the sampling date of the 20th of May.

Carbon and Nitrogen sampling:

Total aboveground plant N of harvest samples was determined using a CN analyser. Sampled were sorted into stems and leaves; rachi and chaff; and grain. The samples were then dried at 70 °C for approximately 12 hours to remove any moisture. After drying, samples were blended then finely ground in a ball mill. The finely ground samples were

analysed in a CN analyser in order to determine the N content of each. N content of soil was also determined. 10 g of soil was weighed out for each sample, this was then dried at 70 °C for approximately 12 hours to remove any moisture and ground using a pestle and mortar. A Flash EA 1112 Series CN analyser (Thermo Finnigan) fitted within a MAS 200R autosampler and thermal conductivity detector was used with a gas flow of carrier helium 140 mL/min, reference helium 100 mL/min. Sample introduction coincided with a pulse of oxygen at 250 mL/min for 10 s (delay of 12 s). The oxidation reactor contained copper oxide and silvered cobaltous oxide and was held at 950°C. The reduction rector contained reduced copper wires and was held at 840°C. Samples and standards (~6 mg for plants and ~15 mg for soils) were weighed out to 6 decimal places into tin foil capsules (8x5 mm). The instrument was calibrated using a birch leaf reference standard. Run time per sample was 5 min.

Once N of aboveground plant biomass was determined, N use efficiency and N uptake efficiency of crops was determined. N use efficiency was calculated as:

AGB_gSS/AGB_NSS.

i.e., aboveground standing stock of biomass (g/m^2) divided by aboveground standing stock of nitrogen (mg/m^2).

N uptake efficiency was determined as the efficiency of crops to recover N fertiliser and was calculated as:

$(AGB_NSS(x) – AGB_NSS^{Control}(\bar{x}))*100/$Volume of applied fertiliser.

i.e., the aboveground nitrogen standing stock of an individual sample (mg/m^2) minus the control average aboveground nitrogen standing stock (mg/m^2), multiplied by 100 then divided by the volume of fertiliser applied (kg/ha).

Microbial and Soil N:

To determine microbial biomass N, our soil samples were fumigated with 40ml chloroform for 24 hours in order to burst microbial cell walls, the N content of these samples could be compared to N content in non-fumigated samples (which would represent soil TNb) in order to calculate microbial biomass N following the methods outlined by Brookes (1985).

We weighed out two samples of 4g of soil for each collar (one to be fumigated and one to not be). After fumigation (or not), potassium sulphate (K_2SO_4) extraction was carried

out and samples were frozen until analysis occurred. Our potassium sulphate extraction methodology consisted of adding 20ml of 0.5M K_2SO_4 to each sample (fumigated or not), we also added 20ml of 0.5M K_2SO_4 to six empty test tubes to serve as 'blanks'. After the potassium sulphate was added, samples were places in a centrifuge at 200rpm for 30 minutes. After centrifugation, samples were filtered through 3µm ashless filter paper. After filtration, samples were frozen until the time of analysis. TNb (total bound nitrogen) and DOC (dissolved organic carbon) were determined using a TOC analyser. Before this analysis, samples underwent further filtration through 0.45µm Nylon syringe filters so that dissolved organic carbon and nitrogen could be inferred. The TNb values of soil represents the content of inorganic N (ammonium + nitrate) and DON (dissolved organic nitrogen). The non-fumigated samples were also analysed in an auto-analyser where ammonium and nitrate content of the samples could be determined, representing soil ammonium and nitrate. Vance and colleagues (1985) developed a method to calculate microbial biomass N using a constant conversion factor (Kx). The difference between the fumigated and unfumigated TNb values was determined and then divided by the constant conversion factor of 0.54. A similar method outlined in Brookes (1985) was used to determine microbial biomass C. The difference between the fumigated and unfumigated DOC values was calculated and then divided by the constant conversion factor of 0.38.

Statistical Analysis:

All data analysis was carried out using Excel 2022 (Microsoft Office 365) and R Studio (Version 1.4.1103; RStudio Team 2020). Prior to conducting statistical analysis of data, normality tests were performed using Shapiro-Wilks tests. Levene's tests were carried out to test whether variances were equal. The RStudio library 'car' was used to perform Levene's tests (Fox & Weisberg 2019). If variables were non-normal or the variances were not equal, the data was transformed and retested. Log transformations were used for all data except for microbial N concentration where arcsine transformations were used as this was the most appropriate transformation. The aov() function in RStudio was used to perform repeated measures two-way analysis of variance (ANOVA) to determine if there was an effect of treatment or time or an interaction between the two for our dependent variables. If an interaction between these variables was found, the data was split by date and one-way

ANOVA tests were carried out to determine the effect of treatment at each sampling date. One-way ANOVA tests were carried out after finding significant effects following two-way ANOVA so that the effect of time could be accounted for and treatment could be analysed independently of time. If a significant effect of treatment was found by the one-way ANOVA analyses, post-hoc Tukey tests were carried out using the TukeyHSD() function in RStudio. If variables did not meet the requirements of ANOVA testing after transformation, Kruskal-Wallis tests were used as a non-parametric alternative.

Chapter 3: The effect of little and often fertiliser application on soil N and microbial N and standing stocks compared to the regularly treated and untreated soils

Aim and Hypothesis:

Our hypotheses are that the little and often treatment will decrease total microbial and soil N when compared to the regularly treated and untreated soils. Based on previous studies, we predict that, in fertilised plots, crops will be better competitors for applied N, especially in the long term (i.e., over the whole length of the growing season). We also predict that the little and often treatment will increase the competitive ability of the crops relative to the regular and control treatment and therefore total soil and microbial N will be even further reduced under this treatment. We expect levels of inorganic fertiliser N in microbes and the soil to be lower in the control treatment as they have had no fertiliser applied.

Results:

Soil N – Ammonium, Nitrate, TNb and total N

Two-way ANOVA results show a significant effect of time and treatment but not of their interaction for soil ammonium standing stock (Figure 3A; Table 1). Significant effects of time, treatment and their interaction were found for soil nitrate standing stock (Figure 3B; Table 1). One-way ANOVA analyses conducted for each sampling date found that soil nitrate standing stock was significantly affected by LO treatment for all sampling dates except for the 11th of April (Table 2).

For soil TNb concentration and soil TNb standing stock, an insignificant effect of treatment but a significant effect of time and time-treatment interaction was observed (Figure 3C; Figure 3D; Table 1). One-way ANOVA analysis of total soil N standing stock at harvest revealed a significant effect of treatment (Figure 4; Table 3) with Tukey analysis finding a significant difference between the LO treatment and the control (Table 3) but no significant difference between the LO treatment or the regular treatment and no significant

difference between the regular treatment and the control (Table 3). There was no significant effect of treatment found for total soil N concentration (Figure 4; Table 3).

Table 1 – The results of two-way ANOVA analysis on soil ammonium, nitrate and TNb standing stocks and concentrations to the fertiliser treatments over time. Significant results ($P < 0.05$) are shown in green.

Variable	Source	F value	DF	P value
Ammonium Standing Stock	Treatment	23.34	2	< 0.001
	Time	5.24	4	0.001
	Interaction	1.96	8	0.059
Nitrate Standing Stock	Treatment	41.01	2	< 0.001
	Time	8.54	4	< 0.001
	Interaction	2.32	8	0.025
TNb Standing Stock	Treatment	60.26	2	< 0.001
	Time	15.24	4	< 0.001
	Interaction	5.90	8	< 0.001
TNb Concentration	Treatment	68.28	2	< 0.001
	Time	12.96	4	< 0.001
	Interaction	6.62	8	< 0.001
DOC Standing Stock	Treatment	1.04	2	0.358
	Time	126.19	4	< 0.001
	Interaction	2.95	8	0.005
DOC Concentration	Treatment	0.94	2	0.393
	Time	149.64	4	< 0.001
	Interaction	2.89	8	0.006

Table 2 – The results of one-way ANOVA analysis on soil ammonium, nitrate, TNb and DOC at individual sampling dates. Post hoc tests performed were Tukey tests. Significant results ($P < 0.05$) are shown in green. LO stands for little and often, R for regular and C for control.

	Variable	Test statistic value	P value	Post hoc test	P value
04/04/2022	Ammonium Standing Stock	6.65	0.006	(LO + R) > C	>0.05
	Nitrate Standing Stock	6.55	0.006	LO > C	0.007
	TNb Concentration	6.40	0.007	(LO + R) > C	>0.05
	TNb Standing Stock	8.32	0.016	LO > C	0.010
	DOC Concentration	6.295	0.008	(LO + R) < C	>0.05
	DOC Standing Stock	4.925	0.018	LO < C	0.019
11/04/2022	Ammonium Standing Stock	3.19	0.203		
	Nitrate Standing Stock	5.16	0.076		
	TNb Concentration	1.79	0.191		
	TNb Standing Stock	3.40	0.183		
	DOC Concentration	4.964	0.017	(LO + R) > C	>0.05
	DOC Standing Stock	4.058	0.032	R > C	0.042
26/04/2022	Ammonium Standing Stock	6.04	0.008	(LO + R) > C	>0.05
	Nitrate Standing Stock	14.02	0.001	(LO + R) > C	>0.001
	TNb Concentration	19.81	< 0.001	(LO + R) > C	>0.001
	TNb Standing Stock	14.44	0.001	(LO + R) > C	>0.001
	DOC Concentration	0.675	0.520		
	DOC Standing Stock	0.462	0.636		
20/05/2022	Ammonium Standing Stock	13.35	< 0.001	(LO + R) > C	>0.05
	Nitrate Standing Stock	16.64	< 0.001	(LO + R) > C	>0.001
	TNb Concentration	17.07	< 0.001	(LO + R) > C	>0.001
	TNb Standing Stock	17.07	< 0.001	R > LO > C	>0.001§
	DOC Concentration	4.535	0.104		
	DOC Standing Stock	3.620	0.164		
29/07/2022	Ammonium Standing Stock	9.79	0.008	LO > C	0.011
	Nitrate Standing Stock	13.55	0.001	(LO + R) > C	>0.05
	TNb Concentration	9.54	0.001	(LO + R) > C	>0.05
	TNb Standing Stock	7.39	0.004	(LO + R) > C	>0.05
	DOC Concentration	1.520	0.468		
	DOC Standing Stock	0.995	0.608		

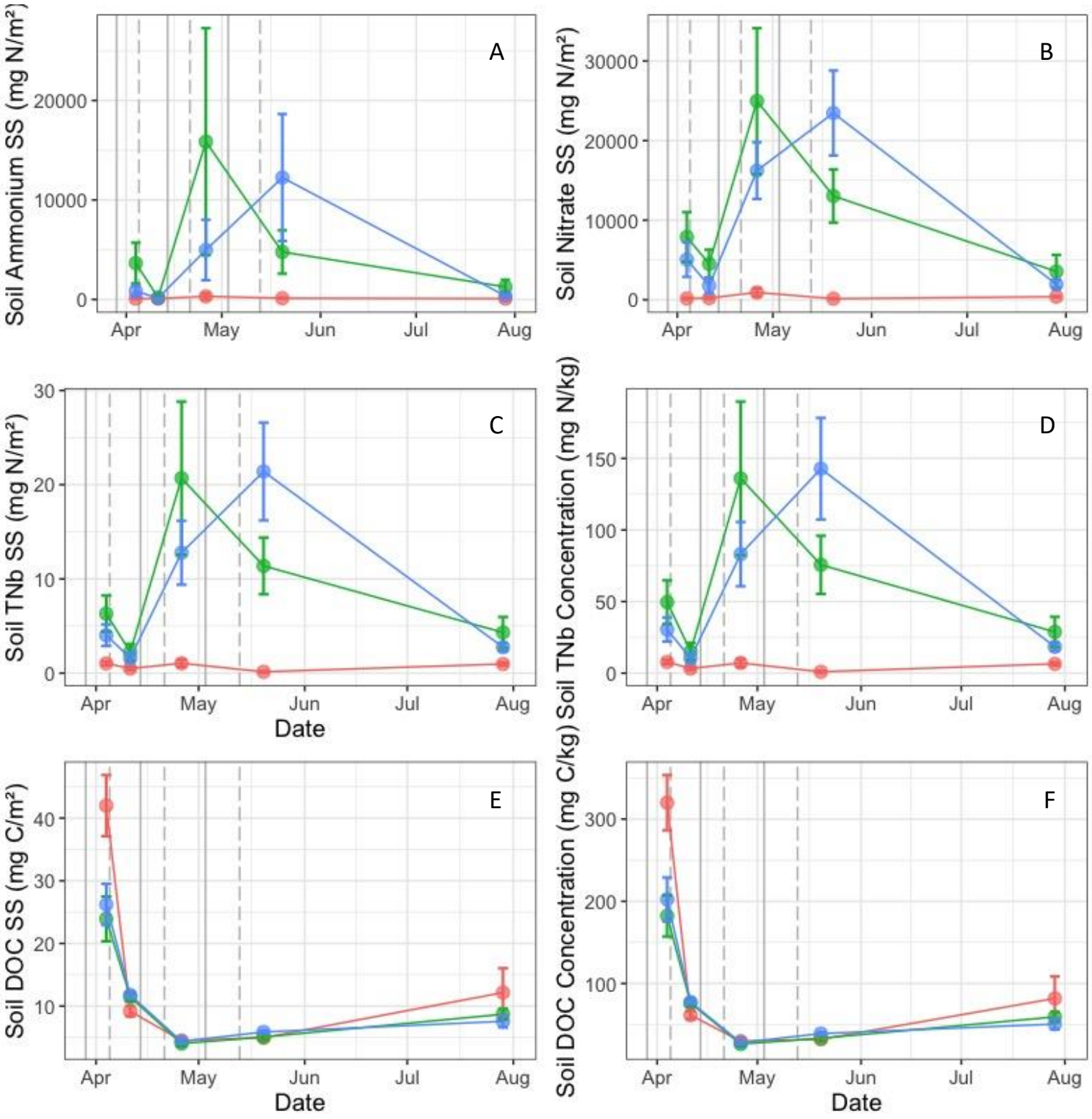

Figure 3 - Mean soil ammonium, nitrate, TNb and DOC standing stocks and concentrations in response fertiliser treatment. Error bars represent standard error. The control treatment is shown in red, little and often in green and regular in blue. Grey lines represent fertilisation events, solid lines show regular application and dashed little and often application.

Table 3 – The results of one-way ANOVA analysis of total soil N and total soil C standing stock and concentrations and C/N ratio in response to the N fertiliser treatment at harvest. Post hoc tests performed were Tukey tests. Significant results ($P < 0.05$) are shown in green. LO stands for little and often, R for regular and C for control.

Variable	Test statistic value	P value	Post hoc test	P value
Soil N Standing Stock	4.015	0.033	LO < C	0.034
Soil N Concentration	4.313	0.270		
Soil C Standing Stock	0.510	0.608		
Soil C Concentration	0.044	0.650		
Soil C/N Ratio	6.315	0.043	(LO + R) < C	>0.05

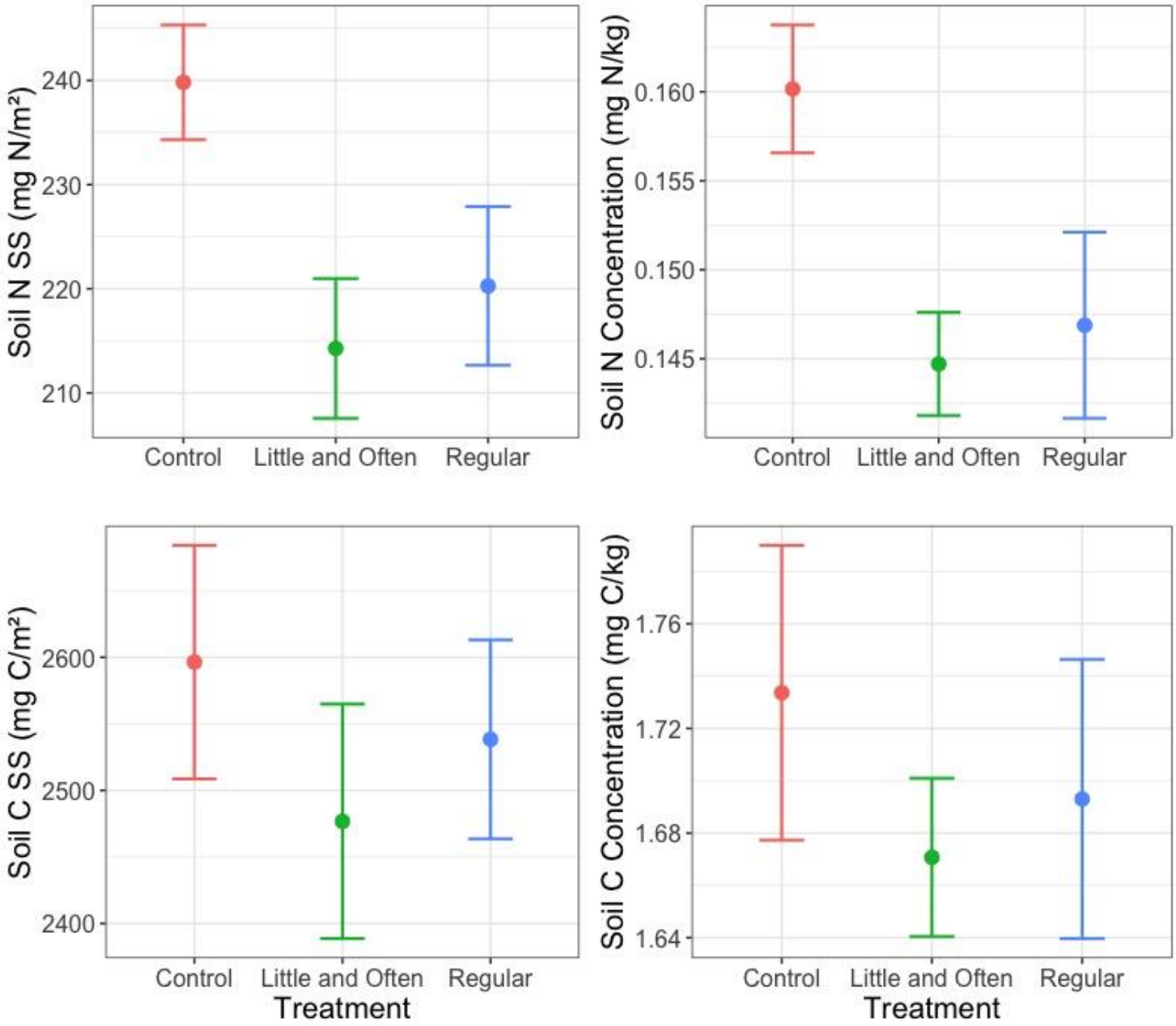

Figure 4 - Mean total soil N and C standing stocks and concentrations at harvest. Error bars represent standard error. The control treatment is shown in red, little and often in green and regular in blue.

Soil C – DOC, total C and C/N ratio at harvest

Soil DOC concentration and standing stock were found to be significantly affected by time but not treatment with a significant interaction between the two (Figure 3E; Figure 3F; Table 1). For the first two sampling dates, one-way ANOVA analysis revealed a significant effect of treatment on soil DOC concentration and standing stock (Table 2), however, from the 26th of April onwards, no significant effect of treatment was observed (Table 2). Additionally, no significant effect of treatment was found for soil C standing stock, soil C concentration or soil C/N ratio at harvest (Figure 4; Figure 5; Table 3). The LO treated soil has insignificantly less soil C standing stock and soil C concentration (Figure 4; Table 3).

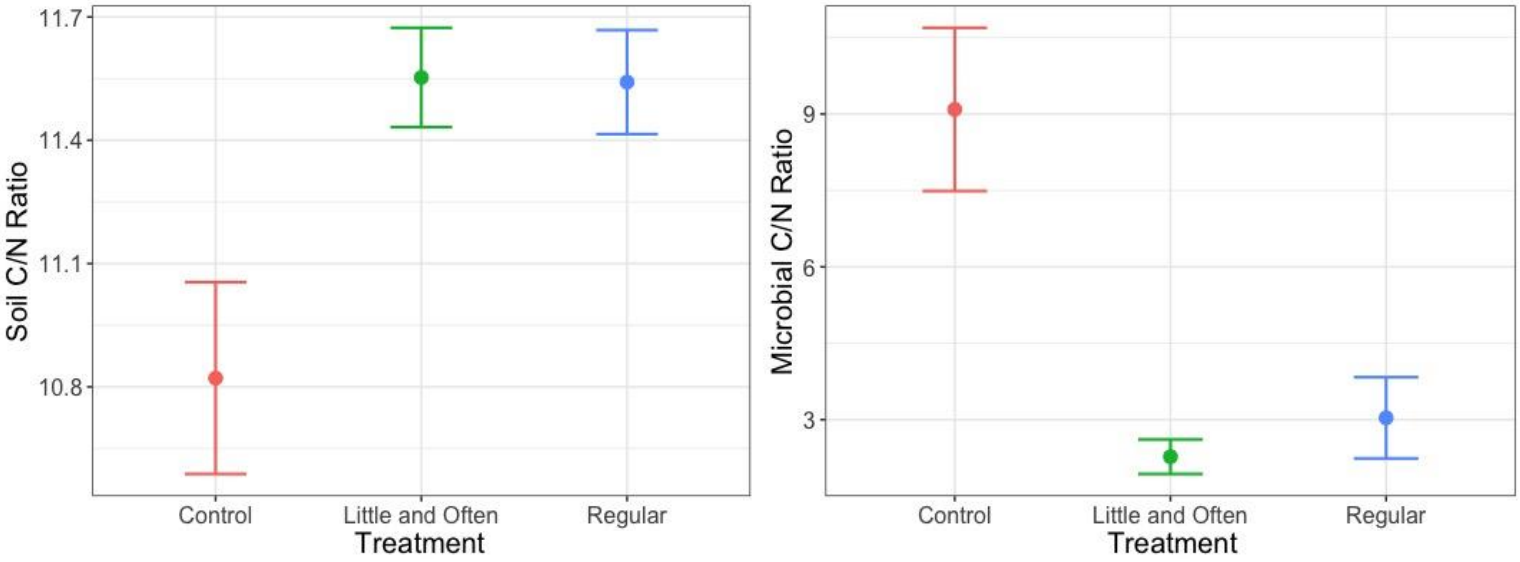

Figure 5 – Mean C/N ratios for soil and microbes. Error bars represent standard error. The control treatment is shown in red, little and often in green and regular in blue.

Microbial C + N – biomass N, biomass C and C/N ratio over time

Two-way ANOVA results show a significant effect of time and treatment but not of their interaction for microbial biomass N standing stock and concentration (Figure 6; Table 4). Fertiliser treatment is shown to significantly reduce microbial biomass N standing stock and concentration. For microbial biomass C standing stock and concentration, a significant effect of time and the interaction between time and treatment was found but not of treatment itself (Figure 6; Table 4). A significant effect of time, treatment and their interaction were found for microbial C/N ratio (Figure 5; Table 4).

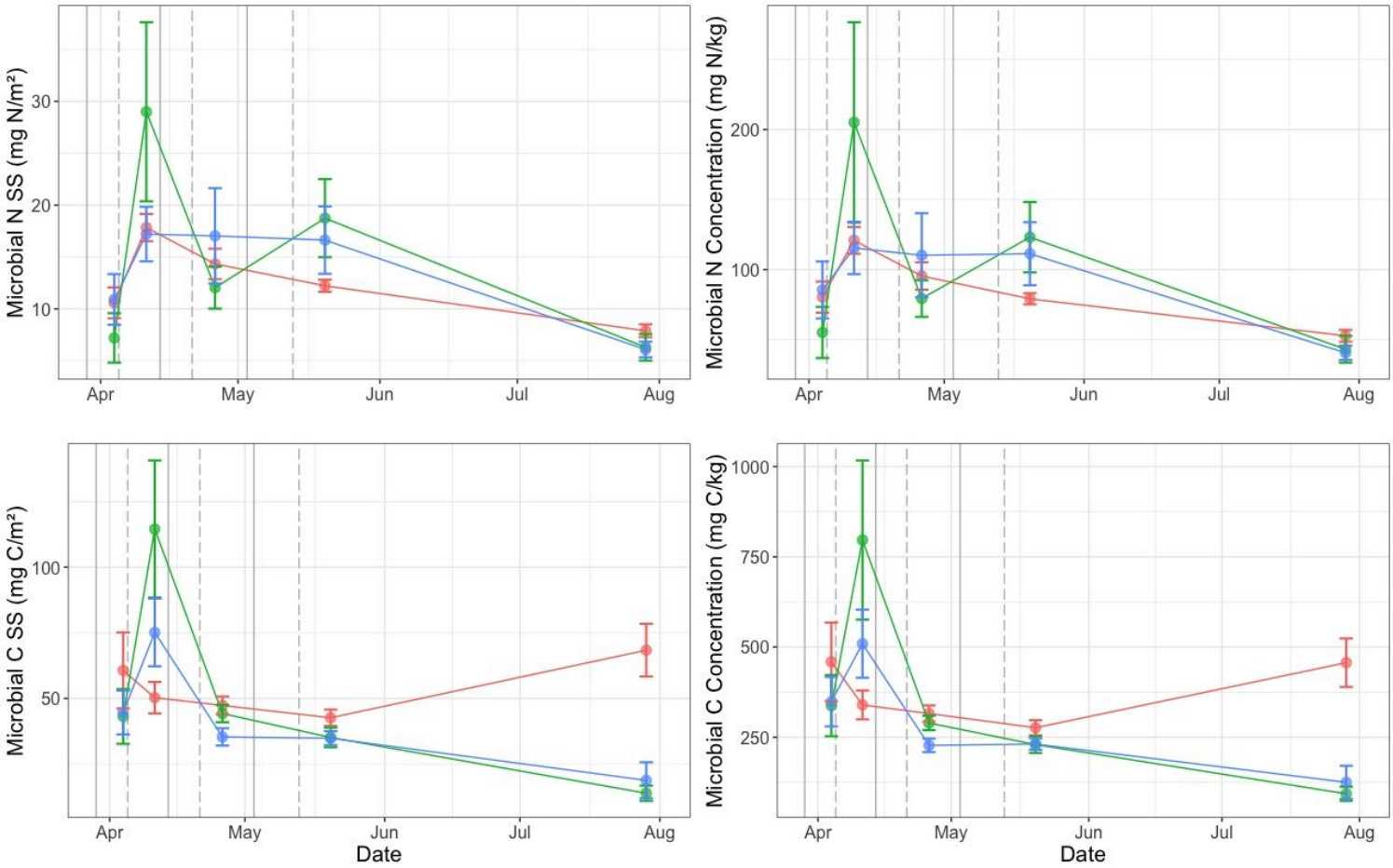

Figure 6 - Mean microbial C and N values. Error bars represent standard error. The control treatment is shown in red, little and often in green and regular in blue. Grey lines represent fertilisation events, solid lines show where fertiliser was applied to both the little and often and regularly treated crops, the dashed line shows the 'extra' doses only received by the crops treated little and often.

Table 4 – The results of two-way ANOVA analysis on microbial N and C. Significant results ($P < 0.05$) are shown in green.

Variable	Source	F value	DF	P value
Microbial N Standing Stock	Treatment	0.45	2	0.642
	Time	12.62	4	< 0.001
	Interaction	1.20	8	0.305
Microbial N Concentration	Treatment	7.42	2	0.001
	Time	2.96	4	0.027
	Interaction	0.46	8	0.877
Microbial C Standing Stock	Treatment	4.17	2	0.018
	Time	11.33	4	< 0.001
	Interaction	4.12	8	< 0.001
Microbial C Concentration	Treatment	3.24	2	0.044
	Time	7.98	4	< 0.001
	Interaction	3.06	8	0.004
Microbial C/N Ratio	Treatment	1.45	2	0.241
	Time	4.17	4	0.004
	Interaction	3.04	8	0.004

Table 5 – The results of one-way ANOVA analysis on total microbial C and N and C/N ratios at harvest. Post hoc tests performed were Tukey tests. Significant results ($P < 0.05$) are shown in green. LO stands for little and often, R for regular and C for control.

	Variable	Test statistic value	P value	Post hoc test	P value
04/04/2022	Microbial N Concentration	1.977	0.175		
	Microbial C Concentration	0.492	0.619		
	Microbial N Standing Stock	0.808	0.461		
	Microbial C Standing Stock	0.640	0.538		
	Microbial C/N Ratio	1.629	0.443		
11/04/2022	Microbial N Concentration	1.357	0.507		
	Microbial C Concentration	5.098	0.016	LO > C	0.012
	Microbial N Standing Stock	1.355	0.508		
	Microbial C Standing Stock	6.196	0.008	LO > C	0.006
	Microbial C/N Ratio	5.729	0.011	LO > C	0.009
26/04/2022	Microbial N Concentration	2.832	0.118		
	Microbial C Concentration	4.890	0.018	R < C	0.016
	Microbial N Standing Stock	0.625	0.545		
	Microbial C Standing Stock	3.531	0.048	R < C	0.046
	Microbial C/N Ratio	0.723	0.697		
20/05/2022	Microbial N Concentration	1.659	0.231		
	Microbial C Concentration	1.723	0.203		
	Microbial N Standing Stock	1.050	0.592		
	Microbial C Standing Stock	1.913	0.172		
	Microbial C/N Ratio	6.709	0.035	LO = R = C	<0.05
29/07/2022	Microbial N Concentration	0.956	0.401		
	Microbial C Concentration	7.868	0.003	(LO + R) < C	>0.05
	Microbial N Standing Stock	1.166	0.332		
	Microbial C Standing Stock	8.488	0.002	(LO + R) < C	>0.05
	Microbial C/N Ratio	6.826	0.033	(LO + R) < C	>0.05

Discussion:

Effect of treatment on Soil N + C

Our hypothesis states that soil N would decrease under the little and often treatment compared to the regular and unfertilised treatments. Our data show that average levels of ammonium, nitrate and TNb are higher in the soil of the treated crops (Figure 3). This result is expected as we did not apply any inorganic N to the control treatment. We can see that by the end of the growing season, almost all of the inorganic N applied as N fertiliser has been taken up by the treated crops or microbes (Figure 3). Our figures show that levels of soil TNb are highly variable for treated soils throughout the growing season and eventually taper off towards harvest time, this variability is caused by fertiliser application events (Figure 3). Our figures show fertiliser application events and their relation to TNb variability, after four applications of little and often treatment, when the regularly treated crops have received two fertiliser applications (26th April) there is a large spike in the little and often soil TNb standing stock and concentration, on the 20th of May when the regularly treated crops have had another treatment, the regularly treated soil has a spike in TNb standing stock and concentration (Figure 3). For untreated soil, there is much less variability throughout the growing season but there is a peak in TNb at the same sampling event as the treated crops on the 26th of April (Figure 3). The variability in TNb in the unfertilised soils could be caused by a number of reasons. The most likely is that N mineralisation was being encouraged around this time by climatic conditions such as increased air temperature of soil moisture content.

Our total soil N data encompasses total soil N including organic and inorganic sources of N. At harvest, the control plots had higher average total soil N than the treated crops (Figure 4). This result was only significant when comparing the little and often and untreated crops, there was no significant difference between the regularly treated crops and the untreated crops (Table 3). Our data suggest that despite higher average levels of soil ammonium, nitrate and TNb in the fertilised plots (i.e., regular and little and often treatment), overall, the average levels of N in the soil are lower for the little and often treatment when compared to the other treatments (Figure 3; Figure 4). This suggests that

there must be a higher organic N status of the untreated soil, suggesting that the addition of inorganic N to the little and often crops in the form of fertiliser could have enabled crops to reduce rates of root exudation or could have had an impact on release of organic N by microbes.

Our hypothesis states that we expect soil N to be decreased following little and often treatment. Overall, TNb and inorganic N were lower for untreated crops throughout the growing season, by harvest the difference between treatments was reduced due to crop assimilation of available N (Figure 3). Our total soil N at harvest shows the control treatment to have higher average total N, with a significant difference found between the little and often treatment and the untreated crops (Table 2; Figure 3). Our data supports our hypothesis and implies that there is an effect of little and often fertiliser addition on all soil N variables due to stronger N assimilation by crops and thereby reduction of soil N under this treatment.

Effect of treatment on Microbial C + N

We found no significant effect of treatment on microbial biomass N standing stock but a significant treatment effect on microbial biomass N concentration, no interaction between time and treatment was found for either standing stock or concentration (Table 4; Figure 6). This suggests that despite fertilisation events occurring, microbes did not take up a significant quantity of fertiliser N in treated soils. The literature suggests that microbes are better competitors for N in the short term but crops outcompete them in the long term i.e., over the whole duration of the growing season, this is because the lifespan of microbes is much shorter than crops enabling crops to uptake any N released by microbes when they die (Inselsbacher *et al*., 2010; Kuzyakov & Xingliang 2013; Ouyang *et al*., 2016). Despite this, our analysis found no significant effect of treatment for microbial biomass N standing stock (Table 4; Figure 6).

Analyses did find, however, a significant effect of treatment for both microbial biomass C concentration and standing stock, this disparity between C and N is reflected in the C/N ratio of microbes under the control treatment (Table 4; Figure 5; Figure 6). Microbes in untreated soils have significantly greater microbial biomass C, this suggests that microbes are stronger competitors in untreated soils. The literature supports this, arguing

that under low N conditions, plant-microbial competition is increased (Dunn *et al*., 2006). The literature also states that microbes are strong competitors for N even when available N is low (Kuzyakov & Xu 2013). This suggests that microbes benefit from the reduced plant productivity in unfertilised soils. Although there is no significant difference in microbial biomass N across treatments, the greater microbial biomass C suggests that microbes are able to amass much greater biomass in unfertilised soils when crops are weaker competitors (Figure 6).

Our results show a significantly higher carbon content of microbes in the control treatment but only a significant effect of fertiliser for N standing stock, this increase in carbon cannot be explained by the addition of fertiliser but by the plant-microbe competition for other resources. Other studies have also found no effect of fertiliser addition on microbial N and found microbes to prefer organic sources of N to inorganic sources, which could explain the high levels of microbial biomass C and low levels of microbial biomass N in our control treatment (Dunn *et al.,* 2006; Koch *et al*., 2021; Ma *et al*., 2019). Another explanation could come from competition for other nutrients, such as phosphorus and potassium, which have not been studied in this experiment.

We expected to observe lower microbial biomass N in treated crops as suggested by our hypothesis. Our results found no significant differences in microbial biomass N across the treatments (Table 5). However, significant differences between treatments in microbial biomass C and microbial C/N ratios were found (Table 5). Despite not observing differences in microbial biomass N, the differences in microbial biomass C show a clear effect of treatment. Our data does not support our hypothesis but does suggest that microbes are stronger competitors than crops in low N environments and are able to produce more biomass when N is limiting.

Chapter 4: The effect of split fertiliser application aboveground plant N and grain N standing stocks, nitrogen use efficiency and nitrogen uptake efficiency.

Aim and Hypothesis:

We propose that by applying N fertiliser more often, N is likely to be available to plants for longer periods of time. Therefore, we expect to observe higher N standing stock and concentration in the aboveground biomass of little and often treated crops than the regularly fertilised and unfertilised crops. Furthermore, the nitrogen use and uptake efficiency of crops should therefore be increased by the little and often treatment compared to the regular treatment and control treatment.

Results:

Plant N

Aboveground N standing stock and grain N standing stock of the crop at harvest time were significantly affected by the N fertiliser treatments (Figure 7; Table 6). For both N standing stocks; the two N fertiliser treatments did not significantly differ but they were significantly higher than the control (Table 6; Figure 7). Analogous to the N standing stocks, the N concentrations of the grain and aboveground biomass were significantly higher for the fertiliser treatments than for the control and did not differ between the two fertiliser treatments (Figure 7; Table 6).

Table 6 – Results of one-way ANOVA and post hoc analyses studying the effect treatment dependent variables for hypothesis 2 at harvest. P values in green are statistically significant. Tukey tests were used as post hoc tests. LO stands for little and often, R for regular and C for control.

Variable	Test statistic value	P value	Post hoc test variable	P value
AGB C Standing Stock	16.64	< 0.001	(LO + R) > C	>0.001
AGB N Standing Stock	110.90	< 0.001	(LO + R) > C	>0.001
Grain C Standing Stock	16.81	< 0.001	(LO + R) > C	>0.001
Grain N Standing Stock	111.70	< 0.001	(LO + R) > C	>0.001
AGB C/N Ratio	15.38	< 0.001	(LO + R) < C	>0.001
Grain C/N Ratio	30.81	< 0.001	(LO + R) < C	>0.001
N Use Efficiency	66.94	< 0.001	(LO + R) < C	>0.001
N Uptake Efficiency	0.53	0.479		

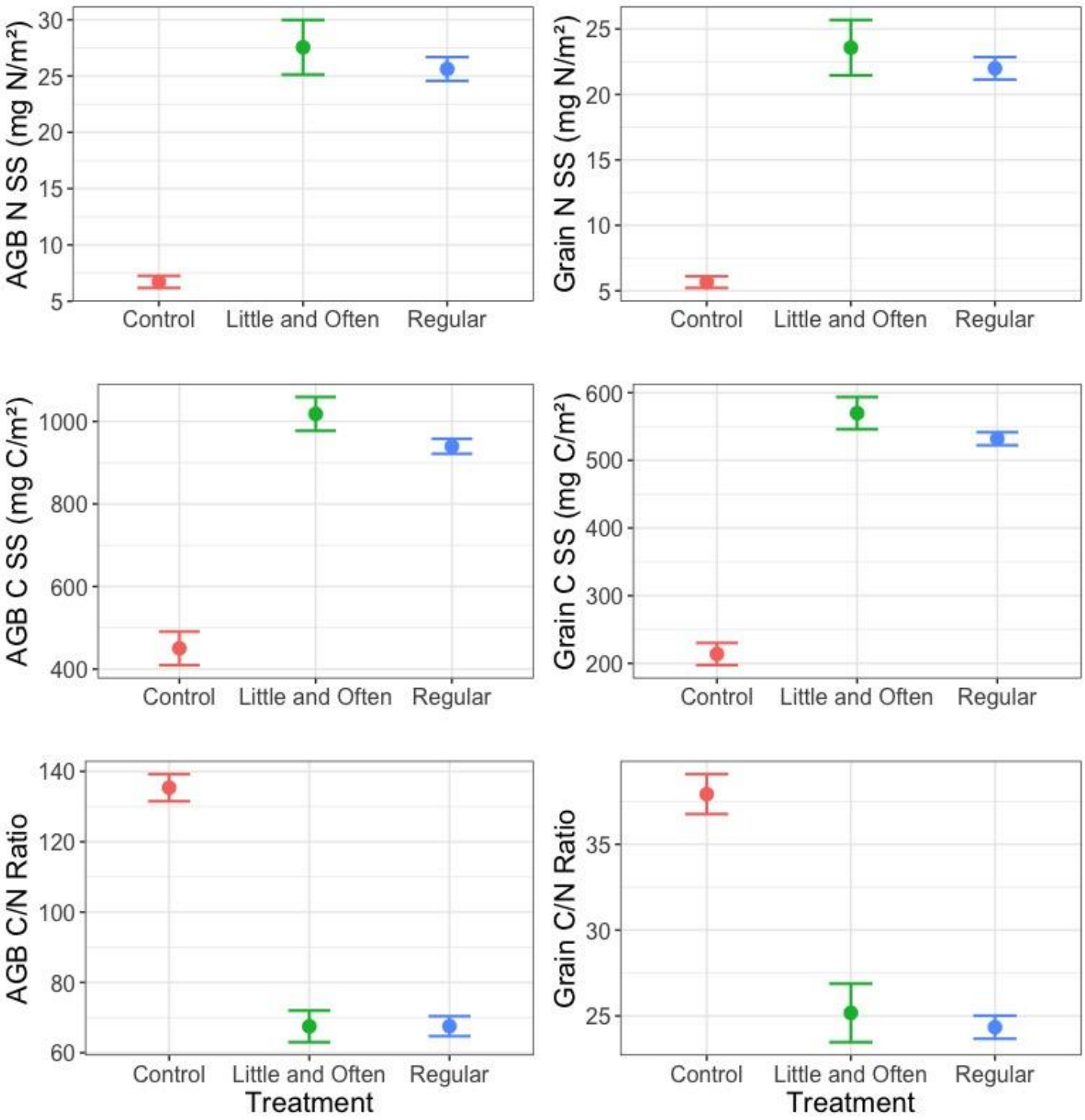

Figure 7 – Mean aboveground C values and C/N ratios. Error bars represent standard error. The control treatment is shown in red, little and often in green and regular in blue**.**

Aboveground biomass C and C/N ratios

No significant treatment effect was observed on the C concentration of the aboveground biomass and the grain at harvest time (Figure 7; Table 6). There was a

significant effect of treatment on grain C concentration and grain C/N ratio at harvest (Figure 7; Table 6). Post-hoc Tukey analysis found, for grain N standing stock at harvest, the difference between treated crops (either little and often treatment or regular treatment) and untreated crops to be significant (Figure 7; Table 6), however no difference was found in grain N standing stock between the little and often treatment and the regular treatment (Figure 7; Table 6).

Plant N/microbial N ratio at harvest

At harvest, plant/microbial N ratio was found to be significantly affected by treatment ($P < 0.05$; Figure 8). A significant difference was found between the regularly treated crops and the control treatment ($P < 0.05$) but not between the little and often treated and the untreated crops or the little and often treated and the regularly treated ($P > 0.05$; Figure 8).

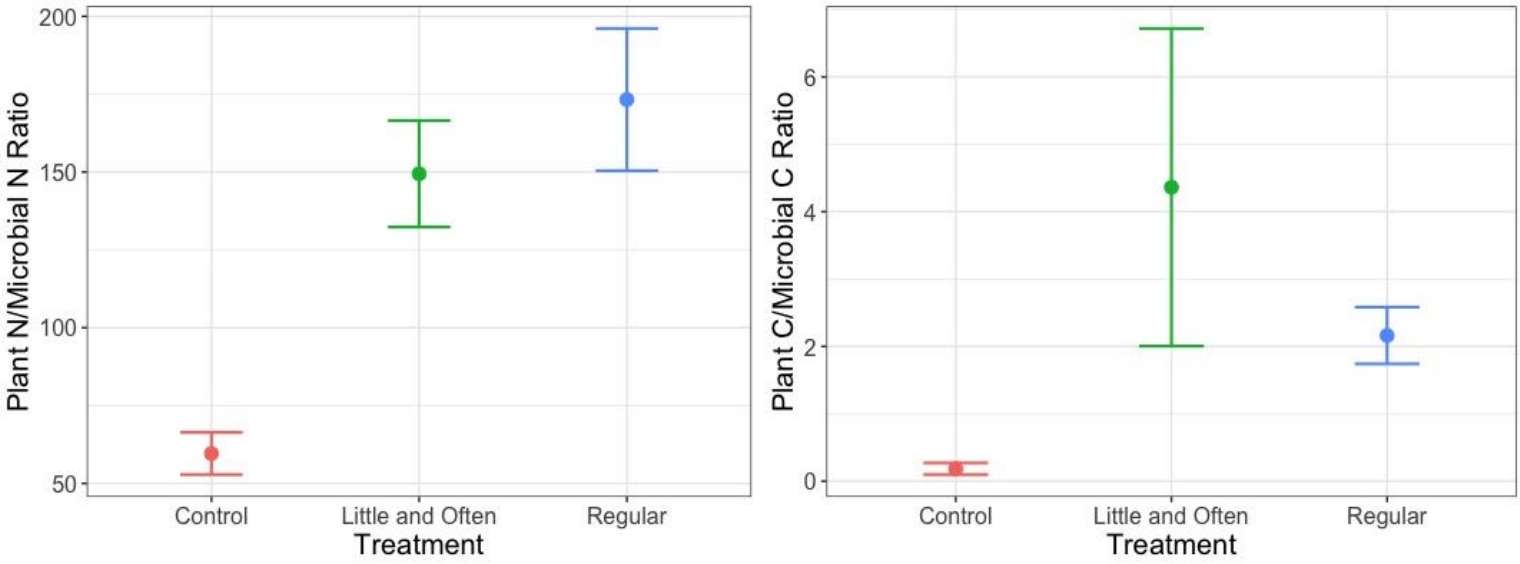

Figure 8 – Mean C/N ratio values and plant to microbial ratios. Error bars represent standard error. The control treatment is shown in red, little and often in green and regular in blue.

Nitrogen use efficiency and nitrogen uptake efficiency

One-way ANOVA reveal that the effect of treatment was significant for N use efficiency (Figure 9; Table 6) but not for N uptake efficiency (Figure 9; Table 6). Tukey tests show that there was a significant difference in nitrogen use efficiency between treated and untreated crops but no difference was found between the little and often treated and the regularly treated crops (Table 6).

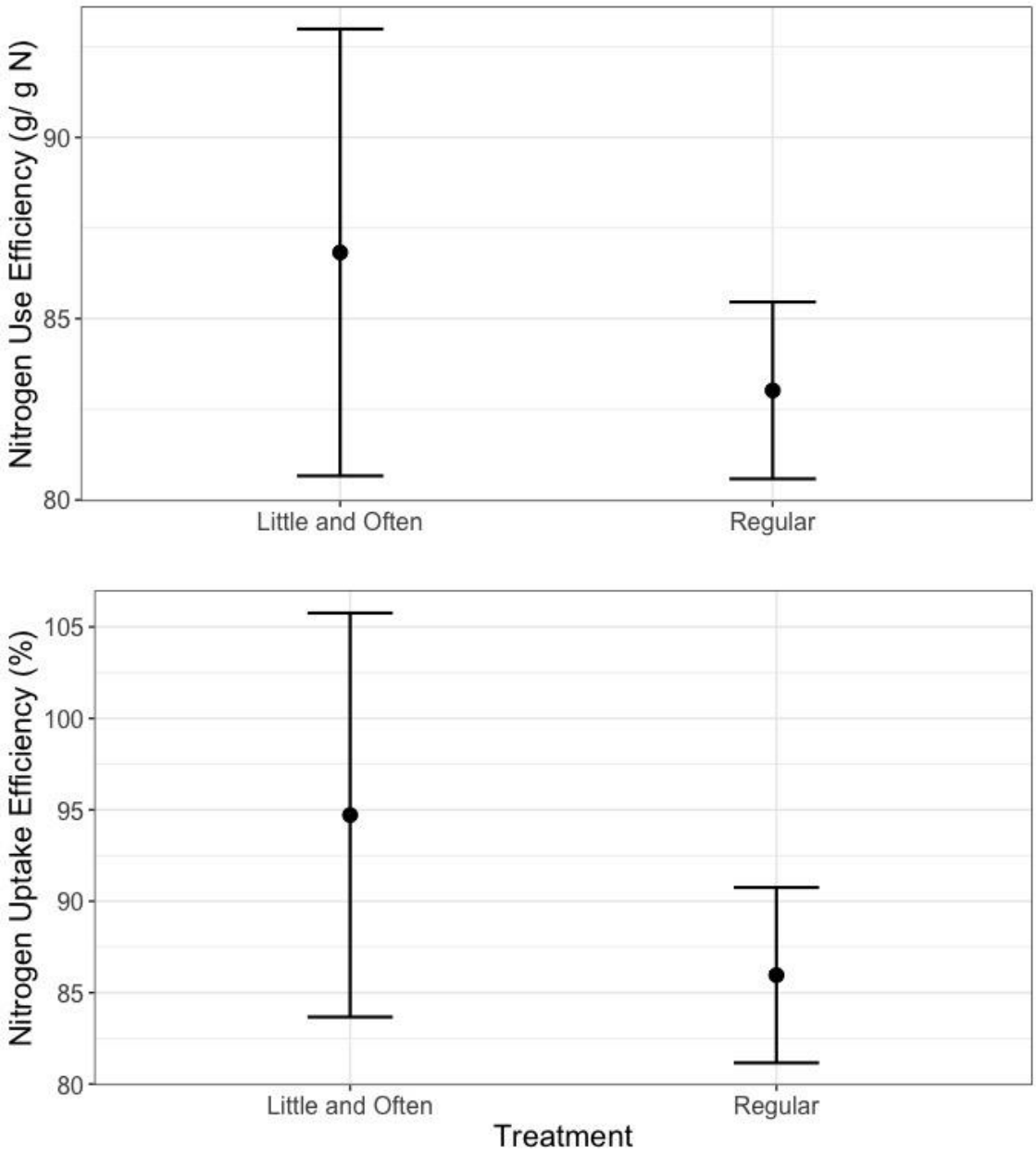

Figure 9 – Mean values for nitrogen use efficiency and nitrogen uptake efficiency. Error bars represent standard error.

Discussion:

Effect of fertilisation on Plant N

We expected that the little and often treatment would result in an increased aboveground plant N, grain N, nitrogen use efficiency and nitrogen uptake efficiency when

compared to the regularly treated and untreated crops. Our results show a strong effect of treatment on plant N but no significant difference between the two types of treatment (Figure 7; Table 6). We expected to find a difference between the types of treatment as the literature suggests that little and often treatments should lead to a reduction in N losses during application, either to the atmosphere or through competition with microbes, enabling crops to access more N (Bell *et al*., 2015; Jiménez *et al.,*2019; Sitthaphanit *et al.,* 2009). We argue that no significant difference in plant N is seen between the two treatments due to the timings of fertiliser application events.

In our study fertilisation events were concentrated towards the early stages of crop development, between GS30 and GS40 during the stage of stem elongation (Figure 10). The AHDB produced Nutrient Management guide (RB209) recommends fertilisation of winter wheat between GS32 and GS39, in line with our application (AHDB 2023). The recommendation also states that additional later fertilisation applications are likely to increase N uptake further but are not recommended if dry conditions are expected (AHDB 2023). This is because applying fertiliser when soils and conditions are dry can lead to the loss of N through volatilisation, this is when applied N is converted into gas and is then rendered unavailable to crops. A study on cotton plants found that application of fertiliser at flowering was most significant and contributed the most to plant N (Yang *et al*., 2013). Delogu and colleagues (1998) state that the vast majority of N in winter cereals comes from fertilisation events pre anthesis (GS60) but that post-anthesis N applications can be crucial as they can contribute to grain N content. Another study found late-stage N application (near anthesis) to be effective, arguing that fertiliser recovery is highest around this time (Kirda, Derici & Schepers 2001). Based on our findings and the literature, we suggest that focusing the majority of fertilisation events during the stages recommended by RB209, i.e., during stem elongation, and adding an additional fertilisation event later in the growing season is likely to have the strongest impact on plant N uptake. It is worth noting that the year (2022) and area of this study (North Yorkshire) were unusually droughty during the experiment and even if later stage fertilisation occurred, it is likely that due to the climate, the effects of fertilisation would have been limited due to N volatilisation (Figure 11).

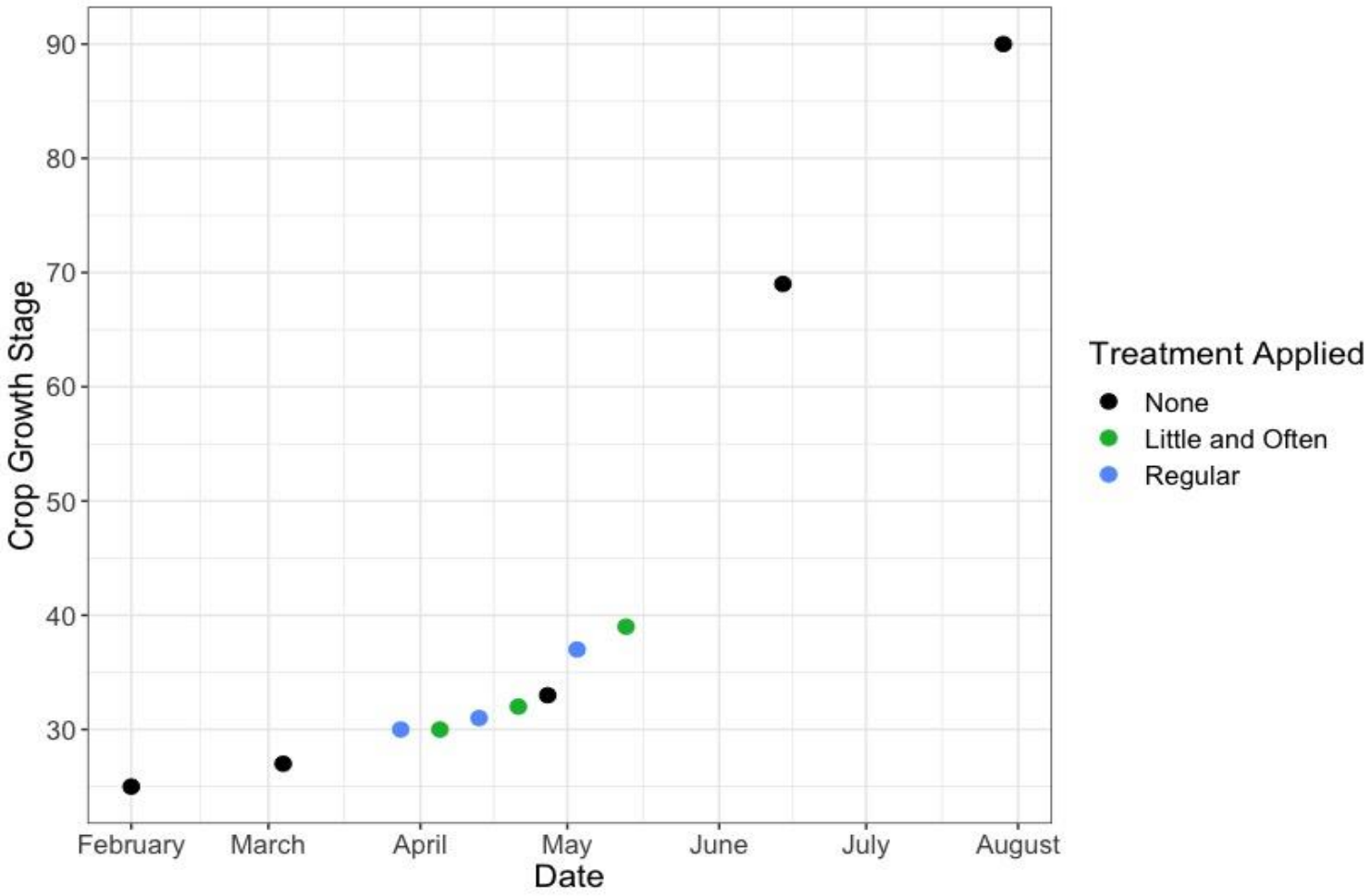

Figure 10 – Timings of fertilisation events throughout the growing season of the crop. Where treatments are described as regular, the regularly and little and often treated crops received fertilser.

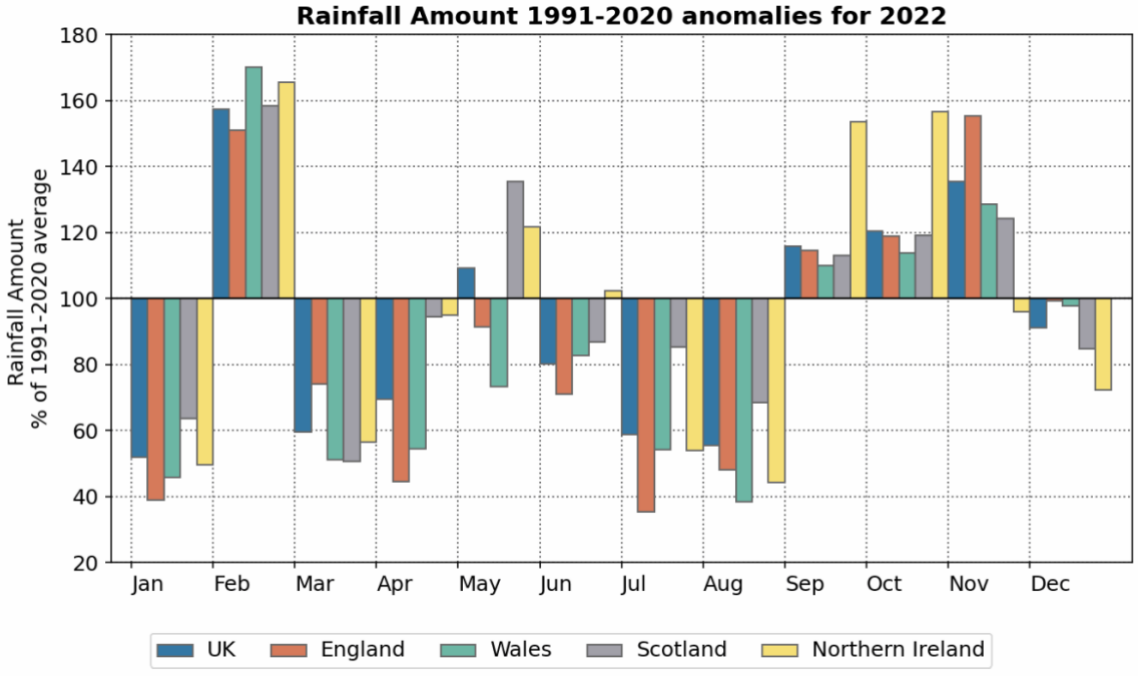

Figure 11 – Monthly variation in rainfall in 2022 compared to the standard 30-year average from 1991-2020 (Met Office 2023).

Effect of fertilisation on Aboveground plant C and C/N ratio

There is a significant improvement of aboveground C following fertilisation (Figure 7; Table 6). Although our analysis did not find a significant difference between the little and often treatment and the regular treatment, the mean little and often values are higher (Figure 7). Our C/N ratios were significantly affected by treatment (Table 6) but show a minimal difference between the two fertiliser treatments (Figure 7). A higher AGB and grain C/N ratio in the control treatment were found as these crops were able to generate relatively high biomass despite having very low levels of N. We would expect to have observed a significant difference in the C/N ratios of the little and often treated crops and the regularly treated crops, the lack of a difference suggests that the treatment did not affect the ability of crops to generate biomass relative to the amount of N taken up by plants. This result is not too surprising and does not directly discredit our hypothesis.

Plant/Microbial C and N

When reviewing the results of microbial N in this study, it is important to consider that at this site the previous crop was N-fixing beans, this is likely to have impacted microbial dynamics of the soil and to have had an effect on nitrogen cycling at this site.

Although we see no significant difference in microbial biomass N across treatments, we do observe greater microbial C in unfertilised soils (Figure 6). In unfertilised soils, we found lower plant N and C (Figure 7). These results strongly suggest that plant-microbe competition is amplified by lack of fertiliser. Under these conditions, microbes are able to outcompete crops and have a much greater N and C content relative to crops i.e., plant N/microbial N and plant C/microbial C (Figure 8). The literature suggest that microbes prefer organic sources of N whilst crops prefer inorganic sources (Dunn *et al.,* 2006). When fertiliser is not applied, total N and inorganic N levels of the soil are lower, therefore, competition becomes fiercer, with microbes being able to outcompete crops as they are more able to uptake organic sources of N. In fertilised soils, microbes are able to enjoy organic N in soils and crops are able to uptake applied inorganic N, reducing the competition

between plants and microbes. Our analysis did not find a significant difference in plant N/microbial N or plant C/microbial C between the little and often and regular treatments (Figure 8). We propose that the application of fertiliser in little and often doses is likely to reduce competition between crops and microbes in the initial stages of application. It is well documented that microbes are better competitors for inorganic N following application (Inselsbacher *et al*., 2010). By applying smaller doses of fertiliser, microbes will be able to better uptake inorganic N and can act as a store of N in soils which crops will be able to uptake following microbial death. The dynamics of plant-microbe competition following little and often fertiliser application require further study in order to understand whether or not this application method enables crops to outcompete microbes in the long term and whether more N is able to be taken up by crops because of this.

Effect of fertilisation on Nitrogen Use Efficiency and Nitrogen Uptake Efficiency

Our nitrogen uptake efficiency values represent how efficient crops were at recovering fertiliser. A significant effect of treatment on nitrogen uptake efficiency was not discovered by our analysis. Our analysis did, however, find a significant effect of treatment on nitrogen use efficiency, this is because the unfertilised crops had a high nitrogen use efficiency as they were able to generate a relatively high biomass without any fertiliser being applied (Table 6). Our nitrogen use efficiency represents how much aboveground biomass crops were able to obtain given the amount of nitrogen in aboveground biomass of the crop and was calculated as AGB_gSS/AGB_NSS. Split application of fertiliser addition has been well documented as increasing N use efficiency (Abbasi, Tahir & Rashim 2013; Gezahegn *et al*., 2021; Velasco *et al*., 2012). However, most existing studies have studied the effect of splitting fertiliser compared to not splitting (i.e., two to four doses compared to one large dose). We argue that by further splitting applications, as we have done, gains in N efficiency are likely to be seen.

Chapter 5: The effect of little and often fertiliser application on aboveground plant biomass standing stocks and grain standing stock compared to the regularly treated and untreated crops

Aim and Hypothesis:

Application of fertiliser is known to improve crop growth and biomass (Wang *et al*., 2011). We expect that by applying fertiliser more often and thereby providing nutrients to crops more frequently, it is more likely that N is available to plants when it is needed by plants. Plants use this available N to synthesise amino acids, proteins and enzymes which are used to encourage cell division, we therefore expect that cell growth and crop biomass will be greater for the little and often treatment (Gezahegn *et al*., 2022). Our hypothesis states that the little and often fertiliser treatment should increase aboveground plant biomass and grain standing stock relative to the regular treatment and control treatment.

Results:

Belowground biomass

For root standing stock, a significant effect of time was observed ($P < 0.05$) but not of treatment or of the interaction between time and treatment ($P > 0.05$; Figure 12).

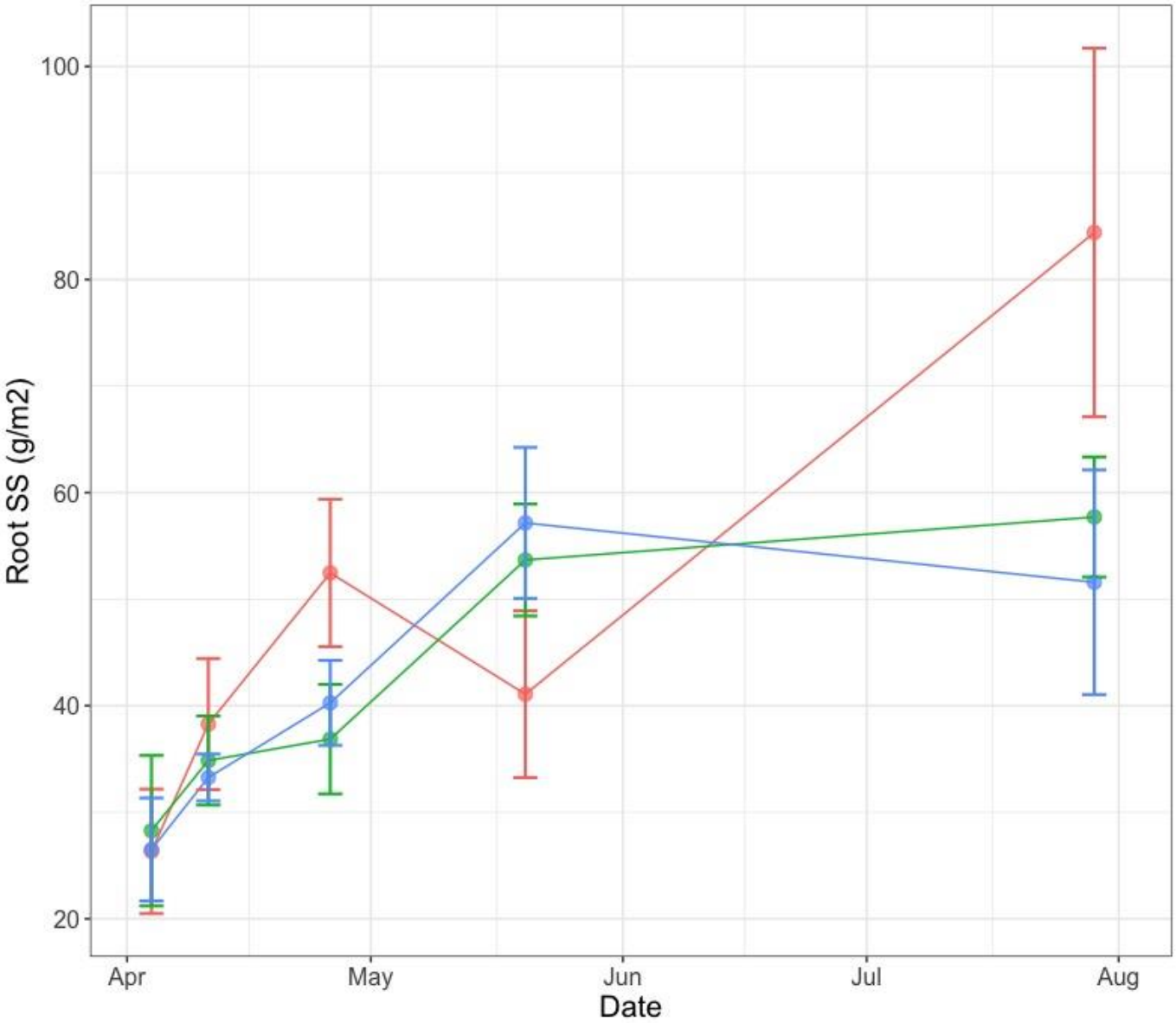

Figure 12 – Mean root standing stock (SS) for the three treatments over the growing season. Error bars represent standard error. The control treatment is shown in red, little and often in green and regular in blue.

Aboveground biomass metrics

A significant effect of time and treatment but not of their interaction was found for tiller count and tiller density (Figure 13; Table 7). Two-way ANOVA results show significant effect of treatment, time and their interaction on plant height, stem standing stock, AGB standing stock and AGB per tiller (Figure 13; Table 7). Two-way ANOVA results show significant effect of time and the interaction between time and treatment but not of treatment itself for stem biomass per tiller (Table 7). From the 26th of April onwards, the

was a significant effect of treatment on plant height at every sampling date (Table 8). Stem standing stock and stem biomass per tiller were only significantly affected by treatment at harvest ($P < 0.05$). Tukey tests found that treated crops had significantly greater stem standing stock and stem biomass per tiller than the untreated crops but that there was no difference between the two treatments (Table 8). From the 26th of April, there was a significant effect of treatment on AGB standing stock, however there was only a significant effect on AGB per tiller at harvest (Table 8). Where these differences were significant, the treated crops were had greater biomass than the untreated crops with no difference found between treatments (Table 8).

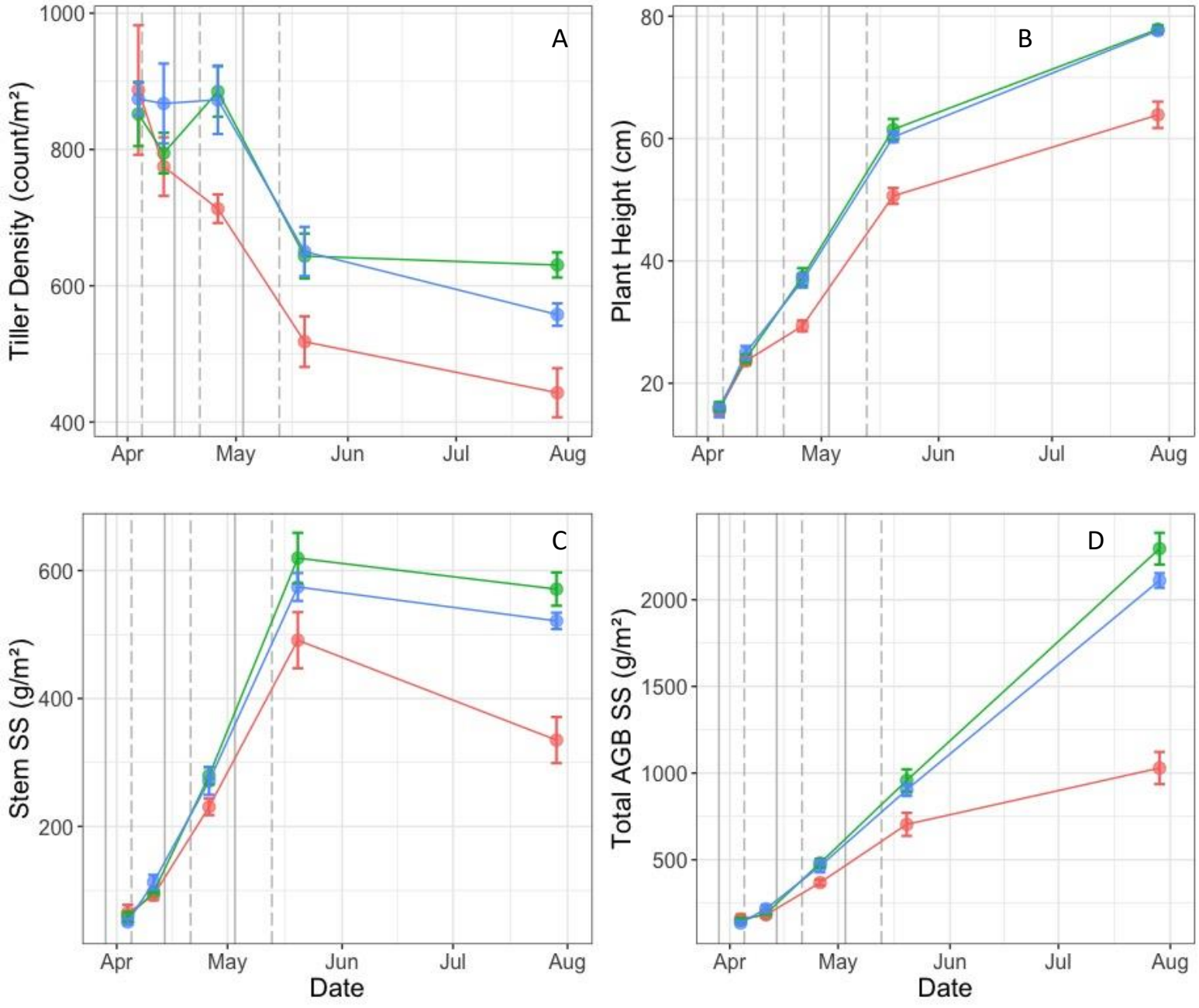

Figure 13 – Mean tiller density, plant height, stem and aboveground standing stock. Error bars represent standard error. The control treatment is shown in red, little and often in green and regular in blue. Grey lines represent fertilisation events, solid lines show where fertiliser was applied to both the little and often and regularly treated crops, the dashed line shows the 'extra' doses only received by the crops treated little and often. SS stands for standing stock.

Table 7 – The results of two-way ANOVA analysis on aboveground biomass metrics. Significant results ($P < 0.05$) are shown in green.

Dependent Variable	Source	F value	DF	P value
Tiller Count	Treatment	13.29	2	< 0.001
	Time	40.92	4	< 0.001
	Interaction	1.94	8	0.061
Tiller Density no./m2	Treatment	8.17	2	0.001
	Time	34.61	4	< 0.001
	Interaction	1.50	8	0.168
Plant Height	Treatment	21.91	2	< 0.001
	Time	877.36	4	< 0.001
	Interaction	2.90	8	0.006
Stem Standing Stock	Treatment	8.21	2	< 0.001
	Time	401.42	4	< 0.001
	Interaction	2.55	8	0.014
Stem g/tiller	Treatment	2.94	2	0.057
	Time	962.65	4	< 0.001
	Interaction	4.58	8	< 0.001
AGB Standing Stock	Treatment	22.49	2	< 0.001
	Time	551.06	4	< 0.001
	Interaction	6.17	8	< 0.001
AGB g/tiller	Treatment	36.98	2	< 0.001
	Time	2914.98	4	< 0.001
	Interaction	26.86	8	< 0.001

Table 8 – Results of one-way ANOVA and post hoc analyses studying the effect of treatment on dependent variables for aboveground biomass metrics. P values in green are statistically significant (<0.05). Tukey tests were used as post hoc tests. LO stands for little and often, R for regular and C for control.

	Dependent Variable	Test statistic value	P value	Post hoc test	P value
04/04/2022	Plant Height	0.16	0.853		
	AGB Standing Stock	0.31	0.740		
	AGB per tiller	1.00	0.386		
	Stem Standing Stock	0.38	0.688		
	Stem g/tiller	0.96	0.399		
	Tiller Density no./m^2	0.08	0.923		
	Tiller Density	0.06	0.945		
11/04/2022	Plant Height	0.64	0.536		
	AGB Standing Stock	1.46	0.255		
	AGB per tiller	0.45	0.645		
	Stem Standing Stock	3.52	0.173		
	Stem g/tiller	0.76	0.480		
	Tiller Density no./m^2	1.16	0.334		
	Tiller Density	0.95	0.405		
26/04/2022	Plant Height	16.20	< 0.001	(LO + R) > C	>0.001
	AGB Standing Stock	5.96	0.009	(LO + R) > C	>0.05
	AGB per tiller	0.48	0.624		
	Stem Standing Stock	2.79	0.085		
	Stem g/tiller	0.31	0.740		
	Tiller Density no./m^2	6.80	0.005	(LO + R) > C	>0.05
	Tiller Density	7.16	0.004	(LO + R) > C	>0.05
20/05/2022	Plant Height	20.14	< 0.001	(LO + R) > C	>0.001
	AGB Standing Stock	5.43	0.013	LO > C	0.014
	AGB per tiller	1.35	0.282		
	Stem Standing Stock	3.22	0.060		
	Stem g/tiller	0.62	0.545		
	Tiller Density no./m^2	4.42	0.025	R > C	0.039
	Tiller Density	4.42	0.025	R > C	0.039

27/09/2022	Plant Height	15.57	< 0.001	(LO + R) > C	>0.001
	AGB Standing Stock	16.64	< 0.001	(LO + R) > C	>0.001
	AGB per tiller	15.68	< 0.001	(LO + R) > C	>0.001
	Stem Standing Stock	21.78	< 0.001	(LO + R) > C	>0.001
	Stem g/tiller	11.53	< 0.001	(LO + R) > C	>0.05
	Tiller Density no./m^2	14.11	< 0.001	(LO + R) > C	>0.001
	Tiller Density	14.11	< 0.001	(LO + R) > C	>0.001

Leaf biomass

Two-way ANOVA results show significant effect of treatment, time and their interaction on green leaf index, green leaf standing stock, green leaf biomass per tiller, total leaf standing stock, total leaf biomass per tiller (Table 9; Figure 14). A significant effect of time and the interaction between time and treatment but not of treatment itself was found for brown leaf standing stock and brown leaf biomass per tiller (Table 9; Figure 14). From the 26th of April, green leaf index, green leaf standing stock, green leaf biomass per tiller, total leaf standing stock and total leaf biomass per tiller were significantly affected by treatment at each sampling date. For all of these metrics application of fertiliser significantly increased biomass but there were no significant differences between the two fertilisation application methods (Table 10).

Table 9 – The results of two-way ANOVA analysis on leaf biomass metrics. Significant results ($P < 0.05$) are shown in green.

Dependent Variable	Source	F value	DF	P value
Green Leaf Index	Treatment	35.35	2	< 0.001
	Time	361.20	4	< 0.001
	Interaction	8.77	8	< 0.001
Green Leaf Standing Stock	Treatment	27.17	2	< 0.001
	Time	2749.11	4	< 0.001
	Interaction	7.13	8	< 0.001
Green Leaf g/tiller	Treatment	36.71	2	< 0.001
	Time	545.11	4	< 0.001
	Interaction	13.71	8	< 0.001
Brown Leaf Standing Stock	Treatment	2.26	2	0.109
	Time	166.66	4	< 0.001
	Interaction	5.58	8	< 0.001
Brown Leaf g/tiller	Treatment	1.86	2	0.161
	Time	620.55	4	< 0.001
	Interaction	12.85	8	< 0.001
Total Leaf Standing Stock	Treatment	22.58	2	< 0.001
	Time	68.60	4	< 0.001
	Interaction	3.75	8	0.001
Total Leaf g/tiller	Treatment	15.26	2	< 0.001
	Time	301.31	4	< 0.001
	Interaction	3.26	8	0.002

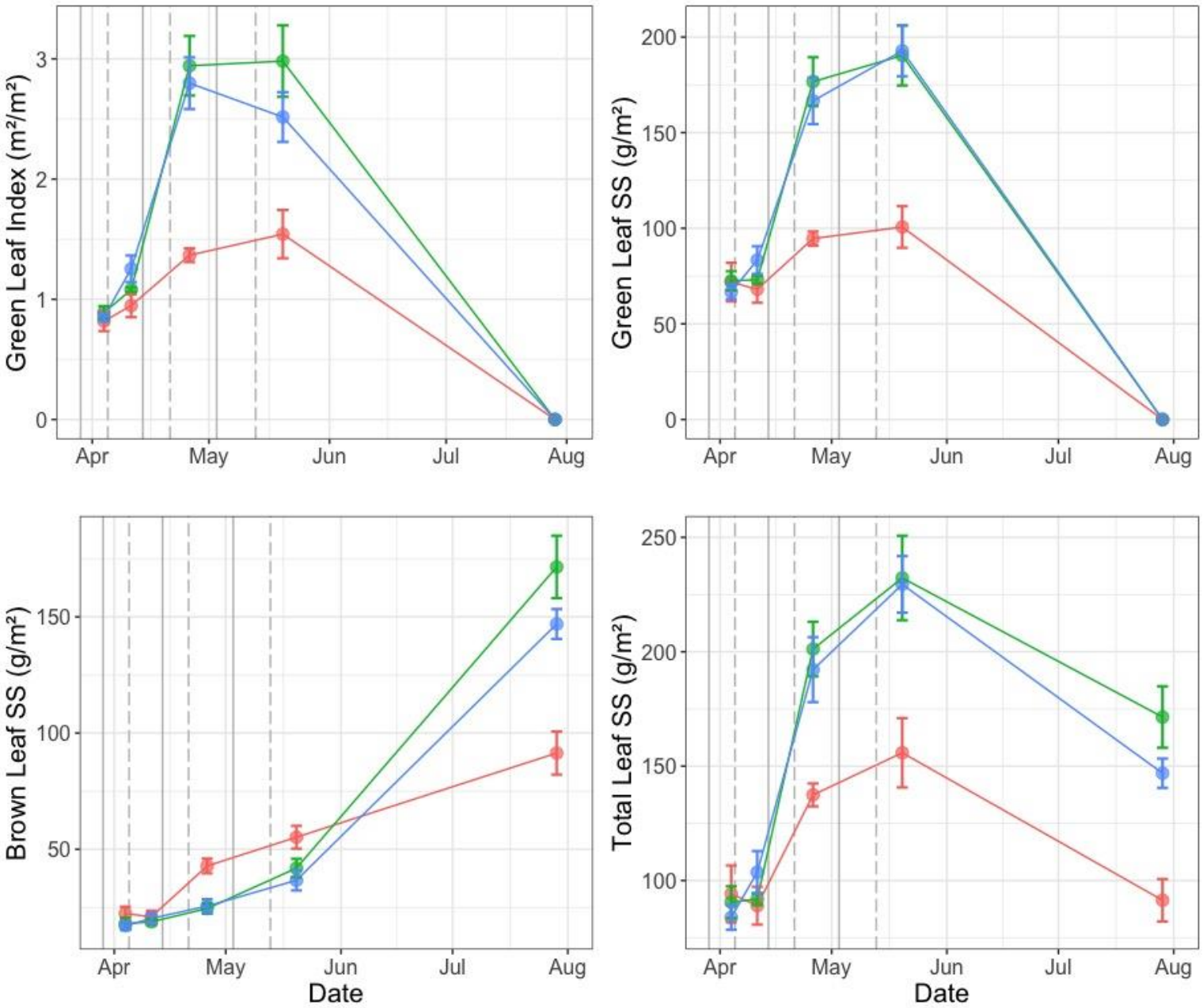

Figure 14 – Mean leaf standing stock. Error bars represent standard error. The control treatment is shown in red, little and often in green and regular in blue. Grey lines represent fertilisation events, solid lines show where fertiliser was applied to both the little and often and regularly treated crops, the dashed line shows the 'extra' doses only received by the crops treated little and often. SS stands for standing stock.

Table 10 – Results of one-way ANOVA and post hoc analyses studying the effect of treatment on dependent variables for leaf biomass metrics. P values in green are statistically significant (<0.05). Tukey tests were used as post hoc tests. LO stands for little and often, R for regular and C for control.

	Dependent Variable	Test statistic value	P value	Post hoc test	P value
04/04/2022	Green Leaf Index	0.46	0.640		
	Green Leaf Standing Stock	0.21	0.813		
	Brown Leaf Standing Stock	0.78	0.470		
	Total Leaf Standing Stock	0.21	0.816		
	Green Leaf g/tiller	1.21	0.547		
	Brown Leaf g/tiller	1.47	0.252		
	Total Leaf g/tiller	0.81	0.457		
11/04/2022	Green Leaf Index	5.80	0.055		
	Green Leaf Standing Stock	2.04	0.155		
	Brown Leaf Standing Stock	0.12	0.890		
	Total Leaf Standing Stock	1.14	0.339		
	Green Leaf g/tiller	0.83	0.449		
	Brown Leaf g/tiller	1.01	0.381		
	Total Leaf g/tiller	0.17	0.848		
26/04/2022	Green Leaf Index	33.14	< 0.001	(LO + R) > C	>0.001
	Green Leaf Standing Stock	29.33	< 0.001	(LO + R) > C	>0.001
	Brown Leaf Standing Stock	10.78	0.001	(LO + R) > C	>0.05
	Total Leaf Standing Stock	12.89	< 0.001	(LO + R) > C	>0.001
	Green Leaf g/tiller	26.16	< 0.001	(LO + R) > C	>0.001
	Brown Leaf g/tiller	25.97	< 0.001	(LO + R) > C	>0.001
	Total Leaf g/tiller	5.66	0.011	(LO + R) > C	>0.05
20/05/2022	Green Leaf Index	11.12	0.001	(LO + R) > C	>0.001
	Green Leaf Standing Stock	15.29	< 0.001	(LO + R) > C	>0.001
	Brown Leaf Standing Stock	4.96	0.017	R < C	0.014
	Total Leaf Standing Stock	7.78	0.003	(LO + R) > C	>0.05
	Green Leaf g/tiller	20.16	< 0.001	(LO + R) > C	>0.001
	Brown Leaf g/tiller	20.37	< 0.001	(LO + R) > C	>0.001
	Total Leaf g/tiller	5.99	0.009	(LO + R) > C	>0.05

27/09/2022	Brown Leaf Standing Stock	16.44	< 0.001	(LO + R) > C	>0.001
	Total Leaf Standing Stock	16.44	< 0.001	(LO + R) > C	>0.001
	Brown Leaf g/tiller	7.17	0.004	(LO + R) > C	>0.05
	Total Leaf g/tiller	7.16	0.004	(LO + R) > C	>0.05

Ear and grain biomass

Ear density and standing stock were significantly affected by treatment (Table 11; Figure 15). Tukey tests show that there was a significant positive effect on ear density and standing stock following fertiliser treatment but that the method of application was insignificant (Table 11). Treatment was also found to be significant for grain standing stock, grain count per ear, grain biomass per ear and individual grain biomass (Table 11; Figure 15). Tukey tests showed that grain standing stock, grain biomass per ear and grain count per ear were significantly improved by fertiliser application (Table 11; Figure 15) but that the type of fertiliser application method was insignificant (Table 11; Figure 15). For individual grain biomass, there was a significant difference between regularly treated crops and untreated crops (Table 11; Figure 15) but there was no difference between little and often treated crops and regularly treated crops or untreated crops (Table 11).

Table 11 – Results of one-way ANOVA and post hoc analyses studying the effect of treatment on ear metrics at harvest. P values in green are statistically significant (<0.05). Tukey tests were used as post hoc tests. LO stands for little and often, R for regular and C for control.

Dependent Variable	Test statistic value	P value	Post hoc test	P value
Grain biomass/ear	16.81	< 0.001	(LO + R) > C	>0.001
Grain no./ear	15.44	< 0.001	(LO + R) > C	>0.001
Individual grain biomass	5.05	0.016	R > C	0.013
Grain Standing Stock	16.49	< 0.001	(LO + R) > C	>0.001
Ear Density no./m^2	20.67	< 0.001	(LO + R) > C	>0.001
Ear Standing Stock	16.98	< 0.001	(LO + R) > C	>0.001

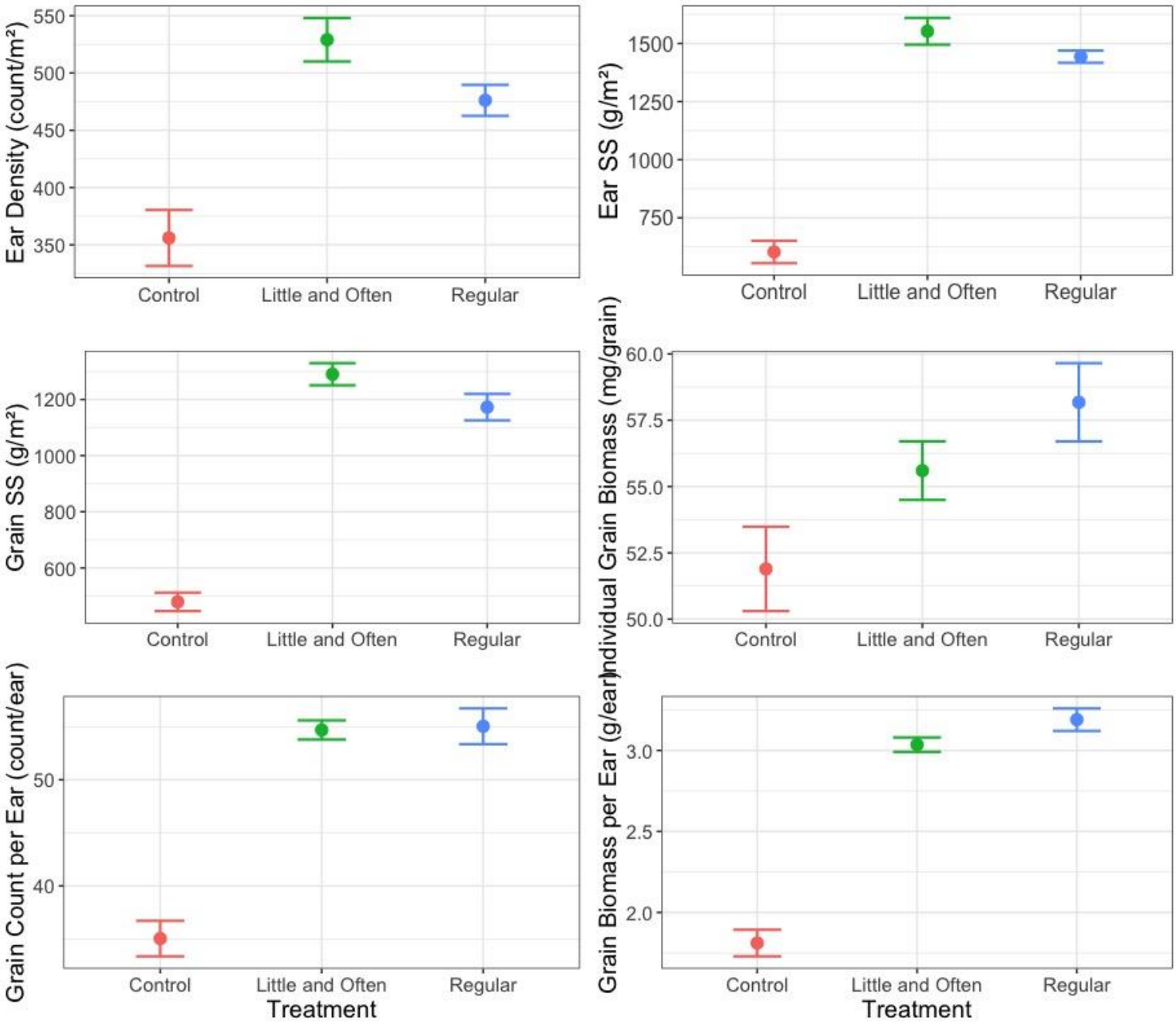

Figure 15 – Mean values for ear and grain metrics. Error bars represent standard error. The control treatment is shown in red, little and often in green and regular in blue.

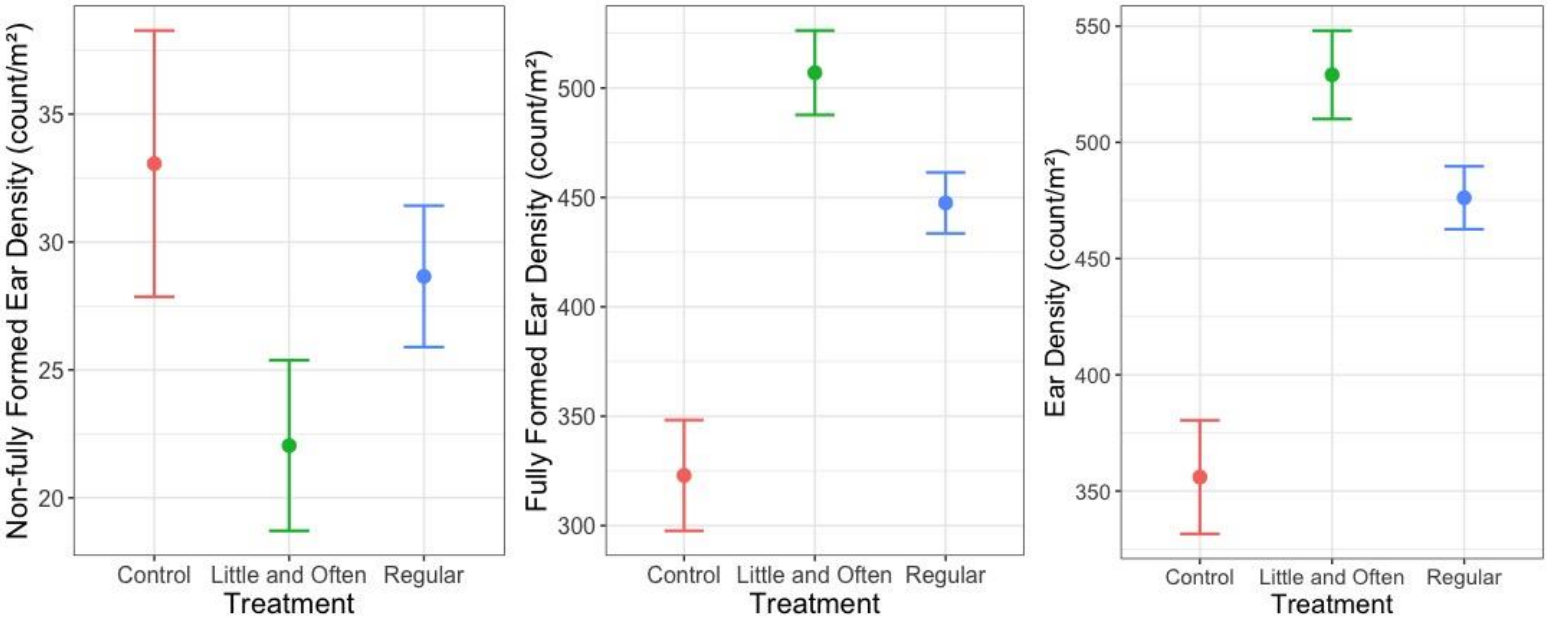

Figure 16 – Mean values for ear densities. Error bars represent standard error. The control treatment is shown in red, little and often in green and regular in blue.

Discussion:

Effect of treatment on crop roots

Despite not finding a significant difference between treatments for root standing stock, our data shows that the untreated crops on average had greater root biomass (Figure 12). This suggests that the untreated crops were investing more energy into root development than the treated crops. Interestingly, the literature suggests that fertiliser enrichment should promote root growth leading to greater root biomass in fertilised wheat (Tian, Zhang & Ju 2023). A study on soybeans found that organic N sources were more effective at increasing root biomass than inorganic N (Arslanoglu 2022), our results may be due to this effect as our unfertilised soils had greater organic N. The increased root biomass in our unfertilised crops suggests a redirection of energy investment into root development that we do not observe for treated crops. From this, it could be inferred that fertiliser addition enables crops to invest more energy into aboveground (i.e., ear and grain) development.

Soil C/N ratios are low (below 30) for all treatments suggesting the main microbial processes in the soil for all treatments is N mineralisation, i.e., the conversion of organic forms of N into ammonium (Figure 5; Kumar *et al*., 2020). The soil C/N ratio is significantly

lower in the control treatment implying that N mineralisation is even more abundant in these soils than other microbial processes. This increased N mineralisation in unfertilised soils could be a contributing factor to the higher root biomass observed. N mineralisation leads to the production of N forms (i.e., ammonia) that can be more easily assimilated by crops (Hodge, Robinson & Fitter 2000). The greater volume of production of ammonia by microbes in untreated soils could encourage stronger root development as unfertilised crops would be strongly N limited.

Effect of treatment on aboveground biomass:

We expected that we would observe greater aboveground crop biomass in the plants treated with the little and often fertiliser doses than those untreated or treated regularly. A significant interaction between the effects of treatment and time on aboveground biomass metrics throughout the growing season of the crops was found, however, no significant effect between the little and often and regular fertiliser treatments were observed for any of the aboveground biomass and root variables (Figure 13; Figure 14; Table 7; Table 9). In the early stages of the growing season, no significant differences between the treatments were found (Figure 13; Figure 14). The most significant effects of treatment were found from the 26th of April onwards, this was when the crop was in the growth stage of stem elongation (Figure 10; Figure 13; Figure 14; Table 8; Table 10). A significant investment from crops into growth was observed until the 20th of May, after this point the crops are entering growth stage 40 (booting) (Figure 10; Figure 13; Figure 14). At this point, the little and often crops have a greater average green leaf index than both the regularly and untreated crops (Figure 14). A study on the effect of fertilisation on winter wheat found fertilisation to increase leaf surface area and chlorophyll content (Khan *et al*., 2019). We suggest that the effect on leaf surface area and chlorophyll is further improved by little and often fertilisation methods. This implies that their photosynthetic ability and therefore energy production is greater than the other treatments.

Our hypothesis states that we expect a greater aboveground biomass following little and often fertiliser application, we are unable to accept this hypothesis as we did not observe a significant difference between the two fertiliser application methods. We suggest

that by altering the timings of fertiliser application, as mentioned previously, the differences between the two treatments may become significant.

Effect of treatment on ear and grain biomass:

We found similar results for ear and grain biomass metrics as for aboveground biomass. There was a significant effect of treatment but no significant difference between the two types of treatment (Figure 15; Table 11). However, for a number of our ear and grain metrics, we do observe a greater mean value for the little and often treatment. Our data suggests that the ear density, ear standing stock and grain standing stock are all greater under the little and often treatment (Figure 15). Additionally, our ear densities show that the little and often treatment had the least non-fully formed ears, on average, when compared to the other treatments (Figure 16). This suggests even if a significant affect was not found, there is an effect of the little and often fertiliser.

Timings of fertiliser application can be a key factor in determining the effect of fertiliser on crop yield (Ayoub *et al*., 1994). In our study fertilisation events were concentrated towards the early stages of crop development, between GS30 and GS40 (Figure 13). As previously mentioned, the Nutrient Management guide (RB209) recommends fertilisation of winter wheat between GS32 and GS39, in line with our application (AHDB 2023). The recommendation also states that additional later fertilisation applications are likely to increase yield but are not always recommended (AHDB 2023). There is plenty of evidence in the literature to support the benefits of later stage fertiliser application. A study on Summer Maize found that by applying split doses of fertiliser at later stages of development, yield was increased (Deng *et al*., 2023). Fertilisation at booting (GS40) and milk ripening stages (GS70) have also been found to have the most prominent effect on winter wheat yield (Vaguseviciene & Juchneviciene 2015). We argue that by concentrating fertiliser application early in the development of the crop, the differences between the two fertiliser treatments were masked and despite being observable, are not significant.

Similarly to aboveground biomass, we are unable to accept our hypothesis as we do not observe a significant difference between any ear or grain characteristics for the little and often treatment and the regular treatment. Our results also show higher means for some variables under the little and often treatment suggesting an impact of this fertilisation

method but without any statistical significance. As mentioned previously, by altering fertiliser application timings, we suggest that these differences may become significant.

Chapter 6: Concluding Remarks

We found no significant effect of the little and often treatment on any of our dependent variables. Despite this, we suggest that there is still a strong case to be made for the benefits of little and often fertilisation methods. We propose that no significant differences were observed between the regular and little and often crops due to timings of fertiliser addition. Fertiliser application events were concentrated towards the start of the growing season for both the little and often and regular application. It was beyond the scope of this study to investigate the effectiveness of fertiliser timings and any potential interaction of fertiliser timings and splitting of fertiliser application. However, we recognise that there is a clear gap in the literature regarding this and believe that a study into this will validate the effectiveness of little and often fertiliser regimes.

The literature suggests that a late-stage application of fertiliser, around the growth stage of anthesis is likely to improve crop yield (Deng *et al*., 2023). In a regular fertiliser application system, to apply fertiliser at this stage may not be beneficial. Crops have their highest demand for N during the start of the growing season (AHDB 2023) and there is a benefit to supplying N at this stage which is why traditional recommendations, such as RB209, recommend applying at this stage. Under a regular treatment, it is logical to focus fertiliser applications early in the growing season. However, when applying fertiliser in smaller more frequent doses, the majority of fertiliser could still be applied at this stage, meeting the demand needed by crops and a final dose could be applied later on in the growing season to 'top up' the soil N supply which will have been drained by crops as they have developed. N requirements at this late stage in crop development are likely to have an effect on yield as this is when crops begin investing energy into ear development. By resupplying N around anthesis, we can mediate any potential loses in yield and provide the plants with a 'boost' of productivity to assist with strong ear and grain development.

There is very little literature concerning the effect of split fertiliser application on plant-microbe competition. We found a clear effect of fertiliser application on the competition between plants and microbes, but were unable to establish a significant difference between the little and often and regular fertiliser treatments. We suggest that further study into the specific plant-microbe dynamics following little and often fertilisation are crucial in understanding the effectiveness of the little and often method. The

importance of microbial processes involved in fertiliser application cannot be overlooked. Not only is microbial diversity beneficial for agricultural output of soils (Lankau, George & Miao 2022) and efforts to maintain microbial diversity should be encouraged, microbes are responsible for the emissions of N_2O from soils (Bateman & Baggs 2004; Hernandez-Ramirez, Ruser & Kim 2021). Monitoring the effects of agricultural practice on microbial processes is crucial for any efforts to increase crop yield and nitrogen use efficiency as well as to meet GHG emission targets.

Our study shows that there is a clear potential for little and often fertiliser application to have a beneficial impact on crop biomass and crop yield. This has the prospect to be hugely influential in the agricultural sector, saving farmers financially, especially as fertiliser prices continue to rise, but also providing ecosystem benefits, by reducing fertiliser run off and reducing GHG emissions from soils. We propose further study focuses on whether altering timings of little and often applications can provide benefits to crop biomass and yield, as well as nitrogen use efficiency and nitrogen uptake efficiency. Additionally, further study should focus on plant-microbe competition throughout the growing season, monitoring the effects of fertiliser application and competition strategies of crops and microbes over the period of the whole of the growing season.

References

Abbasi MK, Tahir MM & Rahim N. 2013. Effect of N fertilizer source and timing on yield and N use efficiency of rainfed maize (Zea mays L.) in Kashmir–Pakistan. *Geoderma*. **195-196**. pp 87-93.

Adesemoye A & Kloepper JW. 2009. Plant–microbes interactions in enhanced fertilizer-use efficiency. *Applied Microbiology and Biotechnology*. **85**. pp 1-12.

AHDB. 2023. Nutrient Management Guide (RB209): Section 4 Arable crops. [*Updated June 2023*].

Amani A & Behzad MA. 2020. Effect of split application of nitrogen on growth and yield of wheat (Triticum aestivum L.). *Journal of Experimental Agriculture International*. **42**(6). pp 44-48

Arslanoglu S. 2022. The effects on the root and plant development of soybean of organic fertilsier application. *Bioscience Journal.* **38**.

Aulakh MS & Malhi SS. 2005. Interactions of Nitrogen with Other Nutrients and Water: Effect on Crop Yield and Quality, Nutrient Use Efficiency, Carbon Sequestration, and Environmental Pollution. *Advances in Agronomy*. **86**. pp 341-409.

Ayoub M, Guertin S, Lussier S & Smith DL. 1994. Timing and Level of Nitrogen Fertility Effects on Spring Wheat Yield in Eastern Canada. *Crop Science*. **34**(3). pp 748-756.

Azeem B, KuShaari K, Man ZB, Basit A & Thanh TH. 2014. Review on materials & methods to produce controlled release coated urea fertilizer. *Journal of Controlled Release*. 181. pp 11-21.

Bateman EJ & Baggs EM. 2005. Contributions of nitrification and denitrification to N_2O emissions from soils at different water-filled pore space. *Biology and Fertility of Soils*. **41**. pp 379-388.

Bell MJ, Hinton N, Cloy JM, Topp CFE, Rees RM et al. 2015. Nitrous oxide emissions from fertilised UK arable soils: Fluxes, emission factors and mitigation. *Agriculture, Ecosystems and Environment*. **212**. pp 134-147.

Brookes PC, Landman A, Pruden G & Jenkinson DS. 1985. Chloroform fumigation and the release of soil nitrogen: a rapid direct extraction method to measure microbial biomass nitrogen in soil. *Soil Biol Biochem*. **17**(6). pp 837-842.

Cardenas LM, Bhogal A, Chadwick DR, McGeough K, Misselbrook T et al. 2019. Nitrogen use efficiency and nitrous oxide emissions from five UK fertilised grasslands. *Science of the Total Environment*. **661**. pp 696-710.

Castellano-Hinojosa A, Correa-Galeote D, Gonzalez-Lopez J & Bedmar EJ. 2020. Effect of nitrogen fertilisers on nitrous oxide emission, nitrifier and denitrifier abundance and bacterial diversity in closed ecological systems. *Applied Soil Ecology*. **145**.

Chislock MF, Doster E, Zitomer RA & Wilson AE. 2013. Eutrophication: Causes, Consequences, and Controls in Aquatic Ecosystems. *Nature Education Knowledge*. **4**(4).

Coventry D, Yadav A, Poswal R, Sharma R, Gupta R et al. 2011. Irrigation and nitrogen scheduling as a requirement for optimising wheat yield and quality in Haryana, India. *Field Crops Research*. **123**(2). pp 80-88.

Delogu G, Cattivelli L, Pecchioni N, Falcis D, Maggiore T et al. 1998. Uptake and agronomic efficiency of nitrogen in winter barley and winter wheat. *European Journal of Agronomy*. **9**(1). pp 11-20.

Deng T, Wang J, Gao Z *et al*. Late Split-Application with Reduced Nitrogen Fertilizer Increases Yield by Mediating Source–Sink Relations during the Grain Filling Stage in Summer Maize. *Plants*. **12**(3). p 625.

Dunn RM, Mikola J, Bol R & Bardgett RD. 2006. Influence of microbial activity on plant–microbial competition for organic and inorganic nitrogen. *Plant and Soil*. **289**. pp 321-334.

Efretuei A, Gooding M, White E, Spink J & Hackett R. 2016. Effect of nitrogen fertilizer application timing on nitrogen use efficiency and grain yield of winter wheat in Ireland. *Irish Journal of Agricultural and Food Research*. **55**(1). pp 63-73.

FAO. 2021. FAOSTAT: Fertilizers by Nutrient. http://www.fao.org/faostat/en/#data/RFN [Accessed 23/6/2022].

Fernández L. 2021. Agricultural fertilizer market in the UK – statistics & facts. *Statistica*. https://www.statista.com/topics/4588/agricultural-fertilizer-market-in-the-uk/#topicHeader__wrapper [Accessed 20/06/2022].

Flessa H, Ruser R, Schilling R, Loftfield N, Munch JC et al. 2002. N_2O and CH_4 fluxes in potato fields: automated measurement, management effects and temporal variation. *Geoderma*. **105**(3-4). pp 307-325.

Fox J, Weisberg S. 2019. An R Companion to Applied Regression. Third Edition. *Sage*.

Gezahegn AM, Eshetu S, Tafes B, Feleke G. 2021. Timing and Splitting of Nitrogen Application to Increase Tef (Eragrostic Tef (Zucc.) Trotter) Yield and Nitrogen Use Efficiency at Central Highlands of Ethiopia. *Communications in Soil Science and Plant Analysis*. **52**(10). pp 1100-1114.

Gezahegn AM, Desta BT, Takel A & Eshetu S. 2022. Split Application of Nitrogen Fertilizer for Optimum Yield of Durum Wheat (Triticum turgidum L. var. Durum) at Central Ethiopia. *Communications in Soil Science and Plant Analysis*. **53**(14).

Hénault C, Grossel A, Mary B, Roussel M & Léonard J. 2012. Nitrous Oxide Emission by Agricultural Soils: A Review of Spatial and Temporal Variability for Mitigation. *Pedosphere*. **22**(4). pp 426-433.

Hernandez-Ramirez G, Ruser R & Kim D-G. 2021. How does soil compaction alter nitrous oxide fluxes? A meta-analysis. *Soil & Tillage Research*. **211**.

Hodge A, Robinson D & Fitter A. 2000. Are microorganisms more effective than plants at competing for nitrogen? *Trends in Plant Science*. **5**(7). pp 304-308.

Huang WY & Uri ND. 1995. A note of nitrogen fertilizer application timing. *Applied Mathematical Modelling*. **19**(3). pp 182-191.

Inselsbacher E, Hink-Najera Umana N, Stange FC, Gorger M, Schuller E et al. 2010. Short-term competition between crop plants and soil microbes for inorganic N fertilizer. *Soil Biology & Biochemistry*. **42**(2). pp 360-372.

IPCC. 2014. Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change.

IPCC. 2022. Climate Change 2022: Impacts, Adaptation, and Vulnerability. Contribution of Working Group II to the Sixth Assessment Report of the Intergovernmental Panel on Climate.

Jiménez JG, Daly K, Roberts W, Healy M. 2019. Split phosphorus fertiliser applications as a strategy to reduce incidental phosphorus losses in surface runoff. *Journal of Environmental Management*. **242**. pp 114-120.

Kaur B, Kaur G & Asthir B. 2017. Biochemical aspects of nitrogen use efficiency: An overview. *Journal of Plant Nutrition*. **40**(4). pp 506-523.

Kaye JP & Hart SC. 1997. Competition for nitrogen between plants and soil microorganisms. *Trends in Ecology & Evolution*. **12**(4). pp 139-143.

Kenea WB, Reidsma P, Decheemaeker K, Rurinda J, Balemi T et al. 2021. Variability in yield responses, physiological use efficiencies and recovery fractions of fertilizer use in maize in Ethiopia. *European Journal of Agronomy*. **124**.

Khan A, Muhammad A, Khan A, Anwar S & Hollington P. 2019. Nitrogen Affects Leaf Expansion and Elongation Rates During Early Growth Stages of Wheat. *International Journal of Agriculture and Biology*. **21**(6). pp 1117-1122.

Kirda C, Derici MR & Schepers JS. 2001. Yield response and N-fertiliser recovery of rainfed wheat growing in the Mediterranean region. *Field Crops Research*. **71**(2). pp 112-122.

Koch M, Akshalov K, Carstens JF et al. 2021. Competition of Plants and Microorganisms for Added Nitrogen in Different Fertilizer Forms in a Semi-Arid Climate. *Agronomy*. **11**.

Kronenberg L, Yu K, Walter A & Hund A. 2017. Monitoring the dynamics of wheat stem elongation: genotypes differ at critical stages. *Euphytica*. **213**(157).

Kumar A, Medhi K, Fagodiya RK, Subrahmanyam G, Mondal R et al. 2020. Molecular and ecological perspectives of nitrous oxide producing microbial communities in agro-ecosystems. *Reviews in Environmental Science and Bio/Technology*. **19**. pp 717-750.

Kuzyakov Y & Xu X. 2013. Competition between roots and microorganisms for nitrogen: mechanisms and ecological relevance. *New Phytologist*. **198**(3). pp 656-669.

Lankay RA, George I & Miao M. 2022. Crop performance is predicted by soil microbial diversity across phylogenetic scales. *Ecosphere*. **13**(5).

Li GH, Cheng Q, Li L, Lu DL, Lu WP. 2021. N, P and K use efficiency and maize yield responses to fertilization modes and densities. *Journal of Integrative Agriculture*. **20**(1). pp 78-86.

Liu C, Wang K, Meng S, Zheng X, Zhou Z et al. 2011. Effects of irrigation, fertilization and crop straw management on nitrous oxide and nitric oxide emissions from a wheat-maize rotation field in northern China. *Agriculture, Ecosystems & Environment*. **140**(1-2). pp 226-233.

Liu XA, Groenigen KJ, Dijkstra P & Hungate BA. 2017. Increased plant uptake of native soil nitrogen following fertilizer addition – not a priming effect? *Applied Soil Ecology*. **114**. pp 105-110.

Luo HD, Zhu CF, Wang JW, Liu SM, Guo XM et al. 2018. Effects of different fertiliser types on the yield of Camellia Oleifera Abel and nitrogen utilization efficiency. *Fresenius Environmental Bulletin*. **27**(11). pp 7425-7432.

Ma BM, Zhou X, Zhang Q, Qin M, Hu L et al. 2019. How do soil micro-organisms respond to N, P and NP additions? Application of the ecological framework of (co-)limitation by multiple resources. *Journal of Ecology*. **107**(5). pp 2329-2345.

Martin RJ, Craighead MD, Williams PH, Tregurtha CS. 2001. Effect of fertiliser rate and type on the yield and nitrogen balance of a Pukekohe potato crop. *Agronomy New Zealand*. **31**. pp 71-80.

Met Office. 2023. UK Temperature, rainfall and sunshine anomaly graphs. https://www.metoffice.gov.uk/research/climate/maps-and-data/uk-temperature-rainfall-and-sunshine-anomaly-graphs [Accessed 06/09/23].

Müller R. 2021. The impact of the rise in atmospheric nitrous oxide on stratospheric ozone. *Ambio*. **50**. pp 35-39.

Nkebiwe PM, Weinmann M, Bar-Tar A, Muller T. 2016. Fertilizer placement to improve crop nutrient acquisition and yield: A review and meta-analysis. *Field Crops Research*. **196**. pp 389-401.

Otie V, Ping A, John NM, Eneji AE. 2016. Interactive effects of plant growth regulators and nitrogen on corn growth and nitrogen use efficiency. *Journal of Plant Nutrition*. **39**(11). pp 1597-1609.

Ouyang S, Tian Y, Liu Q, Zhang L, Wang R et al. 2016. Nitrogen competition between three dominant plant species and microbes in a temperate grassland. *Plant and Soil*. **408**. pp 121-132.

Ritchie H & Roser M. 2013. Fertilisers. *Our World in Data*. https://ourworldindata.org/fertilizers [Accessed 23/06/2022].

RStudio Team. 2020. R Studio: Integrated Development for R. *RStudio*.

Sitthaphanit S, Limpinuntana V, Toomsan B, Panchaban S, Bell R. 2009. Fertiliser strategies for improved nutrient use efficiency on sandy soils in high rainfall regimes. *Nutrient Cycling in Agroecyosystems*. **85**(2). pp 123-139.

Smith KA. 2017. Changing views of nitrous oxide emissions from agricultural soil: key controlling processes and assessment at different spatial scales. *European Journal of Soil Science*. **68**. pp 137-155.

Stehfest E & Bouwman L. 2006. N_2O and NO emission from agricultural fields and soils under natural vegetation: summarizing available measurement data and modeling of global annual emissions. *Nutrient Cycling in Agroecosystems*. **74**. pp 207-228.

Thorman RE, Williams JR, Misselbrook TH, Rollett AJ, Bowden et al. 2013. Nitrification inhibitor and nitrogen fertiliser application timing strategies to reduce nitrous oxide emissions from winter wheat land.

Tian X, Zhang C & XT J. 2023. Crop responses to application of optimum nitrogen fertilizers on soils of various fertilities formed from long-term fertilization regimes. *European Journal of Agronomy*. **148**.

Tilahun CB, Gebrekidan H Kibret TK & Debele DT. 2017. Effect of rate and time of nitrogen fertilizer application on durum wheat (Triticum turgidum Var L. Durum) grown on Vertisols of Bale highlands, southeastern Ethiopia. **5**(1). pp 39-56.

Vaguseviciene I & Juchneviciene A. 2015. The effect of nitrogen fertilisers on the grain yield of different cultivars of Winter Wheat. *Rural Development*.

Vance ED, Brookes PC & Jenkinson DS. 1987. An Extraction Method for Measuring Soil Microbial Biomass C. *Soil Biology and Biochemistry*. **19**. pp 703-707.

Velasco JL, Rozas HS, Echeverría HE & Andrés P. 2012. Optimizing fertilizer nitrogen use efficiency by intensively managed spring wheat in humid regions: Effect of split application. *Canadian Journal of Plant Science*. **92**. pp 847-856.

Wang XB, Cai DX, Hoogmoed WB, Oenema O. 2011. Regional distribution of nitrogen fertilizer use and N-saving potential for improvement of food production and nitrogen use efficiency in China. *Journal of the Science of Food and Agriculture.* **91**(11). pp 2013-2023.

Xing YY, Jiang WT, He XL, Fiaz S, Ahmad S et al. 2019. A review of nitrogen translocation and nitrogen-use efficiency. *Journal of Plant Nutrition*. **42**(19). pp 2624-2641.

Xing YY, Mi FY & Wang XK. 2022. Effects of different nitrogen fertilizer types and application rates on maize yield and nitrogen use efficiency in Loess Plateau of China. *Journal of Soils and Sediments*.

Yang G, Chu K, Tang H, Nie Y & Zhang X. 2013. Fertilizer ^{15}N Accumulation, Recovery and Distribution in Cotton Plant as Affected by N Rate and Split. *Journal of Integrative Agriculture.* **12**(6). pp 999-1007.

Zhang S, Zheng Q, Noll L, Hu Y & Wanek W. 2019. Environmental effects on soil microbial nitrogen use efficiency are controlled by allocation of organic nitrogen to microbial growth and regulate gross N mineralization. *Soil Biol Biochem.* **135**. pp 304-315.