Molecular gene markers for nitrification and denitrification and comparison with agricultural soil nitrous oxide emissions

Pierre-Alain van Griethuysen

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University of York

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Abstract

Nitrous oxide (N_2O) is an important greenhouse gas that accounts for 6% of total global forcing. Soils produce 70% of yearly global emissions and arable agriculture contributes significantly to this. Most N_2O production is from microbial activity and soil microbes produce N_2O from two main pathways, nitrification and denitrification. It is important to understand the relationship between N_2O production and its microbial origins under different crop regimes for better mitigation of N_2O emissions in arable agriculture. Due to the phylogenetic spread of nitrifiers and denitrifiers, a functional gene approach is preferable for studying the microbial origins of these processes.

Open gas chamber sampling was carried out on open-bottomed soil cores filled with agricultural soils. Cores were either unplanted or filled planted with either *Triticum aestivum* or *Brassica napus*. Nitrogen was added as urea solution to some of the cores to simulate fertilizer addition. Soil-atmosphere fluxes of N_2O were quantified and soil samples were taken for DNA extractions and analysis of the functional genes.

Flux data suggested evidence for differences in N_2O emissions between *T. aestivum* and *B. napus*. N_2O fluxes were significantly lower in cores planted with *T. aesitivum* compared to unplanted and *B. napus* cores when treated with nitrogen. There was no significant difference in presence of functional genes with nitrogen addition or between different planting regimes. This study found that different crops respond differently to N addition, causing significant changes in N_2O emissions. It offers an additional tool to make decisions about soil and agricultural management, such as N addition and will enable more sustainable use of soils.

Contents

List of Figures

List of Tables

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More than anyone, thanks to Annabel, for always being there, no matter what the situation and always being there with charm, patience, love and no small amount of standing in a field in the rain. I couldn't have done this without you.

Author's declaration

I, Pierre-Alain Guillaume van Griethuysen declare that all the material contained within this thesis except for the work outlined below is a result of my own work and has been written solely by myself. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.

The preliminary design for the field experiment was done in collaboration with Phil Ineson's undergraduate project student (Luke Cox). The soil collection and filling of cores at the University was carried out by Pierre, Luke and Phil Ineson. The transportation of the cores to the field site and the digging in of the cores was carried out by Phil Ineson and Luke. Changes and amendments to that initial design were done by Pierre.

Gas sampling and transportation of equipment mostly carried out by Pierre and Phil Ineson's summer project student (Renato Albernaz de Carvalho), some transportation was carried out by James Stockdale. Most runs were carried out by Pierre and Renato and some by Pierre and Annabel Gipp. Soil samples collected, weighed and DNA extracted by Pierre. All molecular work designed and carried out by Pierre. SAS script for selecting and tidying data from the LGR written by James Stockdale and Phil Ineson. All statistical analysis and writing up carried out by Pierre.

Section 1: Introduction

World population is predicted to grow from 7 billion in 2013 to a projected 9.6 billion by 2050 (United Nations Population Division). As demand rises for increasingly diverse diets as consumer purchasing capacity increases, especially in the developing world, it's been predicted that food production must increase by at least 70% (FAO 2009). Most remaining potential farmland are crucial habitats for wild flora and fauna, so most of this increase must come from improving productivity on pre-existing agricultural land (Connor *et al.* 2011). Tempering this with combating the increase in greenhouse gas emissions from agriculture (IPCC 2013) is a great challenge. Nitrous oxide is a potent greenhouse gas (GHG) with a global warming potential 298 times greater than that of CO_2 over 100 years (IPCC 2013), responsible for 6% (IPCC 2013) of total global forcing. Soils contribute around 70% of global N_2O emissions with agriculture being the single largest global source (6.8 Tg N_2O-N yr^{-1}, Baggs and Philippot, 2011).

Much of this comes from nitrogen enrichment from fertilisers and biological nitrogen fixation (e.g. green manure legumes grown between crops). N_2O is produced mainly by soil microbes through nitrification and denitrification (Fig. 1). Emissions from nitrate ammonification seem unlikely to contribute a great deal to the global emissions as measured rates are quite low (Schmidt *et al.* 2010). Similarly, chemodenitrification, which is non biological, is known to occur at pH<5.5 and may contribute to N_2O emissions in those conditions (Mørkved *et al.* 2007, Van Cleemput and Samater, 1996) but is less of a concern in most agricultural crop systems.

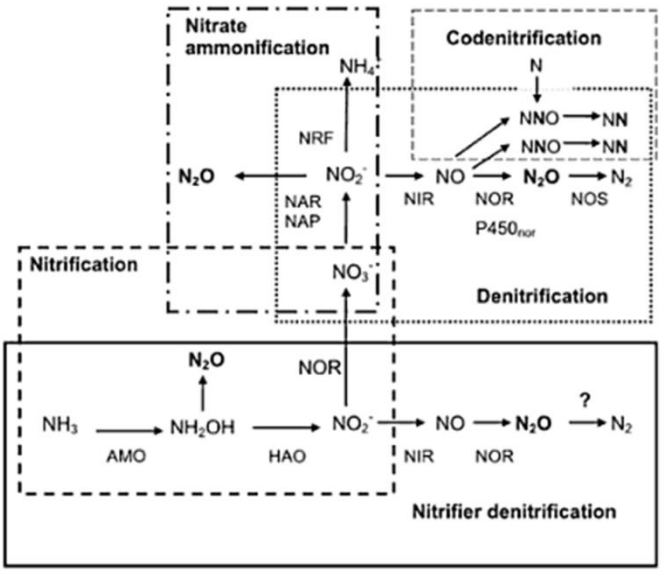

Figure 1. The pathways in which N_2O can be produced by soil microbes and the enzyme responsible. The same enzymes are involved in denitrification and nitrifier denitrification although the organisms are different. Enzymes: AMO=ammonia monooxygenase, HAO= hydroxylamine oxidase, NIR= nitrite reductase, NOR=nitric oxide reductase, NAR/P= nitrate reductase, NOS= nitrous oxide reductase. (From Baggs and Philippot 2011 copyright , modified from Baggs 2008)

1.1 Nitrification and Denitrification

Nitrification is the oxidation of ammonia to nitrate via nitrite. All known bacterial autotrophic ammonia oxidisers belong to the genera *Nitrosomonas and Nitrosospira* that form a monophyletic cluster. Many heterotrophic microbes are known than can also use organic substances as well as ammonia; however data about the contribution of heterotrophic nitrification to soil process rates is inconclusive (Bateman and Baggs, 2005). Recent findings have shown that mesophilic crenarchaea, (now thaumarchaea, to distinguish them from the extreme thermophiles of the rest of crenarchaea [Brochier-Armanet *et al.* 2008]) are also capable of oxidising ammonia. They are often far more abundant than ammonia oxidising bacteria (Leninger *et al.* 2006, Chen *et al.* 2008) although this is not always the case (Petersen *et al.* 2012).

Nitrification can lead to N_2O production when there is less available oxygen, usually due to waterlogged soils. The oxidation of ammonia to nitrite is a two-step process. First, ammonia is oxidised to hydroxylamine by the enzyme ammonia mono-oxygenase (*amo*), then this is oxidised to nitrite by hydroxylamine oxido-reductase (*hao*). In limited oxygen, *hao* can convert hydroxylamine to N_2O in a branched reaction (Kostera *et al.* 2008). Studies have shown that at 60% water-pore filled space (WPFS) nitrification can account for 81% of N_2O emitted (Bateman and Baggs, 2005). So whilst traditionally nitrification is thought of an aerobic process, its contribution to N_2O emissions may be higher under more anoxic conditions.

Denitrification is the reduction of nitrate or nitrite to nitric oxide, nitrous oxide and dinitrogen under anaerobic conditions. This ability is very widespread and common in micro-organisms. Denitrifiers have been found in over 60 genera (Baggs and Philippot 2011) and can represent up to 5% of the total soil microbial community (Henry *et al.* 2006). This is a heterotrophic process, where N oxides are used as terminal electron acceptors and so requires a C source. N_2O is produced in the penultimate step, when nitric oxide is reduced by nitric oxide reductase (*nor*) before being further reduced by nitrous oxide reductase (*nos*) to form dinitrogen. Whilst all of these enzymes are suppressed by high O_2, *nos* is particularly sensitive and will be suppressed first with increasing concentrations of O_2. It will also be the last to be switched back on when soils become anaerobic following an aerobic period. So high fluctuations in soil moisture, possibly due to heavy rain and then extended periods of dryness should increase the ratio of N_2O/N_2 emissions.

1.2 Functional Gene Abundance and SP Ratio

This project aimed to quantify the real time process rates for nitrification and denitrification by looking at functional gene abundances. Functional genes are groups of genes across organisms and enzymatic pathways that have been shown to have a functional role in a process we are interested in. For example *amoA* was found to code for the active site of ammonia mono-oxygenase (McTavish *et al.* 1993) in *Nitrosomonas europaea* but has since been found in many other bacteria, and archaeal variants also exist. Rather than searching for abundances of organisms that are known to be nitrifiers such as *N. europaea*, studies have looked for abundances of *amoA* genes, irrespective of what species they are found in, and comparing them with nitrification rates (Bernhard *et al.* 2010, Petersen *et al.* 2012).

There are good reasons for using functional genes. Firstly some of these genes (such as *amoA*) have been used extensively as molecular markers in many studies (Wang *et al.* 2014, Restrepo-Ortiz *et al.* 2014, Henry *et al.* 2006, Chen *et al.* 2008) and been found to correlate well with potential nitrification rates (Petersen *et al.* 2012). Secondly, denitrifiers are spread sporadically and widely across phylogenetic groups; which makes a functional gene approach more sensible as all can be accounted for at the same time.

The relative contributions of nitrification and denitrification to N_2O emissions in agricultural soil and whether there is any evidence of a relationship with the functional gene abundances is also of interest. Previous techniques have used inhibitors such as acetylene (C_2H_2) to inhibit nitrification in some treatments combined with bulk stable isotope analysis to distinguish between nitrification and denitrification (Bateman and Baggs 2005) or by isotopically labelling an N source to use as a tracer (Groffman *et al.* 2006). Both of these approaches can be problematic for a variety of reasons (see Ostrom and Ostrom, 2011). A more recent technique is to analyse the intramolecular distribution of ^{15}N in N_2O rather than looking at bulk isotopes (Toyoda and Yoshida 1999, Brenninkenmeijer and Rockman 2000, Yoshida and Toyoda 2000). The structure of N_2O (Fig. 2) is such that there is a central and outer N atom denoted α and β respectively, (Toyoda and Yoshida 1999). Sutka *et al.* in 2006 comprehensively evaluated the intramolecular distributions of ^{15}N in N_2O for both processes. This intramolecular distribution is often expressed as the site preference (SP) which is the difference in $\delta^{15}N$ between the central and outer atoms (Toyoda and Yoshida 1999). The authors found that the SP values for these processes were distinct and did not overlap (nitrification: 33-37‰, denitrification: -10-0‰).

Therefore analysis of the SP of N_2O emissions enables discrimination between these processes without altering the soil environment in a way that could affect the results.

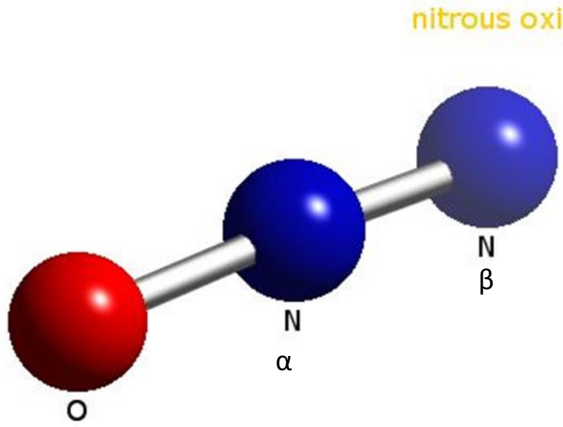

Figure 2: Structure of an N_2O molecule showing α and β N atoms. Modified from: https://www.webelements.com/compounds/nitrogen/nitrous_oxide.html

1.3 Aims and Hypotheses

The aim of this project is to find evidence of links between information on functional gene abundance and GHG emissions. If it possible that functional gene abundance can be used to predict N_2O emissions, and by what process, and suitable on-site diagnostic tests developed then farmers and policy makers could use this information to optimise e.g. watering and fertilisation regimes to minimise impact on the environment whilst trying to maximising yields responses.

The initial hypotheses for this project are:

1. Abundance of denitrification genes will accurately predict N_2O emissions with lower SP values (-10-0 ‰).
2. Abundance of nitrification genes will accurately predict N_2O emissions with higher SP (33-37‰)
3. Addition of an N source will increase abundances of functional genes for nitrification and denitrification.
4. Addition of an N source will increase N_2O emissions from soils.

Large pulses of emitted N_2O are expected just after addition of N, lasting up to two weeks. It's expected that successive pulses will be larger than the first, as the microbial community will be more established after the first.

Section 2: Field Investigation

2.1 Introduction

The goal of this work was to investigate how the interaction between molecular markers and N_2O emissions changes with different crops and agricultural setups, in order to simulate realistic farming practices. Two of the most widely grown arable crops in the UK (Fig. 3), *Triticum aestivum* (common wheat) and *Brassica napus* (oilseed rape) were chosen for this investigation. Although barley (*Hordeum vulgare* L.) is the second most commonly grown crop in the UK, *B. napus* was selected instead to try and identify any differences in the N_2O emissions and soil microbial community between cropping with *Poaceae* (grasses) and *Brassicaceae* (brassicas). A consistent response from these crops would suggest the conclusions drawn from this investigation could be applied to a wide range of crops. Furthermore, *T. aestivum* has been shown to form interactions with arbuscular mycorrhizal fungi (AMF) (Al-Kharaki *et al.* 2003, Sharma *et al.* 2011, Daniell *et al.* 2001) whereas *B. napus* like other brassicas does not. Although more work is required on the topic, it has been suggested that AMF may have a role in reducing N_2O emissions by competing with soil microbes for NH_4^+ (Veresoglou *et al.* 2012) which would limit the amount available for nitrification (see Fig 1). As N requirements for AMF is often quite high, this can occur even when the plant is not receiving a benefit in growth (Hodge and Fitter 2010). There have been some studies on the effects of AMF on both the nitrifier and denitrifier communities but these seem inconclusive (see Veresoglou *et al.* 2012) but a consensus has yet to emerge. The varieties used were *T. aestivum* var. Cadenza (KWS Seeds, Royston UK) and *B. napus* var Ability(DSV UK Ltd, Downham Market). Both of these are spring sowing varieties.

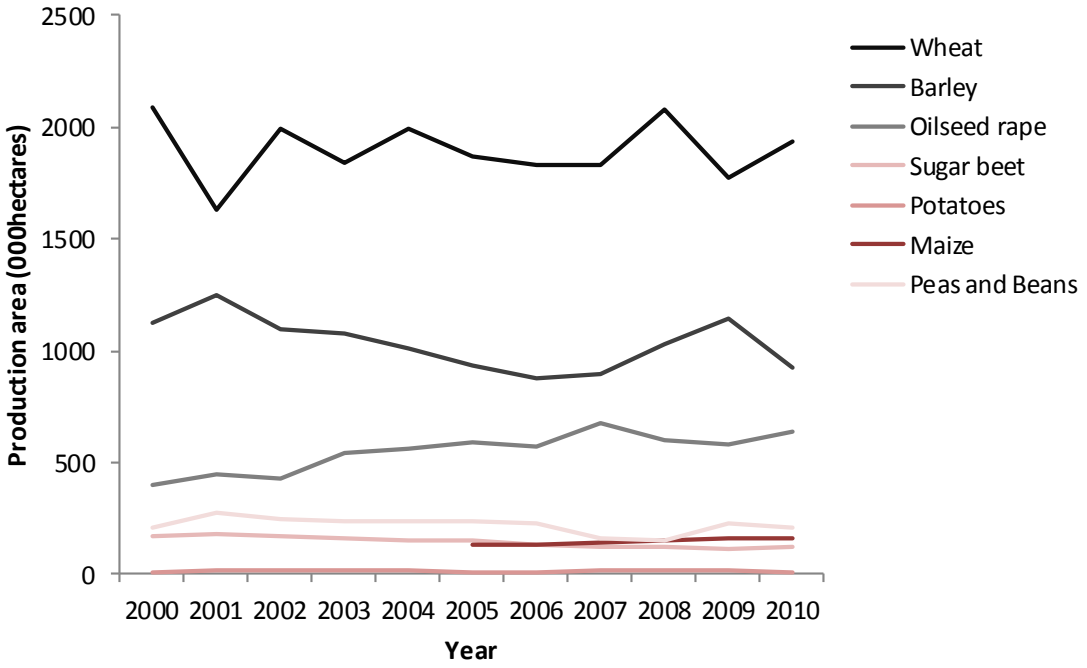

Figure 3: The total production area of the most common UK crops from 2000-2010. Wheat, barley and oilseed rape are the three most widely grown. Data from Living Countryside

2.2 Study Site

The study site selected was near Heslington, North Yorkshire, UK, part of the University of York's Heslington East campus (Latitude: 53.94412166, Longitude: -1.03181212) because it is the location of SkyGas (Ineson 2014), a fly-by-wire array that allows automated GHG measurements over complex land and water systems. It allows a chamber (and other equipment) to be moved to any location within the 3D space of its sampling area (Fig. 4). This investigation aimed to use this equipment in order to reduce the amount of temporal variation in the GHG data (Chadwick *et al.* 2014). Soil at the site is mixed with rubble left over from construction work, so soil from a nearby field was used, a sandy loam that had previously been used to grow winter wheat during the growing season 2012-2013 and was left fallow in 2013-2014. It was collected in November 2014. The annual average air temperature (average from 2010-2013) was 9.5°C and the average total annual rainfall (2010-2013) was 541mm (Department of Electronics, University of York).

Figure 4: The SkyGas chamber system. At the back of the picture is shown one of four towers in the tower and wire system which allows the chamber to be moved to any point in the experimental area enclosed by the towers. Not shown is the computer control centre where analysis equipment can also be stored. Photo: Phil Ineson

2.3 Molecular Markers

Four genes were selected for this study based on published primer sets including archaeal and bacterial homologs where appropriate (Table 1). Petersen *et al.* (2012) found that bacterial *amoA* abundance explained, with soil ammonium concentration, 79% of the variability in potential nitrification rates (PNR) across five soil types. For denitrification, abundance of *nosZ* (the gene that codes for the active site of nitrous oxide reductase) alone explained 79% of the variability in potential denitrification rates (PDR), however *nirK/S* (two variants of nitrite reductase) explained most of the *nosZ* variation. This suggests that these are the rate determining steps of the two processes. Much of the literature has focussed on the genes when looking at these processes in soils and sediments (e.g. Wang *et al.* 2014, Restrepo-Ortiz *et al.* 2014, Henry *et al.* 2006, Chen *et al.* 2008). Archaeal *amoA* is also included because of the huge variability in soils of ammonia oxidising archaea and bacteria (Petersen *et al.* 2012, Leninger *et al.* 2006, Chen *et al.* 2008). For both bacteria and archaea, pairs for a section of the 16S ribosomal RNA were also used to compare the N cycling microbial community with the total bacterial and archaeal community in the soil. These genes are used as they are ubiquitous in nearly all microbes and provide a proxy for total number of bacteria/archaea.

Table 1: Functional gene primers used in this investigation. Other universal (16S) primer pairs were tested (27F/338R, Bac V3-F/Bac V3R) but discarded in favour of those listed below. M=A or C, Y= C or T, K= G or T, S= C or G, R= A or G, V= A, C or G,

Role	Gene	Forward Primer	Reverse Primer	References
16S RNA				
bacterial 16S		27F AGAGTTTGATCMTGGCTCAG	338R CATGCTGCCTCCCGTAGGAGT	(Duncan et al. 2004)
archaeal 16S		Ar9r CCCGCCAATTCCTTTAAGTTTC	rSAf CCTAYGGGGCGCACCAG	(Jurgens et al. 1997 & Nicol et al. 2005)
Nitrification				
ammonia monooxygenase	*amoA-AOB*	*amoA*-1F GGGGTTTCTACTGGTGGT	*amoA*-2F CCCCTCKGSAAAGCCTTCTTC	(Rotthauwe et al. 1997)
ammonia monooxygenase	*amoA-AOA*	Arch-*amoA*F STAATGGTCTGGCTTAGACG	Arch-*amoA*R - GCGGCCATCCATCTGTATGT	(Francis et al. 2005)
Denitrification				
nitrite reductase	*nirS*	Cd3a- F GTSAACGTSAAGGARACSGG	R3cd - GASTTCGGRTGSGTCTTGA	(Throback et al., 2004; Yergeau et al., 2007)
nitrite reductase	*nirK*	*NirK*876 -ATY GGC GGV CAY GGC GA	*NirK*1040 GCC TCGATCAGR TTRTGGTT	(Henry et al., 2004)
nitrous oxide reductase	*nosZ*	*nosZ*2F CGCRACGGCAASAAGGTSMSSGT	*nosZ*2R-CAKRTGCAKSGCRTGGCAGAA	(Henry et al., 2006)

2.4 Experimental Set-up

A fully factorial experiment of cropping type and N-additions was carried out resulting in a total of six treatments: *T. aestivum*, *B. napus* and bare soil (unplanted) cropping treatments, each with and without a nitrogen addition. The addition of nitrogen was done twice, once at the start of the experiment (25th June) and a second time five weeks into the experiment (1st August). The soil of interest was loaded into plastic open bottomed cores, 20cm in diameter and 40cm deep. These were placed into holes dug at the site, with a collar above ground level where the chamber could form an adequate seal. The chamber was connected to the analysis equipment with 4mm PTFE tubing. Due to this design, repeated-measures methods were used when analysing the data. It allowed the soil to be mixed thoroughly prior to being placed in the cores, in an attempt to reduce the innate heterogeneity of soil which must be taken into consideration when sampling soils (Chadwick *et al.* 2014, Stoyan *et al.* 2000). The SkyGas chamber would seal automatically when coming into contact with the plastic lip of the core. The treatments were laid out in a randomised block design, 5 rows of 6 cores, each treatment present once in every row (Table 2)

The nitrogen addition was calculated based on Defra guidelines (Defra 2010). The soil was light/sandy, in an area of low rainfall (<500mm per annum) and had previously been used to grow cereals giving a Soil Nitrogen Supply (SNS) index of 0. The recommended N addition for spring sown *T. aestivum* and *B. napus* is 160 and 120 kgN ha^{-1} respectively. The higher value was used on all cores requiring a nitrogen addition. This was added in two doses; 80 kgN ha^{-1} at the start of the experiment, and the remaining 80 kgN ha^{-1} after five weeks. The literature suggests that there will be strong "pulses" of N_2O emissions for 7-14 days after application (Scheer *et al.*2008, Bai *et al.* 2014) which will then decrease but the five week duration was chosen to allow any lag in changes of the gene copy numbers of the functional genes to become apparent. The N was added as 18% N solution in the form of 51% ammonium nitrate solution. This was made up to 50ml with distilled water. Non N treatments received 50ml of distilled water at the same time to avoid any effects of increased moisture.

Table 2: Description of the blocks and treatments with the core numbers.

Core Number	Block	Treatment	Core Number	Block	Treatment
1	1	*B. napus*	16	3	unplanted
2	1	unplanted	17	3	*B. napus*+160kg N ha^{-1}
3	1	unplanted+160kg N ha-1	18	3	unplanted+160kg N ha^{-1}
4	1	*B. napus*+160kg N ha-1	19	4	unplanted+160kg N ha^{-1}
5	1	*T. aestivum*	20	4	Unplanted
6	1	*T. aestivum* +160kg N ha-1	21	4	*B. napus*
7	2	unplanted	22	4	*T. aestivum*
8	2	*B. napus*+160kg N ha-1	23	4	*B. napus*+160kg N ha^{-1}
9	2	*T. aestivum* +160kg N ha-1	24	4	*T. aestivum* +160kg N ha^{-1}
10	2	unplanted+160kg N ha-1	25	5	*B. napus*+160kg N ha^{-1}
11	2	*T. aestivum*	26	5	*T. aestivum* +160kg N ha^{-1}
12	2	*B. napus*	27	5	*T. aestivum*
13	3	*T. aestivum* +160kg N ha-1	28	5	unplanted+160kg N ha^{-1}
14	3	*T. aestivum*	29	5	*B. napus*
15	3	*B. napus*	30	5	Unplanted

2.5 Experimental Hypothesis

The specific hypotheses for the field investigation were as follows:

- Abundance of *nirK/S* genes will accurately predict N_2O emissions with lower SP (-10-0‰) values.
- Abundance of bacterial and archaeal *amoA* will accurately predict N_2O emissions with higher SP (33-37‰).
- Abundances of bacterial and archaeal *amoA*, *nirK*, *nirS* and *nosZ* will be greater in treatments with an N source.
- Addition of an N source will increase the likelihood of presence of the genetic markers for functional genes involved in nitrification and denitrification appearing on the end point gel, but not for the 16SrRNA genes.
- Addition of an N source will increase N_2O emissions from soils.
- Treatments with *B. napus* will show greater N_2O emissions than treatments with *T. aestivum*, possibly due to competition with soil microbes for N by AMF
- Treatments with no plants and *B. napus* will show similar patterns in N_2O emissions, whilst the plants are competing with the microbes for the N, they are also providing a C source through root exudates for many denitrifiers, promoting their growth.

2.6 DNA Sampling, Extraction and PCR

Soil samples were taken every week from the cores, flash frozen in liquid N_2 and stored at -85°C until extraction. No molecular data was present for Runs 2 and 4. Four 1cm diameter cores were taken from each collar to a depth of 15cm and the subsamples pooled prior to DNA extraction to minimise the effect of soil heterogeneity. Extraction was carried out using PowerSoil™ DNA Isolation Kits (MoBio, USA) according to the manufacturer's instructions. Extracted DNA concentration and purity was assessed using a NanoDrop 8000 Spectrophotometer (NanoDrop Products, Wilmington DE, USA) before storage at -85°C. The PCR reactions were performed in the presence of 0.1 U/μl *Taq* DNA polymerase, 0.2 mM dNTPs, 12.5 pmols of each primer at 0.5μM, 1.5mM $MgCl_2$ and the manufacturer's reaction buffer (Invitrogen™). All PCRs carried out on a Techne TC-512 thermo cycler (Bibby Scientific Ltd. UK). Bacterial PCRs were run at 30 cycles of 30 seconds at 94°C, 30 seconds at 52°C and 50 seconds at 72°C. The initial denaturation was 5 minutes at 95°C with a heated lid at 105°C and

the final extension 10 minutes at 72°C. Samples were held at 15°C before being stored at -85°C. Archaeal PCRs were run at 30 cycles 1 minute at 94 °C, 1 minute at 62°C and 1 minute at 72°C. The initial denaturation was 12 minutes at 95°C. End point PCR gels were ran on 1-1.5% agarose gels depending on the size of the fragments, to check for successful PCR before moving onto quantitative PCR.

2.7 N_2O Sampling and Amendments

The N_2O data were collected using a Los Gatos Research Isotopic N_2O Analyzer (site-specific $\delta^{15}N$, $\delta^{18}O$ and N_2O) (Los Gatos Research, Mountain View CA, USA). Isotope measurement failed, despite attempts at repair, and data presented are therefore only of N_2O concentrations. Similarly, completion of automated SkyGas measurement was not completed in time for this investigation, so the data had to be collected by manually moving the gas chamber between the collars and fixing it to each collar with a rubber gasket.

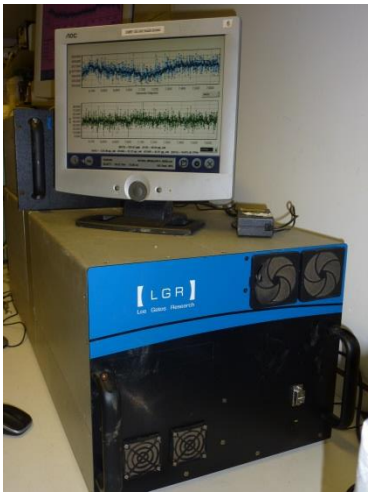

Figure 5: LGR N_2O analyser in operation, showing total N_2O emissions in blue and bulk ^{15}N in green.

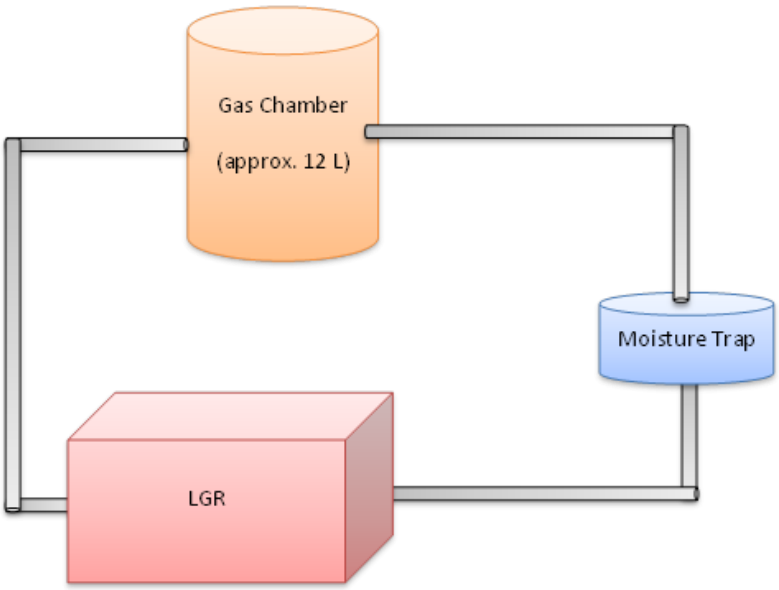

Figure 6: LGR Initial closed chamber setup for N_2O analysis. Gas would flow from the chamber to a water trap, to prevent damaging the LGR if any water got into in the tubing. The moisture trap and LGR would be contained in the computer control centre.

Because of manual data collection, all samples needed to be taken in a single working day, and speeding up sampling required a pump to drive enough gas around the system for measurement. As the LGR operates under a specific pressure, if air was forced into the LGR at 600ml min^{-1} it would prevent the analyser operating properly and risk damaging it. Because of this, the original closed chamber system (Fig. 6) was abandoned for an open chamber system (Fig. 7), where most of the air coming from the chamber was allowed to escape and the LGR "sipped" off the incoming air at 95ml min^{-1}.

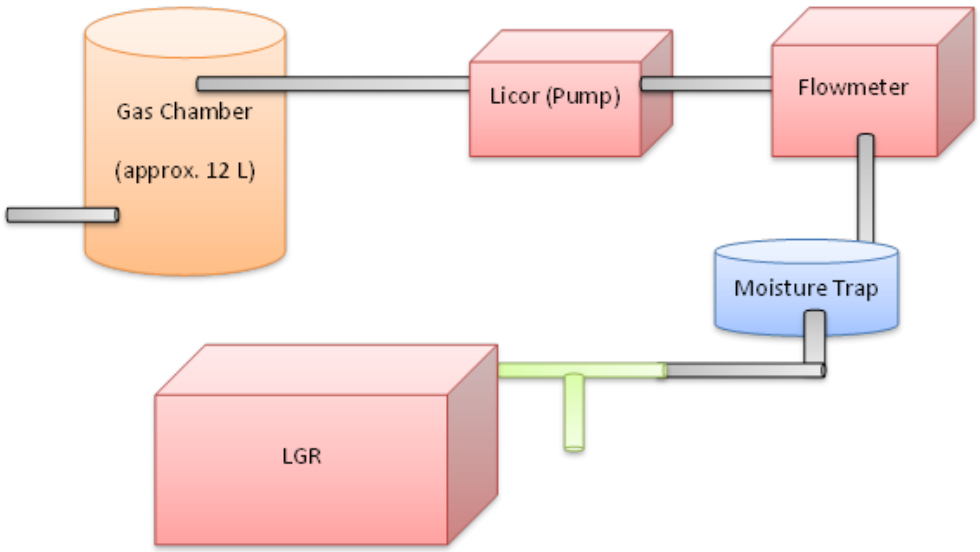

Figure 7: The amended layout of the N_2O sampling equipment. A Licor was used as a pump, set to 600ml min^{-1} which was regulated with a flowmeter. A water trap protected the LGR and most of the air was allowed to escape, with the LGR analysing a subsample of this (in green).

Table 3: Timetable of the modified experiment.

Run	Date	Sample	Run	Date	Sample
1	25/06	Pre-treatment+ Application	-	01/08	2nd application
2	26/06	1 day after 1st application	7	02/08	1 day after 2nd application
3	01/07	1 week after 1st	8	06/08	1 week after 2nd
4	09/07	2 weeks after 1st	9	15/08	2 weeks after 2nd
5	20/07	3 weeks after 1st	10	22/08	3 weeks after 2nd
6	25/07	4 weeks after 1st	11	29/08	4 weeks after 2nd

2.8 Calculating N_2O Fluxes

Only the end point PCRs were carried out (due to time constaints), giving presence/absence data for the functional genes. The N_2O data from the LGR was first treated in SAS 9.4 (SAS Institute Inc, Cary CE, USA) and follow up analysis and statistics were implemented in R 3.12 (R Core Team). With an open chamber method (Norman *et al.* 1997) the flux is usually calculated by taking the final concentration of N_2O when the change plateaus, subtracting the ambient concentration and using this difference to calculate flux. An example of this is shown below (Fig. 8). The ambient concentration at the start of the experiment is 320 ppb (parts per billion) and the increase in concentration seems to plateau at 357ppb after 40 minutes. As the chamber is open, there is no build-up of N_2O in the system so the increase can be attributed solely to N_2O production from the core. Therefore:

$$N_2O \text{ conc. Total (ppm)} - N_2O \text{ conc. Ambient (ppm)} = N_2O \text{ production by core (ppm)}$$

For this example, 0.357-0.320= 0.037 ppm (parts per million) N_2O increase can be attributed to the core.

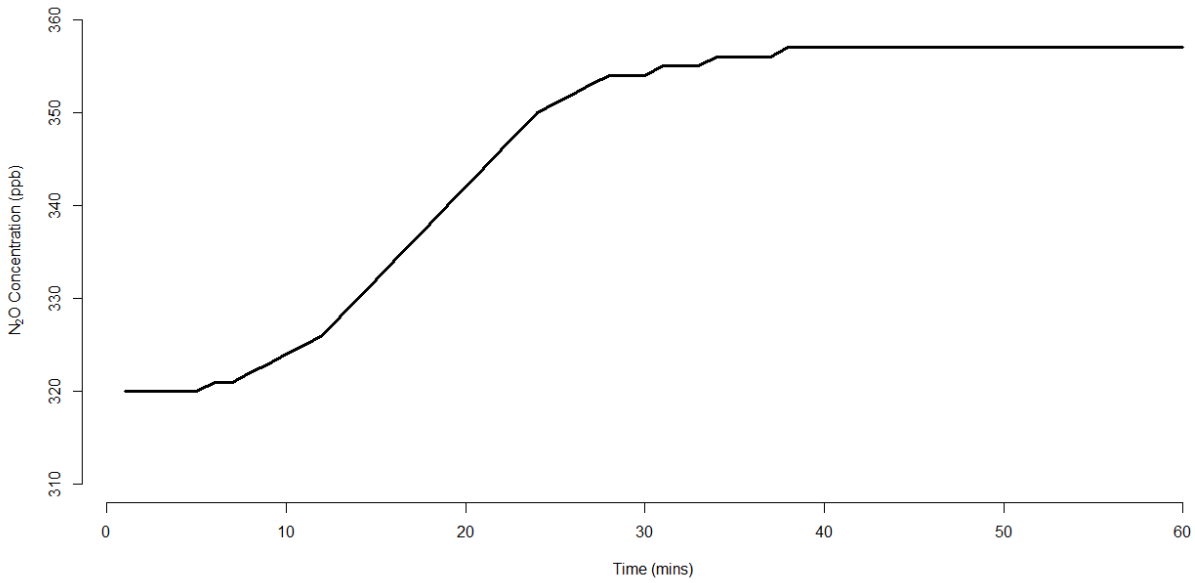

Figure 8: Sampling from a theoretical open core until plateauing is reached. In this example, the core has been placed on five minutes after the start of measuring to get a value for the ambient concentration.

A preliminary test showed that each core would have to be sampled for at least 40 minutes (Fig. 9) before an appropriate end point could chosen with any accuracy. To sample each core for 40 minutes would have taken 20 hours each sampling run, which would not have been feasible. Splitting the runs over multiple days would introduce a confounding variable in comparisons between cores among the time points. Due to time constraints imposed by the project and access to the LGR, each core was sampled for 10 minutes, the maximum time possible for sampling in one day.

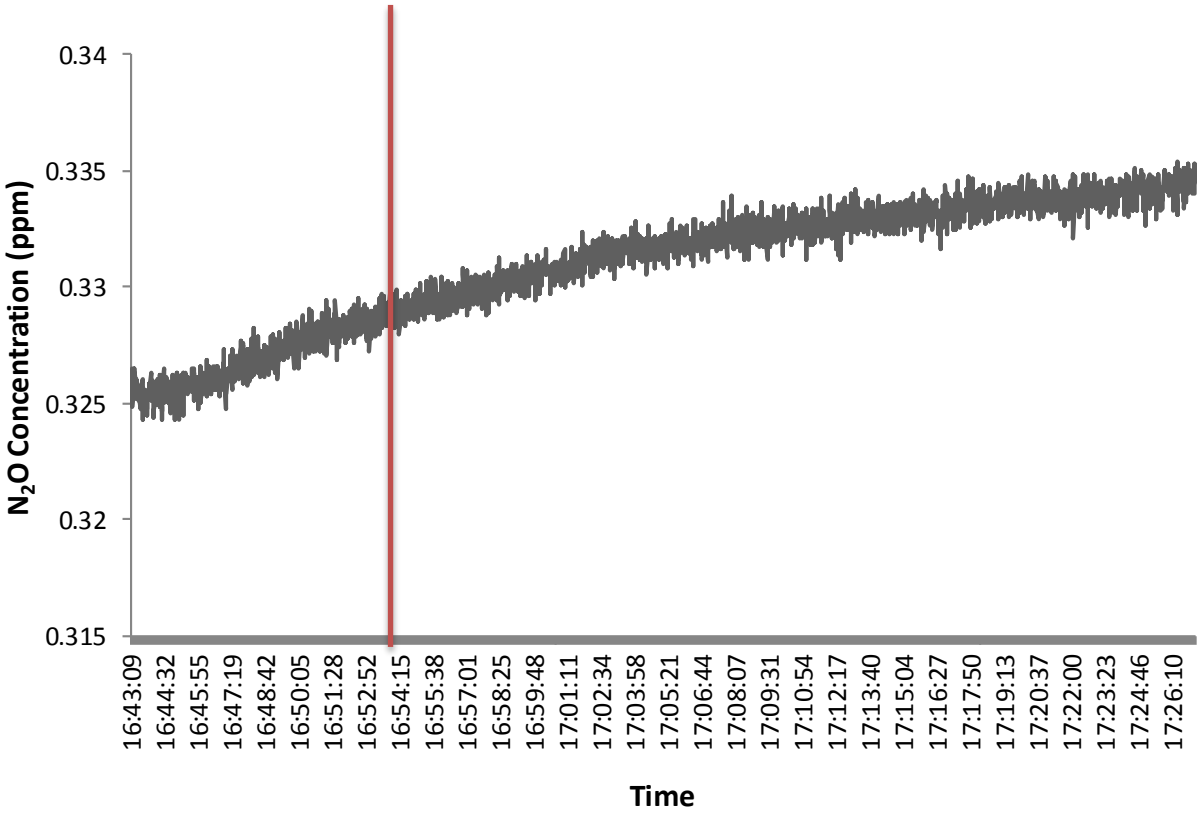

Figure 9: A core at random was selected to test how long it would take to plateau with the amended experimental setup. A clear plateau was not reachable after 43 minutes. The red line shows the 10 minute cutoff point for sampling.

The concentrations measured after 10 minutes of sampling are lower than the true final concentrations after 30+mins. An additional complication emerged in the first two runs (25Jun and 26Jun) that were carried out with a pump that was far too powerful, causing the measured concentration to fall after a few minutes of sampling (Fig. 10). This was suggested to be because the flow rate was so great that it was actively pulling air out from the soil as well as through the small opening in the chamber (see Fig. 7). So the initial increase was the amount of N_2O in the air spaces in the soil being measured but then production of N_2O could not match the flow rate. Therefore, the final N_2O concentrations were lower than concentrations in the middle of the sample run. To compare the N_2O production on those days with the rest of the experiment, the arithmetic mean N_2O concentration, was taken rather than the final N_2O concentration to calculate the "flux" as a compromise. At this point, the pseudofluxes calculated would not be appropriate for comparing flux values in a wider context; however comparisons within the experiment and any treatment effects are still valid.

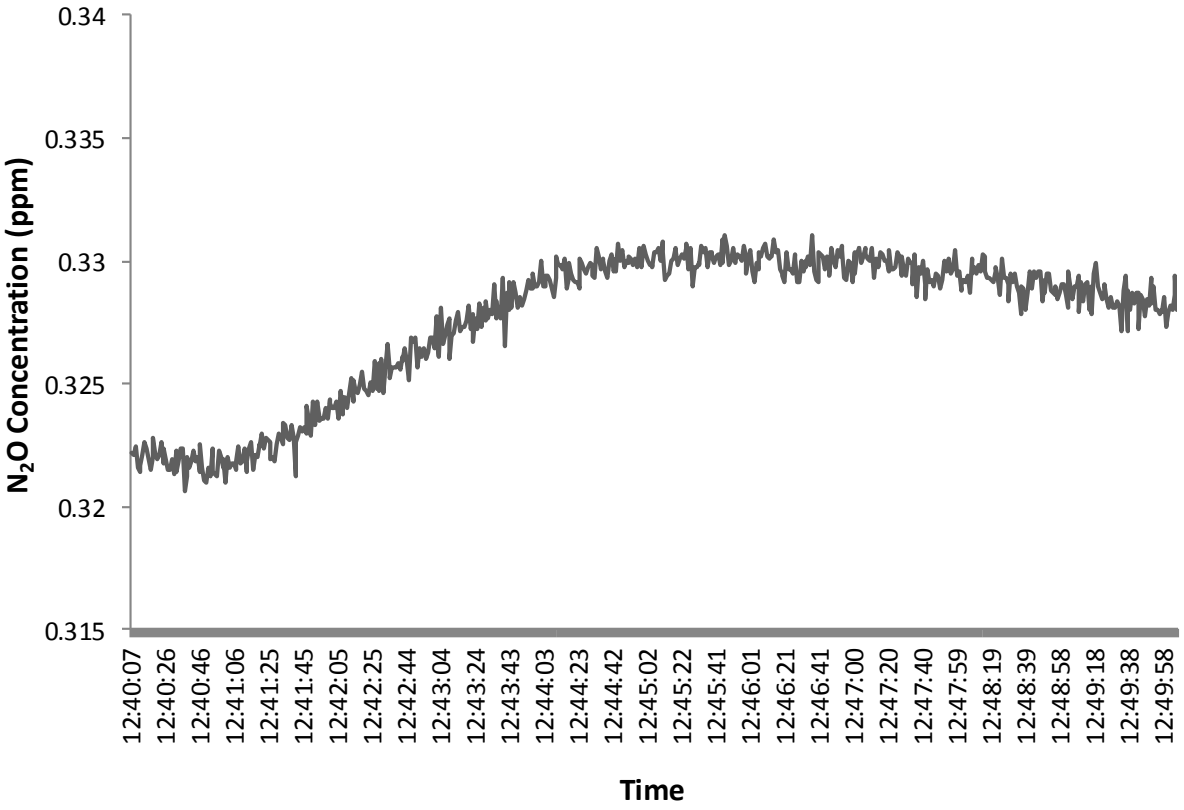

Figure 11: Core 16 (no plant N-) on Run 2 (26Jun) selected as an example to show the dropping off of N_2O production halfway through sampling. This pattern is repeated for many of the cores on Runs 1&2.

The fluxes were calculated using a rearrangement of the Clapeyron's Ideal Gas Law:

$$PV=nRT$$

Where P= pressure (atmospheres), V=volume (litres), n=number of moles, R= universal gas constant and T=temperature (K). This can be rearranged to:

$$n=PV/RT$$

In this case, the volume at the end of the sampling period is given as the (FR) flow rate (in litres/min) * (t) time (mins) so *V* can be replaced by *FR*t*. Data from the University weather station shows that the atmospheric pressure around York fluctuates very closely around 1 atm (Department of Electronics, University of York) to the point where *P* can be considered =1.

$$n=(FR*t/RT)*(N_2O \text{ conc.}/10^6)$$

The flow rate also gives us unit time (1 minute). Mean N2O concentration expressed in ppm and converted from moles to grams gives the flux per core:

$$\text{Flux } N_2O(g/\text{min per core}) = (FR*t/RT)*(\text{mean } N_2O \text{ conc.}/10^6)*44.01$$

Dividing by the surface area of the core gives the flux per m^2. As the fluxes are quite low due to being underestimated, they are given in μg rather than g:

$$\text{Flux } N_2O\ (\mu g/\text{min}/m^2) = (FR*t/RT)*\text{mean } N_2O \text{ conc.(ppm)}*44.01/(\pi*0.1^2)$$

2.9 Soil Characteristics

Soil temperature and moisture were collected for each core immediately after gas sampling to see if there was any effect of these on the N_2O flux readings and gene presence/absence that may influence any pattern seen within the treatments. Data were collected using a Delta-T GP-1 data logger with ThetaProbes and temperature probes (Delta-T Devices Ltd., Cambridge, UK). The probes were left in the soil for 5 minutes and an average of the temperature (°C) and soil moisture (m^3m^{-3}) were taken. Air temperature was also measured at this time for the purposes of calculating N_2O fluxes.

Section 3: Results

3.1 Soil Temperature and Moisture

Soil temperature and moisture readings were collected on five of the sampling runs. Soil temperature had a bimodal distribution (Fig 12a), the average soil temperature dropping by around 4°C in the last two sampling runs at the end of August (from 22.3±0.82 °C on Run 8 and 18.67±0.37 °C and 18.15±0.66 °C on Runs 9 and 10 respectively, Fig 12b) so is not normally distributed. As predicted, there were no differences in soil temperature across treatments (Kruskal-Wallis, χ^2 (5) =1.1, p=0.95), however there were significant differences among the sampling runs (Kruskal-Wallis, $\chi 2$ (4) =130.5, p<0.001). Pairwise t-tests were carried out (adjusted with the Bonferroni correction (Dunn 1959) to avoid type 1 errors) for individual sampling dates, as the soil temperature values are normally distributed when grouped by run. In fact, all the runs were significantly different from each other (Table 4) and the difference between Runs 9 and 10 and the others is the greatest.

Table 4. The t and p values from the pairwise Welch's t-tests performed with a Holm-Bonforrini adjustement on soil temperature across the runs. All tests have 29 degrees of freedom. All t values given to 1 d.p.

	Run 4	**Run 6**	**Run 8**	**Run 9**
Run 6	t=-9.1, p= $4.9*10^{-10}$	-	-	-
Run 8	t=5.0, p=$2.5*10^{-5}$	t=15.6, p=$1.3*10^{-15}$	-	-
Run 9	t=21.7, p=$2.2*10^{-16}$	t=31.1, p=$2.2*10^{-16}$	t=26.0, p=$2.2*10^{-16}$	-
Run 10	t=28.7, p=$2.2*10^{-16}$	t=28.8, p=$2.2*10^{-16}$	t=29.2, p=$2.2*10^{-16}$	t=3.7, p=$7.9*10^{-4}$

There was a significant difference in soil moisture both across runs (ANOVA, $F(4, 145)= 19.75$, $p<0.001$) and treatments (ANOVA, $F(5, 144)=3.31$, $p<0.05$). The difference across treatments was unexpected as all treatments were unwatered and received the same volume of solution when ammonium nitrate was added, even though this was quite a small amount. *Post hoc* testing showed that only *T. aestivum* N- ($0.118\ m^2m^{-2} \pm 0.03$) and *B. napus* N- ($0.088\ m^2m^{-2} \pm 0.03$) were significantly different from one another (TukeyHSD, $p<0.05$).

If soil moisture or temperature are significantly correlated with N_2O flux, then they are potential covariates in the analysis of N_2O fluxes across treatments. As the temperature and the N_2O flux data are both non-parametric (see below for N_2O), the Spearman's rank order correlation coefficient was used for both comparisons. For soil temperature, there was a significant correlation ($r_s[377148]=0.33$, $p<0.001$) but a weak interaction (Fig. 12). There was no significant correlation found for moisture ($r_s[546470]=0.02$, $p=0.7$). As the relationship with temperature was weak and there was no relationship found with moisture, neither was used as a covariate in the subsequent N_2O flux data analysis.

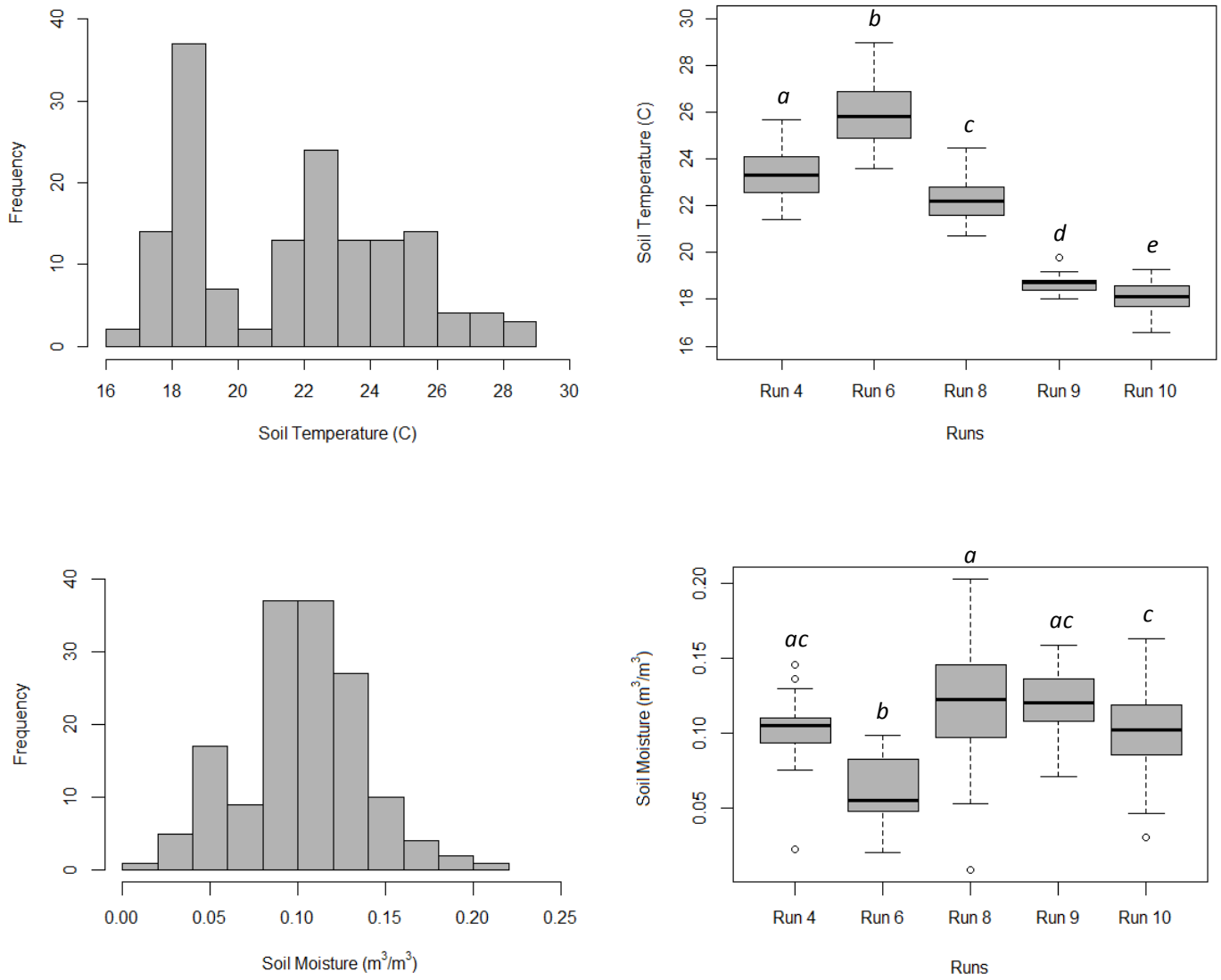

Figure 12: Soil temperature and moisture across the five runs measured. The histograms illustrate the bimodal distribution of soil temperature, driven by the colder sampling Runs 9 and 10 and the more normal unimodal distribution of soil moisture. On the plots, the letters show which groups are significantly different from each other. Post hoc tests for soil temperature carried out with pairwise Boneferroni adjusted paired t-tests and Tukey HSD for soil moisture. Boxes represent the interquartile range with the whiskers showing the range. Temperature in degrees Celsius and soil moisture in m^3 water per m^3 soil.

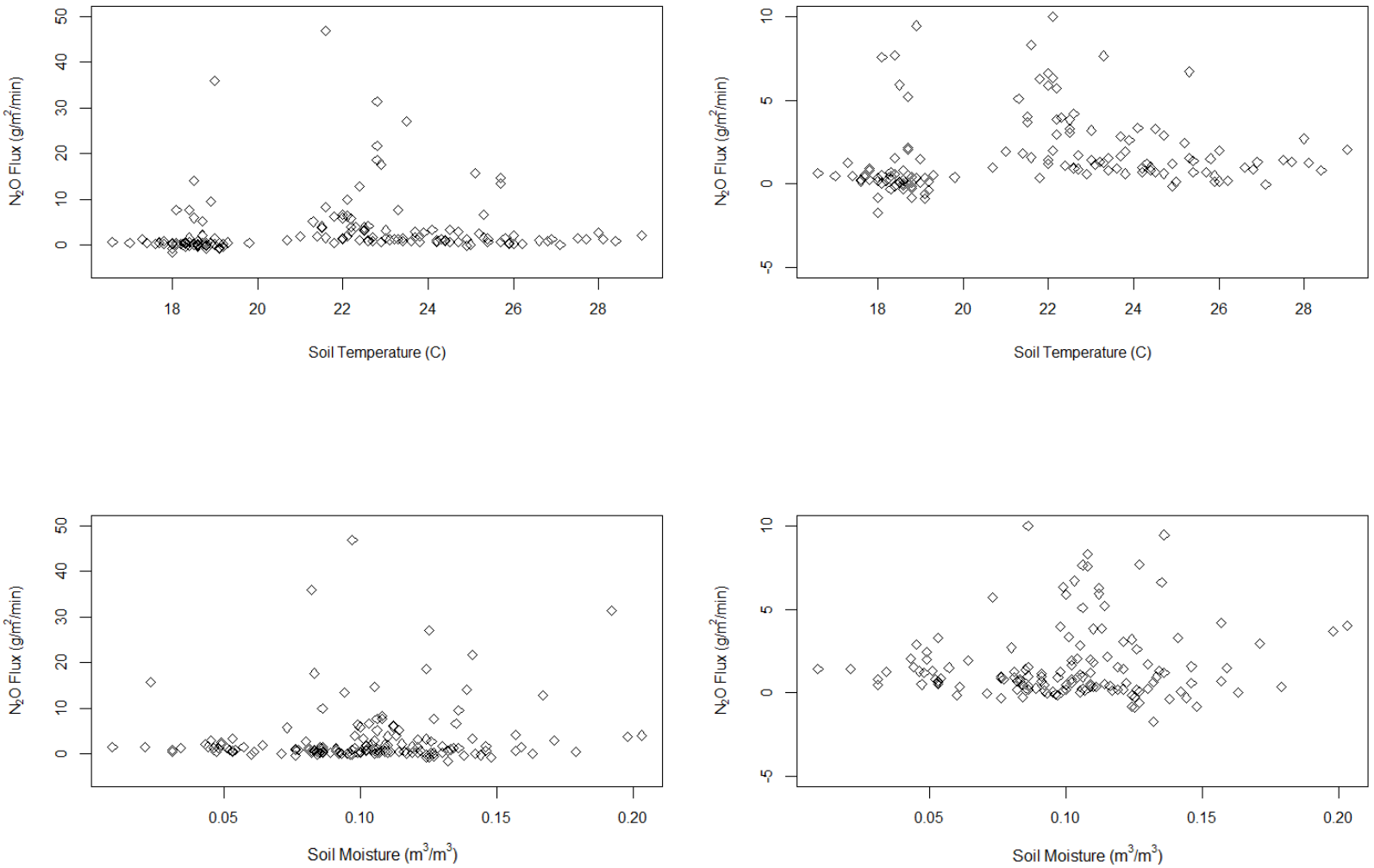

Figure 13: Relationship between N_2O fluxes and (top) soil temperature and (bottom) soil moisture. The figures on the right show a more zoomed in view of the main clusters of points without the "outliers" of high N production. Soil temperature seems to show a s light positive relationship with N_2O flux but as shown by the test, it's a weak association. There was no relationship found with soil moisture. Temperature in degrees Celsius and soil moisture in m^3 water per m^3 soil.

3.2 N_2O Flux

N_2O concentration data were successfully collected on the 25th and 26th June, the 9th, 20th and 25th July and the 2nd, 6th, 15th and 29th August. Run 3 (1st July) was not collected. When converted into fluxes, values ranged from -1.71 $gN_2O\ min^{-1}m^{-2}$ (Untreated, unplanted Run 9) to 46.88 $gN_2O\ min^{-1}m^{-2}$ (treated *B. napus* Run 8). Highest overall production was on Run 8, where the largest difference in means was between treated *B. napus* (22.0±15.9) and untreated *T. aestivum* (1.5±1.0). There is large variability in the data, especially when large N_2O fluxes were recorded for some treatments as not all cores responded to the treatment. Some very small negative fluxes were also recorded, on Runs 1 and 9.

The flux data was not normally distributed as most cores across the sampling period did not produce much N_2O resulting in a low mean with a high standard deviation due to the few cores that had a lot of N_2O production (2.6 g $N_2O/min/m^2$ ±5.3). This resulted in a large positive skew (Fig.13). As the data is from repeated measures, the only non-parametric test applicable was the Friedman's test which does not allow more than one observation per variable so would have been carried out on the means of the five replicates of each treatment per sampling run without the information about the spread around those means.

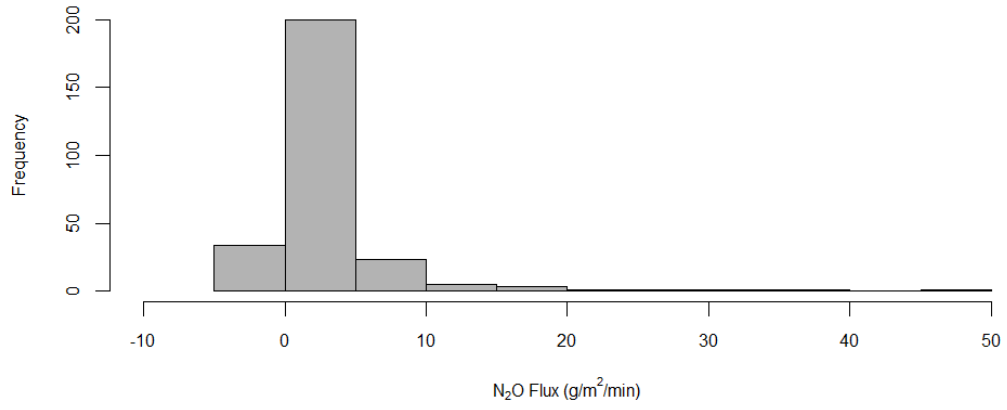

Figure 13: The few cores with high N production caused a large positive skew of the flux data. Negative fluxes were caused when ambient air N_2O concentrations were not measured before starting a sample and an average had to be taken from measurements around that period. No true N_2O uptake by soils was measured.

Instead, the data was transformed in order to use parametric tests. As the flux values straddle 0, but never lower than -1.71 (no plant N-, Run 9), first 2 was added to all values to make them all positive. Two transformations were used: $\log_{10}$ and square root. Log_{10} was preferred over ln as it is possible to see the magnitude of the original value easily. The data from both transformations were tested for normality (Kolmogorov-Smirnov [K-S] test) and equal variance (Levenes test). Levene's was chosen because although it is less powerful than Bartlett's test, it is less sensitive to departures from the normal distribution (Gartside 1972). Although neither transformation allowed the data to pass the K-S test, when the data were transformed by $\log_{10}$, the *p* value was much closer to the threshold value of 0.05 (F= 0.12, p=0.0005) than either the untransformed data or the square root transformed data (F=2.66, $p<2.2*10^{16}$ and D=0.19, $p= 2.92*10^{-9}$, respectively [Fig.14]). When the data were transformed with $\log_{10}$, the variances were no longer significantly different (F (53,216) =0.88, p=0.74), compared to the square root data (F= (53,216) = 1.35, p=0.07) or the untransformed data (F (53,216) = 1.98, $p<0.001$). Although the transformed data still violated the assumption of normality that would allow a repeated-measures ANOVA to be used to test the data, the literature suggests that whilst ANOVAs are sensitive to differences in variance, the procedure is relatively robust when normality is violated (Glass *et al.*1972, Keselman *et al.* 1998). ANOVAs are used on this data and as type I error may increase, results with significance close to p=0.05 were treated with caution. When reporting means and standard deviations of flux data, the backtransformed values are given.

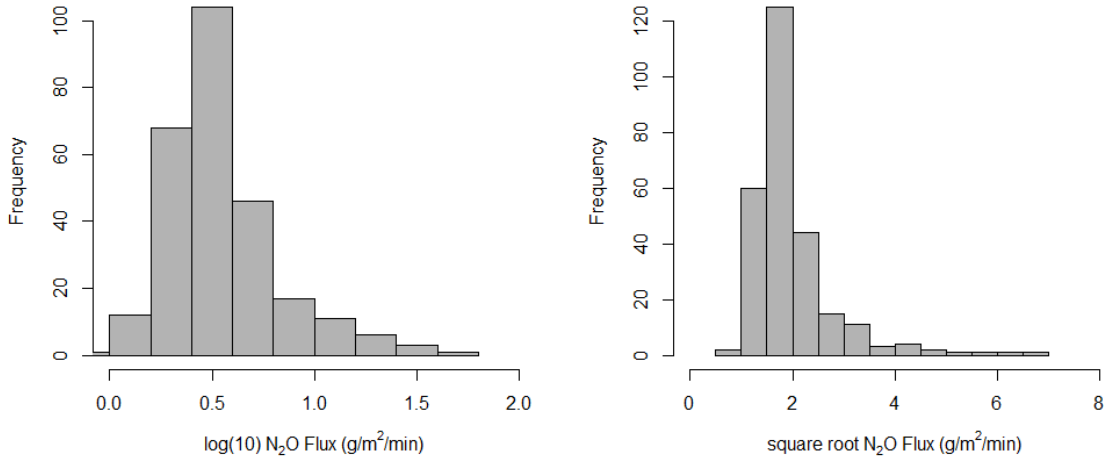

Figure 14: Both transformations reduced the extreme positive skew although the effect was more pronounced with the $\log_{10}$ transformation.

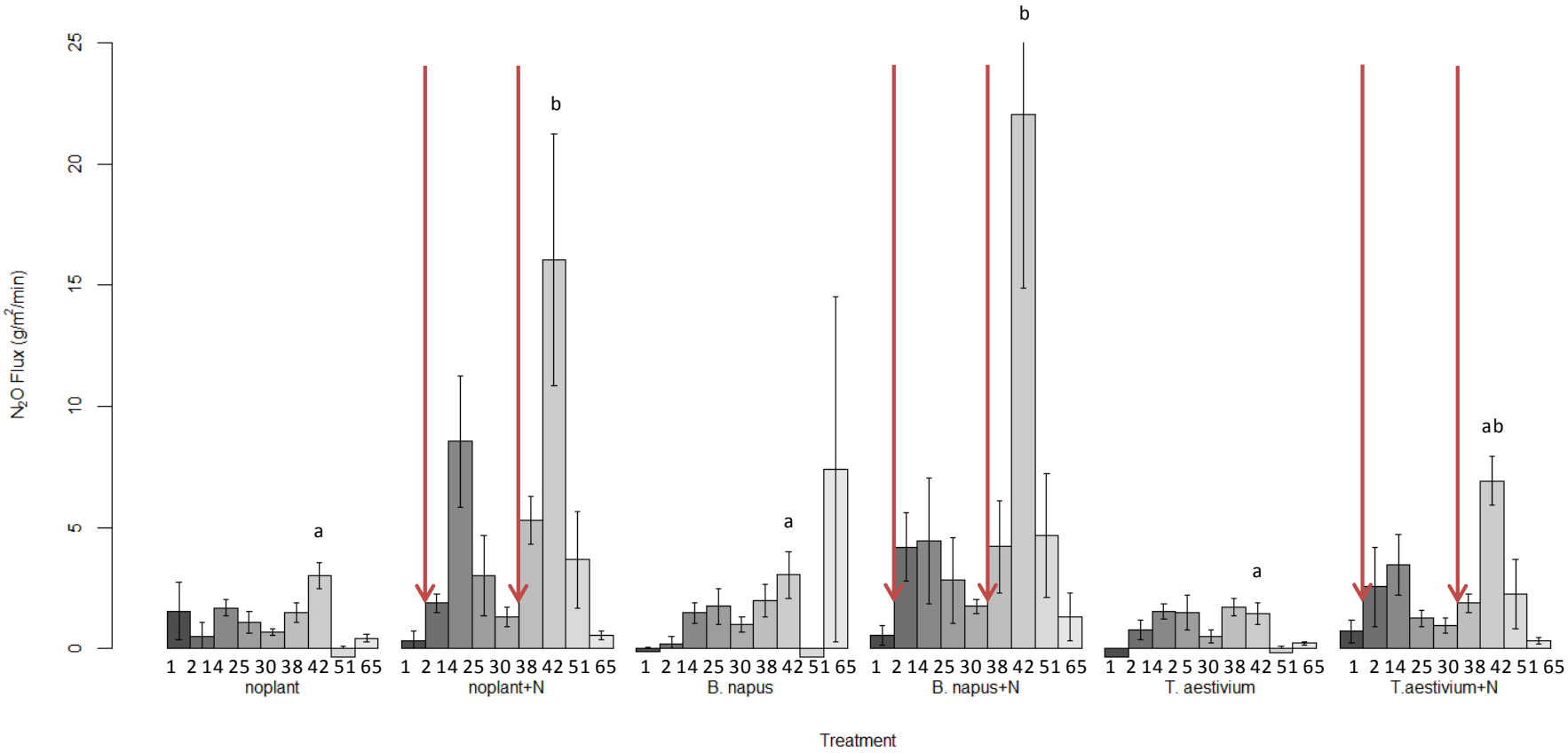

Figure 15: The effect of N addition on N_2O fluxes from cores. The red arrows show when N was added to the +N treatments. Error bars are standard error. The letters show significantly different treatments for Run 8 when the log transformed data is analysed. Runs 1,2 and 4-10 are shown. Data for Run 3 (day 6) is missing. Runs given as days from start of experiment.

There was no significant difference in $\log_{10}$ flux between N treated cores and non-N treated cores immediately after first application (Run 2, Fig14) and the data for one week after first application (Run 3) is missing. Two weeks later (Run 4) there was a significant difference between treatments (ANOVA, $F(5,24)=2.67$, $p=0.0468$) with post hoc testing using Tukey's HSD suggests that N treated unplanted cores (6.75±0.13tend towards higher fluxes than untreated *B. napus* cores (1.39±0.27) and untreated *T. aestivum* cores(1.46±0.22) but this was not significant (p values = 0.058 and 0.067 respectively). After this two week period, any trend in flux increase in treated cores seems to stop (Fig.15) until the second N application just before Run 7.

After the second application, there is an immediate flux increase in some of the treated cores, in contrast to the first application. There is a significant difference between treatments on Run 7 (ANOVA $F(5,24) =3.39$, $p=0.019$): N treated unplanted cores (5.03±0.34) have significantly higher fluxes (Tukey's HSD, $p<0.05$) than untreated unplanted cores (1.40±0.30). The great differences between treated and untreated cores is seen a week after application on Run 8 (Fig.13) where there is a strongly significant difference between treatments (ANOVA, $F(5,24) = 10.15$, $p<0.001$). There was a significant difference between plant species: N treated unplanted (12.53±0.18) and *B. napus* (17.97±0.01) cores have significantly higher N_2O fluxes than all untreated cores, whilst treated *T. aestivum* cores (6.70±0.29) do not differ significantly from any other treatment (Fig.14). On Run 9, there was a slight significant difference between treatments (ANOVA, $F(5,24) =3.31$, $p=0.02$), with treated *B. napus* cores (2.99±0.35) having slightly significantly greater N_2O fluxes than untreated unplanted cores.

A two way ANOVA with replication was used to compare $\log_{10}N_2O$ fluxes across the entire sampling period. The test found that there was a significant difference between treatments ($F(5,163) = 10.92$, $p<0.001$) and between runs ($F(8,163) =14.81$, $p<0.001$). The interaction between treatments and runs was also significant ($F(40,163)=3.89$, $p<0.001$) suggesting that the treatments are not responding the same way across the runs. Post hoc testing was carried out using pairwise paired t-tests with a Bonferroni adjustment. The untreated cores were not significantly different from each other and treated *T. aesitivum* was not significantly different from either the unreated cores or the remaining treated cores (Fig.16). N treated *B. napus* (2.88±0.20) and unplanted (2.69±0.14) cores had significantly different production than all the other cores except treated *T. aestivum* (1.62±0.01). Among runs, there are significantly greater N_2O fluxes on Run 8 (5.62±0.21) than any other run.

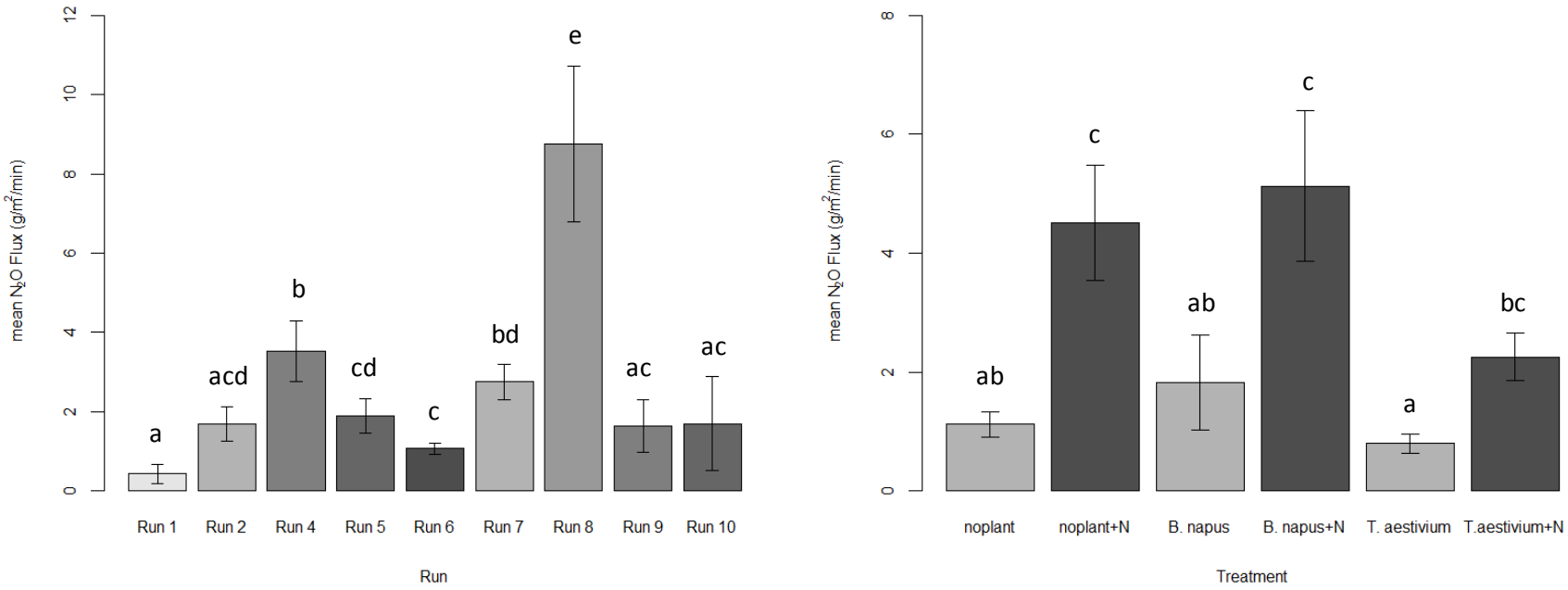

Figure 16: Differences across the runs and treatments of N_2O fluxes. Bars are standard error. The letters above the bars denote significant differences between the log_{10} transformed fluxes across those variables, as produced by pairwise paired t.tests with Boneferroni adjustement.

3.3 Molecular Presence/Absence

All genes of interest successfully amplified and their presence varied among the cores. A molecular marker present on the end point gel was counted as present in that core (Fig.17).

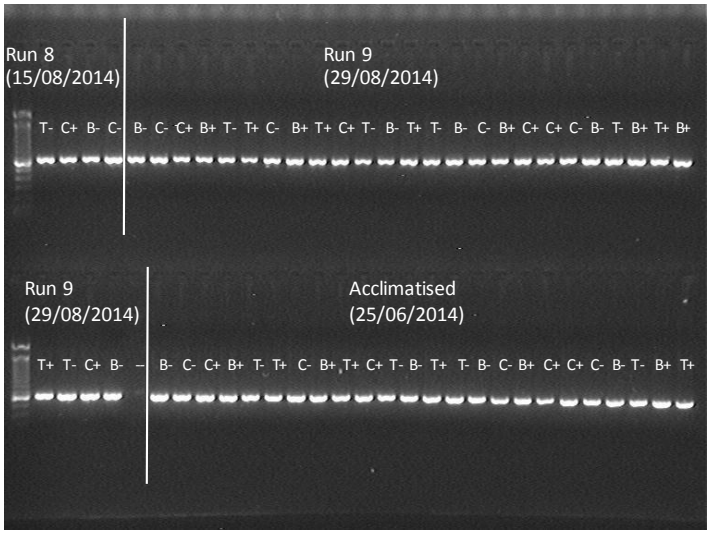

Figure 17: An agarose gel for the arch-amoA PCR product. Top row is core 27 of Run 8 to core 25 of Run 9.

Bottom row is core 26-29 of Run 9 (core 30 is missing), then core 1-24 of Run 1. The blank space is a negative control.

All of these bands would have been counted as presence of this marker in the respective cores.

T is Triticum aestivum, B is Brassica napus and C is the no plant control +/- refers to with/without N addition.

Both bacterial and archaeal 16S presence levels are low and there were cores where the gel showed bands for other genes but no band for 16S genes. This was especially the case for arch 16S and arch *amoA*. This is unexpected, because the 16S primers should pick up any bacteria and archaea present in the sample and all carriers of the arch *amoA* gene will also have a copy of 16S RNA.

χ^2 tests of association were used to test the hypothesis that molecular markers presence/absence is non-random among treatments. Expected values were based on an even distribution of total presence counts across all treatment types if there was no effect of treatment on the molecular markers. There was no significant difference found for any of the molecular markers across the treatments (Table 5). *NirS* showed a difference between unplanted cores (mean presence count= 16) compared to planted cores (mean presence count= 21.5), although this was not significant .There was no similar trend in differences of 16S and *amoA*, both bacterial and archaeal, when looking at pairs of treated/untreated (e.g. comparing untreated *B. napus* with treated *B. napus*), with some increasing presence with the addition of N (e.g. bac-16S in *B. napus*)

and some decreasing (e.g. bac-16S in *T. aestivum*) and many staying the same (arch-16S in *T. aestivum* and most bac-*amoA* and arch-*amoA*). *NirS* and *nirK* presence increased slightly with the addition of N (*nirK*: 25 to 26 for unplanted, 24 to 27 for *B.napus* and 25 to 29 for *T. aestivum*) and presence of *nirS* in unplanted cores was lower than other treatments for both treated and untreated cores.

Table 5: Counts of presence of molecular markers across treatments. Total number of cores sampled= 210. Null hypothesis was that there was an even distribution of presence across treatments and this cannot be rejected for any of the molecular markers. Chi-square tests had 4 d.f. as the expected parameter was estimated from the sample.

Treatment	bac-16S	arch-16S	bac-*amoA*	arch-*amoA*	*nirK*	*nirS*	*nosZ*
Unplanted N-	25	22	30	35	25	15	32
Unplanted N+	24	20	31	35	26	17	30
***B. napus* N-**	22	21	31	35	24	20	32
***B. napus* N+**	25	22	31	35	27	24	31
***T. aestivum* N-**	26	19	31	35	25	20	31
***T. aestivum* N+**	25	19	31	35	29	22	33
Total counts	147	123	185	210	156	118	189
Expected	24.5	20.5	30.8	35	26.0	19.7	31.5
Chi-square	0.39	0.46	0.03	0.00	0.62	2.71	0.17
p value	0.98	0.98	1.0	1.0	0.96	0.61	1.0

The same was done across the different runs and for some molecular markers there was a significant difference between runs (Table 6). Bacterial 16SrRNA ($\chi^2(5)=46.19$, $p<0.001$), archaeal 16SrRNA ($\chi^2(5)=54.5$, $p<0.001$), *nirK* ($\chi^2(5)=19.09$, $p<0.005$) and *nirS* ($\chi^2(5)=48.69$, $p<0.001$) all showed significant differences across runs. These differences are due to runs with very low presence of certain markers (e.g. Runs 6 and 9 [1 and 0 counts, respectively] for *nirS*). Runs 1 and 9 have low counts for three of the markers (bac-16S, arch-16S and *nirK* for Run 1, bac-16S, arch-16S and *nirS* for Run 9) and Runs 6 and 10 each have low values for 1 marker apiece (*nirS* and arch-16S respectively).

Table 6: Counts of presence of molecular markers across runs. Total number of cores sampled= 210. Null hypothesis was that there was an even distribution of presence across treatments and this can be rejected for four markers. Chi-square tests had 5 d.f. as the expected parameter was estimated from the sample.

Run	**bac-16S**	**arch-16S**	**bac-*amoA***	**arch-*amoA***	***nirK***	***nirS***	***nosZ***
1	2	6	30	30	6	25	30
5	28	27	29	30	18	20	30
6	26	29	29	30	28	1	30
7	30	30	29	30	21	28	30
8	28	24	27	30	24	26	30
9	3	6	12	30	30	0	22
10	30	1	29	30	29	18	17
Total counts	147	123	185	210	156	118	189
Expected	21.0	17.6	26.4	30	22.3	16.9	27.0
Chi-square	46.19	54.50	9.37	0.00	19.09	48.69	6.30
***p* value**	<0.001	<0.001	0.0951	1.0000	<0.005	<0.001	0.2784

When presence counts were compared against soil moisture, there was a significant difference found for archaeal 16rRNA (Wilcoxon rank sum, W=2326, $p<0.01$) and *nirS* (Wilcoxon rank sum, W=1295, $p<0.05$). Cores where archaeal 16SrRNA was absent were on average ~16% wetter than where it was present. Cores where *nirS* was absent were ~16% drier than where it was present. This was calculated by treating presence and absence of each gene as factors and assigning cores to either group for each gene of interest. As a note of caution, apart from archaeal 16S rRNA, most splitting by gene presence caused unequal group sizes (Fig.18).

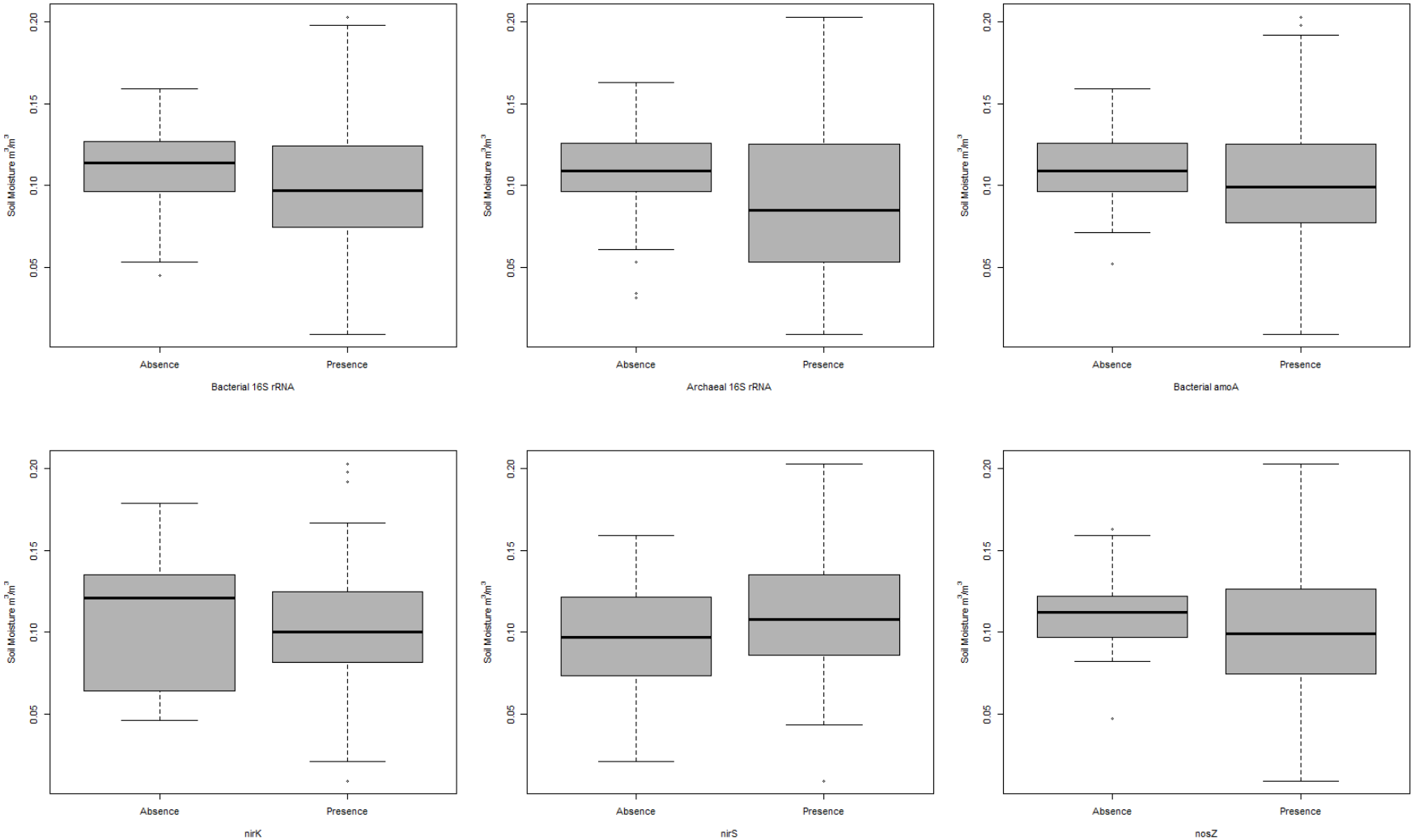

Figure 18: Differences in soil moisture between the cores that showed presence/absence of functional gene markers. Archaeal amoA not shown as all cores showed presence. N=120, less than in the previous tables because some of those 210 samples did not have soil moisture data. Most group sizes are uneven: bac 16S(A=33, P=87), arch16S(A=60, P=60), bac amoA(A=23, P=97), nirK(A=9, P=111), nirS(A=75, P=45), nosZ(A=21, P=99). A= absent, P= present

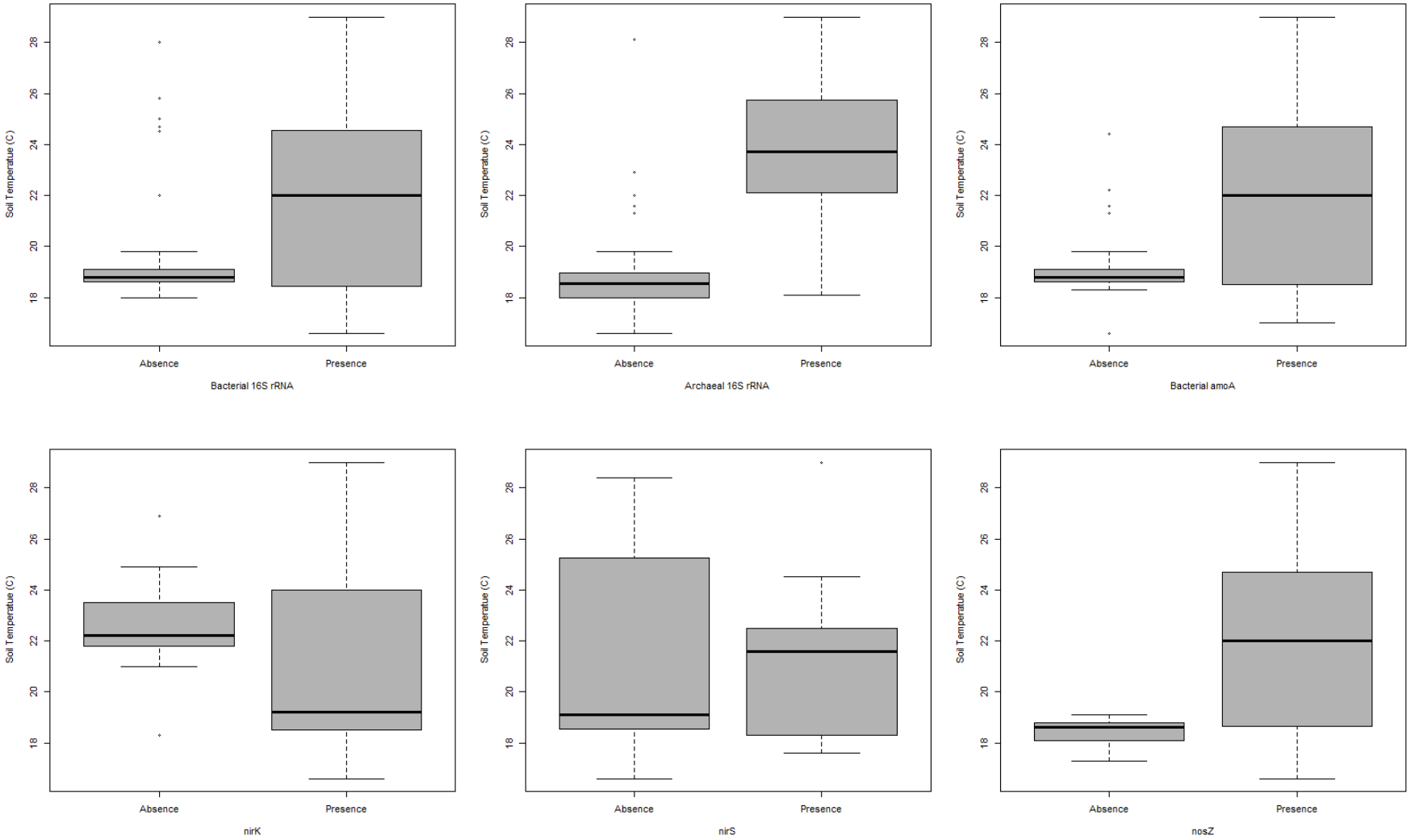

Figure 19: Differences in soil temperature between the cores that showed presence/absence of functional gene markers. Archaeal amoA not shown as all cores showed presence. N=120. Most group sizes are uneven: bac 16S(A=33, P=87), arch16S(A=60, P=60), bac amoA(A=23, P=97), nirK(A=9, P=111), nirS(A=75, P=45), nosZ(A=21, P=99). A= absent, P= present

Gene markers showed a stronger response to temperature. Bacterial 16S (W=1087, $p<0.05$), archaeal 16S (W=262, $p<0.001$), bacterial *amoA* (W=776, $p<0.05$) and *nosZ* (W=438, $p<0.001$) presence/absence were significantly correlated with soil temperature. For all of these, the cores where the gene markers were absent were colder than where they were present.

Section 4: Discussion

4.1 N_2O Fluxes

4.1.1 Treatment Differences

Analysis of the N_2O fluxes showed a marked effect of N addition on N_2O fluxes, in line with predictions. More interestingly, whilst unplanted and *B. napus* cores treated with N showed similar fluxes, N treated *T. aestivum* cores showed a much lower flux response to N addition. Whilst the treated T. *aestivum* fluxes were not significantly lower than the other treated fluxes the fact that it was also not significantly different from the untreated unplanted and *B. napus* cores suggests that there is a difference. This suggests that there is an effect of the *T. aestivum* that is reducing the amount of N_2O emissions that is not present with *B. napus*.

B. napus has higher plant N requirements than *T. aestivum* (Colnenne *et al.* 1998) suggesting that it's N uptake from the soil should be higher reducing availability to nitrifiers and denitrifiers in the soil. *T. aestivum* forms AMF interactions, shown to increase the amount of N available to plants (McFarland *et al.* 2010) including from organic sources (Hodge and Fitter 2010, Hodge *et al.* 2001). Hawkins *et al.* (2000) found that AMF transport 0.2% of organic N to *T. aestivum* plants under low N concentrations and 6% in high concentrations. Whilst this is not huge in terms of the overall plant N budget, Hodge and Fitter (2010) showed that whilst around 3% of *Plantago lanceolata* N came from a patch of organic N that the fungal partner had access to, the fungus acquired 31% of its own N from that patch. Potentially then, if we assume that the fungi require a similar amount of N when forming an interaction with *T. aestivum* as with *P. lanceaolata,* AMF could have taken up between 30% and 40% of the N present in the treated cores, primarily in response to fungal demand but with some N allocation to the plant. This would result in lower N availability in the soil and hence less N_2O flux derived from soil microbes. Untreated *T. aestivum* cores also tended to lower fluxes than other untreated cores. If the lower overall N_2O flux is typical of *T. aestivum* irrespective of treatment. It is possible that AMF could be responsible for the

majority of N uptake, even where little of that is subsequently incorporated into plant tissue (Hawkins *et al.* 2000).

There was little apparent difference between treated *B. napus* and treated unplanted cores. It is plausible to suggest that as the plants grew well, in *B. napus* cores, less N was available to soil microbes compared to unplanted cores, and as a consequence, lower N_2O flux would be predicted. The fact that there is no significant difference between the unplanted and the *B. napus* cores suggests that there is a factor limiting N_2O production in the unplanted cores. Whilst N_2O production in nitrification is carried out by chemoautotrophic ammonia oxidisers (Baggs & Philippot 2011), denitrifiers rely on a C source for their energy. It has long been known that plants can influence the rhizosphere community (Rovira 1956, Marschner *et al.* 2001) including nitrifiers and denitrifiers (Henry *et al.* 2008) and this can vary depending on the plant species (Patra *et al.* 2006). One of these influences is the deposition of C rich exudates into the rhizosphere, which have been demonstrated to stimulate N_2O production by denitrifiers e.g. in *Lolium perenne* grass swards grown under elevated CO_2 (Baggs *et al.* 2003, Baggs & Blum 2004). It may be reasonable to suggest that in N treated *B. napus* pots, N_2O production by nitrifiers may be reduced, due to reduced N availability, whilst production by denitrifiers may be increased through soil priming by C-rich exudates into the rhizosphere. To confirm this, discrimination of denitrifier and nitrifier derived N_2O e.g. with SP ratio information about the N_2O.

4.1.2 Temporal Variation

These treatment effects were mostly driven by the fluxes on Run 8 (Fig.15). This was the only individual run that showed the same pattern between the different treatments as the means pooled from all the runs. These measurements were taken one week after the second application of N_2O and are significantly greater than those taken one day after or two weeks after (Runs 7 and 9, respectively). N_2O emissions peaked after one week of application and no treatment effect was detected more than two weeks after application. This suggests that studies seeking to identify sward level responses to N amendment should focus on "pulse-chase" type approaches rather than regular measurements of the kind undertaken here. As the data from one week after the first application (Run 3) is missing, it is not possible to compare if emissions are greater one week after the second N application than one week after the first, which we could expect if the first application had allowed a proliferation of nitrifiers and denitrifiers in the soil. Aside from Run 8, there was no significant difference in N_2O flux between any of the other runs (Fig. 15).

We suggest that there may be a "priming" effect on the soil, because the N_2O fluxes were significantly lower ($p<0.01$) on Run 9 (backtransformed mean=0.59±0.26), than on Run 4 (backtransformed mean= 2.58±0.24), suggesting that the N had passed through the system more quickly after the second application. However quantitative functional gene data giving an indication of the size of the soil community or other measure of metabolic activity are required to confirm this. In addition, if there was a strong priming effect, we would expect the fluxes to be much greater on Run 7 (2.33±0.05) than on Run 2 (2.12±0.08) but these are not significantly different.

The significant interaction between run and treatment is because the treatments without N addition show no difference across runs, whereas the treatments with N addition obviously responded quite strongly in the runs following N addition.

4.2 Functional Gene Analysis

There was no difference in the presence of genetic markers across any of the treatments despite the prediction that there would be significantly greater presence of the genetic markers for functional genes involved in nitrification and denitrification (Table 5). Although the data was presence/absence rather than quantitative abundances given by qPCR, it would still be expected that significantly more cores treated with ammonium nitrate would show presence of functional genes compared to untreated cores.

There may have been real differences in the counts of the PCR products between the treatments but end point PCR is a relatively crude method for assessing gene abundance, and sensitivity may have simply been insufficient. The difference in *NirS* gene presence between unplanted and planted cores suggests that it may be found in endorhizospheric denitrifies. On the other hand, there was a huge difference in presence for certain genes (bacterial and archaeal 16SrRNA, *nirK* and *nirS*) across the different runs. Many of these genes showed significant decline in abundance in runs 9 and 10, when soil temperatures were cooler (Fig.11) than previously. The data may suggest that ammonia oxidising bacteria (AOB) and denitrifiers are most sensitive to decreased temperatures. The optimum range of growth for nitrifiers is between 20-25°C (Belser 1979, Campbell and Biederbeck 1976, Zhu and Chen 2002, Antoniou *et al.* 2003). The temperatures measured during this experiment however do not seem to be low enough to cause

nitrifiers to reduce in such number that they are no longer identifiable in a soil sample. Even these cooler temperatures are still summer temperatures in late August, warmer than most of the year and it seems unlikely that temperatures between 16 and 20°C would halt ammonia oxidation and N_2O reduction in soils. One possible explanation is that proliferation of other bacterial/archaeal species without these genes proliferated to such an extent that the target genes were diluted beyond the point where they could be detected using a single PCR reaction and end point PCR.

That 16S rRNA genes follow this pattern is less easy to explain especially because some core samples showed an absence of 16S genes but presence of other functional genes, particularly arch-16S and arch-*amoA* in the same core, at the same time point (see Appendix 1). Only one set of DNA was extracted at each sampling point and pooled, from which a subsample was taken for extraction. This extract formed the DNA template for the PCRs for all the genes. Arch-16S and arch-*amoA* PCRs should contain the same mix of DNA; difference seen cannot be due to soil heterogeneity. Any bacteria or archaea present that is holding a copy of those functional genes would also be holding a copy of 16SrRNA genes, hence why they are often used to estimate total counts of bacteria/archaea (Henry *et al.* 2006, Petersen *et al.* 2012), so we would expect to find those genes present in every core that showed presence of functional genes. Similarly there are a wide range of bacteria and archaea in soils that are active to much lower temperatures than those recorded in this investigation (minimum soil temp= 16.6°C)

It is unlikely to be an issue with the PCR; if this were the case, we would expect all of the runs present on that PCR plate to have the same problem and this is not the case. For each primer pair, three separate PCRs were needed to do all the samples, however at least two runs were present on each 96 well primer plate: Plate 1 (Runs 1,2, 5), Plate 2 (Runs 6,7,8) and Plate 3 (Runs 9 and 10). All of the samples from one plate, irrespective of run, were created using the same master mix of buffer, $MgCl_2$, dNTPs, primers and *Taq*. Therefore if it was an issue with the PCR, all samples on that plate should have been affected equally. Aside from Runs 9 and 10 for archaeal 16S rRNA, runs of very low gene presence are from PCRs with at least one run where the gene is well represented. It could be that those sections of DNA were more prone to degradation over time and repeated freeze-thawing of the DNA samples prior to PCR caused increased degradation of those zones, although we still would have expected to see absence not so constrained by runs, as all samples were stored and analysed together and were subjected to the same amount of freeze-thawing.

As it was often an entire sampling day where there was low 16S presence on the gels (Runs 1, 8 &9, see Appendix 1), it is possible that when the DNA was extracted for those runs, not all humic contaminants were removed. It is unlikely that the DNA was not present in the soil, as later samples from the same cores show presence of the genes. Humic contaminants can inhibit DNA detection including the performance of *Taq* in PCR reactions (Tebbe & Vahjen, 1993, LaMontagne *et al.* 2002). It could be that some sections of DNA, maybe those between the 16S rRNA primers used, are less accessible to *Taq* simply due to their locations, and that slightly inhibited *Taq* was unable to amplify enough of those fragments to show up on the gel. If this was the case, humic acid contamination could be estimated for all samples by comparing the A_{230} of the DNA extract with the A_{230} of a humic acid salt solution of known concentration (LaMontagne *et al.* 2002).

It is also possible that there are problems with the primers used. For bacteria, the 16S primers used (27F and 338R) are older primers that may not have the taxonomic coverage to pick up some of the bacterial DNA found in the soil samples. For archaea, when the primers for arch-*amoA* were BLASTed on the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) they produced significant alignment with nine uncultured bacterial *amoA* clones (accession numbers KP272121.1 through to KP272128.1). This suggests that the reason why the presence of arch-*amoA* on the gels was much greater than arch-16S is that much of that is due to accidental amplification of bacterial 16S, and in fact there are very few archaea at all in these soil samples, and far fewer ammonia oxidising archaea than these results would suggest.

Ammonia oxidising archaea (AOA) have been suggested to be more tolerant to desiccation than AOB (Adair and Schwartz 2008) it's possible that they may be more common in low moisture environments. However AOB presence did not show a response to soil moisture in this investigation.

4.3 Conclusions and Future Research

The flux data suggest that crop species differ in their influence on soil N_2O emissions and that there is a reducing effect of *T. aestivum* on soil N_2O emissions compared to bare soil or *B. napus*, for which the most obvious explanation is an effect mediated by AMF. Recent work on exclusion of ectomycorrhizas in forests suggests that they are vital in reducing N_2O fluxes from

forested soils (Ernfors *et al.* 2010) and Storer (2013) suggested that in *Zea mays,* AMF colonisation reduced N_2O emissions compared to the control pre-harvesting.

Genetic marker information or the SP data would allow the underlying mechanisms determining microbe derived N_2O emissions to be identified. The effect of AMF on the nitrifier and denitrifier communities could be identified by next generation sequencing of the gene markers and quantifying changes in diversity in those genes and comparing these with incidence of AMF colonisation in the plant roots and N allocation to AMF and the plant.

There was no net effect of planting with *B. napus* on N_2O emissions although there may be different processes determining the flux in each system. There was no significant relationship between N_2O fluxes and either soil temperature or moisture so these were not used for further investigation of the flux data. Because of the missing Run 3 data, the potential priming effect could not be properly investigated. Repeating this experiment would allow this to be investigated, but this study suggests the time between N addition events should be shortened, to capture the N_2O signal as it attenuates. Gas sampling would be carried out more often during that two week period to get higher temporal resolution and show whether or not if N passed through the system more readily after a second application.

With further work on samples already available from this project, quantitative PCRs could be run in order to carry out comparisons between the molecular data and the N_2O fluxes and get a better idea of how these functional genes behave under N treatment that is representative of current farming practices in the UK. This study found that different crops show a different response to N addition and that difference manifests itself as significant changes in emission of an important GHG. This study tentatively suggests that this effect is driven in the rhizosphere rather than directly by the plant, mediated by root exudates and AMF. Thus, this is additional evidence that soil biodiversity, increasingly recognised as a vital area we need to understand better. It offers an additional tool to make decisions about soil and agricultural management, such as N addition and will enable more sustainable use of soils.

Appendices

Appendix 1: Table of sorted data

Core	Run	Regime	N_2O Flux ($\mu g/min/m^2$)	Air Temp (°C)	Soil Temp (°C)	Soil Moisture (m^3/m^3)	bac-16S	arch-16S	bac-*amoA*	arch-*amoA*	*nirK*	*nirS*	*nosZ*
1	1	osrN-	-0.53068	NA	NA	NA	0	0	1	1	0	1	1
2	1	noplantN-	-0.16729	NA	NA	NA	0	0	1	1	0	1	1
3	1	noplantN+	0.25299	NA	NA	NA	0	0	1	1	0	0	1
4	1	osrN+	0.102253	NA	NA	NA	0	0	1	1	0	1	1
5	1	wheatN-	-0.97164	NA	NA	NA	0	0	1	1	0	1	1
6	1	wheatN+	0.794964	NA	NA	NA	0	0	1	1	0	1	1
7	1	noplantN-	0.758003	NA	NA	NA	0	0	1	1	0	1	1
8	1	osrN+	0.516228	NA	NA	NA	0	0	1	1	0	1	1
9	1	wheatN+	0.047783	NA	NA	NA	0	0	1	1	0	0	1
10	1	noplantN+	-0.93775	NA	NA	NA	0	0	1	1	0	1	1
11	1	wheatN-	-0.42977	NA	NA	NA	0	0	1	1	0	0	1
12	1	osrN-	0.397187	NA	NA	NA	0	0	1	1	0	0	1
13	1	wheatN+	-0.55358	NA	NA	NA	0	0	1	1	0	1	1
14	1	wheatN-	0.002035	NA	NA	NA	0	0	1	1	0	0	1
15	1	osrN-	-0.137	NA	NA	NA	0	0	1	1	0	1	1
16	1	noplantN-	6.302985	NA	NA	NA	0	0	1	1	0	1	1
17	1	osrN+	2.069476	NA	NA	NA	0	0	1	1	0	1	1
18	1	noplantN+	1.011122	NA	NA	NA	0	0	1	1	0	1	1
19	1	noplantN+	1.279445	NA	NA	NA	0	0	1	1	0	1	1
20	1	noplantN-	0.520956	NA	NA	NA	0	0	1	1	0	1	1
21	1	osrN-	-0.48686	NA	NA	NA	0	0	1	1	0	1	1
22	1	wheatN-	-0.03227	NA	NA	NA	0	0	1	1	0	1	1
23	1	osrN+	-0.16626	NA	NA	NA	0	0	1	1	0	1	1
24	1	wheatN+	2.329031	NA	NA	NA	0	0	1	1	1	1	1
25	1	osrN+	0.109622	NA	NA	NA	1	1	1	1	1	1	1
26	1	wheatN+	0.925261	NA	NA	NA	0	1	1	1	0	1	1
27	1	wheatN-	-0.33743	NA	NA	NA	0	1	1	1	1	1	1
28	1	noplantN+	-0.04919	NA	NA	NA	0	1	1	1	1	1	1
29	1	osrN-	0.073106	NA	NA	NA	1	1	1	1	1	1	1
30	1	noplantN-	0.324362	NA	NA	NA	0	1	1	1	1	1	1
1	2	osrN-	0.840613	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	2	noplantN-	-1.2112	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
3	2	noplantN+	2.167433	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
4	2	osrN+	3.115646	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
5	2	wheatN-	1.419404	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
6	2	wheatN+	2.680506	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Core	Run	Regime	N_2O Flux ($\mu g/min/m^2$)	Air Temp (°C)	Soil Temp (°C)	Soil Moisture (m^3/m^3)	bac-16S	arch-16S	bac-*amoA*	arch-*amoA*	*nirK*	*nirS*	*nosZ*
7	2	noplantN-	1.994526	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
8	2	osrN+	4.030186	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	2	wheatN+	8.766243	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
10	2	noplantN+	1.504224	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
11	2	wheatN-	1.797187	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
12	2	osrN-	0.757964	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
13	2	wheatN+	-0.74545	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
14	2	wheatN-	-0.05028	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
15	2	osrN-	-0.93119	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
16	2	noplantN-	0.792623	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
17	2	osrN+	8.711507	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
18	2	noplantN+	3.162049	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
19	2	noplantN+	1.361687	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
20	2	noplantN-	-0.3422	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
21	2	osrN-	0.049175	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
22	2	wheatN-	0.987027	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
23	2	osrN+	0.025068	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
24	2	wheatN+	0.961758	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
25	2	osrN+	5.086754	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
26	2	wheatN+	1.055677	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
27	2	wheatN-	-0.25793	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
28	2	noplantN+	1.147886	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
29	2	osrN-	0.293702	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
30	2	noplantN-	1.247748	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	4	osrN-	1.200756	25	24.3	0.109	NA	NA	NA	NA	NA	NA	NA
2	4	noplantN-	1.09466	24.2	22.4	0.105	NA	NA	NA	NA	NA	NA	NA
3	4	noplantN+	6.260013	23.6	21.8	0.112	NA	NA	NA	NA	NA	NA	NA
4	4	osrN+	1.541109	25.7	23.4	0.086	NA	NA	NA	NA	NA	NA	NA
5	4	wheatN-	1.699449	26.5	22.7	0.13	NA	NA	NA	NA	NA	NA	NA
6	4	wheatN+	3.820867	24.7	22.5	0.11	NA	NA	NA	NA	NA	NA	NA
7	4	noplantN-	2.813849	26.9	23.7	0.105	NA	NA	NA	NA	NA	NA	NA
8	4	osrN+	1.816742	29.5	21.4	0.11	NA	NA	NA	NA	NA	NA	NA
9	4	wheatN+	7.641806	25.8	23.3	0.106	NA	NA	NA	NA	NA	NA	NA
10	4	noplantN+	15.717	22.4	25.1	0.023	NA	NA	NA	NA	NA	NA	NA
11	4	wheatN-	2.629339	21.3	23.9	0.126	NA	NA	NA	NA	NA	NA	NA
12	4	osrN-	3.200336	27	23	0.124	NA	NA	NA	NA	NA	NA	NA
13	4	wheatN+	0.578708	28.4	22.9	0.146	NA	NA	NA	NA	NA	NA	NA
14	4	wheatN-	1.184511	27.2	22	0.136	NA	NA	NA	NA	NA	NA	NA
15	4	osrN-	1.138657	26.3	23.1	0.091	NA	NA	NA	NA	NA	NA	NA
16	4	noplantN-	1.972535	26.6	22.1	0.109	NA	NA	NA	NA	NA	NA	NA
17	4	osrN+	3.356029	28.3	24.1	0.101	NA	NA	NA	NA	NA	NA	NA

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18	4	noplantN+	13.47651	27.1	25.7	0.094	NA	NA	NA	NA	NA	NA	NA
19	4	noplantN+	6.689811	25.4	25.3	0.103	NA	NA	NA	NA	NA	NA	NA
20	4	noplantN-	1.637968	26.6	23.7	0.102	NA	NA	NA	NA	NA	NA	NA
21	4	osrN-	0.95483	27.5	24.2	0.106	NA	NA	NA	NA	NA	NA	NA
22	4	wheatN-	1.41187	27.9	23	0.121	NA	NA	NA	NA	NA	NA	NA
23	4	osrN+	0.826216	27.2	23.4	0.083	NA	NA	NA	NA	NA	NA	NA
24	4	wheatN+	1.257906	26.1	23.3	0.081	NA	NA	NA	NA	NA	NA	NA
25	4	osrN+	14.63289	28.8	25.7	0.105	NA	NA	NA	NA	NA	NA	NA
26	4	wheatN+	3.969635	24.4	22.3	0.098	NA	NA	NA	NA	NA	NA	NA
27	4	wheatN-	0.683743	22.6	24.5	0.091	NA	NA	NA	NA	NA	NA	NA
28	4	noplantN+	0.612102	25	23.8	0.102	NA	NA	NA	NA	NA	NA	NA
29	4	osrN-	0.891658	21.3	22.7	0.076	NA	NA	NA	NA	NA	NA	NA
30	4	noplantN-	0.905672	21.9	22.6	0.104	NA	NA	NA	NA	NA	NA	NA
1	5	osrN-	0.767745	NA	NA	NA	1	1	1	1	1	1	1
2	5	noplantN-	0.025037	NA	NA	NA	1	1	1	1	1	0	1
3	5	noplantN+	2.458337	NA	NA	NA	1	1	1	1	0	1	1
4	5	osrN+	0.182429	NA	NA	NA	1	1	1	1	1	1	1
5	5	wheatN-	4.016991	NA	NA	NA	1	1	1	1	0	1	1
6	5	wheatN+	2.370991	NA	NA	NA	1	1	1	1	0	1	1
7	5	noplantN-	2.662239	NA	NA	NA	1	1	0	1	0	0	1
8	5	osrN+	0.753776	NA	NA	NA	1	1	1	1	0	1	1
9	5	wheatN+	1.089902	NA	NA	NA	1	1	1	1	1	0	1
10	5	noplantN+	9.571528	NA	NA	NA	1	1	1	1	0	0	1
11	5	wheatN-	2.063903	NA	NA	NA	1	0	1	1	1	0	1
12	5	osrN-	4.582492	NA	NA	NA	1	1	1	1	1	1	1
13	5	wheatN+	0.453073	NA	NA	NA	1	1	1	1	1	1	1
14	5	wheatN-	0.732842	NA	NA	NA	1	0	1	1	1	1	1
15	5	osrN-	1.822458	NA	NA	NA	1	1	1	1	1	1	1
16	5	noplantN-	1.215889	NA	NA	NA	1	1	1	1	1	1	1
17	5	osrN+	2.267933	NA	NA	NA	1	1	1	1	0	1	1
18	5	noplantN+	1.619733	NA	NA	NA	1	1	1	1	1	0	1
19	5	noplantN+	0.860123	NA	NA	NA	1	1	1	1	1	1	1
20	5	noplantN-	0.499123	NA	NA	NA	1	1	1	1	0	0	1
21	5	osrN-	0.903571	NA	NA	NA	1	1	1	1	0	1	1
22	5	wheatN-	0.111608	NA	NA	NA	1	1	1	1	1	1	1
23	5	osrN+	1.148548	NA	NA	NA	1	1	1	1	1	1	1
24	5	wheatN+	1.565483	NA	NA	NA	1	1	1	1	1	1	1
25	5	osrN+	9.745573	NA	NA	NA	1	1	1	1	1	1	1
26	5	wheatN+	0.757921	NA	NA	NA	1	1	1	1	1	1	1
27	5	wheatN-	0.535118	NA	NA	NA	1	1	1	1	1	1	1
28	5	noplantN+	0.528751	NA	NA	NA	1	1	1	1	0	0	1

Core	Run	Regime	N_2O Flux ($\mu g/min/m^2$)	Air Temp (°C)	Soil Temp (°C)	Soil Moisture (m^3/m^3)	bac-16S	arch-16S	bac-*amoA*	arch-*amoA*	*nirK*	*nirS*	*nosZ*
29	5	osrN-	0.636796	NA	NA	NA	0	0	1	1	0	0	1
30	5	noplantN-	1.085538	NA	NA	NA	0	1	1	1	0	0	1
1	6	osrN-	0.110322	31.3	25	0.092	0	1	1	1	1	0	1
2	6	noplantN-	0.709877	31.8	25.7	0.083	1	1	1	1	1	0	1
3	6	noplantN+	1.310605	26.4	27.7	0.051	1	1	1	1	1	0	1
4	6	osrN+	2.040806	31.3	29	0.043	1	1	1	1	1	1	1
5	6	wheatN-	0.904604	30.4	23.6	0.081	1	1	1	1	1	0	1
6	6	wheatN+	0.706149	35.2	24.2	0.053	1	1	1	1	1	0	1
7	6	noplantN-	0.144136	33.9	25.9	0.099	1	1	1	1	1	0	1
8	6	osrN+	2.883719	33.4	24.7	0.045	0	1	1	1	1	0	1
9	6	wheatN+	1.254384	27.5	28.1	0.034	1	0	1	1	1	0	1
10	6	noplantN+	0.500095	32.3	25.9	0.084	1	1	1	1	1	0	1
11	6	wheatN-	1.292264	28.7	26.9	0.046	1	1	1	1	0	0	1
12	6	osrN-	1.204281	28.4	24.9	0.048	1	1	1	1	0	0	1
13	6	wheatN+	-0.13275	27.4	24.9	0.06	1	1	1	1	1	0	1
14	6	wheatN-	-0.02558	30	27.1	0.093	1	1	1	1	1	0	1
15	6	osrN-	1.980236	25.6	26	0.049	1	1	1	1	1	0	1
16	6	noplantN-	1.004324	25.7	24.4	0.086	1	1	1	1	1	0	1
17	6	osrN+	1.510326	31.7	25.8	0.057	0	1	1	1	1	0	1
18	6	noplantN+	2.695907	36.1	28	0.08	0	1	1	1	1	0	1
19	6	noplantN+	1.352198	29.6	25.4	0.085	1	1	1	1	1	0	1
20	6	noplantN-	0.887595	31	26.8	0.054	1	1	1	1	1	0	1
21	6	osrN-	0.807587	29.5	28.4	0.031	1	1	1	1	1	0	1
22	6	wheatN-	0.181837	31.1	26.2	0.082	1	1	1	1	1	0	1
23	6	osrN+	0.959324	25.3	26.6	0.076	1	1	1	1	1	0	1
24	6	wheatN+	1.409747	26	27.5	0.021	1	1	1	1	1	0	1
25	6	osrN+	2.435141	25.9	25.2	0.049	1	1	1	1	1	0	1
26	6	wheatN+	1.556265	25.1	25.3	0.044	1	1	1	1	1	0	1
27	6	wheatN-	0.163888	24.4	26	0.085	1	1	1	1	1	0	1
28	6	noplantN+	0.696021	23.2	25.4	0.082	1	1	1	1	1	0	1
29	6	osrN-	0.812474	27	24.4	0.052	1	1	0	1	1	0	1
30	6	noplantN-	0.616664	23.7	24.7	0.053	1	1	1	1	1	0	1
1	7	osrN-	0.614165	NA	NA	NA	1	1	1	1	1	0	1
2	7	noplantN-	1.884746	NA	NA	NA	1	1	1	1	1	0	1
3	7	noplantN+	3.949807	NA	NA	NA	1	1	1	1	1	1	1
4	7	osrN+	1.955371	NA	NA	NA	1	1	1	1	1	1	1
5	7	wheatN-	0.938994	NA	NA	NA	1	1	1	0	1	1	1
6	7	wheatN+	2.02426	NA	NA	NA	1	1	1	1	1	1	1
7	7	noplantN-	0.524583	NA	NA	NA	1	1	1	1	1	1	1
8	7	osrN+	0.98704	NA	NA	NA	1	1	0	1	1	1	1
9	7	wheatN+	1.244828	NA	NA	NA	1	1	1	1	1	1	1

Core	Run	Regime	N_2O Flux ($\mu g/min/m^2$)	Air Temp (°C)	Soil Temp (°C)	Soil Moisture (m^3/m^3)	bac-16S	arch-16S	bac-*amoA*	arch-*amoA*	*nirK*	*nirS*	*nosZ*
10	7	noplantN+	2.9495	NA	NA	NA	1	1	1	1	1	1	1
11	7	wheatN-	2.682575	NA	NA	NA	1	1	1	1	1	1	1
12	7	osrN-	2.439965	NA	NA	NA	1	1	1	1	1	1	1
13	7	wheatN+	2.462395	NA	NA	NA	1	1	1	1	1	1	1
14	7	wheatN-	1.165593	NA	NA	NA	1	1	1	1	0	1	1
15	7	osrN-	4.301436	NA	NA	NA	1	1	1	1	0	1	1
16	7	noplantN-	2.859228	NA	NA	NA	1	1	1	1	0	1	1
17	7	osrN+	3.493371	NA	NA	NA	1	1	1	1	0	1	1
18	7	noplantN+	8.547016	NA	NA	NA	1	1	1	1	0	1	1
19	7	noplantN+	6.326313	NA	NA	NA	1	1	1	1	0	1	1
20	7	noplantN-	1.366985	NA	NA	NA	1	1	1	1	0	1	1
21	7	osrN-	1.780441	NA	NA	NA	1	1	1	1	0	1	1
22	7	wheatN-	1.29352	NA	NA	NA	1	1	1	1	0	1	1
23	7	osrN+	2.849155	NA	NA	NA	1	1	1	1	1	1	1
24	7	wheatN+	2.903788	NA	NA	NA	1	1	1	1	1	1	1
25	7	osrN+	11.69315	NA	NA	NA	1	1	1	1	1	1	1
26	7	wheatN+	0.720636	NA	NA	NA	1	1	1	1	1	1	1
27	7	wheatN-	2.416209	NA	NA	NA	1	1	1	1	1	1	1
28	7	noplantN+	4.641893	NA	NA	NA	1	1	1	1	1	1	1
29	7	osrN-	0.767426	NA	NA	NA	1	1	1	1	1	1	1
30	7	noplantN-	0.822892	NA	NA	NA	1	1	1	1	1	1	1
1	8	osrN-	1.414842	22.6	22	0.009	1	1	1	1	1	1	1
2	8	noplantN-	4.028808	22	21.5	0.203	1	1	1	1	1	1	1
3	8	noplantN+	3.677698	22.6	21.5	0.198	1	1	1	1	1	1	1
4	8	osrN+	12.78217	30.3	22.4	0.167	1	1	1	1	1	1	1
5	8	wheatN-	0.378026	22.5	21.8	0.179	1	1	1	1	0	1	1
6	8	wheatN+	6.317725	21.6	22.1	0.099	1	1	1	1	1	1	1
7	8	noplantN-	2.927823	23.5	22.2	0.171	1	1	1	1	0	1	1
8	8	osrN+	5.698674	21.4	22.2	0.073	1	1	0	1	1	1	1
9	8	wheatN+	9.9838	26.2	22.1	0.086	1	1	1	1	1	1	1
10	8	noplantN+	31.29628	25.7	22.8	0.192	1	1	1	1	1	1	1
11	8	wheatN-	0.948918	28.6	22.6	0.133	1	1	1	1	1	1	1
12	8	osrN-	6.623075	27.4	22	0.135	0	1	1	1	0	1	1
13	8	wheatN+	4.169986	23.8	22.6	0.157	1	1	1	1	1	1	1
14	8	wheatN-	1.331178	28.5	23.2	0.134	1	1	1	1	1	0	1
15	8	osrN-	3.279174	24.8	24.5	0.053	0	1	1	1	1	1	1
16	8	noplantN-	3.861239	23.7	22.2	0.113	1	1	1	1	1	1	1
17	8	osrN+	27.08999	26.3	23.5	0.125	1	1	1	1	0	1	1
18	8	noplantN+	18.55819	22.3	22.8	0.124	1	1	1	1	1	1	1
19	8	noplantN+	21.59852	23.4	22.8	0.141	1	1	1	1	1	1	1
20	8	noplantN-	3.26886	22.1	22.5	0.141	1	1	1	1	1	1	1

Core	Run	Regime	N_2O Flux ($\mu g/min/m^2$)	Air Temp (°C)	Soil Temp (°C)	Soil Moisture (m^3/m^3)	bac-16S	arch-16S	bac-*amoA*	arch-*amoA*	*nirK*	*nirS*	*nosZ*
21	8	osrN-	1.92995	22.7	23.8	0.102	1	1	1	1	1	1	1
22	8	wheatN-	3.046775	21.7	22.5	0.121	1	1	1	1	0	1	1
23	8	osrN+	17.64326	19.7	22.9	0.083	1	0	1	1	1	1	1
24	8	wheatN+	5.884081	21.4	22	0.1	1	0	1	1	1	1	1
25	8	osrN+	46.87681	19.4	21.6	0.097	1	0	1	1	1	0	1
26	8	wheatN+	8.276937	20.5	21.6	0.108	1	0	0	1	1	1	1
27	8	wheatN-	1.572721	21.1	21.6	0.146	1	0	1	1	1	1	1
28	8	noplantN+	5.071587	20.1	21.3	0.106	1	0	0	1	1	0	1
29	8	osrN-	1.92675	19.6	21	0.064	1	1	1	1	0	1	1
30	8	noplantN-	0.972811	19.2	20.7	0.091	1	1	1	1	1	0	1
1	9	osrN-	0.339221	19.2	18.9	0.11	0	1	0	1	1	0	1
2	9	noplantN-	0.793335	22.6	18.6	0.102	0	1	0	1	1	0	1
3	9	noplantN+	0.393478	17.2	19.8	0.109	0	0	0	1	1	0	1
4	9	osrN+	1.494981	16.2	19	0.159	0	1	0	1	1	0	0
5	9	wheatN-	-0.25933	17.3	18.8	0.125	0	1	0	1	1	0	1
6	9	wheatN+	2.053303	17.8	18.7	0.104	0	0	0	1	1	0	1
7	9	noplantN-	-0.02927	19.7	18.7	0.071	0	0	0	1	1	0	1
8	9	osrN+	-0.31945	21.1	18.3	0.076	0	1	0	1	1	0	1
9	9	wheatN+	1.552919	21.3	18.4	0.119	0	0	0	1	1	0	1
10	9	noplantN+	9.4437	21.2	18.9	0.136	0	0	0	1	1	0	0
11	9	wheatN-	-0.79886	19	18.8	0.124	0	0	0	1	1	0	0
12	9	osrN-	-0.12469	19.5	18.8	0.097	0	0	0	1	1	0	0
13	9	wheatN+	-0.60687	19.2	19.1	0.127	0	1	1	1	1	0	1
14	9	wheatN-	-0.36898	18.5	19.2	0.138	0	0	0	1	1	0	1
15	9	osrN-	-0.2439	18.5	18.8	0.084	0	0	0	1	1	0	1
16	9	noplantN-	0.087143	18.4	18.5	0.142	0	0	0	1	1	0	1
17	9	osrN+	2.179752	19.3	18.7	0.115	0	0	1	1	1	0	1
18	9	noplantN+	0.420043	19.7	18.8	0.116	0	0	0	1	1	0	1
19	9	noplantN+	0.508682	19.5	18.7	0.114	0	0	1	1	1	0	0
20	9	noplantN-	-0.8647	17.6	19.1	0.125	0	0	1	1	1	0	0
21	9	osrN-	-0.28662	18.6	18.6	0.144	0	0	1	1	1	0	1
22	9	wheatN-	-0.09581	18	18.6	0.096	0	0	0	1	1	0	1
23	9	osrN+	5.904104	18	18.5	0.112	0	0	1	1	1	0	0
24	9	wheatN+	0.567159	17.9	18.4	0.122	0	0	1	1	1	0	0
25	9	osrN+	14.09926	18.7	18.5	0.139	1	0	1	1	1	0	1
26	9	wheatN+	7.677983	17.7	18.4	0.127	0	0	0	1	1	0	1
27	9	wheatN-	0.71539	16.3	18.3	0.157	1	0	1	1	1	0	1
28	9	noplantN+	7.568618	16	18.1	0.108	0	0	1	1	1	0	1
29	9	osrN-	-0.80264	15.3	18	0.148	0	0	1	1	1	0	1
30	9	noplantN-	-1.71401	16.5	18	0.132	1	0	1	1	1	0	1
1	10	osrN-	0.490402	20.6	17.4	0.092	1	0	1	1	1	0	1

Core	Run	Regime	N_2O Flux ($\mu g/min/m^2$)	Air Temp (°C)	Soil Temp (°C)	Soil Moisture (m^3/m^3)	bac-16S	arch-16S	bac-*amoA*	arch-*amoA*	*nirK*	*nirS*	*nosZ*
2	10	noplantN-	0.662666	20.2	16.6	0.132	1	0	0	1	1	0	1
3	10	noplantN+	1.270126	21.2	17.3	0.098	1	0	1	1	1	0	0
4	10	osrN+	0.456201	22.8	17	0.031	1	0	1	1	1	0	1
5	10	wheatN-	0.240242	20.8	17.6	0.106	1	0	1	1	1	1	0
6	10	wheatN+	0.352839	20.2	18	0.061	1	0	1	1	1	0	1
7	10	noplantN-	0.910572	19.1	17.8	0.097	1	0	1	1	1	0	1
8	10	osrN+	0.530163	19	17.7	0.053	1	0	1	1	1	1	1
9	10	wheatN+	0.829565	21.5	17.8	0.077	1	0	1	1	1	1	1
10	10	noplantN+	0.519426	25.6	19.3	0.109	1	0	1	1	1	0	1
11	10	wheatN-	0.4759	24.5	18.3	0.1	1	0	1	1	1	1	0
12	10	osrN-	0.169422	27.7	17.6	0.126	1	0	1	1	1	0	1
13	10	wheatN+	0.058653	28.7	18.8	0.095	1	0	1	1	1	1	1
14	10	wheatN-	0.134591	23.3	18.5	0.117	1	0	1	1	1	0	1
15	10	osrN-	0.176016	29	18.2	0.1	1	0	1	1	1	1	1
16	10	noplantN-	0.102426	21.8	19	0.127	1	0	1	1	1	0	1
17	10	osrN+	-0.14173	20.9	18.4	0.124	1	0	1	1	1	1	1
18	10	noplantN+	0.148091	20.2	19.2	0.107	1	0	1	1	1	0	1
19	10	noplantN+	0.363706	20.5	19.1	0.111	1	0	1	1	1	1	0
20	10	noplantN-	0.218034	18.5	18.6	0.121	1	0	1	1	1	0	0
21	10	osrN-	35.86374	19.2	19	0.082	1	0	1	1	1	1	0
22	10	wheatN-	0.021552	19.3	18.6	0.163	1	0	1	1	1	1	0
23	10	osrN+	0.50482	19.8	18.1	0.047	1	1	1	1	1	1	0
24	10	wheatN+	0.272674	18.2	18.3	0.089	1	0	1	1	0	1	0
25	10	osrN+	5.207186	18	18.7	0.114	1	0	1	1	1	1	0
26	10	wheatN+	0.052065	19	18.1	0.105	1	0	1	1	1	1	1
27	10	wheatN-	0.228036	17.7	17.8	0.13	1	0	1	1	1	1	1
28	10	noplantN+	0.409313	17.1	17.7	0.086	1	0	1	1	1	1	0
29	10	osrN-	0.193536	18.2	18	0.086	1	0	1	1	1	1	0
30	10	noplantN-	0.210207	17.6	18	0.119	1	0	1	1	1	1	0

Definitions

AMO: ammonia mono-oxygenase

HAO: hydroxylamine oxidoreductase

N_2O: nitrous oxide

NAR/P: nitrate reductase

NIR: nitrite reductase

NOS: nitrous oxide reductase

NOR: nitric oxide reductase

SP (site preference) ratio: The difference in the ratio of the stable isotopes ^{15}N:^{14}N ($\delta^{15}N$) between the central (α) and outer (β) atoms in nitrous oxide.

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