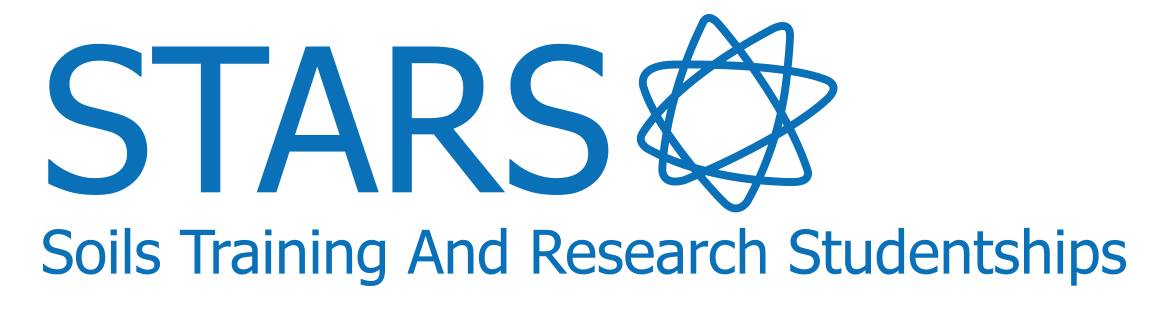
**The influence of antecedent soil moisture conditions and nutrient management on soil nitrous oxide emissions.**

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**Thesis Abstract**

Nitrous oxide (N2O) is a powerful greenhouse gas and ozone depleter, and it is produced in large quantities when a dry soil is rewetted in a phenomenon known as a hot moment. Therefore understanding this phenomena is important for tackling agriculture’s impact on climate change. A literature review and meta-analysis of N2O hot moments was conducted in Chapter 2, revealing that the amount of water added to the soil and how saturated the soil gets during rewetting are the most important controlling factors. For example, rewetting from 50 to 90% water filled pore space (WFPS), would produce more N2O emissions, than rewetting the same soil from 50% to 70%. However, it was clear that the current literature suffered from the lack of a standardised approach, as it was difficult to draw conclusions from experiments with different designs that had different rewetting strategies, controlled temperatures and soil core sizes. Moreover, drought length (i.e. the amount of time the soil stays dry before it was rewetted) had not been adequately investigated

It was still an open question as to how and why the soil’s microbial communities were responding in this manner to soil drought and rewetting, and so Chapter 3 was a lab experiment that aimed to investigate two of the key hypotheses suggested by the current literature. Firstly that drought and the resulting osmotic stress created a selection pressure which favoured the soil microbes that could rapidly denitrify upon rewetting. Secondly, that more carbon (C) and nitrogen (N) would become available from lysed microbes that could not survive the osmotic change. Neither of these were confirmed, as changes in the soil C and N and functional gene abundance could not explain differences in the sizes of the induced hot moments.

Chapter 4 repeated this experiment, with the same aims, and an additional measurement of messenger RNA abundance of key N cycling genes. While functional gene and transcript abundance failed to provide an explanation, ammonium (NH4+) in the driest treatment increased and then reduced in correlation with the largest hot moment. It was concluded that while the soil C and N pools can be enhanced by necromass, this is not the cause of the hot moment. This chapter also included a second experiment to investigate the effect of drought length, which was observed to have an inverted U shaped response, with the least amount of N2O being released when the drought phase was < 6 days or > 24 days. Overall, it was concluded that there are two stages of microbial quiescence that explain N2O hot moments and its response to changing antecedent conditions. The first stage is a state of semi-quiescence as the soil’s microbial communities prepare for the moisture conditions to change, during this stage they can rapidly respond to the changing WFPS by catabolising intercellular osmotic compounds, and the lower the WFPS the more of the soil microbial community are in this state, and therefore the greater the response once the soil is rewetted. However, once the drought becomes too extreme they enter a second stage of quiescence to survive the harsher conditions, which no longer allows a rapid response, explaining the quadratic effect of drought length.

This PhD also conducted a field trial on a long-term experiment based at Rothamsted’s Harpenden site, called Broadbalk, which is split into plots that have had different fertiliser types and quantities. It aimed to investigate how the legacy of fertiliser management, has impacted Broadbalk’s soil and its production of greenhouse gas (GHG) emissions (CO2, N2O, and CH4). This chapter compared differences between the farmyard manure (FYM), inorganic nitrogen and control plots (where only phosphorus and potassium was applied). The FYM treatment had more dissolved organic carbon (+160%) and triple the %C compared to the other treatments, and it had a significantly larger mean CO2 flux. The largest N2O emissions were recorded from the inorganic treatment post fertiliser application, but this did not result in significantly higher mean N2O emissions compared to the FYM treatment, which is likely due to the enhanced microbiological and biochemical activity from continuous organic fertiliser addition. CH4 fluxes were not affected by the different fertiliser regimes in this study, probably because the variables that typically affect CH4 emissions were not impacted by the selected treatments. The control treatment had the lowest GHG emissions, due to the lack of substrates in the soil.

In summary, this PhD quantified how the soil’s antecedent moisture conditions in a controlled environment effect the size of a hot moment, as well as providing new data on the effects of drought length, which due to its quadratic nature will change how researchers interpret the impact of more extreme droughts on N2O fluxes. It also conducted the first field trial on Broadbalk that measured all three of the key GHG greenhouse gases from soil. Concluding that the retention of soil C in the plots is the most important factor affecting CO2e, with CO2 making up the dominant portion of CO2e from all treatments.

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Thank you to my family for supporting me throughout the PhD, and finally a thanks is needed for the soil microbes, for without you, humanity wouldn’t exist.

**Declaration**

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. All other sources are acknowledged by bibliographic references. This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree unless, as agreed by the University, for approved dual awards.

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**Chapter 1**

**Introduction**

**1.1 Why study nitrous oxide emissions and its antecedent drivers?**

Nitrous oxide (N2O) is a potent greenhouse gas with an atmospheric lifespan of 131 ± 10 years (IPPC, 2021), and a global warming potential 298 times higher than carbon dioxide (CO2) over a 100 year time span (Smith 2010). Furthermore, its oxidation products, nitrogen dioxide (NO2) and nitric oxide (NO), deplete stratospheric ozone (Crutzen, 1979). The Intergovernmental Panel on Climate Change’s (IPCC) sixth assessment report estimated that N2O atmospheric concentrations have increased by at least 20% since 1750, averaging 332.1 ± 0.4 ppb in 2019, with significant growth since the 1970s (IPCC, 2021).

The rising atmospheric concentrations of N2O are predominantly due to a marked increase in reactive nitrogen (N) within the biosphere (Gruber and Galloway, 2008). During the pre-industrial era it was chiefly created through the natural processes of lightning and biological nitrogen fixation. The primary source is now anthropogenic, which in 2010 was at least twice that of natural creation (Stocker, 2014). There are three key human sources of reactive N: the cultivation of legumes, the Haber-Bosch industrial process, which converts unreactive N­2 to ammonia (NH3), and the burning of fossil fuels (Stocker, 2014). The increases in reactive N within the biosphere has resulted in an increase in N2O emissions in the atmosphere, with approximately 80% of N­2O sources believed to be derived from managed food production systems (Stocker, 2014). Humanity can therefore potentially reduce the majority of N2O emissions, however nearly half of the global population relies on a food supply dependent on commercial fertiliser N inputs, and population growth by 2050 is predicted to increase food demand by at least 70% (Fageria, 2014). A balance needs to be struck between providing food while minimising the impact of N fertiliser use and productive agricultural practices.

Overall, agricultural practices produce more than half of the total anthropogenic N2O emissions (Smith, 2010). Wallenstein *et al.* (2006) described two major controls on N2O emissions from soil: distal controls and proximal controls. Distal controls are the long-term controls that change the biological community structure, and proximal controls are the instantaneous conditions that impact nitrogen cycling. In other words, the long-term (distal) conditions of the site e.g. tillage regime, will determine the impact of short-term (proximal) changes e.g. fertiliser input.

Key proximal controls include fertilisation application which can increase emissions substantially, depending on its quantity and type. For example, Cai *et al.* (2013) noted that for two of the years they monitored emissions, the 2 week post fertilisation period accounted for >60% of the annual total N2O emitted. Other proximal factors include the soils temperature and pH. The soil's pH controls biological function as a more acidic pH inhibits nitrous oxide reductase activity, resulting in more N2O, as less is reduced to N2 (Sahrawat & Keeney, 1986). For example, Blackmer and Bremner (1978) recorded an inhibition of N2O reduction of 60% at pH 6.8 and 88% at pH 5.7. Acidic conditions also reduce organic carbon and mineralised nitrogen, affecting their availability. The soils temperature affects the speed of biochemical reactions and the amount of dissolved oxygen in the soil water films, which decreases with increasing temperatures, impacting the ratio of anaerobic and aerobic activity. For example, Dorland and Beauchamp (1991) measured increasing N2O emissions between 5 and 25 ˚C due to increasing anaerobic denitrification activity.

The fourth important proximal factor is the soil's moisture conditions. The conventional understanding of soil moisture is that as the soil gets wetter, microbial activity switches from aerobic nitrification to anaerobic denitrification (Davidson *et al*., 2000). This typically results in greater N2O, unless the soil stays anaerobic for prolonged periods of time, in which case N2O will likely be reduced to N2 (Ciarlo *et al*., 2007). However, soil moisture can also act as a distal control, the idea for this thesis originated from the Bergstermann *et al.* (2011) experiment, that investigated these effects in the DENIS lab, at Rothamsted's North Wyke site.

Bergstermann *et al*. (2011) packed arable soil cores (143 mm diameter and 120 mm height) and induced two different pre-incubation conditions. One set of soil cores were kept at 25% waterfilled pore space (WFPS) and the other set were kept at 75% WFPS for 1 month. Then both were wetted to 90% WFPS. Past research would suggest that both cores should produce similar quantities of N2O and N2, given they have similar anaerobicity and are at the same moisture (and it's the same soil at the same temperature). However the soil cores pre-incubated at 25% WFPS before rewetting (known as the pre-dry treatment), produced almost double the quantity of N2O emissions. This has been labelled as an N2O hot moment due to the rapid biological activity that is induced from the rewetting over a short period of time (McClain *et al*., 2003). Bergstermann *et al*. (2011) measured changes in soil dissolved organic carbon (DOC), nitrate (NO3-) and ammonium (NH4+), as well as changes in the N2O and N2 ratio. They also added 400 kg C ha-1 as glucose and 75 kg N ha-1 as KNO3- to determine the N cycling processes using isotopologue and isotopomer analysis. Despite this, they could not find significant differences between the treatments, other than a slighter higher DOC for the pre-dry at the end of the pre-incubation phase, which disappeared after the first day of rewetting (yet emissions were significantly different for 10 days post wetting). They concluded anaerobic denitrification was a key process in both treatments, however this was clearly selected for by the addition of nitrogen and glucose. Overall, why these differences were observed remained a mystery.

The first summary of this phenomena was published a year later. Wherein Kim *et al.* (2012) explored both the freeze-thaw and the hot moment phenomena. However the focus in this review paper was mainly on CO2 and freeze-thaw affects, touching only briefly on N2O hot moments. The review left open questions regarding the extent of this phenomena and its contribution to *in-situ* N2O soil emissions. This is true for the wider literature (which is explored in greater detail in chapter 2), as the research has focussed on the proximal impact of soil moisture, with no definitive explanations for its antecedent impacts. This inspired this PhD to focus on the soil's moisture, and provide a clearer picture of its complex and large antecedent impact on N2O emissions. As this PhD is based at North Wyke in Devon, local soils and native soil conditions will be used, aswell as the same DENIS system outlined in Bergstermann *et al.* (2011). Given the extent of the knowledge gap, controlled lab conditions were selected over measuring *in-situ* to reduce variance, and increase the probability of observing statistically significant differences between treatments.

The PhD's proposal also stated that the antecedent impact of management practices on soil N2O emissions would be explored. At the time of the proposal, it was not decided which management strategies would be investigated, however there were several clear directions. Firstly conservation tillage practices have become increasingly popular, and it is known that they have a long-lasting impact on soil properties, especially GHG emissions (Boardman *et al*., 2018). Secondly, North Wyke has three field scale treatments which differ according to fertiliser application rates and grass species. Finally, there are numerous long-term field experiments at Rothamsted's Harpenden site. After analysing the feasibility of each of the options, and examining the literature, the Broadbalk long-term field trial based in Harpenden, was selected for several reasons.

Firstly, Rothamsted had no long-term field sites that investigated tillage, and due to resource constraints, we wanted to focus on experiments where the cost could be reduced by Rothamsted’s internal pricing. Secondly, Rothamsted had a large research project called Soil2Nutrition (S2N), and if this PhD aligned itself to this programmes objectives, the scope of the work could be supported by its budget. This left two options, the Fosters field trial or Broadbalk, both of which had experiments planned for S2N. The Foster’s field trial investigates the impact on soil health when land is changed from grassland to arable, and has been going since the 1950s. Whereas Broadbalk has been going for more than 150 years, and investigates the impact of fertiliser type and quantity on yields and soil health. Broadbalk was chosen due to the lack of GHG measurements on its plots, and while N2O had been measured once previously (Harrison *et al*., 1995), it missed measurements in the winter and autumn, when farm-yard manure was applied. It also did not measure CO2 or CH4 emissions. Furthermore, this PhD was limited to static chambers for GHG measurements, and when analysing the field layouts, Fosters random design would make gas sampling by a single individual difficult, due to the time constraints of chamber sampling and the size of the field. Therefore Broadbalk was selected, for practical reasons, and because this PhD could help add important insights in terms of GHG soil emissions from a field site that has over a century of consistent management with contrasting treatments.

**1.1.1 The aims of this PhD**

The development of effective mitigation strategies requires an understanding of the major controls and mechanisms behind the release of N2O from soil. Most studies focus on the current state of the soil as explanation for emissions, typically looking at the mineral N content, the water content, the pH, and soil structure (Cardenas *et al.*, 2019). While this approach has helped limit N pollution, with solutions focussed on changing the timing and quantity of N fertiliser application, to improve the nitrogen use efficiency (NUE) (Lyu *et al.*, 2021; Rees *et al.,* 2020; Rees *et al.,* 2013); this is not always effective, due to the nature of N2O as a trace gas with many different possible redox pathways (Butterbach-Bahl *et al.*, 2013). A complicating factor, is how the microbial community which are responsible for a significant proportion of emissions, are affected by soil management over longer periods of time, as well as fluctuating weather patterns, which have been happening with increasing frequency in the UK since 1961 (Dodd *et al.,* 2021). Distal controls are less understood in terms of their impact on the quantity of N2O released, due to the cost and labour implications with setting up and maintaining long-term experimental sites, and the difficulties of sustained soil and gas measurements over months and years. Yet it has been established by the literature that fluctuating soil water content and the legacy of certain management practices can dramatically change the amount of N2O produced.

This PhD aimed to address these gaps in the research by exploring the distal controls of N2O emissions. Particular focus was given to the impact of the moisture legacy, due to lack of research in this area. In this regard, there are 3 main questions that this PhD aimed to answer:

1. What is the current state of research regarding historic soil moisture conditions, and the release of N2O?
2. How does adjusting these antecedent moisture variables impact the resulting N2O emissions from soils?
3. What is the mechanism behind the release of these N2O emissions?

Rothamsted Research has several long-term trials that aim at analysing the impact of different fertiliser regimes (<https://www.rothamsted.ac.uk/long-term-experiments>, accessed April 2022). This PhD selected Broadbalk, a long-term field trial, to compare the legacy of management practices between organic and inorganic use over the timescale of 140 years. The research was framed to answer to the following questions:

1. How does long-term application of organic fertiliser compare to inorganic fertiliser in relation to soil properties and soil GHG emissions?

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**Chapter 2**

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**The impact of drought and rewetting on N2O emissions from soil in temperate and Mediterranean Climates**

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**Key words:** Soil Moisture, Nitrous Oxide, Denitrification, Dry Wet Cycles, Drought, Legacy

**Highlights:**

1. We investigated the main causal mechanisms of N2O hot moments after soil is rewetted.
2. This is the first meta-analysis and synthesis of the literature regarding N2O hot moments.
3. The more anaerobic the soil and the more C and N substrates, the larger the hot moment.
4. We provide recommendations for future studies and outline key gaps in the research.

**Authors contributions:**

**H.A. Barrat:** Conducted the literature searches, formatted the papers for the meta-analysis, interpreted the results and wrote all drafts.

**J. Evans:** Conducted the meta-analysis in R and helped design the statistical approach.

**D.R. Chadwick:** Helped with minor edits.

**I.M. Clark:** Helped with minor edits.

**K. Le Cocq:** Helped with minor edits, and the experimental design.

**L. Cardenas:** Helped with minor edits, and the experimental design.

**Abstract**

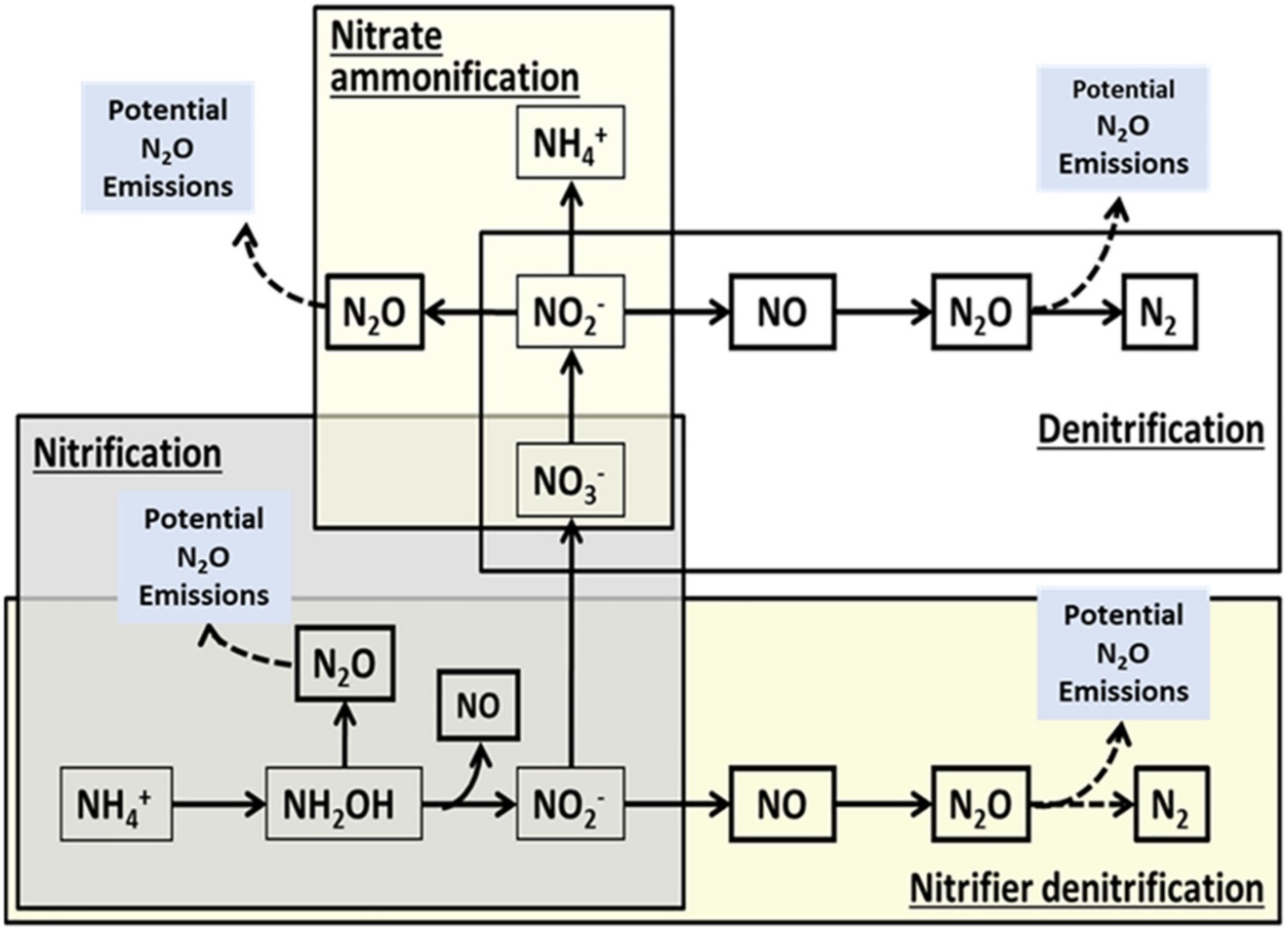
The potential for soils to produce nitrous oxide (N2O) is impacted by past moisture conditions; however, the extent of this impact is not fully understood. We conducted the first meta-analysis of this, using two literature searches. We found 36 studies out of a possible 735 that described experiments where soil moisture conditions had been controlled, such that the impact of antecedent moisture levels could be separated from contemporary moisture levels and attributed to N2O emissions. Of those studies, 14 (130 data points) used the appropriate experimental design and presented suitable data that could be standardized for the meta-analysis. We found that the degree to which the soil was rewetted and the water filled pore space (WFPS) the soil was brought to were significant explanatory variables (p = < 0.0001). The larger the difference between the dry and wet states of the soil and the higher the WFPS of the soil after rewetting, the larger the hot moment, with an exponential increase once the soil is anaerobic. Substrate availability and fertiliser quantity and type were also important controls on the amount of N2O emitted during the hot moment (p = < 0.0001). However, controls with a constant WFPS can have the same anaerobicity and substrate concentrations, yet much lower emissions, so we suggest that it is the bioavailability and how the substrates are utilized by the microbial community, and thus how they are primed by the drought, that is the main causal mechanism. Unfortunately, there is still a large uncertainty regarding how microbial population structure, relative gene abundances and gene expression profiles change according to antecedent dry/wet cycles. We suggest several areas of improvement for future studies and the development of drought-impact curves. These would show the relationship between N2O emissions and the length of drought (we found no studies that have investigated this) and N2O emissions and the severity of drought (e.g. the difference between 20% and 40% WFPS).

**2.1 Introduction**

Nitrous oxide (N2O) is a potent greenhouse gas with a global warming potential 298 times larger than carbon dioxide (CO2) (Stocker, 2014). Baggs (2008) cites four major biological processes that generate N2O: nitrification, nitrifier denitrification, denitrification, and nitrate ammonification; Figure 1 summarises these processes. N2O predominantly originates from oxidation and reduction processes via biological catalytic enzymes produced by microorganisms (Smith, 2010). Soil moisture impacts oxygen availability, and oxygen concentrations determine the processes responsible for N2O production and therefore the quantity of N2O produced (Smith, 2010). Soil moisture also affects gas diffusion and substrate mobility (Guo, 2014). Therefore, soil moisture is considered one of the most important factors controlling soil N2O emissions. Anthropogenic climate change is predicted to increase the intensity and frequency of extreme weather events such as flooding, storms, and droughts (Stocker, 2014). This has implications for biogenic greenhouse gas emissions, as the hydrological regime of soil is increasingly likely to fluctuate (Sánchez-Rodríguez *et al.*, 2017).

Long-term studies of N2O emissions from field sites have noted that emissions can suddenly increase orders of magnitude above the average seasonal flux (Parkin & Kaspar, 2006; Scanlon & Kiely, 2003) with rapid changes in temperature and soil moisture often due to spring rains or winter thawing. Some authors have labelled these as hot moments, which were first defined by McClain *et al.* (2003) as a disproportionately high reaction rate over a relatively brief period of time. In the case of N2O emissions, these hot moments can last several days, forming significant proportions of the annual flux. For example, Molodovskaya *et al.* (2012) recorded hot moments that equalled 51% of the total N2O emissions for the year, but they occurred over < 7% of the monitored period. Similar events have been noted in incubation and plot experiments (Harrison-Kirk *et al.*, 2013; Leitner *et al.*, 2017). It is important to note that these are different from hot spots, which can be defined as a higher reaction rate in one location compared to the surrounding area. Hot spots are therefore used to describe changes spatially, whereas hot moments describe changes temporally (McClain *et al.*, 2003).

It is worth noting that Bernhardt *et al.* (2017) reviewed the use of the hot moment and hot spot (HMHS) concepts within the scientific literature, concluding that they should be replaced by a different concept that they defined as ecosystem control points. Using this new framework, N2O hot moments that are driven by historic moisture conditions could be defined as an activated ecosystem control point, because high emissions only occur when changes in water content activate biological activity. In this review we kept the use of ‘hot moments after rewetting’ as Bernhardt *et al.* (2017) state that ‘nitrogen and denitrification are among the ten most frequently used words in titles of papers citing McClain and others (2003)’. Therefore, the term is still popular amongst those researching this phenomena, and therefore relevant.



***Figure 2.1.*** *Soil processes showing the most relevant microbiological nitrogen substrate transformations for nitrous oxide emissions, modified from Baggs (2008).*

The size of a hot moment depends on the geo-physicochemical nature and the historical conditions endured by the soil because this determines the composition of the native microbial community and therefore their response to stress events, such as flooding (Wallenstein *et al.,* 2006). For example, flooding soil following extended periods of drought stress produces a larger pulse in CO2 and N2O emissions, compared to soils which were already moderately wet before the flood event (Flechard *et al.*, 2007). There are several hypotheses for why this is the case. Firstly, hydrologically isolated substrates during a drought period concentrate at separated microsites within the soil matrix, which are then reconnected upon wetting (Borken & Matzner, 2009). Microorganisms then rapidly utilise these substrates and undergo biomass growth and activate extracellular enzymes (Rudaz *et al.,* 1991). This can further induce anoxic conditions due to the rapid consumption of oxygen, further favouring conditions for denitrification. Moreover, it is suggested that in historically dry conditions the production of N2O reductase may have a longer lag phase of production compared to pre-wet conditions (Bergstermann *et al.*, 2011). Thus, when microorganisms resort to denitrification for respiration, it is likely that there will be a higher ratio of N­2O:N­2 produced. It is also possible that the drought period affects the population dynamics of the microbial community, leading to a different response to higher soil water contents than a soil that never experienced the drought (Morales *et al.,* 2015).

As far as we are aware there has been no meta-analysis of the literature regarding N2O hot moments. Yet what is currently known so far indicates that it is an important contributor to greenhouse gas emissions from soil, and it is going to become more important as weather patterns become more extreme in temperate and Mediterranean climates due to climate change. However, the majority of studies only consider soil moisture as a controlling factor of emissions at the time of N application and thereafter. We aim to further the understanding of the role of soil moisture in this review via the synthesis of the current knowledge of how past soil moisture conditions impact the release of N2O from temperate and Mediterranean climates, and assess the validity of the proposed hypotheses:

H1: The more water added to the soil the larger the N2O hot moment, especially once the soil becomes anaerobic.

H2: The length and severity of the drought affects the build up of substrates and therefore the size of the N2O hot moment.

H3: The length and severity of the drought affects the production of N2O reductase once the soil is rewetted.

H4: The duration of the drought period affects the microbial population and therefore the size of the N2O hot moment. We then aim to identify key gaps in the literature, and suggest experiments that would help further the understanding of the mechanisms behind this large pulse in N2O.

**2.2. Methods and Materials**

**2.2.1 Literature search**

Two systematic searches of the literature were conducted. It is worth noting that we have excluded papers that relate to the freeze-thaw release of N2O due to the large difference in mechanisms that drive this effect (Wagner-Riddle *et al.*, 2017). This was achieved by analysing a selection of papers which meet a specific methodological criterion (see Supplementary Material for the list of papers and relevant criteria, we did not have a cut-off date).

The two searches were carried out as follows:

The 1st search was carried out on the 12/05/20 in Web of Science [v.5.35] using the search terms were Soil moisture AND nitrous oxide AND denitrification. This resulted in 674 papers, 28 of which were accepted.

The 2nd search was carried out on the 12/05/20 in Web of Science [v.5.35] using the search terms ‘Dry wet cycles’ AND ‘nitrous oxide’ AND ‘denitrification’. This resulted in 61 papers 15 of which were accepted, however only 8 were unique from the previous results. Overall this gave 36 unique papers from both searches. Denitrification was included in the search criteria as we aimed to explore data collected when after a drought there is a significant rewetting event likely to result in denitrification as the dominant process. It is worth noting that these papers do not have to conclude that denitrification is a key process, and many of the papers within both searches discussed the role of other processes as outlined in Figure 2.1

**2.2.2 Meta-analysis inclusion criteria and screening**

Using the data from the literature searches, we carried out a meta-analysis in order to explore the key explanatory variables that affect the size of the hot moment. Although the literature review resulted in 36 suitable studies, only 14 had the right experimental design (see rules below), and presented suitable data that could be standardised for the meta-analysis, this resulted in 130 data points (see supplementary material).

Studies were accepted: (i) if they measured N2O emissions after rewetting and recorded the soil moisture content, (ii) if the emissions could be represented as an accumulation of emissions during the peak period in terms of mg m-2, and (iii) if the soil moisture content could be calculated in terms of % water filled pore space (WFPS). WFPS was selected because it is the most accurate representation of soil water content (as opposed to volumetric or gravimetric), as it accounts for the bulk density, particle density and volume allowing standardising when comparing different soil types. The emission measurement of mg m-2 was selected because of its suitability at comparing field and lab studies (typically field trials do not know how much soil is beneath the surface of the area they are measuring and so mg kg-1 is unsuitable), and because most of the latest studies used this metric.

**2.2.3 Meta-analysis standardisation and assumptions**

Emissions were recalculated if needed, from g kg-1 to mg m-2 (Bergsma *et al.*, 2002; Guo *et al.*, Drury *et al.*, 2014; Guo *et al.*, 2010; Li *et al.*, 2008; Liu *et al.*, 2018). For Li *et al.* (2008) we assumed a diameter of 33 mm from 125 ml wide mouthed bottles, and for Liu *et al.* (2018) we assumed a diameter of 13 mm from 22 ml GC vials. For Burger *et al.* (2005), Ruser *et al.* (2006), Bergstermann *et al.* (2011), Chen, Whalen, and Guo (2014), Rabot, Hénault, and Cousin (2016), Leitner *et al.* (2017) and Liu *et al.* (2018) we had to calculate the cumulative emissions post wetting by reading each studies figures to get the individual data points using [www.graphreader.com](http://www.graphreader.com), similarly for Priemé and Christensen (2001) we recalculated the Finland ploughed and woodland treatment in order to get appropriate standard error (SE) values.

***Table 2.1.*** *Description of explanatory variables collected for the final meta-analysis.*

|  |  |
| --- | --- |
| Variable name | Description |
| **Factor explanatory variables** | |
| Wholepeak | Did they observe the N2O going back to baseline levels (captured whole peak: yes/no) |
| Texture | Soil texture (category: the 12 soil textures) |
| Land use | The land use where the soil was collected from (category: grassland, arable, woodland) |
| Fertiliser | Fertiliser added before the experiment, no means no fertiliser was added, N means nitrogen was added, C means carbon was added, N&C means both nitrogen and carbon was added |
| **Continuous explanatory variables** | |
| Peakhrs | The length of the peak observed post treatment (hours) |
| Hrsmeasured | The length of time the study monitored post treatment (hours) |
| WFPSbeforepeak | WFPS before the N2­O peak (%) |
| Length of drought | Period of drying before wetting (days) |
| WFPSduringpeak | WFPS during the N2­O peak (%) |
| WFPSchange | WFPS change from before the peak to during (%) |
| Claypercent | The amount of clay in the soil (%) |
| Bulkdensity | The soil’s bulk density (g cm-3) |
| TempC | Air temperature (degrees Celsius) |
| pH | The soil’s pH (pH scale) |
| beforepeakDOC | The dissolved organic carbon (DOC-C) before the peak (mg kg-1) |
| duringpeakDOC | The dissolved organic carbon (DOC-C) during the peak (mg kg-1) |
| beforepeakNO3N | The nitrate (NO3-N) before the peak (mg kg-1) |
| duringpeakNO3N | The nitrate (NO3-N) after the peak (mg kg-1) |
| beforepeakNH4 | The ammonium (NH4-N) before the peak (mg kg-1) |
| duringpeakNH4 | The ammonium (NH4-N) after the peak (mg kg-1) |
| QuantityNfert | Quantity of fertiliser added (kg N ha-1) |
| QuantityCfert | Quantity of fertiliser added (kg C ha-1) |

Leitner *et al.* (2017) presented soil moisture content as volumetric, these were recalculated using the bulk density provided and assuming a particle density of 2.65 g cm-3. Similarly, Burger *et al.* (2005) and Bergsma *et al.* (2002) presented soil moisture as gravimetric, we used the bulk density provided and the volume of the core to convert to WFPS. For Liu *et al.* (2018) we assumed 40% WHC was the same as 40% WFPS. For Ruser *et al.* (2006) we assumed the prolonged artificially hot drying period reduced the WFPS to 1%, we assumed the same for Bergsma *et al.* (2002). A range of explanatory variables were also recorded and standardised, these are outlined in Table 2.1.

**2.2.4 Statistical analysis**

R version 3.6.1 was used for all statistical analysis.

**2.2.4.1 Standard Error (SE) and cumulative emissions**

If studies did not include an SE, then it was estimated using other information available from the study. Calculating missing standard errors was necessary in order to weight the studies according to variance. If we calculated the accumulated emissions from a treatment using a figure, then we recorded the SE from each data point that made up the emission curve. Then the SE was used to calculate confidence intervals at each of the given timepoints and these confidence intervals allowed upper and lower confidence limits to be calculated for each accumulated emission value. The standard error of each accumulated emission value was then estimated by subtracting the lower limit of this confidence interval from the upper limit and dividing this difference by 4.

For Bergstermann *et al.* (2011) the SE was only available at a single point on the curve for each of their treatments, so we assumed this standard error was the same for all points in order to estimate the standard error of the cumulative emissions as above. For Liu *et al.* (2018) the only error term available was the LSD so the SE had to be estimated based on this value. If no error term was given, then we gave the study a large standard error based on the coefficients of variation (CV) observed in all of the other studies. The largest observed CV was 35 so these studies were given a SE equal to 35 times their mean.

**2.2.4.2 Meta-analysis**

Analysis was carried out in using the metafor package version 2.1-0 in R version 3.6.1. Data was analysed on the log10 scale to satisfy the homogeneity of variance and normality assumptions. Standard errors on the log10 scale were estimated using the delta method. A random effects meta-regression was fitted to assess the relationship between the peak cumulative N2O emissions and other reported variables. A single explanatory variable model was initially used to assess each potential explanatory variable, to give an indication of which variables might have a statistically significant relationship (p ≤ 0.1) with the response (log10 N2O). We choose a higher cut off of p ≤ 0.1 in this initial step to allow more variables into the next step, and to make sure we did not miss any possible relationships. Each model included a random structure accounting for between and within study variation and observations were weighted by the inverse of their standard errors.

Then the glmulti package was used to compare the full set of possible models that include subsets of the chosen variables found to be statistically significant in step 1. The glmulti function ranks all possible models based on their AIC (Akaike information criterion), which allows the best model to be selected. This model was then selected and presented in the results section.

**2.3. Results**

**2.3.1 Single explanatory model**

The single explanatory variable models revealed 10 key variables that showed a statistically significant relationship with log10 N2O p ≤ 0.1 (see Table 2.2 and 2.3). However, 5 variables (peakhrs, hours measured, claypercent, QuantityNfert, QuantityCfert) were excluded from the next step due to large amounts of missing values, as too few distinct values observed or large gaps in the range of observed values could bias fitted models. Therefore, the following 5 variables were selected for further analysis: WFPSduringpeak, WFPSchange, pH, Landuse and Fertiliser. Table 2.2 shows the results of the individual analyses.

***Table 2.2*** *Results table from single explanatory model. For the factor explanatory variables, the estimates of effects is the mean log10N2O value for that variable group, the p value indicates whether there is evidence of a difference in log10N2O values for different levels of the factor. For the continuous explanatory variables, the estimate of effect is the slope of the straight line fitted to the data i.e. the amount the log10N2O changes when you increase the variable by one unit. A p value of less than 0.1 was taken to indicate that the slope was statistically different from 0. Values of p ≤ 0.1 have been highlighted in bold. Both the factor and explanatory variables are explained and described in Table 2.1.*

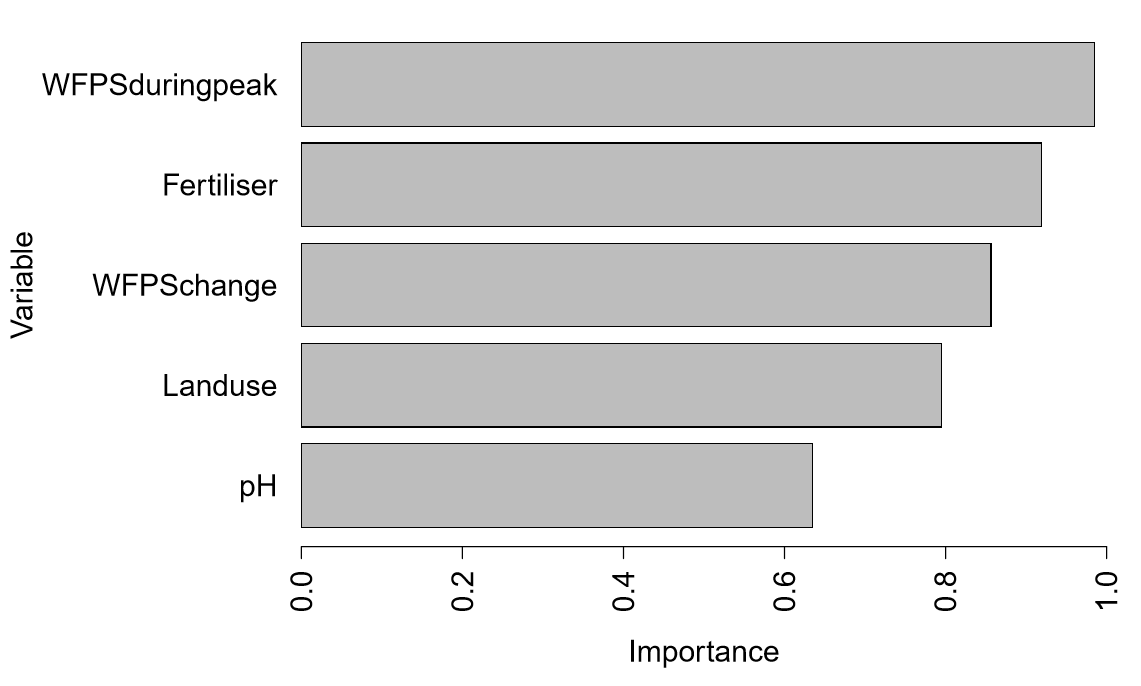
|  |  |  |
| --- | --- | --- |
| **Factor Variables** | **Estimate(s) of effects mean standard error** | **P value for test of variable** |
| Observed whole peak | No 1.1471 (0.4340)  Yes 1.3253 (0.2650) | 0.6988 |
| Soil Texture | Unknown 0.4177 (0.5388)  Clay 1.1321 (0.9351)  Clay loam 1.3771 (0.4710)  Fine loamy 1.6310 (0.6263)  Sandy 1.1117 (1.0127)  Silt loam 1.6201 (0.5261)  Silt clay 1.2529 (0.5682)  Clay loam 2.2502 (0.9617) | 0.6148 |
| **Land use** | Arable 1.2111 (0.2549)  Grassland 1.7268 (0.3533)  Woodland 1.0439 (0.3782) | **0.0651** |
| **Fertiliser** | no 0.8787 (0.2315)  N 1.1152 (0.2388)  N&C 2.4055 (0.4020) | **0.004** |
| **Continuous Variables** | **Estimate of effect (slope (SE))** | **P value for test of moderator** |
| **Peakhrs** | 0.0066 (0.0018) | **0.0003** |
| **Hrsmeasured** | 0.0007 (0.0004) | **0.0916** |
| WFPSbeforepeak | 0.0037 (0.006) | 0.5363 |
| Length of drought | 0.0014 (0.0179) | 0.9392 |
| **WFPSduringpeak** | 0.0287 (0.0054) | **<0.0001** |
| **WFPSchange** | 0.0242 (0.0055) | **<0.0001** |
| **Claypercent** | -0.0922 (0.0154) | **<0.0001** |
| Bulkdensity | 0.4790 (0.5376) | 0.3729 |
| TempC | 0.0132 (0.0517) | 0.7981 |
| **pH** | 0.2273 (0.1201) | **0.0583** |
| beforepeakDOC | -0.0001 (0.0001) | 0.4175 |
| duringpeakDOC |  | Excluded – too few observations |
| beforepeakNO3N | 0.0000 (0.0019) | 0.9898 |
| duringpeakNO3N | 0.0554 (0.0371) | 0.1359 |
| beforepeakNH4N | 0.0000 (0.0014) | 0.9738 |
| duringpeakNH4N | 0.0108 (0.0433) | 0.8037 |
| **QuantityNfert** | 0.0013 (0.0008) | **0.0794** |
| **QuantityCfert** | 0.0012 (0.0005) | **0.0119** |

***Table 2.3.*** *Model summary of predicted values (back-transformed) for the accumulative N2O emissions from a hot moment, it shows each combination of fertiliser and landuse at average observed values of WFPSduringpeak (66.62) and WFPSchange (46.99). The variables fertiliser, landuse, WFPSduringpeak and WFPSchange are described in Table 2.1.*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Fertiliser | Landuse | Mean predicted value | Higher confidence interval boundary | Lower confidence interval boundary |
| no | Grassland | 14.9 | 3.1 | 67.1 |
| N | Grassland | 26.0 | 5.3 | 128.1 |
| N&C | Grassland | 129.1 | 15.0 | 1107.7 |
| no | Arable | 4.2 | 1.3 | 13.6 |
| N | Arable | 7.4 | 2.1 | 24.2 |
| N&C | Arable | 36.7 | 4.9 | 276.4 |
| no | Woodland | 5.2 | 1.1 | 25.5 |
| N | Woodland | 9.2 | 1.1 | 46.4 |
| N&C | Woodland | 45.6 | 4.1 | 445.5 |

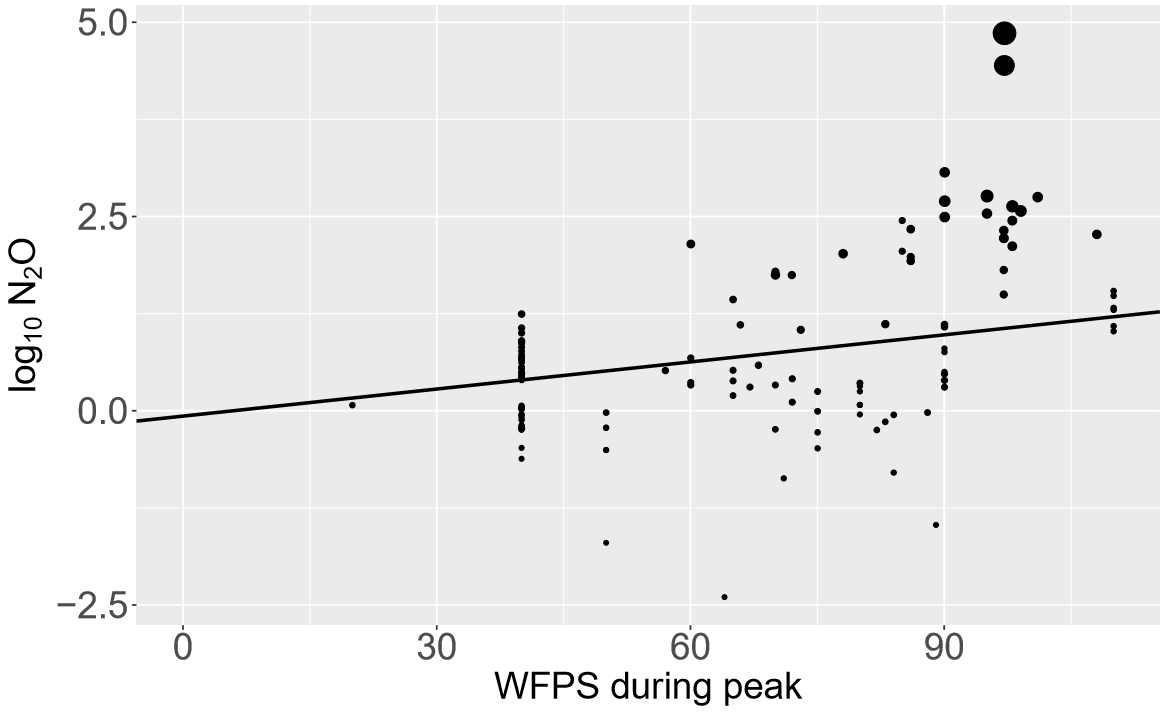
**2.3.2 Multi explanatory variable model**

Following the selection of key explanatory variables, the glmulti package was then used to consider models containing combinations of the 5 variables selected in step 1. All possible models were fitted and ranked based on their AIC, which represents the best models in terms of Information Criterion, the lower the value the better the fit (see supplementary material for the glmutli package output). The importance of each individual variable was also ranked in terms of its prediction power (see Figure 2.2). The final model selected (model 1) is a mixed effects model with random structure (study/observation) to account for between and within study variation and observations weighted by the inverse of their standard errors. The fixed structure is: Fertiliser+Landuse+WFPSduringpeak+WFPSchange.



***Figure 2.2.*** *The relative importance of variables in predicting N2O emissions. The glmulti package output indicates the “importance” of each variable in terms of its ability to predicate N2O emissions, this is shown above, an explanation of each variable is found in Table 2.1. A variable that appears in lots of models with large weights will receive a high importance value.*

Out of 32 possible models, model 1 was ranked as second best by the glmulti function, which included the top four most important terms shown in Figure 2.2. The highest ranked model included pH as well as those included in the chosen model. We excluded pH in model 1 as no studies within the meta-analysis investigated the effect of pH, and the fit with N2O seems circumstantial with changes in fertiliser and WFPS being the main experimental drivers. The difference in AIC between these two models was very small (0.86) so we do not lose much information by excluding pH. In addition, the slope of pH, if the highest ranked model were selected, was not significantly different to 0 (0.1164, SE=0.1750, p=0.5059).



***Figure 2.3.*** *The effect of peak soil water filled pore space on N2O emissions when the soil is rewetted. The WFPSduringpeak is the effect of soil saturation when the soil is rewetted on the log10 N2O values, points are observed values from data, size indicates the contribution of each point (larger points had smaller standard errors and therefore make a larger contribution). The estimate of effect of the slope for WFPSduringpeak is 0.0196 ± 0.0063, this is the amount the log10 N2O changes when you increase the WFPSduringpeak by one unit. Black line indicates the predicted value for each possible value of WFPSduringpeak at an average observed value for the WFPSchange (46.99) and averaged over all possible combinations of fertiliser and landuse. The WFPSchange stands for the degree of rewetting, the higher the value the larger difference between the WFPS before wetting and after rewetting. A description of the variables WFPSchange, WFPSduringpeak, fertiliser and landuse is described in Table 2.1.*

There was significant residual heterogeneity (p ≤ 0.0001) suggesting that there may be other variables that were not available that could have explained more of the variability in log10 N2O. However, the model did explain a significant proportion of the variability (p ≤ 0.0001). The effect of WFPS change and the WFPS end value is presented in Figures 2.2 and 2.3. The effect of fertiliser is presented in Table 2.3.

**2.4 Discussion**

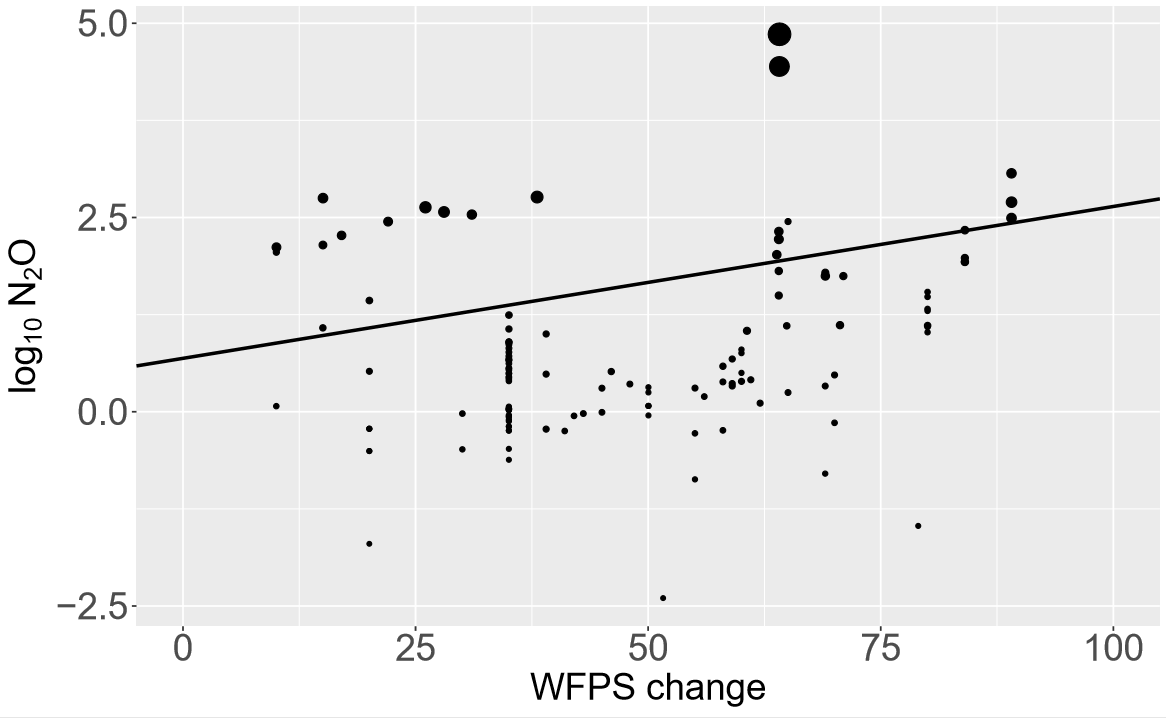
**2.4.1 Limitations of the dataset**

Although studies have observed hot moments under field conditions (Cao *et al.*, 2019; Molodovskaya *et al.*, 2012; Mumford *et al.* 2019). The meta-analysis dataset is missing measurements from plot and field trials, we have found that only Leitner *et al.* (2017) and Kostyanovsky *et al.* (2019) investigated hot moments under natural conditions with cover vegetation using frequent gas and soil measurements (at least every 10 hours) over a representative time scale (beyond 24 hours after rewetting). Also, these plots were under semi-arid woodland (Leitner *et al.*, 2017) and winter wheat (Kostyanovsky *et al.*, 2019). It is therefore still unknown how representative the current set of experiments are of hot moments under grassland. A lack of high temporal resolution measurements and manipulation of soils *in-situ* may be due to the difficulty of accurately manipulating the water content of soils, and keeping it constant at larger scales. While some progress has been made on the impact of the wetting intensity, we have found no study that has investigated how the period of drying affects the size of a hot moment, or whether this priming effect plateaus after a certain time period.

Bergsma *et al.* (2002) and Liu *et al.* (2018) did not record data for long enough periods of time after rewetting (they were less than 24 hours), therefore it is possible that they missed an even larger response to rewetting, as other studies have seen responses that last at least 100 hours (Bergstermann *et al.*, 2011; Li *et al.*, 2008; Priemé & Christensen, 2001; Ruser *et al.*, 2006). Only Bergstermann *et al.* (2011) recorded soil nitrogen and carbon before the rewetting, during rewetting, and after. No studies in this meta-analysis had a control ‘treatment’ for both the rewetting WFPS and the previous WFPS, so it is unknown whether the pulse in emissions recorded would be any larger than if the soil was kept at the constant high WFPS. Harrison-Kirk *et al.* (2013) did have such controls, but they presented no N2O data over time, just cumulative N2O emissions for drying and wetting cycles compared to the control, so it is impossible to tell how much a single drying and wetting cycle would compare to the control, and how this relationship changed over time. Therefore, we could not use their data in this meta-analysis.

**2.4.2 Key mechanisms that drive hot moments**

Within natural and controlled settings, large N2O fluxes have been recorded after rewetting dry soil (Burger *et al.*, 2005; Leitner *et al.*, 2017; Li *et al.*, 2008; Liu *et al.*, 2012; Mummey *et al.*, 1994; Rudaz *et al.*, 1991; Ruser *et al.*, 2006; Wulf *et al.*, 1999). This meta-analysis confirms the first hypothesis - that the size of the N2O hot moment depends on how much water is added to the soil, and how saturated the soil is after rewetting. We found that the larger the WFPS change and the higher the WFPS end value, the larger the N2O pulse (see Figure 2.3 and 2.4). Although the relationship is not linear, and unsurprisingly the largest emissions occur at 70% WFPS and above, when the soil is mostly anaerobic. Similarly Ruser *et al.* (2006) showed that highly compacted soils which are also more likely to be more anaerobic, produced higher emissions when rewetted. Although this meta-analysis indicated that the WFPS end value is the most important predicator, only Bergstermann *et al.* (2011) and Guo *et al.* (2014) controlled the WFPS end value and changed the degree the soil was wetted. Therefore, within this meta-analysis a large WFPS change is mostly just an indication that the WFPS end value was particularly high. Consequently, more research is needed to uncouple the impact of drought severity from the degree the soil is saturated, as it is already well established that saturated soils are more likely to produce more N2O. The development of two different drought impact curves would provide greater insight into the nature of the priming effect. The first should be created from various drought severities (WFPS at 10%, 20%, 30%, etc) with the same target saturation level (e.g. 90% WFPS). The second should explore the period of drought, we found no study that held constant the degree the soil was wetted and the soil saturation level, and only modified the drought period, and so the second hypothesis could not be investigated.



***Figure 2.4.*** *The effect of the change in soil water filled pore space from rewetting, on N2O emissions. The higher the value the larger difference between the soil moisture before wetting and after rewetting. Points are observed values from data, size indicates the contribution of each point (larger points had smaller standard errors and therefore make a larger contribution). The estimate of effect of the slope for WFPSchange is 0.0116 ± 0.0058, this is the amount the log10 N2O changes when you increase the WFPSchange by one unit. Black line indicates the predicted value for each possible value of WFPSchange at an average observed value of the WFPSduringpeak (66.62) and averaged over all possible combinations of fertiliser and landuse. A description of the variables WFPSchange, WFPSduringpeak, fertiliser and landuse is found in Table 2.1.*

However, it is theorised that upon rewetting dead cells lyse, and the microbes that survive release solutes to equilibrate to the new water potential (Borken & Matzner, 2009). This, combined with the physical disruption of soil aggregates, results in a sudden flush of mineralised N and C, which is quickly capitalised by the surviving microorganisms (Borken & Matzner, 2009; Guo *et al.*, 2014; Morales *et al.*, 2015). Clearly, substrate availability is an important control on the amount of N2O produced during the hot moment, the studies that recorded the largest hot moments were either from soils fertilised before measurements, or recorded high NO3- and NH­4+ concentrations during the peak, and whether the soil was fertilised was a significant predicator of a larger hot moment (see Figure 2.2) (Chen *et al.*, 2014; Li *et al.*, 2008; Rabot *et al.*, 2016; Ruser *et al.*, 2006). However, only Bergstermann *et al.* (2011) recorded N and C concentrations within the soil before rewetting, during the peak, and after the peak, and there was no significant difference between the two treatments in terms of C and N concentrations, but there was a large and significant difference in N2O emissions. Moreover, total mineralisation rates can be higher in a continuously wet control compared to a wet and dry cycle, yet the controls have significantly lower emissions (Borken & Matzner, 2009; Guo *et al.*, 2010; Harrison-Kirk *et al.*, 2013). This suggests that it is not just quantity of nutrients that’s important, but how the C and N substrates are used.

The utilisation of substrates within a soil depends on the state of the microbial community, which will be affected by the degree of drought, and other important distal controls, such as plant cover and land management practices, as well as soil type and physical properties (Morales *et al.*, 2015; Wallenstein *et al.*, 2006). This meta-analysis revealed that the type of land use was a significant predicator of large hot moments, with grassland soils seemingly more prone to large hot moments, followed by woodland and arable which were similar; this is in agreement with the current experimental evidence (Liu *et al.*, 2018; Priemé & Christensen, 2001). Considering the high WFPS post wetting, it is likely that anaerobic denitrification is the main source process of N2O emissions, and it is possible that on average grassland soils have a larger denitrifying community (Bijay *et al.*, 1989). Another indicator that microbial community structure is an important factor, is the sudden increase in variance between replicates at the height of the emission peak. It could be that hot spots were created in different replicates, as sieving and packing soil cores does not necessarily homogenise the microbial community (Chen *et al.*, 2014; Priemé & Christensen, 2001).

There is some evidence that the third hypothesis could be an important factor, wherein dry periods limit the production of N2O reductase, resulting in a higher ratio of N2O:N2O+N2 when the soil is rewetted (Bergsma *et al.*, 2002; Bergstermann *et al.*, 2011). Indeed, higher N2O:N2O+N2 ratios have been recorded from the more intense treatments of drying and rewetting (Bergsma *et al.*, 2002; Bergstermann *et al.*, 2011; Burger *et al.*, 2005; Guo *et al.*, 2014), although there are mixed results, e.g. Li *et al.* (2008) observed that higher WFPS end values had lower ratios. It is worth noting that Bergstermann *et al.* (2011) observed no significant change in N2 emissions, but rather it was the quantity of N2O that caused the significant change in the N2O:N2O+N2 ­ratio. Differences in the N2O:N2O+N2 ratio could be for a range of reasons other than a lack of N2O reductase. For example, it could be that nitrate ammonification (see Figure 2.1) is the main community process when there is drought stress followed by rewetting, or it could be that the part of the microbial community that reduces N2O is more susceptible to drought (Baggs, 2011). Within the papers we found there is currently not enough amplicon evidence to support a particular hypothesis, including the idea that N2O reductase is inhibited by the drought legacy.

Unfortunately, there are very few studies (three were found out of 735 within our search) that recorded changes in the abundance of N cycling genes and the microbial population, following drying and rewetting (Banerjee *et al.*, 2016; Chen *et al.*, 2014; Radl *et al.*, 2015), and only Chen *et al.* (2014) simultaneously recorded N2­O emissions. Both Radl *et al.* (2015) and Chen *et al.* (2014) found no significant change in the abundance of denitrifier genes between a wet control and the dry and wet cycles, or a significant difference in denitrifer enzyme activity (DEA); Chen *et al.* (2014) measured *nosZ* and *nirS,* Radl *et al.* (2015) measured *nirK,* *nirS* & *nosZ*. It is not known if Radl *et al.* (2015) induced a hot moment, however Chen *et al.* (2014) observed a typical hot moment, with over 1000 times higher accumulative N2O emissions from the dry and wet treatment than the continuously wet treatment. Banerjee *et al.* (2016) provides a comprehensive explanation of why this might be the case, as transcription abundance, not gene abundance seems to provide the best correlation with N2O emissions. It is not clear if Banerjee *et al.* (2016) created a hot moment. However, they observed that an increase in WFPS from 60% to 80% and then to 100% had an effect on transcription abundance, with *nosZ* copy numbers increasing from 80% to 100%, *norB* copy numbers reduced from 60% to 80%, and then increased to the original abundance at 100%, both *hao* and *amoA* increased in copy number from 60% to 100% WFPS. It seems that 80% WFPS is a key switch point, likely related to when their particular soil became anaerobic. Banerjee *et al.* (2016) found that both community diversity and evenness increased from 60% to 80%, but decreased from 80% to 100%. Although Chen *et al.* (2014) treatments showed no significant differences in 16S rRNA between a high WFPS control and the wetting and drying cycle, Radl *et al.* (2015) found that dry and wet cycles altered the nitrification enzyme activity (NEA), and drought stress significantly reduced the ratio of ammonia oxidising bacteria (AOB) to ammonia oxidising archaea (AOA). This directly affected the nitrification potential, because there was a significant positive relationship between AOB and NEA. Interestingly, there was not a significant correlation between AOA abundance and NEA, suggesting that AOB might have been a more active nitrifier, converting more NO2 into N2O. An effect on nitrification can indirectly affect denitrification as it will modify the supply of NO3-, the substrate for this process. Therefore, there is not enough evidence to confirm the fourth hypothesis that the drought period significantly impacts the microbial population and therefore the size of the hot moment. Future work should focus on inducing a hot moment, while also measuring changes in transcription abundance.

We found no studies showing the impact of viruses on hot moments, however it is possible that mesofauna abundance and diversity could alter the magnitude of the hot moment. Chen *et al.* (2014) showed that earthworm presence can significantly decrease the N2O in wet and dry cycles by 82%, by increasing DEA and N2O reduction to N2. However, earthworms seem to increase the mineralisation rates, increasing the availability of carbon, which favours denitrification and N2O production. Although complete denitrification typically occurs under anaerobic conditions, and so under wetter conditions worms can encourage the reduction of N2O (Rizhiya *et al.*, 2007).

**2.4.3 Future data reporting and experimental design**

There is significant unexplained variance within this meta-analysis (p ≤ 0.0001). This is likely due to the large range of experimental designs, from different soil preparation methods, to the length of measurement, and the size of the cores used, as well as the lack of certain measurements like soil carbon and nitrogen before, during and after rewetting. The following factors should be recorded in future studies for ease of comparison and a better understanding of the causal mechanisms, and to be able to include more studies in future meta-analyses:

1. N2O measurements for at least 4 days following rewetting, coupled with a presentation of emission data and standard errors.
2. The inclusion of controls where the soil moisture is kept constant.
3. The measurement of nitrogen (NO3-, NH4+) carbon substrates (in the dissolved organic carbon fraction) before, during and after rewetting.
4. The determination of WFPS before, during and after rewetting.
5. Incorporation of oxygen probes at different soil depths to correlate WFPS with overall soil aeration and at microsites at the pore scale.
6. Reporting the soil’s bulk density pH, and temperature.
7. Investigating the effect of the length of drought (we found no studies that have investigated this) and the severity (e.g. the difference between 20% to 90% WFPS and 40% to 90% WFPS).
8. Changes in molecular biology, specifically nitrogen cycling genes and transcripts, before, during, and after rewetting, as well as changes in community composition.
9. High resolution measurements under natural conditions (plot and field trials), which have vegetation cover, we found only two studies that did this (Kostyanovsky *et al.*, 2019; Leitner *et al.*, 2017).

**2.5 Conclusions**

We conducted the first review and meta-analysis to better understand the mechanisms behind N2O hot moments caused by large changes in soil moisture, which is increasingly more likely due to climate change. It is clear that rewetting soil after a drought can cause a significant release of N2O. The larger the difference between the soil’s dry state and its wet state, the larger the hot moment. Moreover, the more saturated the soil is after rewetting the larger the hot moment, with an exponential increase once the soil is anaerobic. It is hypothesised that the accumulation of substrates from the drought and their subsequent accessibility after rewetting is the main cause of a hot moment. From our meta-analysis it is clear that nitrogen and carbon substrates are a significant controlling factor, however, controls with a constant WFPS can have just as much carbon and nitrogen and a similar anaerobic status, and they typically have much lower emissions (Bergstermann *et al.*, 2011; Borken & Matzner, 2009; Chen *et al.*, 2014; Harrison-Kirk *et al.*, 2013). The priming effect is likely related to changes in microbial community and behaviour, unfortunately only one study (Chen *et al.*, 2014) that induced a hot moment, measured N2­O emissions, and measured gene abundance related to N cycling. There is still a large uncertainty regarding how microbial population structure, relative gene abundances and gene expression profiles change according to antecedent dry/wet cycles, and whether these can be manipulated by land management practices to reduce N2O emissions. Moreover, the lack of data from soils *in-situ* means that it is unknown if the hot moments observed in controlled settings are a good analogue for soils in field settings with plant cover.

We suggest a novel approach, wherein future experiments should work on developing two different drought impact curves to help better predict the potential emissions from drying and rewetting, one would show how the drought period influences emissions (we found no study that has investigated this), and one would determine how the relative difference between the soil’s dry state and wet state influences emissions (currently only Bergstermann *et al.* (2011) and Guo *et al.* (2014) have investigated this). This would provide a clearer understanding of how the relationship between soil moisture and N2O emissions is influenced by the drought legacy, allowing the creation of better predictive models and mitigation strategies.

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**Chapter 2 Supplementary material**

The tables for this chapter’s supplementary material are large (over 60 pages and 20,000 words) and do not fit into a A4 format. Therefore, to access these tables please visit the online version on this article: <https://doi.org/10.1111/ejss.13015> or email the corresponding author.

***Table 2.S1.*** *The search results from the two search terms outlined within the methods section and as headers within the table itself; accepted papers are highlighted in yellow.*

***Table 2.S2.*** *Data used for the meta-analysis, description of variables are found within the methods section of the main text.*

***Table 2.S3.*** *Factors included in the models and resulting Akaike information criterion and weight (inverse of their standard errors).*

**Chapter 3**

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**N2O Hot Moments Were Not Driven by Changes in Nitrogen and Carbon Substrates or Changes in N Cycling Functional Genes**

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**Key words:** Soil Moisture, Denitrification, Rewetting, Drought, Legacy

**Highlights:**

1. Rewetting following drought caused a significant release of N2O

2. The pre-dry soil produced higher N2O fluxes but contained less key substrates (TOC, NH4+ and DOC) than the pre-wet soil

3. The pre-dry and pre-wet soil had a similar quantity of denitrification functional genes

4. Differences in soil chemistry and denitrification functional genes between the pre-dry and pre-wet soil cannot explain the differences in N2O emissions

**Authors contributions:**

**H.A. Barrat:** Led and designed the experiment, interpreted the results, and wrote all drafts.

**A.F. Charteris:** Helped to conduct the experiment in the DENIS lab.

**K. Le Cocq:** Helped with minor edits, and guided the DNA extractions.

**M. Abadie:** Helped with minor edits, and guided the DNA extractions and qPCR analysis.

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**Abstract**

If a dry soil is wetted it can produce a large flush of nitrous oxide (N2O), which can be 10 times above the background rate, this phenomenon is called a hot moment. However there is uncertainty in the literature regarding the mechanisms that cause hot moments. Therefore this study aimed to induce two different hot moments on soil cores to investigate changes in nitrogen (N) cycling functional genes, and correlate these changes with subsequent N2O emissions, and transformations in soil chemistry. We induced a ‘pre-dry’ treatment which wetted soils from 50% to 90% waterfilled pore space (WFPS) and ‘pre-wet’ treatment which wetted soils from 70% to 90% WFPS to analyse changes in gas fluxes (N2O, nitric oxide [NO], and carbon dioxide [CO2]), soil chemistry (pH, dissolved organic carbon [DOC], total oxidised nitrogen [TON], ammonium [NH4+]) and microbiology (bacterial 16S, fungal ITS and nitrogen cycling functional genes associated with denitrification). The soil produced emissions of N2O that were significantly higher for the pre-dry treatment compared with the pre-wet treatment (p=<0.001; 9 times that of the pre-wet). The pre-dry treatments did not prime the soil any more than the pre-wet treatments for CO2 and NO emissions, and the results showed that the soil’s TON and DOC concentrations were significantly higher in the pre-wet conditions despite its lower N2O emissions. This suggests that the cause of the hot moment was not as dependant on the soil’s substrate concentrations as previously thought. Moreover, the amount of denitrification genes in the pre-dry soil was statistically similar to the pre-wet (*nirK*, *norB,* *nifH*, *nosZI*, *nxrA*), apart from *nirS* which was marginally higher (p=0.03). Contrary to other experiments, this study provides evidence that changes in soil substrates and functional gene abundance are not the cause of N2O hot moments. We therefore suggest that future studies should investigate changes in mRNA abundance related to N cycling genes.

**3.1 Introduction**

Long periods of drought in a soil followed by a rewetting event that brings it to a high water filled pore space (WFPS) can produce a significant increase in nitrous oxide (N2O) emissions (Priemé and Christensen, 2001; Burger *et al.,* 2005; Ruser *et al.,* 2006; Harrison-Kirk *et al.,* 2013). For example, in an incubation experiment, Bergstermann *et al.* (2011) flooded a wet soil (70% WFPS) and a dry soil (20% WFPS) after 4 weeks to 90% WFPS. The soil that was previously dry produced N2O emissions three times that of the pre-wetted soil. These have been called hot moments, defined as moments of rapid biological activity producing a pulse in N2O (McClain *et al.,* 2003). A recent review and meta-analysis of hot moments by Barrat *et al.* (2020) outlined the relationship between drought intensity (the starting WFPS) and the rewetting intensity (the end WFPS) and the size of the N2O emission pulse, as well as the importance of other factors such as land use, and the quantity of carbon (C) and nitrogen (N) substrates in the soil. They concluded that the higher the rewetting intensity and the higher the drought intensity, the larger the hot moment. However, there is still uncertainty regarding the underlying mechanism, although there are several hypotheses.

Firstly, it could be that very dry soils that are rewetted undergo a pulse in N mineralisation, resulting in an increase of available substrates (Borken and Matzner, 2009). Although compared with controls that are kept continuously wet, there is some evidence that the mineralisation rates of both N and C can be suppressed by dry and wet cycles (Harrison-Kirk *et al.,* 2013). Alternatively, drier conditions could create isolated pockets where substrates accumulate until they are reconnected when the soil is wetted, and then this flush of mineralised C and N is quickly utilised by microbes (Harrison-Kirk *et al.,* 2013; Leitner *et al.,* 2017).

Secondly, the drought before rewetting could affect the diversity and composition of microbial communities, and therefore the abundance of functional genes related to N cycling. The microbial community plays an essential role in N2O emission dynamics under changing soil conditions, but there is limited literature on the changes to the microbial community in soil cores during a N2O hot moment. Banerjee *et al.* (2016) observed that wetting soils from 60% to 80% WFPS caused both an increase in microbial diversity and N2O emissions, although they did not induce a hot moment. Radl *et al.* (2015) measured changes in the abundance of N cycle functional genes during wet and dry cycles as part of a control to study the impact of antibiotics in manure. Ammonia oxidising bacteria (AOB) were negatively affected by drought, but they recovered during rewetting, while ammonia oxidising archaea (AOA) showed little change. Moreover, Radl *et al.* (2015) recorded no significant changes in denitrifying functional genes (*nirK nirS* & *nosZ*), so overall the changes in functional gene abundance were unlikely to impact N2O emissions.

On the contrary, Snider *et al.* (2015) recorded significant changes in denitrification gene abundance, (*nirS*, *nosZ*) which increased after a large rainfall event raised the WFPS from <40% to >70%, coinciding with a large flux of N2O. Bacteria seemed to dominate the microbial response, with bacterial 16S significantly increasing and remaining elevated throughout the measured period, whereas archaeal 16S decreased overall. Radl *et al.* (2015) used water holding capacity to measure water content whereas Snider *et al.* (2015) used WFPS, making it difficult to compare moisture regimes, which in turn might explain why they observed different changes in denitrification gene abundance. The experimental set-up was also fundamentally different, as Radl *et al.* (2015) used packed soil cores and a pre-determined wetting and drying regime of 7 days, while Snider *et al.* (2015) measured emissions from field plots that had been naturally dry for an unspecified period of time (at least 12 days).

Thirdly, drying and rewetting cycles could affect the transcription of key enzymes that are used to oxidise or reduce N substrates. This was suggested by Bergstermann *et al.* (2011), who hypothesised that the dry period may inhibit the production of N2O reductase. Barrat *et al.* (2020) noted that there was some evidence of this based on changes in N2O:N2 ratios post wetting after drought, although there is yet to be a study that has measured changes in the mRNA transcription of *nosZ* in a soil that has undergone a hot moment.

There has been considerably more literature on the Birch effect, wherein rewetting a dry soil causes a pulse of CO2 emissions (Barnard *et al.,* 2020). If the mechanisms are similar for N2O hot moments, then there are several changes in the microbiology and soil chemistry that we would expect to observe. Firstly there should be higher dissolved organic carbon (DOC) concentrations in the soil post wetting, which could be from both abiotic and biotic sources (Blankinship and Schimel, 2018). This new pool of DOC would decrease over time as it is utilised by the active microbial community after rewetting. This could be from the solubilization of carbonates in the soil, and from the necromass of microbes that did not survive the drought phase (Blazewicz *et al.,* 2020). If the source of substrates is necromass then we might see a decrease in microbial DNA in the soil post wetting, although this would depend on the residency time of eDNA in the soil. Drought might also result in higher N concentrations. It could also be due to the desorption of organic compounds from the mineral surface, due to changes in pH and ionic strength in the soil solution after the soil is rewetted (Blankinship and Schimel, 2018). An important factor that affects the size of the Birch effect, is the relative abundance and diversity of microbes post wetting, and it seems likely that this would also impact N2O emissions (Evans and Wallenstein, 2012). If the antecedent conditions impact the abundance and diversity of microbes to a point where this changes the soil’s N cycling function, then we would expect to see changes in the abundance of functional genes from DNA and RNA.

While many studies have investigated the relationship between soil WFPS and N2O emissions, few have explored how historical soil moisture conditions impact the abundance of denitrifiers and their ability to manipulate the N cycle, which can lead to these hot moments. Overall, this study aimed to understand how the associated N cycling functional genes changed within different rewetting patterns, and correlate these changes with subsequent N2O emissions, transformations in total soil oxidised nitrogen (TON, includes nitrate and nitrite) and ammonium (NH4+) concentrations, as well as dissolved organic carbon (DOC). We hypothesised that:

H1: A soil that undergoes drought before flooding will have a higher abundance of denitrification genes after it is rewetted, compared to a wetter soil that is then flooded to the same WFPS, due to the drought and rewetting favouring the growth of denitrifiers.

H2: More intense drought and rewetting events would result in higher concentrations of C and N substrates, as suggested by the literature investigating the Birch effect.

**3.2 Method**

**3.2.1 Soil sampling**

The soil (clayey pelostagnogley, clayey throughout, greyish colours and mottled throughout) was collected from Rowden grassland field site at North Wyke in Devon, England (50°46'47.9"N 3°55'13.3"W). The site’s mean annual temperature is 9.6 °C and its mean annual rainfall is 1055.7 mm from 1961-2000 (Harrod 2008). Approximately 2 kg of soil was sampled from each point in a 9 point W design in a 1 ha field at 4 to 10 cm depth using a trowel, and it was sieved to < 9 mm. A larger sieve size was selected to minimise soil disturbance, while still generating some homogeneity. The soil was bulked by combining the sieved field samples. This was kept refrigerated at 4 °C for 7 days before it was used to pack 88 cores (7.6 cm height by 4.5 cm diameter, with sealed bases, ~130 g per core) to a soil bulk density of 0.8 g cm-3. Packing density was determined from the mean bulk density of 9 sample points (0.76 g cm-3, which was rounded to 0.8 g cm-3). See Table 3.1 for soil properties.

**3.2.2 Soil pre-treatment**

Two different pre-treatments were imposed for 14 days; 44 cores were kept at 50% WFPS and the other 44 cores were kept at 70% WFPS (Figure 3.1), 38 cores from each treatment were then flooded, while the other 6 cores from each treatment were kept as controls (see section 2.5). Target moistures were maintained by adding deionised water to maintain a specified weight. Values of 50% and 70% WFPS were selected as reasonable reflections of moisture conditions that would occur over a 2-week period in the summer/spring time at the site. All cores were maintained at a temperature of 15.5 °C, based on the site’s mean July air temperature (1961 to 2000) throughout the pre-incubation period.

***Table 3.1.*** *Soil properties of the pooled soil used to pack all cores, see the methods section for how they were determined. Error is represented using standard error. N=6*

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **TON mg N kg-1 dry soil** | **NH4+-N mg N kg-1****dry soil** | **pH** | **DOC mg C kg-1****dry soil** | **Vegetation cover** | **Soil texture** |
| 43.7 ± 0.7 | 3.5 ± 0.3 | 5.42 ± 0.04 | 147.5 ± 1.8 | Grassland, Permanent pasture  (*L. perenne*) | Clay |

**3.2.3 Flooding Incubation**

After 12 days of the pre-incubation, 36 cores were placed in the Denitrification Incubation System (DENIS). This system is described in Cárdenas *et al.,* (2003) and Cardenas *et al.,* (2017); it contains 12 vessels, with each vessel holding three small cores, the vessels are sealed into a flow-through system which is automatically connected to two gas chromatographs and a nitric oxide chemiluminescence analyser (see section 2.4). Six vessels in the DENIS were randomly allocated to each treatment (pre-dry at 50% WFPS; and pre-wet at 70% WFPS). Simultaneously, 52 cores were placed in a separate temperature-controlled cabinet for parallel incubation and destructive soil sampling to assess changes in soil chemistry and nitrogen cycling functional genes over time. Destructive soil samples were taken before packing the cores, at 2 and 14 days pre-flooding, and 1, 2, 6 and 14 days post flooding. The temperature was kept at 15.5 °C during the incubation. After flushing the soil headspaces with 80:20 He:O2 for 2 days to remove atmospheric N2, the soil moisture in all the cores in the DENIS system and the parallel incubation was increased to 90% WFPS by adding deionised water using a 50 ml syringe (after 14 days of preincubation, marked as day 0). Note that for the parallel incubation, the flooding was delayed by 2 days, so that destructive sampling times could be informed through observation of the DENIS N2O emission data. We aimed to sample these soil cores when the N2O emissions started to increase, at the N2O maximum and at the end of emission pulse. The flooded conditions were 90% rather than 100% WFPS because there were concerns that the flooded soil might develop a water cap, impeding gas flow.

**3.2.4 Greenhouse Gas Measurements**

Gas samples were automatically taken every 8 minutes sequentially from the incubated cores. The DENIS is a flow-through system that directs the gas from one vessel to a sample loop that injects the sample to the GC and NO analyser via automated valves within a 8 minute cycle, it then repeats the process with a different vessel. Fluxes of N2O and CO2 were quantified using a Pye Unicam 500 equipped with an electron capture detector (ECD) for N2O and CO2. NO concentrations were determined by chemiluminescence (Sievers NOA280i, GE Instruments, Colorado, USA) (Cárdenas *et al.,* 2003; Loick *et al.,* 2017). All the gas concentrations were corrected according to the flow rate through the vessel, which was measured daily, and fluxes were calculated on a mg N or C kg-1 h-1 of dry soil basis. Baseline gaseous emissions were determined by two days of flushing cores before treatment application (see section 2.3). The nitrogen (N2) background was especially high, probably due to the use of sealed-base cores, which prevented thorough flushing of the soil pores. This was necessary however to prevent leaching, while maintaining the 90% WFPS flooded conditions required for the experiment. Such high background N2 levels prevented the assessment of N2 production, usually possible using the DENIS during this experiment.

**3.2.5 Soil chemistry measurements**

Destructive soil samples were collected at the following stages:

1. Before packing the cores, 100 g samples (n=6) were scooped from the bulk soil
2. After 2 days’ pre-incubation (day -12), dry treatment (n=5) and wet treatment (n=5)
3. After 14 days’ pre-incubation (day 0 as all samples were flooded on this day after sampling), dry treatment (n=5) and wet treatment (n=5)
4. 1 day post flooding (day +1), pre-dry treatment (n=5) and pre-wet treatment (n=5)
5. 2 days post flooding (day +2), pre-dry treatment (n=5) and pre-wet treatment (n=5)
6. 6 days post flooding (day +6), pre-dry treatment (n=5), pre-wet treatment (n=5), dry controls which were never flooded (50% WFPS; n=3) and wet controls which were also never flooded (70% WFPS; n=3).
7. 14 days post flooding (day +14), end of incubation, 12 samples representing each vessel from DENIS were created by pooling the three cores from each vessel, pre-dry (n=6) and pre-wet (n=6), plus dry (n=3) and wet (n=3) controls which were not flooded.

Soil inorganic N concentrations (TON and NH4+) and the DOC concentration of the samples were determined after extraction (20 g soil, fresh weight) using 100 ml 0.5 M potassium sulphate (K2SO4); they were shaken for 30 minutes and centrifuged, the supernatant was removed and stored at -20 °C (Jones and Willett 2006). Nitrate (NO3-) and nitrite (NO2-) comprise TON, but as NO2- is highly reactive, it is considered that most of the TON is in the form of NO3-. An Aquakem 250 discrete photometric analyser was used for inorganic N concentrations (Jokinen 2013; Searle 1984). For DOC concentrations, the samples were diluted by a factor of 10 (to prevent precipitation in the instrument) and determined using a Shimadzu TOC-L CPN total organic carbon analyser. Gravimetric moisture was determined using a 50 g sub-sample in the oven at 105 °C for 24 hours. Fresh soil pH was determined using a pH meter with 5 g of soil to 12.5 ml of water using a Jenway 3320 pH meter.

***Figure 3.1.*** *Wetting regime imposed on 88 soil cores, 44 had the pre-wet treatment and 44 the pre-dry. Day zero indicates when all cores apart from controls were flooded. Destructive samples were taken at -15 days, -12 days, 0 days, +1 day, +2 days, +6 days, and +14 days, marked with an arrow.*

**3.2.6 Soil microbial DNA extraction and quantitative PCR**

To test changes in functional gene abundance, DNA was extracted from soil samples taken at the following time points (which were based on the observed N2O data):

1. After 2 days’ pre-incubation (day -12), dry treatment (n=5) and wet treatment (n=5)
2. After 14 days’ pre-incubation (day 0 as all samples were flooded on this day after sampling), dry treatment (n=5) and wet treatment (n=5)
3. 1 day post flooding (day +1), pre-dry treatment (n=5) and pre-wet treatment (n=5)
4. 6 days post flooding (day +6), pre-dry treatment (n=5), pre-wet treatment (n=5), dry controls which were never flooded (50% WFPS; n=3) and wet controls which were also never flooded (70% WFPS; n=3).
5. 14 days post flooding, end of incubation, 12 samples representing each vessel from DENIS were created by pooling the three cores from each vessel, pre-dry (n=6) and pre-wet (n=6), plus dry (n=3) and wet (n=3) controls which were not flooded.

Upon destructive soil sampling, 15 g soil samples were wrapped in foil and frozen in liquid nitrogen. Following storage at -80 °C, samples were freeze-dried to create a homogenous sample size that could be easily weighed and poured into falcon tubes for DNA extractions. DNA was extracted using the RNeasy PowerSoil DNA Elution Kit using 2.0 g samples. The concentrations of DNA were measured using Qubit dsDNA BR Assay Kit (ThermoFisher) and the quality assessed on a Nanodrop spectrophotometer (ThermoFischer). Samples were then stored at −80°C prior to further analysis.

qPCR analysis was performed as described in de Sosa *et al.,* (2018) for the timepoints at 2 and 14 days pre-incubation, and 1, 6 and 14 days post flooding. Specific primers (see Table 3.2) were employed to quantify gene abundance of microbial kingdoms and genes associated with denitrification N cycling using the QuantiFast SYBR® Green PCR Kit (Qiagen) and a Biorad CFX384 Touch Real-Time PCR Detection System. Using a 384 plate, each run contained 3 different genes, 24 blanks, 2 no-template blanks per gene, 8 x 3 standards per gene, and 2 positive controls using a standard created from a mix of grassland and arable soil. Several studies have concluded that anaerobic denitrification is the major process producing N2O post wetting (Ruser *et al.,* 2006; Bergstermann *et al.,* 2011; Kim *et al.,* 2012; Harrison-Kirk *et al.,* 2013) and due to the high WFPS upon rewetting in this study we hypothesised that this would be the case. Therefore we focussed on changes to denitrification gene abundance. Total bacterial (16S) (Glaring *et al.,* 2015), fungal populations (ITS) (Vilgalys and Hester 1990; Gardes and Bruns 1993), and the denitrification genes nitrite reductase (*nirK* and *nirS*) (Henry *et al.,* 2004; Throbäck *et al.,* 2004), nitrate reductase (*nxrA*) (Fu *et al.,* 2018), nitric oxide reductase (*norB*) (Braker and Tiedje 2003) and nitrous oxide reductase (*nosZ* clade I) (Henry *et al.,* 2006), and a gene for nitrogen fixation (*nifH*) (Poly *et al.,* 2001) were measured. DNA copy numbers are represented on a per gram of dry soil basis (cn g-1).

***Table 3.2.*** *List of the primers used to target each gene*

|  |  |  |  |
| --- | --- | --- | --- |
| Target gene | Primer | Sequence 5'-3' | References |
| *16S rRNA*  *Bacteria* | 341F | CCT AYG GGR BGC ASC AG | Glaring *et al.,* (2015) |
| 806R | GGA CTA CNN GGG TAT CTA AT |
| *Fungal*  *ITS* | ITS1f | TCC GTA GGT GAA CCT GCG G | Gardes and Bruns (1993) Vilgalys and Hester (1990) |
| 5.8s | CGC TGC GTT CTT CAT CG |
| *nirK* | nirK876F | ATY GGC GGV CAY GGC GA | Henry *et al.,* (2004) |
| nirK1040R | GCC TCG ATC AGR TTR TGG TT |
| *nirS* | cd3aF | GTS AAC GTS AAG GAR ACS GG | Throbäck *et al.,* (2004) |
| R3cdR | GAS TTC GGR TGS GTC TTG A |
| *nosZI* | nosZ1F | CGC RAC GGC AAS AAG GTS MSS GT | Henry *et al.,* (2006) |
| nosZ1R | CAK RTG CAK SGC RTG GCA GAA |
| *norB* | qnorB5R-F | TGG TGG GTN GTN CAY CTN TGG GT | Braker and Tiedje (2003) |
| qnorB7R | GGN GGR TTD ATC ADG AAN CC |
| *nifH* | PolF | TGC GAY CCS AAR GCB GAC TC | Poly, Monrozier, and Bally (2001) |
| PolR | ATS GCC ATC ATY TCR CCG GA |
| *nxr-Nitrospira-* | nxr-spira-for5 | CAR TCS AAC TTC CGG TAY GG | Fu *et al.,* (2018) |
| nxr-spira-rev6 | AGC CAC TTG ATC ATG AAY TC |
| *nxr-Nitrobacter* | nxr-bacter-for1 | GAC SCG YAC CCC SGA CGT GCA CYT CAT |
| nxr-bacter-rev3 | ATG ACG TGR TTG RCC GCC ATC CA |

**3.2.7 Statistics**

Genstat 20th edition was used for all statistical analysis. The cumulative emissions of NO, N2O and CO2 were estimated by calculating the area under the curve of the fluxes, post wetting. Differences between the pre-dry and the pre-wet treatment for gas data were determined using two sample t-tests. Differences in soil chemistry and functional gene abundance from DNA was determined using ANOVA. To analyse differences according to treatment, the treatment structure of treatment\*time was used. To assess the difference between controls and treatment, a different treatment structure was used: Trt1\*Trt2\*Time, with Trt1 = dry or wet factor and Trt2 = treatment or control factor. Unfortunately, a replicate for -1.0 day for the pre-wet treatment for the chemistry and DNA data had to be omitted, due to accidentally adding too much water to the sample during the antecedent period. The variability of the data is represented using standard error (SE).

**3.3.0 Results**

**3.3.1 Gas data**

Flooding of both the pre-dry and the pre-wet treatment created a hot moment for N2O and NO (see Figures 3.2 and 3.3) with a larger maximum for the pre-dry treatment. The highest N2O flux was observed at 2.3 days (55 hours) in the pre-dry (0.85 mg N m-2 h-1) and at 1.6 days (38 hours) in the pre-wet (0.14 mg N m-2 h-1) post flooding (see Figure 3.2). The pre-dry N2O fluxes did not return to baseline values (zero emissions) during the 14-day observation period. The pre-wet treatment returned to baseline after 10 days. There were significantly larger cumulative N2O emissions in the pre-dry than the pre-wet treatment (p=<0.001; 9 times that of the pre-wet). The mean cumulative emissions of the pre-dry treatment (n=6) was 121.92 ± 12.48 mg N m-2 whilst for the pre-wet (n=6) was 14.16 ± 3.84 mg N m-2.

The highest NO flux occurred at 0.15 days (4 hours) in the pre-dry (0.0014 mg N m-2 h-1) and at 0.07 days (2 hours) in the pre-wet (0.0011 mg N m-2 h-1 ) treatment post flooding (see Figure 3.3). Cumulative NO emissions were not statistically different between the treatments (p=0.108). The mean cumulative emissions for the pre-dry treatment (n=6) was 0.161 ± 0.012 mg N m-2 and for the pre-wet treatment (n=6) it was 0.134 ± 0.010 mg N m-2.

At t=0, prior to flooding, CO2 fluxes were higher from the pre-wet treatment compared with the pre-dry (see Figure 3.4). Rewetting did not result in a flush of CO2, and the pre-wet and pre-dry treatments were statistically similar in terms of cumulative CO2 emissions post flooding (p=0.078). The mean for the pre-dry cumulative emissions (n=6) was 279.5 ± 3.44 mg C m-2 h-1 and the mean for the pre-wet cumulative emissions (n=6) was 289.4 ± 3.62 mg C m-2 h-1.

***Figure 3.2.*** *Mean N2O fluxes from two antecedent moisture treatments. Pre-dry soil was kept at 50% WFPS and then flooded to 90% WFPS on day zero; pre-wet soil was kept at 70% WFPS and then flooded to 90% WFPS on day zero. Error bars represent standard error. N=6.*

***Figure 3.3.*** *Mean NO fluxes from two antecedent moisture treatments. Pre-dry soil was kept at 50% WFPS and then flooded to 90% WFPS on day zero; pre-wet soil was kept at 70% WFPS and then flooded to 90% WFPS on day zero. Error bars represent standard error. N=6.*

***Figure 3.4.*** *Mean CO2 from two antecedent moisture treatments. Pre-dry soil was kept at 50% WFPS and then flooded to 90% WFPS on day zero; pre-wet soil was kept at 70% WFPS and then flooded to 90% WFPS on day zero. Error bars represent standard error. N=6.*

**3.3.2 Soil chemistry**

Soil pH averaged across all time points (-12, 0, +1, +2, +6 and +14 days) showed no significant difference between the pre-dry treatment (n=31, mean pH 5.72 ± 0.02) and the pre-wet treatment (n=31, mean pH 5.70 ± 0.02) p=0.523. Priming the soil for 14 days at different WFPS levels (0 days, n=5 for dry, mean pH 5.75 ± 0.052, and n=5 for wet, mean pH 5.82 ± 0.052) resulted in no significant difference between treatments for pH (average LSD=0.15). And the pH post flooding (+1, +2, +6 and +14 days) did not significantly change at any of the time points, (average LSD = 0.15) see Table 3.3. There were also no significant differences (p=0.100) between the controls (6 and 14 days, n=12, mean pH 5.74 ± 0.04) and the treatments (n=22, mean pH 5.64 ± 0.04).

Soil NH4+-N concentrations, averaged across all sampling time points (-12 days, 0 days, +1 day, +2 days, +6 days, +14 days) showed no significant difference (p=0.290) between the pre-dry treatment (n=31, mean 2.2 ± 0.1 mg kg-1) and the pre-wet treatment (n=30, mean 2.4 ± 0.1 mg kg-1). Priming the soil for 14 days at different WFPS levels (0 days, n=5 for pre-dry, mean 2.9 ± 0.3 mg kg-1, and n=4 for pre-wet, mean 2.7 ± 0.3 mg kg-1) resulted in no significant difference between treatments for NH4+ (average LSD=0.6). Soil NH4+ concentration post flooding (+1 day, +2 days, + 6 days, +14 days) did not significantly change at any of the time points (average LSD=0.6) see Table 3.3. For NH4+ there were no significant differences between the controls (n=12, mean 1.2 ± 0.2 mg kg-1) and the treatments (n=22, mean 2.1 ± 0.2 mg kg-1) p=0.537. The dry treatment and controls were not significantly different from the wet treatment and controls (p=0.598).

The soil’s TON concentrations, averaged across all time points (-12 days, 0 days, +1 day, +2 days, +6 days, +14 days) showed a significant difference (p=<0.001) between the pre-dry treatment (n=31, mean 52.4 ± 0.7 mg kg-1) and the pre-wet treatment (n=30, mean 57.6 ± 0.4 mg kg-1). The pre-wet treatment had 10.0% more TON compared with the pre-dry. Priming the soil for 14 days at different WFPS levels (0 days, n=5 for pre-dry, mean 52.7 ± 0.000.9 mg kg-1, and n=4 for pre-wet, mean 55.6 ± 0.9 mg kg-1) resulted in a significant difference between treatments for TON (average LSD=2.5). The pre-wet treatment had 5.5% more TON compared with the pre-dry before the soil was flooded. TON post flooding (+1 day, +2 days, + 6 days, +14 days) was significantly different for all the time points (average LSD = 2.5) see Table 3.3. On average the pre-wet treatment had 11.7% more TON compared with the pre-dry post flooding. For TON there were significant differences between the controls (n=12, mean 61.6 ± 0.7 mg kg-1) and the treatments (n=22, mean 58.7 ± 0.7 mg kg-1) p=0.003. The dry treatment and controls were also significantly different from the wet treatment and controls (p=<0.001).

The soil’s DOC concentrations, averaged across all time points (-12 days, 0 days, +1 day, +2 days, +6 days, +14 days) showed a significant difference between the pre-dry treatment (n=30, mean 130.0 ± 1.6 mg kg-1) and the pre-wet treatment (n=30, mean 137.0 ± 1.6 mg kg-1) p=0.003. The pre-wet treatment had 5.5% more DOC compared with the pre-dry. Priming the soil for 14 days at two different WFPS levels (0 days, n=5 for pre-dry, mean 112.0 ± 3.9 mg kg-1, and n=5 for pre-wet, mean 148.0 ± 3.9 mg kg-1) resulted in a significant difference between treatments for DOC (average LSD=11.1). The pre-wet treatment had 32.1% more DOC compared with the pre-dry, before the soil was flooded (0 days). DOC post flooding (+1 day, +2 days, + 6 days, +14 days) resulted in no significant difference between treatments (average LSD=11.1) see Table 3.3. There were no significant differences between the controls (n=12, mean 129.6 ± 2.3 mg kg-1) and the treatments (n=22, mean 130.0 ± 2.3 mg kg-1) p=0.882. The dry treatment and controls were not significantly different from the wet treatment and controls p=0.150.

***Table 3.3.*** *Changes in ammonium (NH4+), total oxidised nitrogen (TON), dissolved organic carbon (DOC) on a dry weight basis, and pH from two antecedent moisture treatments. Pre-dry soil was kept at 50% WFPS and then flooded to 90% WFPS on day zero; pre-wet soil was kept at 70% WFPS and then flooded to 90% WFPS on day zero. Error is represented using standard error. N=5 for pre-dry and pre-wet for all times points apart from +14 days where N=6.*

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| ***Metric*** | ***Treatment*** | ***-12 days*** | ***0 days*** | ***+1 day*** | ***+2 days*** | ***+6 days*** | ***+14 days*** |
| ***pH*** | *Pre-dry* | *5.90 ± 0.02* | *5.78 ± 0.02* | *5.67 ± 0.02* | *5.70 ± 0.02* | *5.71 ± 0.02* | *5.58 ± 0.02* |
| *Pre-wet* | *5.60 ± 0.05* | *5.82 ± 0.05* | *5.71 ± 0.05* | *5.73 ± 0.05* | *5.70 ± 0.05* | *5.62 ± 0.05* |
| ***NH4+***  mg N kg-1 dry soil | *Pre-dry* | *2.0 ± 0.1* | *2.8 ± 0.1* | *2.2 ± 0.1* | *2.3 ± 0.1* | *2.4 ± 0.1* | *1.9 ± 0.1* |
| *Pre-wet* | *2.7 ± 0.1* | *2.4 ± 0.1* | *2.8 ± 0.1* | *2.4 ± 0.1* | *2.5 ± 0.1* | *1.6 ± 0.1* |
| ***TON***  mg N kg-1 dry soil | *Pre-dry* | *48.0 ± 0.7* | *50.6 ± 0.7* | *53.8 ± 0.7* | *51.8 ± 0.7* | *52.7 ± 0.7* | *55.8 ± 0.7* |
| *Pre-wet* | *47.5 ± 0.4* | *55.6 ± 0.4* | *57.4 ± 0.4* | *58.9 ± 0.4* | *60.1 ± 0.4* | *64.0 ± 0.4* |
| ***DOC***  mg C kg-1 dry soil | *Pre-dry* | *137.6 ± 1.6* | *112.1 ± 1.6* | *124.4 ± 1.6* | *144.9 ± 1.6* | *120.1 ± 1.6* | *138.0 ± 1.6* |
| *Pre-wet* | *137.7 ± 1.6* | *148. ± 1.6* | *128.1 ± 1.6* | *148.3 ± 1.6* | *130.9 ± 1.6* | *129.4 ± 1.6* |

***Figure 3.5.*** *The y axis shows the change in the mean copy number of DNA per gram of dry soil (cn g-1) for 8 functional genes of microbial populations and denitrification processes over time in days. Pre-dry soil was kept at 50% WFPS and the pre-wet soil was kept at 70% WFPS and then both were flooded to 90% WFPS. Day 0 indicates when both treatments were flooded to 90% WFPS, which is marked with a line. At time point -12 days, 0 days, +1 days and +6 days n=5 for each treatment, and at +14 days n=6 for each treatment, with the exception of Bacterial 16S where n=3 for -12 days for both treatments, and for norB where n=3 for the pre-wet treatment at -12 days, this was due to low quality DNA extractions. Error bars represent standard error. Note the Y axes have different ranges.*

**3.3.3 Microbiology**

For DNA the abundance per gram of dry soil (cn g-1) comparing the time points -12 days, 0 days, +1 day, +6 days, and +14 days from rewetting, showed no significant difference according to treatment (p>0.05) for bacterial 16S, fungal ITS and all nitrogen functional genes apart from *nirS* which was significantly different (p=0.03, pre-dry mean 4.04 x 107 ± 5.09 x 106 cn g-1 n=26, and the pre-wet mean 2.61 x 107 ± 3.60 x 106, cn g-1 n=26), see Figure 3.5. Over time fungal ITS and *norB* were statistically similar (p=0.221 and p=0.093 respectively), but bacterial 16S, *nirK*, *nirS*, *nifH*, *nosZI* and *nxrA* showed a significant decrease (p<0.05), once the soil had been rewetted, see Figure 3.5. Comparing the controls, there was no significant difference between the dry controls and the wet controls for any of the genes, and there was also no difference between the treatments (at timepoints +6 days and +14 days) and the controls (data not shown).

**3.4 Discussion**

The rewetting of previously drier soils resulted in a hot moment as defined by McClain *et al.* (2003) for N2O fluxes, without the addition of N or C substrates during the experiment. Our results showed that cumulative N2O emissions were significantly higher for the pre-dry treatment (50% WFPS for 14 days then rewetted to 90% WFPS) compared with the pre-wet treatment (70% WFPS for 14 days then rewetted to 90% WFPS). A recent literature review highlighted that the extent of wetting and the starting point is clearly important in terms of the size of the hot moment (Barrat *et al.,* 2020). Models such as those outlined by Davidson *et al.* (2000), would predict that under the same conditions of bulk density, substrate concentrations, and WFPS for the same soil, the emissions would be the same. Contrary to this, our study indicates the importance of the soil’s moisture legacy as both our soils had the same 90% WFPS post flooding, but the emissions were significantly different (p=<0.001), due to the soil’s WFPS before flooding (50% for pre-dry and 70% for pre-wet). Other studies have recorded similar hot moments regarding N2O emissions. For example, Leitner *et al.* (2017) observed more than a 25-fold increase in NO and N2O emissions after irrigating plots of dry a grassland soil, although the drought period was never specified. Harrison-Kirk *et al.* (2013) rewetted soil cores with different drying and wetting treatments, and they recorded the largest N2O emissions from the cores that were driest before rewetting. Molodovskaya *et al.* (2012) used eddy covariance to detected N2O hot moments from fertilised cropland, with the largest emissions coinciding with the wetting of the dry soil from summer rains, as well as from spring thaws. Simiarly, Rudaz *et al.* (1991) added water to both intact cores and bulked soil samples from a dry grassland and dry oak soil, observing a more than a 30 fold increase in N2O emissions post wetting, however the authors never specified the period of drought.

The statistically similar CO2 and NO emissions from the pre-dry and the pre-wet treatments, indicated that the pre-dry treatment did not prime the soil any more than the pre-wet for these two gases. The lack of statistical difference in NO emissions, suggests the active emission pathway that causes the larger increase in N2O emissions, is not anaerobic denitrification, although clearly there was some activity, given that both soils produced a pulse in NO immediately after rewetting. It is possible that the pre-dry treatment produced more NO within the soil matrix, but it did not diffuse to the soil’s surface as NO is highly reactive and easily reduced by the soil’s microbes (Pilegaard, 2013).

Surprisingly, rewetting did not cause a flush in CO2 emissions, an anticipated phenomena sometimes called the Birch effect (Birch, 1958). The mechanisms behind the Birch effect are currently thought to be from a combination of increasing available C (from both abiotic and biotic sources) with the drought and rewetting favouring those with a rapid response to the change in water potential (Schimel, 2018; Barnard *et al.,* 2020). Due to the higher DOC in the pre-wet treatment at the beginning of the rewetting this study’s results suggest that the Birch effect and N2O hot moments have different causal mechanisms. Presumably, the pre-wet or the pre-dry treatment did not result in a large enough difference in the available carbon pools to produce a flush of CO2 when wetted. The relative C contribution from the biotic and abiotic pools post wetting is dependent on the soil type, so it is possible that a different soil would have caused both an increase DOC and a N2O hot moment (Harrison-Kirk *et al.,* 2014).

We hypothesised (H2) that the pre-dry treatment post wetting would have higher concentrations of extractable C or N between the two treatments. However, our results indicated that the TON and DOC concentrations were significantly higher in the pre-wet treatment compared with the pre-dry before and post flooding, despite its lower emissions. Previous work has shown that higher WFPS levels often correlate with higher mineralisation rates, with the more intense dry periods having the lowest mineralisation rates, despite a pulse in C and N after wetting (Harrison-Kirk *et al.,* 2014; Borken and Matzner 2009). So, it is not unusual that the wetter treatment had higher substrate concentrations. This might be expected to result in greater dentification activity for the pre-wet treatment, as it has more of the key substrates required for anaerobic denitrification, but neither the N2O emission, nor the functional gene data for this study support this. This suggests that the occurrence of the hot moment was not driven by a change in key soil substrate concentrations from the antecedent conditions. It might also indicate that the release of microbial osmolytes from the rewetting was not an important factor in controlling N and C concentrations for this soil, or that the microbial necromass is not significant enough to affect the total available N and C concentrations.

However, it is important to note that extractable N and C are not necessarily representative of the available substrates, as the extraction method is destructive, and does not differentiate how the substrates are distributed in the soil pore network. It could be that there is a change in the distribution of C and N and thus greater bioavailability as the recently resuscitated microbes could be adjacent to dead microbes from the drought allowing immediate activity, which likely has to be supported using anaerobic respiration due to the limited oxygen availability. Secondly, the drought could have affected soil structure and therefore the solute availability. This seems unlikely, given that the treatments used the same soil with similar clay and organic matter content, they also had the same bulk density, which are the major factors that control changes in porosity from drying and wetting (Borken and Matzner 2009; Navarro-García *et al.,* 2012; Diel *et al.,* 2019). Also, the 50% WFPS pre-dry treatment is unlikely to be extreme enough to encourage hydrophobicity and changes in aggregate distribution, especially given that the soil is a mineral soil with comparatively low organic matter content (Müller and Deurer 2011). However, further experimental work will need to be done to discern whether this is the case.

In this study we hypothesised that changes in the N functional genes caused by the antecedent conditions would change the denitrification activity and therefore explain the larger emissions from the pre-dry treatment (H1). Contrary to this, nearly all the N cycling genes (*nirK*, *norB,* *nifH*, *nosZI*, *nxrA*) and fungal ITS and bacterial 16S were statistically similar between the two treatments (see Figure 3.5). Only *nirS* was significantly different (p=0.03), with it having a slightly higher abundance in the pre-dry treatment, which could be due to the growth of nitrifying denitrifiers as previously suggested by the soil’s chemistry. As we did not expect a change in nitrification rates and therefore changes in the abundance of nitrifiers, we did not measure changes in hydroxylamine oxidoreductase (*hao),* ammonia oxidising bacteria (AOB) or ammonia oxidising archaea *(AOA)*. However, given the lack of changes in denitrification genes, and the possibility of nitrifier denitrification, we suggest that future experiments should explore this hypothesis.

There was a significant difference over time for bacterial 16S, *nirK*, *nirS*, *nifH*, *nosZI* and *nxrA* showing a significant decrease in abundance once the soil had been flooded (p<0.05). This is likely due to unfavourable anaerobic conditions for the nitrogen cycling communities (Randle-Boggis *et al.,* 2018). It is hypothesised that the reduction step from N2O to N2 is blocked due to the more intensive drought (Bergstermann *et al.,* 2011), however the functional genes from DNA show no significant change in *nosZI* abundance between the two treatments. Although, further work is needed to determine if the treatments affect the transcription of N cycling genes by measuring changes in mRNA abundance.

We hypothesised that anaerobic denitrification would be the key process producing N2O emissions post wetting, and therefore we focussed our analysis on exploring soil variables related to this process. However, our results do not clearly show this to be the case. Chemodenitrification is possible if there are high concentrations of NH2OH or NO2- and low concentrations of O2, however the pre-wet had higher TON concentrations than the pre-dry, and it is typically observed after N is artificially added (Liu *et al.,* 2019; Liu *et al.,* 2018; Anderson and Levine, 1986). Moreover, it took several days to reach the peak N2O emissions, whereas an abiotic source would produce a peak within several hours (Leitner *et al.,* 2017). Instead, we suggest that the key process causing differences in N2O emissions is nitrifier denitrification. Wherein nitrifiers under oxic shock utilise hydroxylamine and reduce it to N2O, depending on soil conditions this pathway does not produce NO3- (Wrage-Mönnig *et al.,* 2018). It is a process that is likely when there is a sudden change in oxygen availability, in low carbon and low pH conditions, similar to the soils used in this experiment. Although the soil chemistry does not reveal any significant changes in NH4+, the amount required to produce the difference in N2O is less than 0.01% of the observed available NH4+ pool, and some of the NO3- could have been reduced to NO2-. This might explain the slightly higher TON concentrations in the pre-wet soil post wetting, as some of the TON pool in the pre-dry would have been used to produce N2O, which is not replenished due to nitrifier denitrification bypassing NO3- production. However, further microbial work would be needed to determine if this is the main pathway for N2O hot moments under similar conditions as in our experiment. We suggest the use of natural abundance isotope methods, tracer methods or molecular methods as outlined by Wrage-Mönnig *et al.* (2018).

There are some important limitations to this study. Firstly, it only measured the response from one soil type that was bare and at a set bulk density, and the cores do not have the same water movement as large lysimeters or plot scale experiments. The different water movements, especially laterally, might make a difference in the formation of anaerobic microsites, and thus change the response, and different soil types might produce a different biochemical response to rewetting. This study also used bare soil, and if vegetation was grown in the soil cores then the plant roots might have changed the size and nature of the hot moment (Blankinship and Schimel, 2018). In addition, the wetting of soil was incredibly rapid with the new % WFPS reached within ca. 2 minutes, whereas rainfall in the field would likely take a much longer period of time to wet the soil to such an extent. Indeed, the rewetting pattern could change the size of the hot moment, but this has not been studied in a controlled setting to our knowledge. Further work including plants should investigate how plant roots, rewetting patterns, and drought length impact the size of the hot moment. Ideally this would be linked to changes in functional genes related to nitrogen cycling.

**3.5 Conclusions**

Other studies have hypothesised that there is an increase in soil substrate availability to the soil’s microbes following rewetting either from lysed cells or organic matter (Borken and Matzner 2009), with some studies concluding that changes in soil substrate concentrations are an important driver of the resulting N2O flux (Priemé and Christensen, 2001; Ruser *et al.,* 2006; Leitner *et al.,* 2017). This was also suggested in a review by Kim *et al.* (2012). Our experiment investigated the underlying causal mechanism of hot moments, by measuring changes in both soil chemistry and N cycling gene abundance, and contrary to other experiments, our results show that the N2O hot moment can be even larger in a soil with similar or even lower DOC, NH4+ and TON concentrations. This study therefore provides evidence that N2O hot moments are not necessarily dependent on changes in available soil substrates. It is clear that the dynamics of drying and rewetting on mineralisation rates is complex and important, however this study suggests it is not always the cause of a hot moment, although N and C concentrations have been shown to affect the size of hot moment once the soil is sufficiently primed by drought (Bergstermann *et al.,* 2011; Harrison-Kirk *et al.,* 2014). Due to the lack of conclusive data from changes in soil chemistry and DNA, we suggest that changes in transcription is the likely causal mechanism for the hot moments. Future experiments should replicate a similar experimental design to this study, on different soil types and include measurements of the changes in mRNA abundance. Therefore, the mechanism behind the hot moment is likely due to a change in life strategy from the microbes, responding to the rapid changes in WFPS, rather than changes in TON, DOC, pH or NH4+ concentrations, between the two treatments. The slight reduction in TON for the pre-dry treatment, yet the stark difference in N2O emissions, coupled with the oxic shock and acidic soil, suggests that nitrifier denitrification maybe a key mechanism behind N2O hot moments.

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**Chapter 4**

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**The Impact of Drought Length and Intensity on N Cycling Gene Abundance, Transcription and the Size of an N2O Hot Moment From a Temperate Grassland Soil**

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**Key words:** Soil Moisture, Nitrous Oxide, Dry Wet Cycles, Legacy, Water Filled Pore Space

**Highlights:**

1. A greater drying intensity results in larger N2O emissions if soil is sufficiently rewetted
2. Drought length and the size of N2O hot moments are negatively related and non-linear
3. We observed no significant differences in transcription activity for N cycling genes
4. We suggest a two step drought response to explain differences in N2O emissions

**Authors contributions:**

**H.A. Barrat:** Led and designed the experiment, interpreted the results, and wrote all drafts.

**I.M. Clark:** Helped set up the picarro, guided the DNA and RNA extractions and qPCR analysis. Helped with minor edits.

**J. Evans:** Helped design the experiment and the statistical approach.

**D.R. Chadwick:** Helped with minor edits, and the experimental design.

**L. Cardenas:** Helped with some of the gas sampling days, helped with minor edits, and the experimental design.

**Abstract**

This study aimed to investigate the relationship between drought length, drought intensity and the size of the N2O hot moment. It selected two treatments to deduce the main nitrogen cycling process producing N2O (increasing WFPS from 40% to 90%, and from 70% to 90%), by destructively sampling soil cores to analyse gene abundance, transcript abundance, and changes in soil chemistry (TON, NH4+, DOC). Five other drought and rewetting treatments on packed soil cores were selected to create the drought curves described in Barrat *et al.* (2020): these included increases of WFPS from 40% to 90%, 50% to 90%, 60% to 90%, 70% to 90%, and 30% to 60%. For each treatment, drought lengths were imposed from 0 to 30 days. A quadratic linear regression was fitted to the cumulative emissions data. This model explained a significant proportion of the total variation in the data (R2=0.72, p≤0.001). All treatments had an increase in daily N2O emissions post wetting typical of a hot moment apart from the 30% to 60% WFPS treatment. In terms of drought intensity, the 40% to 90% WFPS was significantly larger than rest, probably due to a relatively larger change in water potential compared to the other treatments. The response to drought length followed a quadratic curve with a downward linear trend, with the largest emissions observed between 10 and 15 days of drought, and the smallest at 0 and 30 days. We suggest a 2-stage dormancy strategy to explain this, where microbes under dry conditions store osmolytes which are catabolised upon rewetting, however at prolonged negative water potentials this strategy is no longer effective, and so they enter a deeper state of dormancy where they can no longer rapidly respond to the changing water potential. Given the delayed response after rewetting, and the inverted U shaped curve in terms of drought length, it seems likely that the majority of emissions are of biological origin. The soil’s chemistry data suggested that NH4+ was a key factor controlling the emission flux, but the transcriptional and genomic data were inconclusive. This study therefore suggests that future experiments should focus changes in osmolyte accumulation and catabolism as the key explanation for N2O hot moments, rather than changes in genomic and transcriptomic data or soil substrates, which do not always correlate with emissions.

**4.1 Introduction**

Nitrous oxide (N2O) emissions can be produced by a rapid and large increase in a soil’s water content, resulting in emission events that are many times higher than background levels, defined as hot moments (McClain *et al.*, 2003, Bergstermann *et al.*, 2011, Harrison-Kirk *et al.*, 2013, Leitner *et al.*, 2017). Because N2O is a potent greenhouse gas, understanding the dynamics of hot moments is important particularly in determining how changing weather patterns will affect soil N2O emissions. Dodd *et al.* (2021) demonstrated that there has been a significant increase in the number of extreme weather events in the UK over the last 28 years, including compound events where drought has been followed by large amounts of rainfall. Moreover, it is predicted that the frequency of drought and large amounts of rainfall is going to increase in the UK due to climate change (Burke *et al.*, 2010, Pendergrass and Knutti, 2018). While it is still unknown how this will impact soil emissions, one of the first steps is to determine how the relationship between the length of drought, the degree of rewetting, and the degree of drought before rewetting effects the size of the hot moment.

A recent meta-analysis has made several claims regarding the interaction between the size of the hot moment and the drought and rewetting intensity (Barrat *et al.*, 2020). Using secondary data, this study concluded that the lower the water-filled pore space (WFPS) was before rewetting, and the higher the WFPS was after rewetting, the more likely there would be a larger hot moment due to higher peak emissions and a longer duration. However, it was noted that the studies from which the data for the meta-analysis were extracted lacked consistent methods and a standardized approach, and that no study had investigated how the length of drought impacted N2O emissions after rewetting.

Therefore, this incubation study aimed to test the conclusions of (Barrat et al.)Barrat *et al.* (2020) while using the studies suggestions for experimental design by using a consistent core size with the same bulk density, and with measurements after the soil is rewetted for at least 4 days. It also aimed to produce the drought impact curves suggested by (Barrat et al.)Barrat *et al.* (2020) that show the relationship between drought length and the size of the hot moment (i.e. the magnitude of the flux), and how this changes with the drought intensity in terms of the starting WFPS.

In addition, there is still uncertainty regarding the main nitrogen (N) cycling processes that are dominant post wetting. While it seems probable that anaerobic denitrification is the main process, considering the low oxygen content (Baggs, 2011), the classical assumption of reduction from nitrate (NO3-) has been challenged by new research into nitrifier denitrification (Wrage-Mönnig *et al.*, 2018), chemodenitrification (Liu *et al.*, 2018), and the possibility of antecedent conditions affecting N2O reductase (Bergstermann *et al.*, 2011). Unfortunately the lack of studies that control for antecedent conditions and then measure changes in relative abundance of N-cycling transcripts and gene abundance *in-situ*, have inhibited a better mechanistic understanding of the processes involved (Barrat *et al.*, 2020). Chapter 3 did attempt to discern differences according to functional gene abundance, but no explanatory changes were discovered. There is growing evidence that differences in cell lysis and osmolyte expulsion at the time of rewetting between treatments is unlikely to be the reason for differences in emissions (Barnard *et al.*, 2020, Schimel, 2018, Kakumanu *et al.*, 2013). Instead, the priming effect of the antecedent conditions on the microbial community could be favoring a process that produces more N2O than a wet control.

This study, using one soil type, is comprised of two experiments. The first experiment aimed to reveal which drought durations and intensities produce the smallest and largest hot moment responses. Following the conclusions of Barrat *et al.* (2020) we hypothesize that: (H1), the longer the drought, the larger the hot moment, however this effect will plateau; that (H2), it will take a minimum number of drying days before the largest hot moment will be observed; and that (H3), the more intense the drought the greater the hot moment. Also that (H4), the WFPS after rewetting will have a significant effect on emissions.

The second experiment aimed to reveal which key N cycling processes are dominant at the time of rewetting. As previous work failed to discern differences according to gene abundance and soil chemistry (see chapter 3), this study aimed to replicate that result while also measuring changes transcript abundance to discern the major N cycling processes. We therefore predicted that (H5), there would be statistically similar changes in functional nitrogen cycle genes in terms of microbial DNA between treatments and that (H6), the changes in soil chemistry would not account for differences in the N2O emissions. However, we predicted (H7), that changes in transcription abundance would reveal the key N cycling process driving the N2O emissions.

**4.2 Methods**

**4.2.1.1 Experimental design to create drought impact curves**

Five drought and rewetting treatments were selected based on likely values to be measured in summer at the North Wyke Farm Platform (NWFP, <https://nwfp.rothamsted.ac.uk/download>): 40% WFPS rewetted to 90%, 50% WFPS to 90%, 60% WFPS to 90%, 70% WFPS to 90%, and 30% WFPS to 60%. A high rewetting WFPS was selected for 4 of the treatments, as a previous meta-analysis showed that this was necessary to induce a hot moment (chapter 2), and a previous experiment using the same soil showed that 90% WFPS should induce a large hot moment (chapter 3). However, this was further tested with the fifth treatment, which was only to 60% WFPS. Each drought and rewetting treatment had 16 different drought lengths (from 0 to 30 days with two-day intervals, e.g. 0 days drought, 2 days drought, 4 days drought… up to 30 days drought) where the soil was kept at its initial WFPS value before being wetted further. https://nwfp.rothamsted.ac.uk/downloadhttps://nwfp.rothamsted.ac.uk/download

Soil was collected and packed in 80 cores to the same bulk density (5 treatments x 16 drought lengths, see section 2.2), and soil cores incubated in a temperature-controlled room at a constant ~18 °C, where the cores were organized for sampling in a randomized design. The WFPS was adjusted every day to keep it consistent to the specified treatment (see section 2.4). N2O emissions from each soil core were measured for 14 days after it was rewetted to capture the entire hot moment (see section 2.4). It is worth noting that this experiment was designed to generate data suitable for linear regression analysis capturing the relationship and variation across a range of drought lengths and intensities, therefore replication at each drought length (e.g. 3 replicates at 2 days) was not required as background variation could be captured across the range of drought lengths and treatments (see section 4.2.6).

**4.2.1.2 Experimental design for the analysis of N processes**

Using the same grassland soil, two antecedent moisture treatments were selected that were shown to produce a hot moment from section 2.1.1. One set of 18 soil cores were kept at 40% WFPS for 14 days, which we defined as the pre-dry treatment, and another set of 18 soil cores were kept at 70% WFPS for 14 days, which we defined as the pre-wet treatment, both were then rewetted to 90% WFPS and held there for 7 days. Destructive sampling of the cores for chemical and biological analysis was informed by the daily N2O emission data (see section 2.5 and 2.6), and at each time point 3 replicates from each treatment were sampled. Soil was collected and prepared and emissions were measured in the same manner as section 4.2.1.1. A visual summary of both experiments is outlined in Figure 1.

**4.2.2.1 Soil collection and preparation**

Soil was collected from Rothamsted Research’s Rowden grassland site at North Wyke, England, at 9 randomly determined points from the 0 to 10 cm depth using a trowel. The soil is a clayey pelostagnogley also known as a Stagni-vertic Cambisol, and it is mottled throughout with greyish colours, see Table 4.1 for other characteristics (Avery, 1973). Roots, stones and vegetation were removed from the soil by hand, and it was air dried for 6 days, reaching a moisture level of 28% WFPS, assuming a packing density of 0.8 g cm-3 and a particle density of 2.43 g cm-3. Then a mixed pool of soil was created and sieved through a 9 mm sieve. It was stored for 2 days at 4 °C before 9.44 kg of the air dried soil was weighed into 80 polyethylene bags (118 g per bag) representing each core. When the soil was initially put into each bag it was wetted to 30% WFPS. Then each bag was wetted to half its starting WFPS a day before packing the soil cores, and then a day later they were completely wetted to the appropriate initial WFPS when the cores were packed. This was done to stagger the rewetting and prevent an initial hot moment for soil cores starting at a higher WFPS. The 5 treatments with 16 different drought lengths resulted in 80 cores that were packed to a height of 7.6 cm (Greiner Bio-One multipurpose container, 150 ml, metal screw cap, clear, aseptic, item 225170), at a bulk density of 0.8 g cm3. The cores were packed by filling and compacting the cores in thirds, so that the bulk density was consistent throughout the core. The bulk density was selected as a replication of a previous experiment (Barrat *et al.* 2021).

For the second experiment, soil was collected again 10 days later from the same site, for the packing of cores that were going to be destructively sampled. This pool of soil was air dried for 3 days to reach a moisture level of 40% WFPS, which was determined by taking 3 x 50 g samples each day to determine gravimetric moisture content and assuming a packing density of 0.8 g cm3 and a particle density of 2.43 g cm3. It was then passed through a 9 mm sieve and stored for 2 days at 4 °C. Then 4.52 kg of the air dried soil was weighed and divided into 36 polyethylene bags (125.5 g per bag) representing each core. Each bag of soil that represented the 70% WFPS treatment was wetted to 55% WFPS a day before packing the soil cores, and then a day later they were completely wetted to the appropriate initial WFPS when the cores were packed, to stagger the rewetting and prevent an initial hot moment. It is worth noting that the second experiment was not a replication of the first, so drying times and sampling times were different due to the different objectives and treatments.

***Table 4.1.*** *Summary characteristics of pooled soil used to pack the soil cores for the first experiment. Variation is represented using standard error. See section 4.2.3 for methods and Figure 4.6 for the soil characteristics for the second experiment.*

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| *Vegetation cover* | *Soil texture* | *Soil bulk density in the field*  *g cm-3 (n=9)* | *Soil pH (n=5)* | *Total oxidized nitrogen on a dry soil basis (TON) mg kg-1 (n=5)* | *Ammonium (NH4+) on a dry soil basis*  *mg kg-1 (n=5)* | *Dissolved organic carbon (DOC) on a dry soil basis*  *mg kg-1 (n=5)* |
| Grassland  Permanent pasture  (*L.perenne*) | Clay | 0.77 ± 0.05 | 6.1 ± 0.1 | 14.0 ± 0.1 | 8.2 ± 0.1 | 146.9 ± 3.1 |

**4.2.3 Incubation and N2O measurements**

The soil cores were kept in a temperature control room at approximately 18 °C to represent a summers day, and throughout the experiment the cores were weighed daily and the WFPS of each core was adjusted when needed to keep it constant by adding deionized water assuming weight loss due to evaporation. When measuring emissions, the cores were sealed using modified metal screw caps containing two rubber septa, 3 cm apart, allowing the headspace (49.3 cm3 at 7.6 cm packing height) to be sampled via PTFE 3.2 mm tubing.

A Picarro cavity ringdown spectrometer G2508 was used to measure the N2O concentrations. The headspace for each core was sealed for 90 seconds before sampling, and then the flux was measured for 90 seconds. The first 10 seconds of measurement were ignored to allow circulation of the cores’ headspace, and the following 80 seconds of measurement were used to calculate a linear regression from T10 to T90 to generate a flux as outlined by Venterea *et al.* (2020). This was converted from parts per million (ppm) into a concentration based on the ideal gas law, the chamber’s dimensions, and the time of measurement (see equation 4.1).

***Equation 4.1.*** *Used to calculate emissions in terms of grams per meter squared per day. Fppm is the ppm flux, atm is the ambient air pressure, K is the air temperature in kelvin, R is the ideal gas constant in L atm mol-1 K-1, m is the moles of N in the N2O molecule (28), Chv is the chamber volume in m3 and Cha is the chamber area in m2, 0.76188 converts the flux from 1 minute 20 seconds to 1 minute, multiplying by 1440 converts it to a daily flux.*

Measurements were taken twice a day (approximately 2 hours apart), and an average from the two samplings was taken for each core creating a daily flux. This was converted into the cumulative emissions over 14 days for each treatment, by adding up all the daily emissions for 14 days post wetting (see supplementary material for a summary of the data).

***Figure 4.1*** *Visual representation of the two experiments conducted in this study. The cylinders represent soil cores, however for experiment 2 there was a range of replication, n=3 for the destructive sampling to determine changes in soil chemistry and microbiology (at days -14, -1, 0, 1, 2, 3 and 7 marked in bold), but the gas sampling used all the cores that were**available**each day****,*** *so the replication starts at n=36 for -4 days and ends at n=3 for 7 days.*

Antecedent WFPS 70%

Rewetting WFPS 90%

Antecedent WFPS 40%

Rewetting WFPS 90%

**7 days**

6 days

5 days

4 days

**3 days**

**2 days**

**1 day**

**0 days**

**-1 day**

-2 days

-3 days

-4 days

-**14 days**

**Experiment 2. Days are numbered according to when the samples were rewetted, both treatments were kept at their antecedent WFPS for 14 days**

**Experiment 1. Days indicate time spent at antecedent WFPS before being rewetted to its designated WFPS**

Antecedent WFPS 30%

Rewetting WFPS 60%

Antecedent WFPS 70%

Rewetting WFPS 90%

Antecedent WFPS 60%

Rewetting WFPS 90%

Antecedent WFPS 50%

Rewetting WFPS 90%

Antecedent WFPS 40%

Rewetting WFPS 90%

30

days

22

days

26

days

24

days

20

days

28

days

18

days

16

days

14

days

12

days

10

days

8

days

4

days

6

days

2

days

0

days

**4.2.4 Soil chemical analyses**

Soil chemical characteristics were determined by destructive sampling the soil cores on days: -14, -1, 0, 1, 2, 3 and 7. Day 0 marks the day of rewetting, on this day measurements were taken after the soil was rewetted. Dissolved organic carbon (DOC), total extractable oxidized nitrogen (TON) and ammonium (NH4+), was determined on supernatant following 0.5 M potassium sulphate (K2SO4) extractions with 10 g of fresh soil in 50 ml of extractant. Samples were shaken for 30 minutes and then centrifuged (14,000 rpm, 30 minutes), the supernatant was removed and frozen until later analysis (Jones and Willett, 2006). Inorganic N concentrations were then determined using an Aquakem 250 discrete photometric analyzer. DOC concentrations were determined using a Shimadzu TOC-L CPN total organic carbon analyzer, the samples were diluted by a 10x dilution factor, to prevent precipitation in the instrument. Gravimetric moisture was determined using 50 g samples in an oven at 105 °C for 24 hours. Soil pH was determined using a pH meter with a ratio of 5 g to 12 ml of deionized water using a Jenway 3320 pH meter.

**4.2.5 DNA and RNA extractions and determination**

DNA was extracted from the same time points as the destructively sampled chemical analysis (days -14, -1, 0, 1, 2, 3 and 7) whilst RNA was extracted from all time points post wetting (days 0, 1, 2, 3 and 7). Each time point had 3 true replicates per treatment, and each true replicate consisted of 2 technical replicates. To test changes in functional gene abundance, 2 sub samples of soil were taken per soil core replicate and frozen in 15 ml falcon tubes (~20 g of soil per tube) using dry ice, and stored at -80 °C. The soil samples were then freeze-dried to further preserve the integrity of the nucleic acid and improve homogenisation of the samples. In summary, DNA was extracted from 84 soil samples, and RNA was extracted from 60 soil samples.

DNA was extracted using and following the instructions in the DNeasy PowerSoil kit (Qiagen) in batches of 24 (and one batch of 12), using 250 mg of freeze-dried soil. 50 µl of autoclaved deionised water was added to each sample at the beginning of each extraction to rehydrate the sample. The concentrations of DNA were measured using a Qubit dsDNA BR Assay Kit (ThermoFisher) and the quality was assessed on a Nanodrop spectrophotometer (ThermoFischer). Samples were then diluted to 20 ng µl-1 and stored at −80°C prior to qPCR analysis.

RNA was extracted using the RNeasy PowerSoil Total RNA kit (Qiagen), in batches of 12, using 2.0 g of freeze-dried soil. Samples were pre-weighed before the day of extraction using a weighing spatula that was heated until it was red hot and then cooled in ethanol. We added 400 µl of autoclaved deionised water to each sample after adding phenol, to rehydrate the sample and improve the extraction success. For acidic soils, we found that modifying step 7 by adding 0.75 ml of SR5 and 0.75 ml of SR3 (instead of 1.5 ml of SR3), dramatically increased the extraction success, and so all extractions included this modified step. The RNA samples were purified using the DNase Max kit (Qiagen), and the concentrations of RNA were measured using a Qubit RNA BR Assay Kit (ThermoFisher) and the quality was assessed on a Nanodrop spectrophotometer (ThermoFischer). Samples were then diluted to 20 ng µl-1 and stored at −80°C prior to qPCR analysis.

For DNA samples, qPCR analyses were performed as described in de Sosa *et al.* (2018). Briefly, using a 384 plate, each run contained 3 different genes, 24 blanks, 2 no-template blanks per gene, 8 x 3 standards per gene, and 2 positive controls using a standard created from a mix of grassland and arable soil. Specific primers (see supplementary Table 4.1) were employed to quantify gene abundance of microbial kingdoms and genes associated with N cycling using the QuantiFast SYBR® Green PCR Kit (Qiagen) and a Biorad CFX384 Touch Real-Time PCR Detection System. DNA copy numbers are represented on a per gram of dry soil basis (cn g-1). Results were standardised assuming 40 ng ul per well.

RT-qPCR analysis on RNA samples were performed using a 384 plate, each run contained 4 different genes, 2 blanks, 2 no-template blanks, 8 x 3 standards per gene, and 2 positive controls using a standard created from a mix of grassland and arable soil. Specific primers (see Supplementary Table 4.1) were employed to quantify gene abundance of microbial kingdoms and genes associated with N cycling using the RT-QuantiFast SYBR® Green PCR Kit (Qiagen) and a Biorad CFX384 Touch Real-Time PCR Detection System. The list of genes analysed and the qPCR extraction efficiency for each gene is stated in Supplementary Table 4.1.

**4.2.6 Data treatment and statistics for the drought curves**

Genstat 20th edition was used for statistical analysis (VSN, 2020). The experimental data were transformed to satisfy the normality and homogeneity of variance assumptions of the analysis by square rooting the cumulative emissions for each treatment. Curves were then fitted to the data to estimate the relationship between the drought days (on the X axis), and the cumulative N2O emissions post wetting (on the Y axis) for each drought intensity. These curves were fitted using linear regression with a quadratic term included to capture the curvature of the relationship (see Figures 4.2 and 4.3). Sequential F tests were used to determine significance (p≤0.05) of the model terms and therefore how complex the final model needed to be to sufficiently describe the relationships.

A one-way ANOVA was used with the cumulative N2O emissions data as the Y variate and WFPS as the Treatment term and the number of days of drying as the block term. In order to determine if on average the different WFPS intensities affected N2O emissions (adjusting for drought lengths). Cumulative emissions for each treatment were calculated by averaging the emissions for each treatment for each day and totaling all the days post wetting.

**4.2.7 Data treatment and statistics for the analysis of N processes**

The N2O experimental data were transformed to satisfy the normality and homogeneity of variance assumptions of the analysis by square rooting the cumulative emissions for each treatment with an offset of +2, due to some of the samples and timepoints before wetting having a small negative value.

A two-way ANOVA was run with the N2O emissions data as the Y variate, and days from wetting and the wetting treatments (pre-dry or pre-wet) as the treatment factors. However, there were two datasets for the emissions data because only a subset of samples were used for microbial and chemical analysis, but N2O emissions were measured from all the cores. The gas data from all the soil cores is shown in the supplementary material. The same two-way ANOVA was used to determine differences in the soil chemistry, and soil microbiology.

**4.3 Results**

**4.3.1 Drought impact curve gas data**

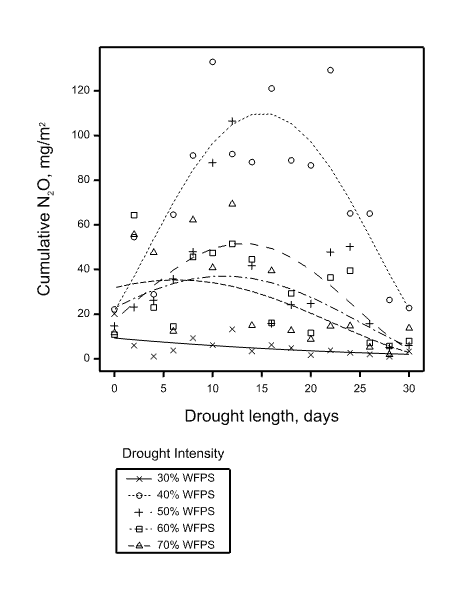
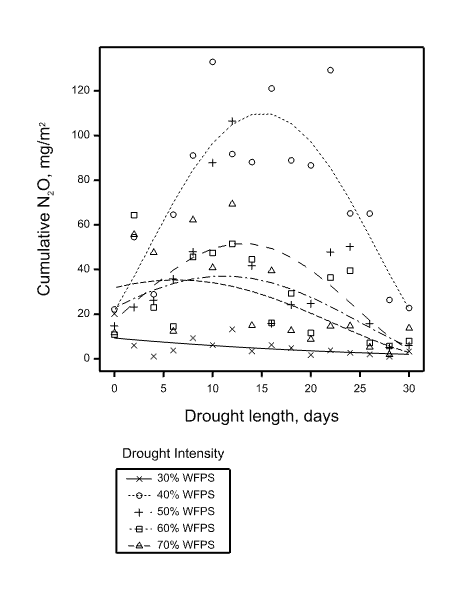
The final model allowed for different slopes and quadratic effects of drought length for each drought intensity. This model explained a significant proportion of the total variation in the data (R2=0.72, p≤0.001). The length of drought had a significant quadratic relationship with the square root of the N2O emissions post wetting (p≤0.001) as well as an overall downward linear trend (p≤0.001). Comparing across all the drought lengths the different wetting intensities had a significant impact on emissions (p≤0.001, 5 treatments, n=16), and cumulative emissions were larger the greater the drought intensity (see Figure 4.2). Although, out of the treatments that were wetted to 90%, only the 40 to 90% (mean 73.68 ± 9.17 mg m-2) was significantly larger, with the other treatments not statistically different (LSD = 13.84 mg m-2, 50 to 90% mean 35.84 ± 7.02 mg m-2 60 to 90% mean 28.44 ± 28.44 mg m-2 and 70 to 90% mean 26.57 ± 5.52 mg m-2). The 30 to 60% (mean 5.57 ± 1.26 mg m-2) had the lowest emissions and this was significantly different to all other wetting treatments.

The response to drought length followed a quadratic curve (see Figures 4.2 and 4.3), with the largest emissions observed between 10 and 15 days of drought. Overall, there was a downward linear trend, with the longest drought durations producing the lowest emissions, which was true for all drought intensities. There is insufficient evidence to suggest that the degree of the downward linear trend changes according to the drought intensity (p=0.19), however the shape of the response to drought length was different according to the drought intensity (p=0.003). This is noticeable in Figures 4.2 and 4.3, with the higher initial WFPS treatments showing a shallower, less pronounced curvature. The daily N2O flux data can be found in the supplementary material.

**4.3.2 N2O emissions for N cycling analysis**

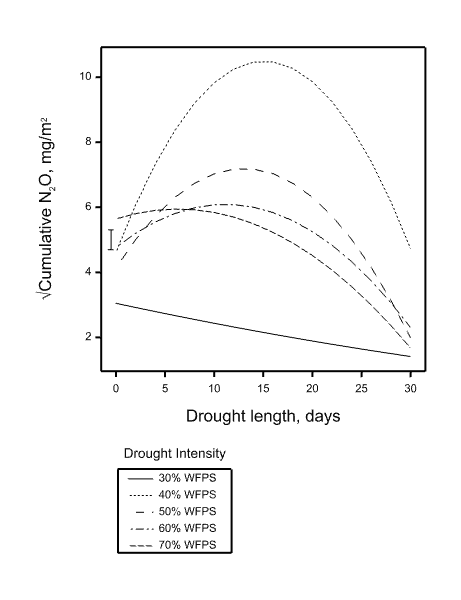
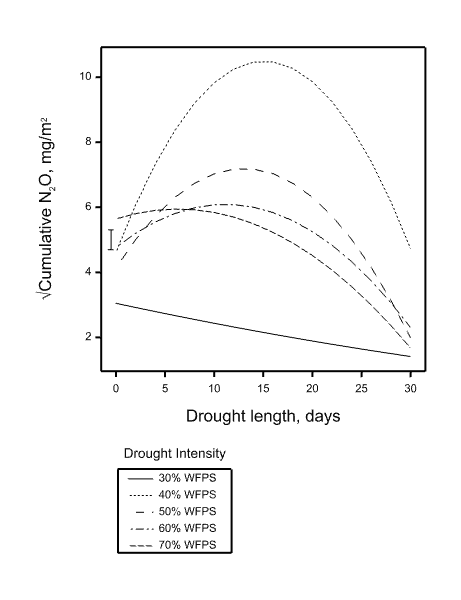
Analyzing N2O-N fluxes at all the timepoints using all the replicates revealed that the pre-dry treatment (n=126, untransformed mean N2O flux 2.21 ± 0.51 mg m-2 day-1) was statistically different than the pre-wet treatment (n=126, untransformed mean N2O flux 0.55 ± 0.12 mg m-2 day-1) p=0.001 (see Supplementary Figure 1). For samples that were destructively sampled, the pre-dry treatment (n=26, untransformed mean N2O flux 3.18 ± 1.31 mg m-2 day-1) was significantly different than the pre-wet treatment (n=26, untransformed mean N2O flux 1.06 ± 0.61 mg m-2 day-1) p=0.037. Peak emissions in the pre-dry treatment occurred on day 2 (n=3, untransformed mean N2O flux 7.32 ± 3.31 mg m-2 day-1), and the peak emissions in the pre-wet treatment also occurred on day 2 (n=12, untransformed mean N2O flux 3.94 ± 2.03 mg m-2 day-1). The untransformed cumulative emissions for the pre-dry soil post wetting were 19.04 mg m-2, which was more than 3 times that of the pre-wet soil post wetting (6.47 mg m-2).

The soil’s N2O flux in relation to days from rewetting regardless of treatment, (time points in days, -4 n=36, -3 n=36, -2 n=36, -1 n=36, 0 n=30, +1 n=24, +2 n=18, +3 n=12, +4 n=6, +5 n=6, +6 n=6,+7 n=6) was significantly different p=0.015. And the interaction between days from rewetting and treatment was not significantly different for the N2O flux (n=6 at each time point in days, -14. -1, 0, +1, +2, +3 and +7) p=0.131. See Figure 4.3.



Cumulative N2O-N mg m-2

***Figure 4.2****. Scatter plot showing the relationship between the different treatments of drought intensity and drought length, and the response in cumulative N2O-N emissions in mg m-2 for 14 days post wetting. All treatments were rewetted to 90% WFPS, apart from 30% WFPS which was wetted to 60%. A linear regression with a quadratic term has been fitted for the different treatments. This was fitted to the square root of the emissions and has been backtransformed.*



Cumulative √N2O-N mg m-2

***Figure 4.3****. Linear model with quadratic term of the relationship between the different treatments of drought intensity and drought length on the X axis, and the response in square root of cumulative N2O-N emissions mg m-2 14 days post wetting. All treatments were rewetted to 90% WFPS, apart from 30% WFPS which was wetted to 60%. The error bar represents the average standard error (1.44) of the predicted responses from the model.*

***Figure 4.4****. The N2O fluxes for the destructively sampled soil cores throughout the experiment. The pre-dry soil was kept at 40% WFPS, and wetted to 90% WFPS at day 0, pre-wet soil was kept at 70% WFPS, and wetted to 90% WFPS at day 0. Error bars represent standard error, n=3.*

**4.3.3 Soil chemistry**

The soil’s pH was significantly different between the pre-dry treatment (n=9, mean pH 5.99 ± 0.05) and the pre-wet treatment (n=9, mean pH 6.17 ± 0.05) p=0.012. The soil’s pH in relation to days from rewetting regardless of treatment, (n=6 at each time point in days, 0, +1, +2, +3 and +7) was significantly different p=0.030. The interaction between days from rewetting and treatment was not significantly different for pH (n=3 at each time point in days, 0, +1, +2, +3 and +7) p=0.537.

The soil’s TON concentrations was not significantly different between the pre-dry treatment (n=26, mean TON 0.020 ± 0.002 mg g-1) and the pre-wet treatment (n=26, mean TON 0.024 ± 0.002 mg g-1) p=0.112. The soil’s TON in relation to days from rewetting regardless of treatment, (n=6 at each time point in days, -14, -1, 0, +1, +2, +3 and +7) was significantly different p=0.001. The interaction between days from rewetting and treatment was not significantly different for TON (n=3 at each time point in days, -14. -1, 0, +1, +2, +3 and +7) p=0.856. See Figure 4.5.

The soil’s NH4+ concentrations were significantly different between the pre-dry treatment (n=26, mean NH4+ 0.036 ± 0.003 mg g-1) and the pre-wet treatment (n=26, mean NH4+ 0.031 ± 0.002 mg g-1) p<0.001. The soil’s NH4+ in relation to days from rewetting regardless of treatment, (n=6 at each time point in days, -14. -1, 0, +1, +2, +3 and +7) was significantly different p<0.001. The interaction between days from rewetting and treatment was significantly different for NH4+ (n=3 at each time point in days, -14, -1, 0, +1, +2, +3 and +7) p=0.009. See Figure 4.5.

The soil’s DOC concentrations was significantly different between the pre-dry treatment (n=26, mean DOC0.297 ± 0.013 mg g-1) and the pre-wet treatment (n=26, mean DOC0.256 ± 0.015 mg g-1) p=0.03. The soil’s DOCin relation to days from rewetting regardless of treatment, (n=6 at each time point in days, -14, -1, 0, +1, +2, +3 and +7) was significantly different p=0.026. The interaction between days from rewetting and treatment was not significantly different for DOC (n=3 at each time point in days, time points -14, -1, 0, +1, +2, +3 and +7) p=0.939. See Figure 4.5.

***Figure 4.5.*** *Soil chemistry changes for total extractable oxidized nitrogen (TON), ammonium (NH4+) and dissolved organic carbon (DOC) on a dry soil basis. The pre-dry soil was kept at 40% WFPS, and wetted to 90% WFPS at day 0, pre-wet soil was kept at 70% WFPS, and wetted to 90% WFPS at day 0. Error bars represent standard error, n=3 for each treatment at each timepoint*

**4.3.4 Microbiology**

Due to the large quantity of variables measured, the ANOVA table for DNA and RNA can be found in the supplementary material. In summary, the soil’s DNA concentrations in terms of copy number per gram of dry soil for targeted genes (Supplementary Table 1) was significantly different between the pre-dry treatment and the pre-wet treatment (n=26, time points in days, -14, -1, 0, +1, +2, +3 and +7) for *16S B* (p<0.026), *ITS* (p<0.001) and *16S P* (p<0.007), see Figure 6 and Supplementary Table 2. In terms of days from rewetting, (n=6 per day, time points -14, -1, 0, +1, +2, +3 and +7) the following genes were significantly different: *16S A* (p<0.001)*, 16S P* (p<0.015)*, ITS* (p<0.001)*, AmoA* (p<0.027)*, nirS* (p<0.0390)*, nirK* (p<0.017)*, nosZI* (p<0.007)*, nosZII* (p<0.001)*, comaB* (p<0.026). In terms of the interaction between treatment and days (n=3), only *ITS* was significantly different (p<0.001).

The following gene transcripts (*AmoA, AmoB, nirS, nirK, nosZI, nosZII* and *norB)* were below the level of detection under these experimental conditions despite increasing the total amount of total RNA per PCR from 20 ng to 40 ng. Only Pseudomonas 16S rRNA gene transcripts, in terms of copy number per gram of dry soil, was significantly different between the pre-dry treatment and the pre-wet treatment (n=15, time points in days, 0, +1, +2, +3 and +7), *16S P* (p=0.021) see Figure 6 and Supplementary Table 2. In terms of days since rewetting, (n=6 per day, time points in days, 0, +1, +2, +3 and +7) the following gene transcripts were significantly different: *16S B* (p=0.021)*, 16S A* (p=0.006)*, 16S P* (p<0.001)*,* and *ComaB* (p=0.015)*.* In terms of the interaction between treatment and days (n=3), *16S A* (p=0.012) and ComaB (p<0.001) were significantly different.

**4.4 Discussion**

This study consisted of two experiments, the first induced a range of antecedent treatments to determine the relationship between the size of an N2O hot moment, and the drought length and drought intensity. The second experiment consisted of two treatments selected from the first experiment to elucidate how changes in soil chemistry and microbiology are driving the higher N2O emissions post wetting. All treatments had an increase in daily N2O emissions post wetting, and all treatments besides the 30% to 60% WFPS treatment had a response that is typical of a hot moment from a unfertilized soil (Priemé and Christensen, 2001, Molodovskaya *et al.*, 2012, Harrison-Kirk *et al.*, 2013, Leitner *et al.*, 2017, Barrat *et al.*, 2021), wherein the daily emissions post wetting are >10 times the daily emissions before rewetting, over a 24 to 72 hour period.

**4.4.1 Drought impact curves**

Drought length had a significant impact on the size of a N2O hot moment, which is shown in Figures 2 and 3, which we have defined as drought impact curves. The relationship that was observed was an inverted U shape, which is contrary to what was hypothesized (H1), as it was assumed to have an increasing positive linear response as drought intensity has been shown to have this relationship (Priemé and Christensen, 2001, Ruser *et al.*, 2006, Harrison-Kirk *et al.*, 2013). Because the size of hot moment increases and then decreases according to drought length, this has significant implications for predicting the size of hot moments, as future studies which will need to determine the degree of moderate drought that will induce the greatest response, and define the limit for when a severe drought will rapidly reduce the emissions post wetting. Moreover, while the model shows a continued curve (see Figure 3), it is clear from the raw data (see Supplementary Table 3) that as predicted (H2) it took >6 days of drying before the hot moment started to increase in size, therefore future incubation studies will also need to determine the minimum drought period required before a hot moment could be induced.

We assumed that increasing drought intensity would increase the size of the hot moment (H3); with the caveat that the final WFPS after rewetting needed to create a sufficiently anaerobic soil for there to be a large increase in N2O emissions (H4), and this was observed, with very low emissions from the 30% to 60% WFPS treatment comparative to the other treatments. It is possible that this explains the results of studies like Pezzolla *et al.* (2019) and Owens *et al.* (2016) that induced drying and wetting cycles, but did not observe an N2O hot moment, as the treatments never created an ideal environment for anaerobic respiration. However, in our study the increase in cumulative emissions as the drought intensity was increased was only significant for 40 to 90% (p<0.05), the other treatments that were wetted to 90% were not significantly different from each other. The large difference in emissions from the 40% WFPS treatment compared to the others, could be due to an important shift in the soil’s matric potential, as although water content is linear, matric potential is extremely non-linear, and the 40% WFPS treatment could be situated either side of the soil’s capillary fringe (Whalley *et al.*, 2013). Although the 50%, 60% and 70% drought curves were statistically similar, we predicate that this is an artifact of the statistical and experimental approach. We therefore hypothesize that if this experiment were repeated with replicates for the drought length treatments between 10 and 20 days, the fitted curves would be more accurate and there would be more power to detect differences between wetting intensities.

**4.4.2 Changes in microbiology**

For the soil cores that were destructively sampled, we observed a decrease in the abundance of most of the functional genes from DNA post wetting, which could be due to cell death as the absolute change in water potential, and the rate of change over time is a stress event that may cause cell death and cell lysis (Schimel, 2018). Clark and Hirsch (2008), showed that the viability of culturable bacteria and extractable DNA decreased in soils that were air dried at ambient conditions prior to long-term archiving. This supports the decrease in functional genes and viability of bacterial soil communities shown in this study under drought conditions. As expected (H5) there was no difference in the quantity of functional N cycling genes between the pre-dry and pre-wet treatments, but there were two population markers that might account for the differences in emissions, fungal ITS and pseudomonas 16S (see Figure 6). Fungal ITS decreased less in the pre-dry soil, and it is well established that fungi are better at surviving changing water potentials (Schimel, 2018, Barnard *et al.*, 2013, Evans and Wallenstein, 2012). Pseudomonas 16S increased post wetting in the pre-dry after a slight decrease. Increases in rRNA population markers could either indicate rapid growth or rapid activity, and many pseudomonas are well known relatively fast growing denitrifiers (Davies *et al.*, 1989), so this population could be the source of N2O from their growth and activity. Moreover, the delay in the response of this population marker, matches the delay in the peak emissions.

***Figure 4.6.*** *The change in different functional genes throughout the experiment which were significantly different (p<0.05) according to treatment.**Soil was rewetted at day 0, error bars represent standard error, n=3.*

**4.4.3 Hot moments and the key nitrogen pathways**

Although we hypothesised (H7) that the transcriptional data could explain the differences in emissions, the lack of differences between the two treatments in terms of mRNA and to some extent DNA, suggests that the active processes are more complex than initially thought. Firstly, N2O does not seem to be produced due to an increase in anaerobic denitrification transcript or DNA abundance via NO2- (*nirS*, *nirK*) or NO (*norB*). Secondly, it does not seem to be due to a change in the rate of reduction of N2O to N2 (*nosZI*, *nosZII*). Chemodenitrification is possible if there are high concentrations of NH2OH or NO2- and low concentrations of O2, however the differences in TON between the treatments were not statistically significant, and it is typically observed after N is artificially added (Liu *et al.*, 2019, Liu *et al.*, 2018, Anderson and Levine, 1986). Moreover, it took several days to reach the peak N2O emissions, whereas an abiotic source would produce a peak within several hours (Leitner *et al.*, 2017). Wang et al (2017), however demonstrated that within short-term waterlogged soils, although the relative abundance of denitrifiers within the soil did not significantly change, the composition of these microbial denitrifiers did change and potentially to more active populations. Analysis of shifts in microbial community structure were not within the scope of this study. However, an increase in microbial activity, although not necessarily picked up in gene abundance, should have been seen in increases in associated N-cycling transcripts, which were not observed in this study.

There were slight and significant differences in NH4+ which contrary to our hypothesis (H6) could be indicating the key process (see Figure 5). In this study some form of nitrifier activity seems the most probable explanation for the differences in N2O emissions, as NH4+ decreased rapidly in both treatments during rewetting (Leitner *et al.*, 2017). There was no significant difference between treatments for DNA or RNA in terms of AmoA, AmoB, and COMAMMOX *AmoB*. Caranto and Lancaster (2017) propose that NH4+ is oxidised to NO via NH2OH, which can produce N2O as a non-enzymatic by-product. This could be likely given the lack of *nirK*, *nirS* or *norB* activity, and it is possible that NH4+ is being oxidised to NH2OH using an, as yet, undetermined gene. It is also possible that the reduction in NH4+ is not related to the increase in N2O, and instead it is being utilised via the ANNAMOX pathway, producing N2. This seems unlikely given that the literature has shown the important contribution NH4+ addition can have on the emission pulse following rewetting (Slessarev *et al.*, 2021, Leitner *et al.*, 2017, Heil *et al.*, 2016, Zhu *et al.*, 2013).

If NH4+ is being oxidised to NH2OH, then further oxidation using hydroxylamine oxidoreductase (*HAO*) can produce large amounts of N2O either via NO or straight to N2O, and this can occur under anaerobic conditions (Caranto and Lancaster, 2017, Caranto *et al.*, 2016, Otte *et al.*, 1999, Hooper and Terry, 1979). Moreover, there is increasing evidence that nitrifiers under oxic shock will utilise NO2- and NH2OH (Liu *et al.*, 2019, Liu *et al.*, 2018, Wrage-Mönnig *et al.*, 2018). Caranto *et al.* (2016) propose a pathway that involves the oxidation of NH2OH which produces N2O, NO and NO2- as a by-product. This could be the case in this study, however we did not measure *HAO* transcription levels or its genetic abundance. Besides NH2OH oxidation, there is also the possibility of nitrifier denitrification which typically involves the use of NO2- as the electron acceptor. It is predicted that NO2- builds up in dry soil and therefore could be readily utilised upon rewetting (Liu *et al.*, 2018). However, we measured no changes in *nirS* or *nirK* in terms of gene expression or in terms of functional gene abundance. In this study the pH was significantly different between treatments, however the difference is biological negligible in terms of affecting N2O (less than 0.2) (ŠImek and Cooper, 2002). The difference was likely caused by the continuous rewetting of the pre-wet samples.

**4.4.4 Future studies and the use of metabolomics**

Given the inconclusive evidence from this study, and the current pool of literature, we suggest a new approach for future studies, which is based on the methods for exploring a similar phenomena known as the Birch effect, where rewetted soils produce a pulse in CO2 emissions.

The work of Warren (Warren, 2020, Warren, 2014a, Warren 2014b) has used metabolomics to investigate the Birch effect, and has made significant progress in understanding how osmolytes fuel the emissions pulse. This could be a relevant analogue for N2O hot moments, as the use of nitrogenous osmolytes is common in cell cultures and in soil (Schimel, 2018, Warren, 2014). Studies exploring the Birch effect and osmolyte accumulation have shown that it does not follow a linear response to drying, as accumulation of osmolytes is observed under moderate dryness, but extreme drying conditions seems to supress this strategy (Warren, 2016, Kakumanu *et al.*, 2013). This is because osmolyte accumulation is costly and at certain water potentials as it no longer provides effective osmoregulation. This matches the inverted U shape response in emissions observed in study. It is therefore possible in this study that the time delay could indicate the mineralisation and then catabolism of osmolytes, but this will need to be further investigated This study used only one soil type, and this soil was sieved and had no vegetation cover, therefore future studies using different soil types with plant cover might observe a different a response to changing antecedent moisture conditions.

**4.5 Conclusions**

In summary, this study outlined the relationship between the size of the N2O response post wetting, and the antecedent conditions of drought length and intensity. For this grassland soil, there is an inverted U shaped response in terms of drought days, with 10 to 15 days of drying showing the largest response, while 0 and 30 days show the smallest. We suggest a 2-stage dormancy strategy to explain this, where microbes under dry conditions store osmolytes which are catabolised upon rewetting, however at prolonged negative water potentials this strategy is no longer effective, and so they enter a deeper state of dormancy, resulting in a dormant microbial community that can no longer rapidly respond to the changing water potential. From this experiment, we hypothesise that the source of the N2O emissions is from the mineralisation of osmolytes. Moreover, given the delayed response after rewetting, and the inverted U shaped curve in terms of drought length, it seems likely that the majority of emissions are of biological origin. Furthermore, given the lack of transcriptional activity in terms of *nirK*, *nirS*, A*moA, AmoB* and *norB*, we suggest that pathways proposed by Caranto *et al.* (2016) and Caranto and Lancaster (2017) seem probable where N2O is a product of nitrifier activity from the oxidation of NH2OH.

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**Chapter 4 Supplementary material**

***Supplementary Table 4.1.*** List of primers and qPCR efficiency

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Target gene | DNA/RNA | Primer | Sequence 5'-3' | qPCR efficiency | References |
| *Bacterial 16S rRNA region*  *(16S B)* | DNA and RNA | 341F | CCT AYG GGR BGC ASC AG | DNA = 84.3% | Glaring *et al.* (2015) |
| 806R | GGA CTA CNN GGG TAT CTA AT | RNA = 100.9% |
| *Archaeal 16S rRNA region*  *(16S A)* | DNA and RNA | Parch519F | CAG CMG CCG CGG TAA | DNA = 104.7% | Øvreas 1997  Reysenbach 1995 |
| Arch1060R | GGC CAT GCA CCW CCT CTC | RNA = 99.5% |
| *Pseudomonas 16S rRNA (16S P)* | DNA and RNA | 16S\_PseuF1  16S\_PseuR1 | CTT CGG GCC TTG CGC TAT CA | DNA = 103.0% | Clark and Hirsch, 2008 |
| GCCCTTCCTCCCAACTTAA | RNA = 93.6% |
| *Fungal Internal transcribed spacer*  *(ITS)* | DNA | ITS1f | TCC GTA GGT GAA CCT GCG G | DNA = 93.2% | Gardes and Bruns (1993) Vilgalys and Hester (1990) |
| 5.8s | CGC TGC GTT CTT CAT CG |  |
| *Nitrite Reductase Gene (nirK)* | DNA and RNA | nirK876F | ATY GGC GGV CAY GGC GA | DNA = 115.1% | Henry *et al.* (2004) |
| nirK1040R | GCC TCG ATC AGR TTR TGG TT | RNA = 109.0% |
| *Nitrite Reductase Gene (nirS)* | DNA and RNA | cd3aF | GTS AAC GTS AAG GAR ACS GG | DNA = 82.1% | Throbäck *et al.* (2004)  Hallin *et al.* 1999 |
| R3cdR | GAS TTC GGR TGS GTC TTG A | RNA = 81.5% |
| *Nitrous Oxide Reductase Clade I (nosZI)* | DNA and RNA | nosZ1F | CGC RAC GGC AAS AAG GTS MSS GT | DNA = 85.4% | Henry *et al.* (2006) |
| nosZ1R | CAK RTG CAK SGC RTG GCA GAA | RNA = 113.3% |
| *Nitrous Oxide Reductase Clade II (nosZII)* | DNA and RNA | nosZIIF\_1162-1178 | CTI GGI CCI YTK CAY AC | DNA = 88.0% | Jones *et al.* 2013 |
| nosZIIR\_1889 -1907 | GCI GAR CAR AAI TCB GTR C | RNA = 84.4% |
| *Nitric oxide reductase subunit B (norB)* | DNA and RNA | qnorB5R-F | TGG TGG GTN GTN CAY CTN TGG GT | DNA = 83.5% | Braker and Tiedje (2003) |
| qnorB7R | GGN GGR TTD ATC ADG AAN CC | RNA =112.3% |
| ammonia monooxygenase Archaeal (AmoA) | DNA and RNA | arch-amoAF | STA ATG GTC TGG CTT AGA CG | DNA = 83.7% | Francis et al 2005 |
| arch-amoAR | GCG GCC ATC CAT CTG TAT GT | RNA = 83.2% |
| Ammonia monooxygenase Bacterial (amoB) | DNA and RNA | amoA-1F | GGG GTT TCT ACT GGT GGT | DNA = 101.2% | Rotthauwe et al (1997) |
| amoA-2R | CCC CTC KGS AAA GCC TTC TTC | RNA = 99.6% |
| Comammox bacteria (ComaB) | DNA and RNA | comaB-244F  comaB-659R | TAY TTC TGG ACR TTY TA  ARA TCC ARA CDG TGT G | DNA = 95.7% | Pjevac et al 2017 |

***Supplementary Table 4.2.*** *p values from ANOVA for the functional genes for DNA and RNA. Treatment represents the pre-dry or the pre-wet treatment, and days the day the soil cores were sampled. For the experimental design see section 2, results where p<0.05 are in bold.*

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **DNA** | | | | | | | | | | | |
| **Source of variation** | *16S B* | *16S A* | *16S P* | *ITS* | *amoA* | *amoB* | *nirS* | *nirK* | *nosZ1* | *nosZ2* | *comaB* | *norB* |
| Treatment | **0.026** | 0.097 | **0.007** | **0.015** | 0.910 | 0.124 | 0.631 | 0.936 | 0.521 | 0.851 | 0.153 | 0.105 |
| Days | 0.258 | **0.001** | **0.015** | **0.001** | **0.027** | 0.138 | **0.039** | **0.017** | **0.007** | **0.001** | **0.026** | 0.768 |
| Treat.Days | 0.465 | 0.635 | 0.281 | **0.001** | 0.281 | 0.487 | 0.587 | 0.095 | 0.059 | 0.310 | 0.373 | 0.096 |

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **RNA** | | | | |
| **Source of variation** | *16S B* | *16S A* | *16S P* | *ComaB* | *amoA* |
| Treatment | 0.959 | 0.755 | **0.021** | 0.084 | 0.403 |
| Days | **0.021** | **0.006** | **0.001** | **0.015** | 0.353 |
| Treat.Days | 0.158 | **0.012** | 0.398 | **0.001** | 0.536 |

***Supplementary Table 4.3.*** *A summary of the daily N2O-N emissions data organized according to the timeline of rewetting in mg m-2 per day. At day 0 samples were rewetted to 90% waterfilled pore space (WFPS) apart from the 30% treatment which was rewetted to 60%. Cumulative emissions are calculated from day 0 to day 14. The missing data at the very beginning are due to constraints on experimental design, as all soil cores had to be packed and wetted on the same day.*

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Treatment | WFPS | Drying days | Day  0 | Day  1 | Day  2 | Day  3 | Day  4 | Day  5 | Day  6 | Day  7 | Day  8 | Day  9 | Day  10 | Day 11 | Day 12 | Day 13 | Day 14 | Cumulative N2O mg/m2 |
| 40W 0 | 40 | 0 |  | -0.2 | 6.0 | 2.3 | 3.6 | 2.9 | 2.1 | 1.4 | 0.6 | 0.6 | 0.4 | 0.6 | 0.7 | 0.6 | 0.4 | 22.1 |
| 40W 2 | 40 | 2 | 1.2 | 17.7 | 13.0 | 6.2 | 4.7 | 2.9 | 1.6 | 1.6 | 0.7 | 1.1 | 1.1 | 1.1 | 0.5 | 0.7 | 0.3 | 54.5 |
| 40W 4 | 40 | 4 | 2.1 | 12.1 | 3.8 | 2.2 | 1.0 | 1.0 | 0.9 | 0.8 | 0.6 | 0.5 | 0.7 | 0.6 | 0.7 | 1.0 | 1.0 | 28.9 |
| 40W 6 | 40 | 6 | 0.0 | 15.5 | 11.7 | 4.7 | 5.7 | 4.4 | 4.2 | 3.5 | 1.5 | 2.5 | 2.8 | 2.3 | 2.0 | 2.3 | 1.7 | 64.5 |
| 40W 8 | 40 | 8 | 0.0 | 22.5 | 24.0 | 11.9 | 6.4 | 4.7 | 3.4 | 3.0 | 2.9 | 2.4 | 2.3 | 2.1 | 1.9 | 1.7 | 1.7 | 91.1 |
| 40W 10 | 40 | 10 | 10.8 | 35.4 | 24.7 | 18.8 | 10.0 | 6.4 | 4.0 | 3.7 | 2.9 | 2.6 | 3.0 | 2.6 | 2.9 | 3.0 | 2.1 | 133.0 |
| 40W 12 | 40 | 12 | 1.0 | 18.5 | 30.6 | 14.3 | 8.0 | 4.0 | 2.9 | 2.5 | 1.7 | 2.2 | 1.2 | 1.4 | 1.0 | 1.5 | 1.1 | 91.7 |
| 40W 14 | 40 | 14 | 1.2 | 18.0 | 23.7 | 12.2 | 6.5 | 5.1 | 2.1 | 3.4 | 3.3 | 2.5 | 2.5 | 2.2 | 2.0 | 2.0 | 1.4 | 88.1 |
| 40W 16 | 40 | 16 | 0.3 | 18.3 | 25.7 | 24.0 | 9.9 | 8.9 | 6.4 | 4.5 | 4.3 | 3.7 | 2.7 | 2.9 | 3.2 | 3.3 | 3.0 | 121.1 |
| 40W 18 | 40 | 18 | 0.3 | 15.4 | 15.2 | 14.7 | 8.1 | 7.0 | 5.2 | 5.4 | 3.9 | 3.0 | 2.3 | 2.2 | 1.8 | 2.3 | 2.1 | 88.9 |
| 40W 20 | 40 | 20 | 0.5 | 7.9 | 19.0 | 12.4 | 11.6 | 9.9 | 4.6 | 4.4 | 2.6 | 2.5 | 1.8 | 2.1 | 4.0 | 1.8 | 1.4 | 86.6 |
| 40W 22 | 40 | 22 | 0.3 | 5.9 | 21.2 | 27.8 | 19.0 | 13.1 | 11.2 | 6.2 | 6.1 | 5.4 | 2.1 | 3.8 | 4.0 | 4.1 | -0.9 | 129.3 |
| 40W 24 | 40 | 24 | 0.2 | 17.0 | 13.4 | 13.4 | 5.1 | 3.6 | 3.1 | 2.8 | 2.0 | 1.4 | 1.5 | 1.2 | -0.7 | 1.1 | 0.3 | 65.1 |
| 40W 26 | 40 | 26 | 0.7 | 5.6 | 15.1 | 14.9 | 11.4 | 6.7 | 3.0 | 1.8 | 1.8 | 1.5 | 1.1 | 0.7 | 0.5 | 0.2 | 0.1 | 65.0 |
| 40W 28 | 40 | 28 | 0.1 | 4.1 | 8.1 | 7.8 | 2.5 | 1.5 | 0.8 | 0.6 | -0.1 | 0.4 | 0.1 | -0.1 | -0.1 | 0.5 | 0.2 | 26.4 |
| 40W 30 | 40 | 30 | 0.2 | 5.4 | 9.5 | 2.7 | 1.8 | 0.9 | -0.1 | 0.5 | 0.3 | 0.3 | -0.1 | 0.2 | 0.1 | 0.2 | 0.7 | 22.7 |
| 50W 0 | 50 | 0 |  | 0.6 | 2.5 | 1.1 | 3.0 | 3.1 | 1.9 | 0.8 | 0.2 | 0.1 | 0.8 | 0.0 | 0.3 | -0.1 | 0.3 | 14.7 |
| 50W 2 | 50 | 2 | 0.6 | 4.4 | 9.3 | 3.6 | 1.6 | 1.0 | 0.4 | 0.2 | 0.5 | 0.2 | 0.4 | 0.2 | 0.3 | 0.1 | 0.3 | 23.1 |
| 50W 4 | 50 | 4 | 1.4 | 12.4 | 7.1 | 1.9 | 0.5 | 0.6 | 0.3 | 0.4 | 0.4 | 0.1 | 0.3 | 0.3 | 0.0 | 0.4 | 0.1 | 26.2 |
| 50W 6 | 50 | 6 | 0.2 | 6.5 | 6.8 | 6.1 | 4.7 | 3.5 | 3.5 | 2.2 | 0.4 | 0.3 | 0.5 | 0.1 | 0.3 | 0.3 | 0.3 | 35.8 |
| 50W 8 | 50 | 8 | 0.1 | 10.6 | 12.8 | 6.5 | 4.6 | 2.8 | 2.2 | 1.9 | 1.3 | 1.0 | 1.1 | 1.0 | 0.7 | 0.7 | 0.6 | 48.0 |
| 50W 10 | 50 | 10 | 1.3 | 10.2 | 21.2 | 14.1 | 14.2 | 8.4 | 7.0 | 3.5 | 1.9 | 1.9 | 1.3 | 1.1 | 0.9 | 0.7 | 0.1 | 87.8 |
| 50W 12 | 50 | 12 | 0.6 | 4.7 | 20.5 | 18.5 | 12.8 | 11.5 | 7.5 | 7.6 | 4.9 | 4.6 | 3.2 | 3.5 | 2.4 | 2.3 | 1.8 | 106.5 |
| 50W 14 | 50 | 14 | 0.8 | 12.3 | 11.4 | 6.1 | 2.8 | 2.0 | 1.9 | 1.4 | 0.8 | 0.4 | 0.4 | 0.5 | 0.6 | 0.1 | 0.2 | 41.7 |
| 50W 16 | 50 | 16 | 0.5 | 2.0 | 3.5 | 4.0 | 0.7 | 1.3 | 0.3 | 0.9 | 0.5 | 0.6 | 0.5 | 0.2 | 0.4 | 0.0 | 0.4 | 15.8 |
| 50W 18 | 50 | 18 | 0.4 | 3.9 | 4.9 | 6.5 | 3.1 | 2.2 | 1.1 | 0.1 | 0.8 | 0.2 | 0.2 | 0.5 | 0.0 | 0.2 | 0.1 | 24.3 |
| 50W 20 | 50 | 20 | 0.5 | 8.7 | 5.4 | 2.9 | 1.3 | 1.5 | 1.3 | 0.7 | 0.5 | 0.4 | 0.3 | 0.4 | 0.1 | 0.3 | 0.3 | 24.7 |
| 50W 22 | 50 | 22 | 0.3 | 3.6 | 9.1 | 8.6 | 5.5 | 4.1 | 3.1 | 3.6 | 2.4 | 1.8 | 1.4 | 1.5 | 1.4 | 1.1 | 0.4 | 47.8 |
| 50W 24 | 50 | 24 | 0.3 | 11.7 | 14.2 | 7.3 | 4.1 | 3.1 | 2.5 | 2.4 | 1.4 | 0.7 | 0.5 | 0.5 | 0.3 | 0.5 | 0.6 | 50.3 |
| 50W 26 | 50 | 26 | 0.4 | 1.6 | 5.0 | 2.7 | 1.8 | 1.5 | 1.3 | 0.4 | 0.4 | 0.2 | 0.0 | 0.1 | 0.3 | 0.1 | 0.1 | 15.7 |
| 50W 28 | 50 | 28 | 0.5 | 0.8 | 1.0 | 0.7 | 0.3 | 0.2 | 0.2 | 0.1 | 0.2 | 0.3 | 0.1 | 0.1 | 0.2 | 0.0 | 0.3 | 5.0 |
| 50W 30 | 50 | 30 | 0.4 | 1.1 | 2.8 | 0.5 | 0.5 | 0.1 | 0.1 | 0.2 | 0.3 | 0.1 | 0.2 | 0.0 | 0.1 | 0.0 | -0.3 | 5.9 |
| 60W 0 | 60 | 0 |  | 0.2 | 2.0 | 2.3 | 0.9 | 2.4 | 1.4 | 0.4 | 0.2 | 0.2 | 0.3 | 0.2 | 0.0 | 0.0 | 0.3 | 10.8 |
| 60W 2 | 60 | 2 | 0.1 | 3.0 | 27.8 | 22.3 | 5.7 | 1.5 | 0.9 | 0.8 | 0.5 | 0.4 | 0.3 | 0.4 | 0.1 | 0.4 | 0.1 | 64.3 |
| 60W 4 | 60 | 4 | 4.6 | 14.6 | 2.4 | 0.5 | 0.2 | 0.2 | 0.2 | 0.0 | 0.1 | -0.1 | -0.1 | 0.2 | -0.2 | 0.3 | 0.1 | 23.0 |
| 60W 6 | 60 | 6 | 0.2 | 3.1 | 2.9 | 2.3 | 1.7 | 1.4 | 0.4 | 0.7 | 0.4 | 0.4 | 0.3 | 0.2 | 0.2 | 0.2 | 0.1 | 14.4 |
| 60W 8 | 60 | 8 | 0.2 | 6.2 | 15.4 | 6.5 | 5.0 | 3.2 | 2.3 | 1.9 | 1.4 | 1.4 | 1.0 | 0.1 | 0.4 | 0.4 | 0.2 | 45.6 |
| 60W 10 | 60 | 10 | 1.5 | 4.1 | 7.0 | 6.2 | 6.5 | 6.1 | 4.5 | 3.6 | 2.0 | 1.9 | 1.5 | 1.0 | 0.7 | 0.3 | 0.6 | 47.4 |
| 60W 12 | 60 | 12 | 0.7 | 5.1 | 11.9 | 8.1 | 5.5 | 4.6 | 3.4 | 3.0 | 2.1 | 2.1 | 1.8 | 1.2 | 0.7 | 0.7 | 0.6 | 51.5 |
| 60W 14 | 60 | 14 | 0.4 | 6.5 | 7.1 | 5.0 | 4.5 | 3.7 | 2.4 | 2.8 | 2.5 | 2.1 | 2.0 | 1.5 | 1.7 | 1.1 | 1.3 | 44.5 |
| 60W 16 | 60 | 16 | 0.5 | 2.5 | 4.9 | 3.3 | 1.1 | 1.1 | 0.3 | 0.5 | 0.5 | 0.6 | 0.1 | 0.0 | 0.2 | 0.0 | 0.5 | 16.1 |
| 60W 18 | 60 | 18 | 0.7 | 2.7 | 5.6 | 3.7 | 2.9 | 2.5 | 1.9 | 1.7 | 1.4 | 1.5 | 1.2 | 0.8 | 0.9 | 0.9 | 1.0 | 29.3 |
| 60W 20 | 60 | 20 | 0.2 | 2.4 | 3.9 | 1.5 | 1.8 | 0.5 | 0.4 | 0.2 | 0.1 | 0.1 | 0.2 | -0.2 | 0.1 | 0.1 | 0.2 | 11.6 |
| 60W 22 | 60 | 22 | 0.3 | 1.9 | 4.1 | 6.3 | 4.0 | 3.0 | 2.6 | 3.7 | 2.3 | 2.4 | 1.9 | 1.0 | 1.4 | 1.7 | -0.3 | 36.4 |
| 60W 24 | 60 | 24 | 0.2 | 4.9 | 7.1 | 5.1 | 3.8 | 2.9 | 3.1 | 3.1 | 2.4 | 1.4 | 2.2 | 1.2 | -0.1 | 1.5 | 0.7 | 39.4 |
| 60W 26 | 60 | 26 | 0.4 | 0.7 | 2.0 | 1.6 | 1.3 | 0.5 | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.0 | 0.1 | 0.0 | -0.4 | 7.1 |
| 60W 28 | 60 | 28 | 0.2 | 0.3 | 1.9 | 1.6 | 0.3 | 0.5 | 0.3 | 0.2 | 0.7 | -0.1 | -0.1 | 0.0 | -0.1 | 0.1 | -0.1 | 5.7 |
| 60W 30 | 60 | 30 | 0.2 | 1.2 | 3.0 | 0.6 | 0.7 | 0.5 | 0.6 | 0.5 | 0.4 | 0.3 | 0.2 | 0.1 | -0.3 | 0.0 | -0.1 | 7.9 |
| 70W 0 | 70 | 0 |  | 0.2 | 0.7 | 1.5 | 2.0 | 3.0 | 1.2 | 0.4 | 0.3 | 0.3 | 0.6 | 0.1 | 0.5 | 0.6 | 0.3 | 11.6 |
| 70W 2 | 70 | 2 | -0.1 | 1.7 | 29.6 | 16.4 | 4.1 | 0.5 | 1.2 | 0.2 | 0.3 | 0.3 | 0.3 | 0.5 | 0.1 | 0.2 | 0.4 | 55.6 |
| 70W 4 | 70 | 4 | 2.9 | 13.4 | 3.1 | 7.1 | 8.2 | 7.5 | 4.7 | -0.7 | 0.0 | 0.1 | 0.2 | 0.0 | 0.4 | 0.3 | 0.5 | 47.6 |
| 70W 6 | 70 | 6 | 0.4 | 2.7 | 2.0 | 2.1 | 1.2 | 0.5 | 0.8 | 0.7 | 0.3 | 0.4 | 0.4 | 0.1 | 0.3 | 0.3 | 0.1 | 12.3 |
| 70W 8 | 70 | 8 | 0.2 | 16.5 | 16.2 | 9.2 | 5.4 | 4.5 | 2.8 | 2.0 | 1.7 | 1.0 | 0.6 | 0.6 | 0.4 | 0.4 | 0.7 | 62.2 |
| 70W 10 | 70 | 10 | 0.4 | 10.6 | 14.0 | 6.6 | 3.5 | 1.1 | 1.2 | 0.7 | 0.6 | 0.5 | 0.6 | 0.4 | 0.3 | 0.2 | 0.1 | 40.8 |
| 70W 12 | 70 | 12 | 0.3 | 3.1 | 10.7 | 12.4 | 10.8 | 10.0 | 5.5 | 4.5 | 2.0 | 2.9 | 1.8 | 1.9 | 1.2 | 0.9 | 1.3 | 69.3 |
| 70W 14 | 70 | 14 | 0.6 | 2.4 | 3.2 | 2.0 | 1.6 | 1.0 | 0.9 | 0.4 | 0.5 | 0.5 | 0.5 | 0.4 | 0.5 | 0.1 | 0.3 | 14.9 |
| 70W 16 | 70 | 16 | 0.3 | 6.6 | 10.9 | 7.1 | 4.0 | 2.5 | 2.0 | 1.8 | 0.9 | 1.1 | 0.6 | 0.5 | 0.5 | 0.4 | 0.3 | 39.4 |
| 70W 18 | 70 | 18 | 0.4 | 1.8 | 3.3 | 2.4 | 1.5 | 1.0 | 0.6 | 0.4 | 0.5 | 0.0 | 0.3 | 0.1 | 0.1 | 0.3 | -0.1 | 12.6 |
| 70W 20 | 70 | 20 | 0.6 | 2.3 | 2.0 | 1.8 | 0.7 | 0.5 | 0.2 | 0.1 | 0.2 | 0.1 | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 | 8.7 |
| 70W 22 | 70 | 22 | 0.2 | 0.7 | 1.7 | 3.9 | 2.6 | 1.0 | 0.9 | 0.9 | 0.5 | 0.6 | 0.3 | 0.6 | 0.3 | 0.3 | 0.1 | 14.6 |
| 70W 24 | 70 | 24 | 0.2 | 2.2 | 2.0 | 2.1 | 1.0 | 0.7 | 0.9 | 1.0 | 0.6 | 1.0 | 0.8 | 0.9 | 0.5 | 0.4 | 0.4 | 14.7 |
| 70W 26 | 70 | 26 | 0.1 | 0.7 | 1.7 | 1.1 | 0.7 | 0.3 | 0.1 | 0.3 | 0.2 | 0.1 | -0.2 | 0.1 | 0.0 | 0.0 | 0.0 | 5.2 |
| 70W 28 | 70 | 28 | 0.2 | 0.2 | 0.5 | 0.6 | 0.3 | -0.1 | 0.2 | 0.0 | -0.2 | 0.1 | 0.0 | 0.2 | 0.0 | -0.2 | 0.2 | 1.9 |
| 70W 30 | 70 | 30 | 0.3 | 1.7 | 2.0 | 2.2 | 2.4 | 3.2 | -0.1 | 0.8 | 0.2 | 0.4 | 0.2 | 0.2 | 0.0 | 0.0 | 0.0 | 13.7 |
| 30W 0 | 30 | 0 |  | 4.8 | 0.1 | 8.8 | 0.2 | 1.1 | 0.5 | 0.9 | 0.4 | 1.7 | 0.9 | 0.7 | 0.1 | 0.1 | 0.0 | 20.2 |
| 30W 2 | 30 | 2 | 0.0 | 2.7 | 0.7 | 0.6 | 0.0 | 0.4 | 0.4 | 1.1 | 0.1 | 0.2 | 0.0 | 0.1 | -0.1 | 0.0 | -0.2 | 6.0 |
| 30W 4 | 30 | 4 | 0.5 | 0.4 | 0.2 | -0.1 | 0.1 | 0.4 | -0.3 | 0.1 | -0.2 | 0.0 | 0.1 | 0.0 | 0.3 | -0.1 | -0.2 | 1.1 |
| 30W 6 | 30 | 6 | 0.1 | 1.0 | 0.7 | 0.5 | 0.5 | 0.4 | 0.1 | 0.1 | 0.1 | 0.0 | 0.1 | 0.2 | 0.0 | 0.2 | -0.1 | 3.8 |
| 30W 8 | 30 | 8 | 0.0 | 1.6 | 5.2 | 0.3 | 0.3 | 0.6 | 0.4 | 0.1 | -0.1 | 0.3 | -0.1 | 0.1 | 0.0 | 0.1 | 0.3 | 9.3 |
| 30W 10 | 30 | 10 | 3.2 | 1.2 | -1.2 | 1.2 | 0.2 | 0.6 | 0.4 | 0.1 | 0.2 | 0.0 | 0.0 | 0.1 | 0.1 | 0.2 | 0.0 | 6.2 |
| 30W 12 | 30 | 12 | 0.2 | 0.6 | 2.2 | 1.0 | 1.5 | 1.9 | 0.9 | 0.5 | 1.6 | 0.4 | 0.4 | 0.3 | 0.6 | 0.9 | 0.2 | 13.3 |
| 30W 14 | 30 | 14 | 0.4 | 0.0 | 0.6 | 0.5 | 0.2 | 0.5 | 0.1 | 0.0 | 0.5 | 0.3 | -0.1 | 0.2 | 0.1 | 0.1 | 0.1 | 3.4 |
| 30W 16 | 30 | 16 | 0.1 | 0.7 | 1.1 | 1.1 | 1.0 | 0.6 | 0.5 | 0.5 | 0.3 | 0.1 | 0.4 | -0.2 | 0.2 | 0.1 | -0.1 | 6.2 |
| 30W 18 | 30 | 18 | 0.4 | 0.5 | 0.9 | 1.2 | 0.6 | 0.1 | 0.1 | 0.3 | 0.2 | 0.3 | 0.3 | -0.2 | 0.0 | 0.1 | 0.1 | 4.9 |
| 30W 20 | 30 | 20 | 0.0 | 1.1 | 0.0 | 0.1 | 0.1 | 0.2 | 0.1 | -0.3 | -0.1 | 0.0 | 0.1 | 0.0 | 0.2 | 0.1 | 0.1 | 1.7 |
| 30W 22 | 30 | 22 | 0.1 | 0.2 | 0.1 | 0.6 | 0.7 | 0.2 | 0.2 | 0.5 | 0.2 | 0.1 | 0.0 | 0.3 | 0.3 | 0.1 | 0.2 | 3.9 |
| 30W 24 | 30 | 24 | 0.0 | 0.2 | 0.3 | 0.5 | 0.2 | 0.2 | 0.2 | 0.0 | 0.0 | -0.1 | 0.3 | 0.2 | 0.2 | 0.4 | 0.2 | 2.8 |
| 30W 26 | 30 | 26 | 0.1 | 0.3 | 0.6 | 0.3 | 0.2 | 0.2 | 0.2 | -0.1 | -0.1 | 0.1 | 0.1 | -0.1 | 0.1 | 0.0 | 0.0 | 2.1 |
| 30W 28 | 30 | 28 | 0.4 | 0.5 | 0.2 | 0.2 | 0.0 | 0.0 | 0.1 | 0.1 | 0.0 | 0.1 | 0.0 | -0.1 | -0.2 | 0.0 | -0.2 | 1.0 |
| 30W 30 | 30 | 30 | 0.3 | 0.3 | 0.7 | 0.2 | 0.5 | 0.4 | 0.8 | 0.1 | 0.3 | -0.1 | -0.1 | -0.1 | 0.0 | -0.1 | 0.1 | 3.3 |

***Supplementary Figure 4.1****. Average daily N2O-N fluxes for all subsamples throughout the experiment. The pre-dry soil was kept at 40% WFPS, and wetted to 90% WFPS at day 0, pre-wet soil was kept at 70% WFPS, and wetted to 90% WFPS at day 0. Error bars represent standard error, varied replicate number each time point, see the methods section for details.*

**Chapter 5**

**The impact of long-term inorganic and organic fertiliser additions on soil properties and GHG emissions from the Broadbalk wheat trial**

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**Key words:** Farmyard Manure, Nitram, Legacy, Nitrous Oxide, Carbon Dioxide, Methane

**Highlights:**

1. GHG emissions were measured for 2 years from 3 fertiliser treatments
2. The FYM treatment had the largest soil carbon pools and the highest CO2 emissions
3. The inorganic treatment recorded the largest N2O emissions but overall is was not significantly different from the FYM treatment
4. CO2 dominated the CO2e ratio, and therefore the FYM treatment produced the most CO2e emissions

**Authors contributions:**

1. **H.A. Barrat:** Led the design, field work, interpreted the results, and wrote all drafts.
2. **L. Cardenas:** Helped with minor edits, and the experimental design.
3. **I.M. Clark:** Helped with field work.
4. **M. Lopez:** Helped with field work.
5. **M. Abadie:** Helped with field work, and the experimental design.
6. **D.R. Chadwick:** Helped with minor edits, and the experimental design.
7. **J. Carter:** Helped with field work.
8. **A. Neal:** Helped with field work and funding.

**Abstract**

The Broadbalk long-term wheat experiment in Harpenden, UK has received applications of organic and inorganic fertilisers for over 140 years, and offers a unique opportunity to compare the differences in greenhouse gas (GHG) emissions resulting from the legacy effects of inorganic and organic fertiliser use. Therefore, this study aimed to compare differences between the farmyard manure (FYM), inorganic nitrogen and control plots (where only phosphorus and potassium was applied). GHG fluxes (CO2, N2O, and CH4) and soil analyses (0-10cm; TON, NH4+, DOC, %C, %N, pH) were made throughout 2019 and 2020, using a clustered design, where sampling frequency increased immediately after fertilisation application. Plots were replicated 4 times for each treatment, and GHG emissions were measured using the static chamber method, with 3 chambers representing each plot. The FYM treatment had more than double the amount of SOC (+160%) compared to the other treatments, and almost triple the amount of %C. The increased carbon content decreased the soil’s bulk density, relative to the other treatments by 0.15 g cm-3 and created a significantly larger mean CO2 flux from the FYM treatment. The largest N2O emissions were recorded from the inorganic treatment post fertiliser application. Moreover, the soil TON and NH4+ concentrations were significantly higher for the inorganic treatment, but this did not result in significantly higher mean N2O emissions overall. It is likely that increased organic matter content from long-term FYM application enhanced microbiological and biochemical activity and provided a carbon source for denitrifiers resulting in statistically similar N2O emissions to the inorganic fertiliser treatment. CH4 fluxes were not affected by the different fertiliser regimes in this study, probably because the variables that typically affect CH4 emissions were not impacted by the selected treatments (the soils are not organic or peaty and did not experienced prolonged periods of flooding). Overall, CO2 emissions were affected by distal controls and N2O emissions by proximal controls. We suggest that future work should focus on how other soil functions have been impacted by the fertilisation legacy of these plots, specifically drought resilience and leaching, as well as meso and micro pore structure.

**5.1 Introduction**

Nitrous oxide (N2O), carbon dioxide (CO2) and methane (CH4) emissions from soil are important contributors to climate change (Stocker, 2014). There are short term and long-term controls which affect how much is emitted from the soil, this has been previously described in terms of distal and proximal controls within the context of tropical soils (Robertson, 1989). More recently, Wallenstein *et al.* (2006) updated this concept for N2O emissions and it is relevant for other biogenic gases. In short, proximal controls are processed through distal controls and distal controls effect the microbial community and therefore how the short-term controls affect emissions (see Figure 5.1). For example the addition of cattle slurry or farmyard manure to a soil, would initially be classified as a proximal control as it adds an immediate source of carbon and nitrogen (Heintze *et al.,* 2017) which is processed by the microbes potentially resulting in greenhouse gas (GHG) emissions. If this practice continues it could become a distal control, as it could change the soil structure, the soil’s carbon pools and the hydrology of the soil (Pagliai *et al*., 2004; Stemmer *et al*., 2000) and therefore the distribution and composition of the microbial community throughout the soil (Stemmer *et al*., 2000). This in turn ultimately controls how the soil reacts to other proximal controls, including further additions of slurry (Heintze *et al.,* 2017).

Distal controls: Climate, geology, vegetation, long-term tilling practices etc

Proximal controls: Temperature, moisture, O2, mineral N etc

GHG emissions

***Figure 5.1.*** *This diagram outlines how biogenic GHG emissions from soil are driven by the interaction between distal controls which affect the composition of the microbial community and their distribution within the soil, and the proximal controls which represent short term variables like temperature that affect the activity of the microbial community, adapted from Wallenstein et al., (2006) and Robertson (1989).*

**5.1.1 Proximal/short term controls**

One of the most significant proximal controls on GHG emissions from soil is carbon (C) and nitrogen (N) availability, which is typically modified by the type and quantity of fertiliser used. An important distinguishing factor of fertilisers is how labile they are. Organic fertilisers with higher C:N ratios (e.g. >20:1) are more stable and mineralize slower and promote nutrient immobilisation, whereas fertilisers with lower C:N ratios (e.g. <10:1) or with no C at all (e.g. ammonium nitrate) will mineralize much more rapidly, and therefore are considered to be much more labile (Brust, 2019; Bhogal *et al.,* 2016; Chadwick *et al.*, 2000). The concept of organic and inorganic fertilisers makes a similar distinction, organic fertilisers typically have higher C:N ratios and are derived from animal or plant by-products, whereas inorganic fertilisers are typically manufactured and have no C at all or much lower C:N ratios. Of course the scale is continuous, and some organic fertilisers like urea can be just as labile as other inorganic fertilisers (Prasad *et al*., 2010).

The more labile the fertiliser, the more likely there will be a large increase in the quantity of available (mineral) N and C in the soil post fertilization (Sosulski *et al.,* 2014; Barton *et al.,* 2016; Piva *et al.,* 2019; Heintze *et al.*, 2017). This has the greatest effect on N­2O emissions, which are typically highest throughout the year post fertiliser application, correlating with large increases in mineral N (Sosulski *et al.,* 2014; Plaza-Bonilla *et al.*, 2014; Kontopoulou *et al.*, 2015; Barton *et al.*, 2016; Krauss *et al.,* 2017). The more N added to the soil the higher the emission peak post fertilization (Ball *et al.,* 2004; Zhang *et al.*, 2012; Carmo *et al.*, 2013; de Urzedo *et al.*, 2013; Nyamadzawo *et al.*, 2017). Moreover, differences in soil ammonium (NH4+) and nitrate (NO3-) concentrations affect the size of the N2O flux (Rabenarivo *et al.*, 2014), and will affect the redox pathway to the N2O flux.

The N2O emissions in the period after fertiliser application can represent a significant proportion of the total annual N2O flux. For example, Cai *et al.* (2013) noted that for two of the years they monitored emissions, the 2 week post fertilization period accounted for >60% of the annual total N2O emitted. Although for the other year it only accounted for <34% of the annual flux, and this was due to a much higher flux later in the year due to a high WFPS (>75%) from a failed drainage system. Higher soil moisture and temperature are usually strongly correlated with N2O emissions (Rabenarivo *et al.,* 2014; Sosulski *et al.,* 2014; Sosulski *et al.*, 2015; Toonsiri *et al.,* 2016; Sosulski *et al.*, 2019; Brenzinger *et al.*, 2018), sometimes more so than mineral N (Brenzinger *et al.,* 2018). This is particularly the case if the fertiliser takes a longer period of time to mineralize. Moreover, temperature is an important controlling factor for nitrogen cycling due to its effect on microbial activity, higher temperatures can increase the nitrification rate (as long as the aeration status is favourable) resulting in more NO3- in the soil (Yin *et al.*, 2017).

The CO2 flux of soils is also increased by higher temperatures and dry and wet cycles (Morell *et al.,* 2010; López-Garrido *et al.,* 2014; Yonemura, 2014). Organic fertiliser application can significantly affect CO2 emissions if the amendment is rich in labile carbon. However, both CO2 and CH4 emissions are more dependent on other proximal environmental variables, like temperature, soil moisture and oxygen status. Higher moisture levels can raise the CO2 flux (Franzluebbers 1999) and the timing of rainfall events also has a significant effect on N2O emissions (Koga *et al.,* 2004; van Bruggen *et al.,* 2017; Boardman *et al.,* 2018; Rahman *et al.,* 2019). It is rare to see changes in CH4 fluxes from non-flooded mineral soils (Carmo *et al.,* 2013; Brenzinger *et al.,* 2018; Bosco *et al.* (2019) and it often forms a small part of the total flux in terms of CO2 equivalents (CO2e). However, organic amendments are more likely to cause short-term peaks in CH4 emissions (Ball *et al*., 2004; Plaza-Bonilla *et al.,* 2014), especially if the soils become flooded (Koga *et al.,* 2004; Xie *et al.,* 2013).

**­5.1.2 Distal/long-term controls**

The long-term controls or distal controls affect the distribution and composition of the microbial community, as well as the structure and hydrology of the soil. Therefore, they ultimately determine the impact of the short-term controls such as adding mineral N. For example, grassland soils, arable soils and woodland soils have significantly different properties, because of the effect of plants roots and the plants life cycle on the soil structure and the residing microbial communities. For arable soils, there are two key distal controls: the fertiliser type and the tilling method (Paustian *et al.,* 2019), which ultimately affect the soil C and N pools. The CO2 flux depends on how easily accessible and large the soil organic carbon (SOC) pool is (Hurisso *et al.,* 2016) and N2O emissions are also affected by long-term changes in N availability and water retention, which is correlated to the amount of OM and SOC in the soil (Rawls *et al.,* 2003).

Larger C pools encourage greater N denitrification rates (Cheng *et al.,* 2016; Barton *et al.,* 2016), and the application of organic fertilisers increases total N pools as well as the amount of organic matter (OM) and therefore the quantity of SOC (Hakala *et al.,* 2012; Ding *et al.,* 2013). Incorporating straw and crop residues, also increases the SOC pool (Lugato *et al*., 2006; Patiño-Zúñiga *et al.,* 2009; Patra *et al*., 2014). For example, after nine years Wang *et al.,* (2019) recorded an increase in SOC of 5-9% from straw addition, whereas Zhang *et al.* (2017) recorded an increase of 52% after 30 years when combined with high fertilisation rates.

The stabilisation of added carbon depends on the soil’s initial carbon pool, the climate and other management practices like tillage method and rotation of crops (Johnson *et al*., 1995; Stemmer *et al*., 2000; Plaza-Bonilla *et al.,* 2014; Nath *et al.,* 2017). In mineral soils the larger the SOC pool the more unstable it is and the less likely that adding organic matter will increase the SOC pool (Chen *et al.,* 2016; Hua *et al.,* 2014). The quality of carbon is important in this regard. The more recalcitrant the carbon source the lower the carbon turnover and therefore the higher likelihood that it will remain in the soil (Xie *et al.,* 2013; Patra *et al*., 2014; Hua *et al.,* 2014; Yan *et al.,* 2015; Cheng *et al.,* 2016; Heintze *et al.,* 2017; Nath *et al.,* 2017). There are examples showing that it can take decades for SOC concentrations to reach an equilibrium (Bolinder *et al.,* 2006; Hua *et al.,* 2014; Dhadli and Brar, 2016; Krauss *et al.,* 2017; Chen *et al.,* 2016). The type of tillage also has significant effects on SOC retention levels (Sandén *et al.,* 2018; Álvaro-Fuentes *et al.,* 2013), with reduced tillage and conservation tillage retaining SOC, and encouraging greater build-up of OM (Marland *et al.,* 2003; Álvaro-Fuentes *et al.,* 2009; Álvaro-Fuentes *et al.,* 2013; Sandén *et al.,* 2018). This in turn affects the soil’s porosity and the movement of water and gas through the soil, as well as providing substrates for the microbial community. Application of higher amounts of inorganic N can result in greater available nitrogen pools in the soil (Lugato, *et al.*, 2006; Lopez-Valdez *et al.,* 2011; Plaza-Bonilla *et al*., 2014) although these do not necessarily manifest into long-term differences.

As mentioned previously, SOC levels are a major control on CO2 emissions, and because the application of organic fertilisers significantly increases the SOC content of the soil (Ryals and Silver, 2013; Ding *et al.,* 2013; Hua *et al.,* 2014), their use can result in a larger overall CO2 flux compared to inorganic fertilisers (Plaza-Bonilla *et al.*, 2014; Dhadli and Brar, 2016; van Bruggen *et al.*, 2017). For N2O emissions, it seems that from field studies, proximal controls are more important, with the amount of mineral N, the soil moisture content and the soil temperature being the main influencing factors.

**5.1.3 Exploring the effect of both distal and proximal controls on GHG emissions**

Understanding how the proximal and distal controls interact with each other is important in understanding the amount of GHG emissions that are emitted from a soil, and which mitigation strategies would be effective at reducing the amount produced. The Broadbalk long-term wheat experiment in Harpenden, UK which has received applications of organic and inorganic fertilisers for 140 years, and it therefore offers a unique opportunity to not only compare the differences in GHG emissions resulting from inorganic and organic fertiliser use, but also the impact of management legacy on soil conditions and ultimately the effects of proximal and distal factors on GHG fluxes.

Therefore, this study aimed to compare differences in GHG emissions between fertiliser types, specifically the difference between application of farm yard manure (FYM) and inorganic nitrogen at a relatively high rate (240 kg N/ha) and a control (where only phosphorus and potassium is applied) from the Broadbalk experiment. An additional aim was to analyse how this difference might be affected by the management legacy. Following on from the previous discussion, we propose the following hypotheses:

H1: There will be a significant difference in the legacy effect of organic fertiliser vs inorganic fertiliser in terms of total annual GHG emissions, expressed as CO2e, and CO2 will form the largest part of the CO2e.

H2: The higher carbon content in the organic fertiliser treated plots will increase the potential for CO2 emissions from respiration compared to an unfertilised control and a treatment given a relatively high rate of inorganic fertiliser.

H3: The inorganic fertiliser treatment will have a higher N2O flux compared to the other treatments, and the highest N2O fluxes will occur within 30 days of fertiliser application.

H4: It is unlikely that CH4 emissions will be significant in organic and inorganic amended plots and the annual GHG emissions will be dominated by CO2 and N2O in terms of CO2e.

**5.2 Methods**

**5.2.1 Site and treatments**

Broadbalk is an arable field and is part of the long-term arable experiments based at Rothamsted Research, in Harpenden, UK (GB Grid Reference: TL123 136). To control for vegetation cover, the continuous wheat sections were selected for this experiment. The farmyard manure (FYM) plot 2.2 (220 kg N ha-1: single application of 35 tonnes of cattle manure, on 18/09/2019 and 22/09/2020) and the inorganic nitrogen plot 15 (240 kg N ha-1: single application of nitram on 12/04/2019 and 21/05/2020) were selected for comparison, plot 5 was also selected as control, as this has had only phosphorus and potassium application. Details of the historical agronomic and nutrient management of these treatments can be found at <http://www.era.rothamsted.ac.uk/experiment/rbk1#datasets>. Times of sampling and fertilisation are noted in Table 5.1. All sections grew winter wheat (Zyatt) in 2019, and due to the very wet winter the sections were not re-sown until the following year where a spring wheat (Tybalt) was planted instead (25/03/2020). Emissions were measured at all stages of wheat growth.

A visualisation of the sections and plots/strips can be found in the supplementary material. Selecting the continuous wheat sections (0, 1, 6 and 9), allows for 4 replicates per plot, which represent the length of the field. Due to the nature of this long-term experiment, the replicates are slightly different from each other, as the sections also have an additional treatment factor. The plots on section 0 and 1 are slightly larger than 6 and 9, and section 0 has the straw incorporated into its plots. Moreover, section 6 has no spring or summer fungicides, and section 9 was re-drained in 1993. Therefore, a block design was used in the statistical analysis (see section 2.4) to account for variation between the replicates. Measurements were taken throughout 2019 and 2020, and due to experimental constraints soil samples were not always taken alongside GHG measurements (see Table 5.1). Therefore, two datasets were generated, one with all the gas measurements (27 sampling dates), and one with both gas and soil measurements (19 dates).

**5.2.2 Greenhouse gas emission measurements**

Greenhouse gas emissions were measured using the static chamber method, the chambers were rectangular 25 cm (height) x 40 cm (length). Chambers were stacked at the appropriate growth stage to incorporate wheat growth, and at their highest the Zyatt variety required 3 stacked chambers. Three static chambers were placed on each plot, to capture the emission variance within plots. These pseudo reps were averaged to represent the whole plot. The timing of the gas sampling following fertiliser and manure applications followed a similar design to that of Cardenas *et al.* (2019) with greater frequency of sampling around the dates of each fertiliser application (see Table 5.1). Headspace samples were taken using a 50 ml syringe and needle, and placed in pre-evacuated 20 ml vials at 0 minutes (T0) and 40 minutes (T40) for all chambers, and for two chambers there was an additional 20 minute (T20) and 60 minute (T60) time point to check the linearity of headspace gas concentrations in the chamber. The flux for each chamber was calculated using linear regression and the ideal gas law (Venterea *et al.,* 2020). Quality of the flux was assessed using a visual inspection of each flux and the R2 values of each chamber. A flux was assumed to be non-linear if a fitted quadratic linear regression was significantly different than the null regression model (p<0.05) (Chadwick *et al.,* 2014).

**Table 5.1.** *List of sampling dates and days from fertilisation for each treatment, note that due to experimental constraints, soil collection was limited as outlined below.*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Sampling Dates | Variables sampled | Days from fertilisation | | |
| Plot 5 (Control) | Plot 2.2 (FYM) | Plot 15 (Inorganic) |
| 11/04/2019 | Gas and soil | n/a | 206 | 364 |
| 16/04/2019 | Gas only | n/a | 210 | 4 |
| 18/04/2019 | Gas and soil | n/a | 212 | 6 |
| 23/04/2019 | Gas and soil | n/a | 217 | 11 |
| 29/04/2019 | Gas and soil | n/a | 223 | 17 |
| 01/05/2019 | Gas only | n/a | 225 | 19 |
| 07/05/2019 | Gas and soil | n/a | 231 | 25 |
| 09/05/2019 | Gas only | n/a | 233 | 27 |
| 21/05/2019 | Gas and soil | n/a | 245 | 39 |
| 18/06/2019 | Gas and soil | n/a | 273 | 67 |
| 20/06/2019 | Gas only | n/a | 275 | 69 |
| 15/07/2019 | Gas and soil | n/a | 300 | 94 |
| 05/08/2019 | Gas and soil | n/a | 321 | 115 |
| 17/09/2019 | Gas and soil | n/a | 364 | 158 |
| 03/10/2019 | Gas and soil | n/a | 15 | 174 |
| 07/10/2019 | Gas and soil | n/a | 19 | 178 |
| 28/01/2020 | Gas and soil | n/a | 132 | 291 |
| 25/02/2020 | Gas and soil | n/a | 160 | 319 |
| 05/03/2020 | Gas and soil | n/a | 169 | 328 |
| 17/09/2020 | Gas and soil | n/a | 365 | 119 |
| 02/10/2020 | Gas and soil | n/a | 10 | 134 |
| 08/10/2020 | Gas and soil | n/a | 16 | 140 |
| 15/10/2020 | Gas and soil | n/a | 23 | 147 |
| 22/10/2020 | Gas and soil | n/a | 30 | 154 |
| 29/10/2020 | Gas and soil | n/a | 37 | 161 |
| 05/11/2020 | Gas and soil | n/a | 44 | 168 |

The static chamber approach used here is prone to errors derived from GC instrumental noise and temperature and pressure changes within the chamber resulting in artificial fluxes (Cowan *et al.,* 2014). For each date the T0 values were used to calculate a confidence interval for a zero flux. The standard error of the T0 values was used to calculate a least significant difference (LSD=SE\*1.96) which was then converted to a flux that indicated the range of fluxes that could be considered to be not statistically different to zero. If the observed fluxes were within this range, we assumed that this was noise and these were zeroed (Cardenas *et al.,* 2010). To test if there were significant differences between using either ambient gas measurements or measurements in the chamber as T0, 6 ambient measurements (3 before and 3 after sampling) were taken at every date and these were averaged and used to calculate a flux.

GHG concentrations were determined in the vials on a PerkinElmer Clarus 500 Gas Chromatograph (GC) equipped with a TurboMatrix 110 automated headspace sampler. Carbon dioxide equivalent (CO2e) for N2O emissions was calculated assuming CO2e = N2O x 298, and for CH4 it was calculated assuming CO2e = CH4 x 25 over a 100 year time horizon (Solomon *et al.,* 2007).

**5.2.3 Soil sampling and chemical analysis**

For each replicate section (4 sections per plot, 3 plots in total) a composite of 3 soil samples were taken at a depth of 0-10 cm using a trowel (~ 2 cm by 2 cm surface area). Immediately after sampling in the field, 20 g of soil from each composite replicate was dried for 24 hours at 105 °C to determine gravimetric water content, which was converted to water filled pore space using equation 1 (WFPS).

***Equation 1.*** *Calculation of the soil’s WFPS, the soil’s water weight is equivalent to the gravimetric water content, the soil’s pore volume is calculated using the soil’s bulk density, particle density and the volume of the soil core that was sampled.*

After removing any stones and flint by hand from each replicate, the remaining soil was refrigerated at 4 °C. Extractions of 0.5 M K2SO4 (10 g to 50 ml) using fresh soil were undertaken the next day. The samples were shaken for 30 minutes and then centrifuged (14,000 rpm, 30 minutes), the supernatant was removed and frozen until later analysis (Jones and Willett 2006). TON, NH4+ and DOC concentrations were determined using a Skalar Continuous Flow Analyser (Skalar SANPLUS System) by spectrophotometry. Soil pH was measured using 5 g of fresh soil to 12.5 ml deionised water on a Jenway 3320 pH meter. Soil was air dried for 1 week, and then sieved and milled (<2 mm) for total carbon and nitrogen analysis, using combustion (Dumas method) and a LECO TruMac Combustion Analyser.

In summary, TON, NH4+ and DOC were analysed from 264 soil samples (n=88 per treatment), pH was measured from 180 soil samples (n=60 per treatment), and total N and C was measured from 96 soil samples (n=32 per treatment). Note that 9 DOC samples had to be omitted due to the plastic storage tubes cracking and splitting when the supernatant was frozen.

**5.2.4 Statistical methods**

Genstat 20th edition was used for all statistical analysis. To satisfy the normality and equal variance assumptions of the statistical methods the following variables were transformed: CO2, CH4, CO2e, TON and NH4 were square rooted, and N2O was logged to the base 10 with an offset of 1. Days after fertilisation application, is the last time the treatment was fertilised in days, and this was included in the statistical analysis. Two datasets were analysed, a gas only dataset (26 sampling dates, n=104) and a gas and soil dataset (19 sampling dates, replicates were varied see Table 5.1). Differences between treatments were determined using ANOVA for each variable, with the following structure: Treatment\*Date, and with a blocking design of Section/Plot. To assess the relationship between the soil variables and gas emissions, all possible subset selection was used to identify linear regression models that sufficiently explain the variation in the data. This was run on the gas and soil dataset for all treatments together (using all variables) and then each treatment separately. Each model was assessed using its AIC and adjusted R2 values. The simplest models that explained the highest amount of variation were selected, with the criteria that a decrease in AIC of 2, or an increase of <5% in the adjusted R2 did not justify adding another explanatory variable. Error values and bars represent standard error, unless stated otherwise.

**5.3 Results**

**5.3.1 Gas only dataset**

Using all time points, the one-way ANOVA (see Table 5.2) showed that there were significant differences in terms of CO2 and CO2e between all the treatments (n=108, p<0.001) with the FYM treatment having the highest CO2 and CO2e mean (29.4 kg ha-1 d-1 and 30.0 kg ha-1 d-1), followed by the inorganic treatment (20.7 kg ha-1 d-1 and 21.1 kg ha-1 d-1), and then the control treatment (6.7 kg ha-1 d-1 and 6.1 kg ha-1 d-1) (see Figure 5.3). In terms of CO2e, CO2 contributed the largest the percentage (mean average of all time points 81% ± 15%, n=104) followed by N2O (19% of the total ± 15%, n=104) and then CH4 (1% of the total ± 1%, n=104). For N2O only the control treatment was significantly different from the inorganic and FYM treatment (p<0.001, n=104). However, there was large variance in the N2O emissions between the different treatments with a significant difference between the interaction of treatment and time (n=104, p=0.01). For example, in the inorganic treatment values were recorded between -66.49 and 77.2 g N ha-1 d-1, for the FYM treatment we recorded values between -54.8 and 26.5 g N ha-1 d-1 and for the control treatment we recorded values between -58.3 to 11.2 g N ha-1 d-1. The largest N2O emissions were observed in the inorganic treatment, within 40 days of fertiliser application. For CH4, all 3 treatments showed no significant difference (p=0.14, n=104). The linearity checks showed that only 2% of the static chamber linearity measurements were significantly quadratic. Comparing the differences in the N2O flux using either T0 or ambient, revealed significant differences across all treatments (see Table 5.3). The degree of difference depended on the size of the N2O flux, if there was a large positive flux, then it was more likely that the ambient and T0 method would generate significantly different results.

**Table 5.2.** *ANOVA on the gas only data set. N2O and CH4 are in g ha-1 d-1 and CO2 and CO2e are in kg ha-1 d-1, n=104.*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| ANOVA | N2O g N ha-1 d-1 | CO2 kg C ha-1 d-1 | CH4 g C ha-1 d-1 | CO2e kg C ha-1 d-1 |
| Treat. | <0.001 | <0.001 | 0.181 | <0.001 |
| Date | <0.001 | <0.001 | <0.001 | <0.001 |
| Treat.Date | <0.001 | <0.001 | 0.543 | <0.001 |
| Ctrl.ave | 0.175 | 2.447 | 0.822 | 2.531 |
| Inorg.ave | 0.526 | 3.915 | 1.528 | 4.247 |
| FYM.ave | 0.489 | 5.026 | 1.203 | 5.232 |
| LSD 5% | 0.1258 | 0.1029 | 0.8 | 0.1205 |

**5.3.2 Gas and soils dataset**

For TON, NH4 and DOC there were significant differences between treatments (p<.001, n=88) with FYM having the highest average DOC (56.6 ± 2.1 mg C kg-1), and inorganic having the highest average TON and NH4 (22.9 ± 1.1 mg N kg-1 and 9.74 ± 0.75 mg N kg-1 respectively, see Table 5.4). There were also significant differences in terms of %C and %N with FYM having the highest averages (3.25 ± 0.07% and 0.30 ± 0.01%, respectively). The soil’s pH showed a significant difference between all treatments, with the inorganic treatment being the closest to neutral (7.30 ± 0.06), followed by the FYM (7.70 ± 0.06) and then the control (7.90 ± 0.06). In terms of the linear regression models (see Table 5.4), CO2 fluxes were best predicted by %C, temperature, and days from fertilisation (AIC=88.8, Adjusted R2=48.1), N2O by WFPS and temperature (AIC=70.0, Adjusted R2=18.4) CH4 by WFPS (AIC=71.2, Adjusted R2=13.0). CO2 emissions correlated best with the soil variables, with the model for FYM having the highest adjusted R2 (86.1%) out of all the modelled gases. However, for N2O the soil variables were poorly correlated with emissions, and this was the same for CH4 (see Table 5.5).

**Table 5.3.** *Comparison of using 6 ambient measurements or measurements inside the chamber as T0, error is expressed as standard error, n=324.*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | All treatment data | FYM treatment only | Inorganic treatment only | Control treatment only |
| Percentage of dates that were significantly different (p<0.05) | 59% | 44% | 52% | 33% |
| Mean difference between ambient and measurements inside the chamber (g ha-1 d-1) for the dates that were significantly different. | 2.97 ± 1.65 | 4.53 ± 2.64 | 3.05 ± 2.42 | 3.34 ± 2.71 |

**Table 5.4.** *p values, predicted means and LSDs from ANOVA from the gas and soil data set. Note that several of the variables have been transformed and the replication is varied, n=88.*

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| ANOVA | **√**TON  mg N kg-1 | **√**NH4  mg N kg-1 | DOC  mg C kg-1 | pH | WFPS % | %N | %C | N2O g  ha-1 d-1 | CO2 kg  ha-1 d-1 | CH4 g  ha-1 d-1 | CO2e kg ha-1 d-1 |
| Treat. | <0.001 | <0.001 | <0.001 | <0.001 | 0.15 | <0.001 | <0.001 | 0.03 | <0.001 | 0.14 | <0.001 |
| Date | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.06 | 0.00 | <0.001 | <0.001 | <0.001 | <0.001 |
| Treat.Date | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.16 | 0.83 | 0.01 | <0.001 | 0.11 | <0.001 |
| Ctrl.ave | 1.01 | 0.44 | 20.80 | 7.90 | 47.00 | 0.10 | 0.93 | -2.31 | 6.73 | 4.09 | 6.10 |
| Inorg.ave | 3.65 | 1.65 | 21.16 | 7.30 | 51.00 | 0.13 | 1.14 | 1.86 | 20.66 | -3.93 | 21.10 |
| FYM.ave | 1.98 | 0.56 | 56.69 | 7.70 | 49.00 | 0.30 | 3.25 | 2.21 | 29.41 | -2.53 | 30.00 |
| LSD 5% | 0.31 | 0.27 | 7.27 | 0.20 | 3.40 | 0.02 | 0.23 | 3.41 | 4.46 | 0.67 | 3.73 |

***Figure 5.2.*** *Changes in %SOC and %N since 1966 for the 3 plots analyzed in this study using secondary data:* [*http://www.era.rothamsted.ac.uk/experiment/rbk1/soilchem#documents*](http://www.era.rothamsted.ac.uk/experiment/rbk1/soilchem#documents)*. The replicates for each plot are from separate sections 0,1,6 and 9. N=4, error bars represent standard error.*

**Table 5.5.** *Selected regression models which had the highest adjusted R2 and the lowest AIC for the different GHG emissions. The models have been applied incorporating all the data, and just the data for a specific treatment.*

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **N2O g ha-1 d-1** | | | | | | | |
| **All treatments** | | **Inorganic** | | **FYM** | | **Control** | |
| Best subset | 2 terms | Best subset | 2 terms | Best subset | 1 term | Best subset | 3 terms |
| WFPS % | 0.002 | WFPS % | 0.025 | WFPS % | 0.271 | Temp | 0.001 |
| Temp | 0.003 | Temp | 0.051 | \* | \* | DOC mg-1 kg-1 | <0.001 |
| \* | \* | \* | \* | \* | \* | NH4 mg-1 kg-1 | 0.007 |
| **AIC** | **Adjusted R2** | **AIC** | **Adjusted R2** | **AIC** | **Adjusted R2** | **AIC** | **Adjusted R2** |
| 70.0 | 18.4 | 21.8 | 29.4 | 19.1 | 1.2 | 29.6 | 58.4 |
| **CO2 kg ha-1 d-1** | | | | | | | |
| **All treatments** | | **Inorganic** | | **FYM** | | **Control** | |
| Best subset | 3 terms | Best subset | 4 terms | Best subset | 4 terms | Best subset | 2 terms |
| %C | <0.001 | Days Fert. | 0.001 | Days Fert. | <0.001 | Temp | 0.012 |
| Temp | <0.001 | Temp | <0.001 | Temp | <0.001 | WFPS | <0.001 |
| Days\_Fert | <0.001 | TON mg-1 kg-1 | 0.02 | TON mg-1 kg-1 | <0.001 | \* | \* |
| \* | \* | WFPS | 0.041 | WFPS\_% | 0.002 | \* | \* |
| **AIC** | **Adjusted R2** | **AIC** | **Adjusted R2** | **AIC** | **Adjusted R2** | **AIC** | **Adjusted R2** |
| 88.8 | 48.1 | 28.8 | 75.4 | 31.0 | 86.1 | 26.8 | 65.4 |
| **CH4 g ha-1 d-1** | | | | | | | |
| **All treatments** | | **Inorganic** | | **FYM** | | **Control** | |
| Best subset | 1 term | Best subset | 3 terms | Best subset | 3 terms | Best subset | 1 term |
| WFPS\_% | 0.002 | DOC | 0.02 | Temp | 0.074 | %N | 0.073 |
| \* | \* | NH4 mg-1 kg-1 | 0.294 | pH | 0.023 | \* | \* |
| \* | \* | WFPS % | 0.013 | WFPS\_% | 0.001 | \* | \* |
| **AIC** | **Adjusted R2** | **AIC** | **Adjusted R2** | **AIC** | **Adjusted R2** | **AIC** | **Adjusted R2** |
| 71.2 | 12.9 | 23.6 | 27.7 | 26.9 | 39.3 | 21.9 | 10.4 |

**5.4 Discussion**

It is clear from this study that different long-term fertiliser management practices alter the soil’s biogeochemical character. This is most apparent in the FYM treatment, which had more than double the amount of DOC compared to the other treatments, and almost triple the amount of %C. The secondary data also indicates that the DOC and %N has remained significantly higher since at least 1966 (see Figure 5.2). This is unsurprising, considering FYM application has been recorded to increase the bioavailable and recalcitrant carbon content, especially when compared to plots with only NPK application (Ghosh *et al.,* 2018). For example Dhadli and Brar (2016) compared a range of fertiliser treatments, and observed that FYM application significantly increased the soil’s SOC by at least 70% when compared to a control, and 25-50% more when compared to plots with only NPK application. In our study, the increased carbon content also had implications for the soil’s bulk density, which was 0.15 g cm-3 less for the FYM treatment, compared to the control and the inorganic treatment. Similarly Manna *et al.* (2005) recorded a decrease in the bulk density of an inceptisol and an alfisol of 0.08 g cm-3 and 0.07 g cm-3 respectively, after 30 years of FYM application, while other treatment plots with inorganic amendments over a similar time period showed no significant differences.

As hypothesised (H2) the increased carbon content of the soil, created a significantly larger mean CO2 flux from the FYM treatment (see Figure 5.3). %C showed the strongest correlation to CO2 emissions when including all treatments, and days from fertilisation application had the strongest correlation when just the FYM treatment was analysed (see Table 5.4). Other fertilisation studies have found similar results (Zhai *et al.,* 2011; Ding *et al.,* 2007) where the C content of the soil significantly changes the CO2 emissions. This is likely because of the positive correlation between available C sources and microbial respiration (Fang and Moncrieff, 2005). Soil TON was part of the final CO2 linear model for the FYM treatment (adjusted R­2=62%) and the inorganic treatment (adjusted R­2=75%), and soil NH4 was part of the final CO2 linear model for the control treatment (adjusted R­2=58%). Although these nitrogen pools are important for microbial activity and growth, they were significantly lower in the FYM treatment compared to the inorganic treatment, despite its significantly higher CO2 emissions. This indicates that there was not a direct relationship between an increase in TON and NH4+ and increase in CO2 emissions.

***Figure 5.3.*** *Differences in the average gas emissions from each treatment plot from all sampling dates, n=4, error bars represent standard error, note the Y axis’s change in scale, and the X axis’s scale is not linear. CO2e is calculated as the summation of the 3 other GHGs. FYM was applied on the 18/09/2019 and 22/09/2020 which is marked by the blue arrows and inorganic nitrogen was applied on 12/04/2019 and the 21/05/2020, which is marked by the orange arrows.*

We hypothesised (H3) that the inorganic treatment would have statistically higher N2O emissions than the FYM treatment due to the higher amounts of bioavailable nitrogen. While TON and NH4+ were significantly higher for the inorganic treatment, this did not result in significantly higher N2O emissions. It is likely that increased organic matter content from long-term FYM application enhanced microbiological and biochemical activity (Valarini *et al.*, 2002) and provided a carbon source for denitrifiers (Jager *et al.*, 2011) resulting in similar N2O emissions to the inorganic treatment, and significantly more N2O emissions than the control. However, it is worth noting that the highest N2O emissions were recorded from the inorganic treatment post fertiliser application, with the inorganic plot having the highest recorded N2O flux within 40 days of application. After inorganic fertilisation N2O emissions are typically the highest compared to the rest of the year (Sosulski *et al.,* 2014; Petersen *et al.*, 2006). Although FYM application has the potential to significantly increase N2O emissions relative to inorganic fertilisation regimes (Zhou *et al.,* 2017), this seems to be highly dependent on soil type and climate. Zhou *et al.* (2017) noted that most of the N2O emissions from manure occurred immediately after application on relatively warm days according to the local climate. However in our study days after fertilisation was not a significant predictor for N2O emissions from the FYM treatment, and beyond 50 days of the inorganic amendment being applied the emissions were relatively low and statistically similar to the FYM treatment, which is probably why they were not significantly different overall.

As predicted (H4) the CH4 emissions were statistically similar between the FYM and inorganic treatment. This is because CH4 emissions are typically very low from mineral soils, and the main factors that would influence this footprint would be very high carbon content (e.g. if it was an organic soil), repeated flooding, and changes in plant cover (Levy *et al.,* 2012). Considering Broadbalk is a mineral soil, which has drainage, and the treatment plots have the same plant cover, it was unlikely that CH4 emissions would be affected. It is worth noting that the inorganic plot did have a CH4 flux that was statistically higher than the control plot (see Table 5.3), however this difference was very small and is likely due to natural variation as none of the independent soil variables measured had a strong correlation with CH4 emissions, and the adjusted R2 values were all less than 16% (see Table 5.4). We would therefore predict that further measurements of CH4 emissions for subsequent years would show no statistical difference between the control and inorganic treatment.

As hypothesised (H1), the CO2e was the highest for the FYM plots and this is because CO2 emissions made up for the majority of the soil emissions, and the FYM had significantly higher CO2 emissions overall. Although the soil’s flux of CO2e were higher from the FYM plots, it is important to note that this study does not assess the wider GHG emissions surrounding the lifecycle of inorganic fertiliser production or FYM production. The static chambers are also opaque and so photosynthesis is not accounted for. From a broader perspective, emissions from soils are only a small part of the wider impact of organic and inorganic farming practices; they are can be less than 40% of total emissions when incorporating a broader life cycle assessment (Hulugalle *et al.*, 2020). The footprint is dependent on a variety of factors, and the energy intensive Haber-Bosch process used to create inorganic fertilizers could result in the overall CO2e being higher for the inorganic treatment (Küstermann *et al.,* 2008; Litskas *et al.*, 2011; Noponen *et al.*, 2012; Prade *et al.*, 2017). Large differences in management practices and the amount of energy used for fuel and fertilizer can result in statistically similar GHG footprints (Astier *et al.*, 2014), or even higher footprints for organic systems. For example Bos *et al.* (2014) found that organic arable and vegetable production in the Netherlands had 0-15% and 35-40% higher emissions than their conventional counterparts, due to the relatively high fertilizer inputs and frequent field operations. Moreover, while this study focussed on changes in soil variables and the subsequent GHG emissions, there are other environmental factors that are just as important as the GHG footprint. Although not in the scope of this study, these include the soil’s nutrient cycles, its structure, and its resilient to pests and diseases.

**5.5 Conclusions**

In summary, this is the first field experiment assessing CO2, N2O and CH4 emissions from the longest agricultural experiment in the world, the Broadbalk’s organic, inorganic, and PK plots. The three different fertiliser management schemes had varied and significant effects on Broadbalk’s soil properties, and the resulting GHG emissions. CO2 emissions were significantly affected by distal controls, as increased recalcitrant carbon content (%C) and changes with the soil’s physical properties from FYM addition resulted in higher CO2 emissions. The N2O emissions were only significantly different when there was significantly higher TON and NH4+ pools because of the inorganic fertiliser addition, which resulted in temperature and WFPS being the overall best predictor for N2O, and fluxes were therefore dictated by proximal controls. CH4 fluxes were not affected by the different fertiliser regimes in this study, likely because the variables that typically affect CH4 emissions were not impacted. This reflects the conclusion of Skinner *et al.,* (2014), who conducted a global meta-analysis of organic and non-organic management practices. We suggest that future work should focus on how other soil functions have been impacted by the fertilisation legacy of these plots, specifically drought resilience and leaching, as well as meso and micro pore structure.

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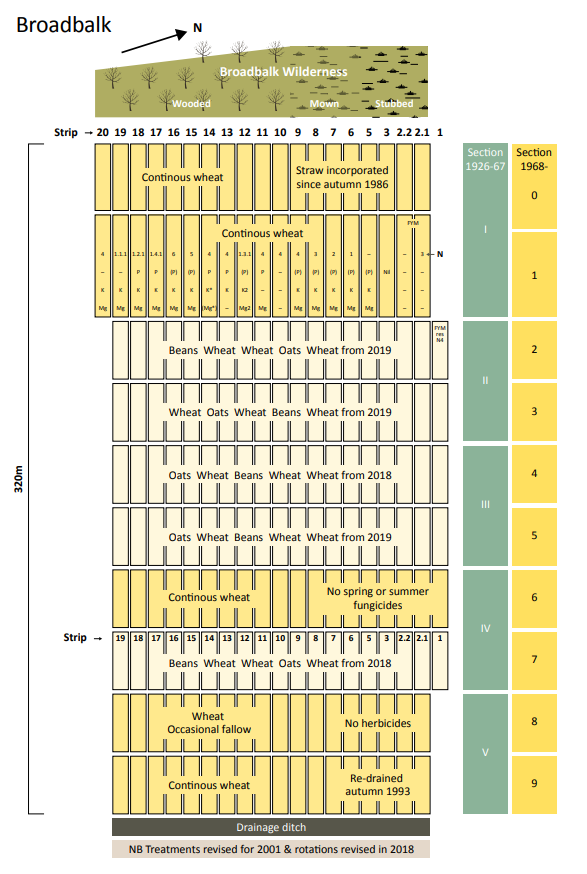
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**Chapter 5 Supplementary Material**

***Supplementary Figure 5.1.*** *The map of the Broadbalk long-term experiment, sections 0, 1, 6, and 9 were selected as replicates for plots/strips 2.2 (FYM), 5 (Control), and 15 (Inorganic).*

**Chapter 6**

**Discussion**

This PhD has focused on two antecedent factors that have an impact on the soil’s function. The first factor is the soil’s water content, which when rapidly changed from a dry state to a wet state causes the soil to produce emissions of nitrous oxide (N2O) that are magnitudes above the soil’s typical background level, commonly cited as a hot moment (McClain *et al.,* 2003). The second factor is use of organic amendments, specifically how Broadbalk’s soil has changed due to the long-term application of farm-yard manure, and inorganic ammonium-nitrate on its wheat plots. Out of the two factors, a larger focus was placed on trying to understand the mechanistic reasons for why hot moments occur, which is reflected in the chapter layout of this thesis, with four experiments and three chapters dedicated to this phenomenon, and one large field trial and chapter dedicated to Broadbalk. A greater emphasis was placed on N2O hot moments for two reasons, firstly the thesis outline originated from previous findings in Bergstermann *et al.* (2011) in which an incubation experiment undertaken in the DENIS lab at North Wyke explored this phenomenon (Scholefield *et al*., 1997). In addition, soil moisture is considered the most important proximal factor affecting emissions and it is clear that there were large gaps in the research that this PhD project could address. A major motivation for this research topic is the increasing frequency of extreme weather events caused by climate change, and therefore the need to better understand how these events affect soil emissions (Dodd et al., 2021). The research on the Broadbalk trial on the other hand, addresses another factor, the long-term application of organic fertilisers. As the Broadbalk field trial formed a single chapter in the thesis, it is unnecessary to repeat the discussion of how the different treatments affected soil properties and the resulting emissions. However, this discussion will explore its limitations and its possible improvements.

**6.1 Research Questions**

**6.1.1 What is the current state of research regarding historic moisture conditions, and the release of N2O?**

Studies which investigate N2O emissions after rewetting the soil have a range of designs, the most basic just measure soil water content and simple edaphic factors like pH, total oxidized nitrogen (TON), ammonium (NH4+) or dissolved organic carbon (DOC) (Barrat *et al.,* 2020). Some experiments will take this further and try and determine which nitrogen cycling processes are dominant, using acetylene inhibition (Rudaz *et al.,* 1991), enzyme activity assays (Radl *et al.,* 2015), or via the assumption that changes in soil nitrogen and carbon pools indicate the key process (Harrison-Kirk *et al.,* 2013; Kessavalou *et al.,* 1998).

However, very few of these studies are designed in a way that can improve our ability to predict the size of a hot moment, or even whether a hot moment will occur. The major problem is the number of confounding variables. Typically between studies the soils are different, the vegetation cover is different, the temperature might not be controlled, the length of drying is different and sometimes this is not controlled, the degree of wetting will likely be different and in lab settings you have range of packing densities, from loose sieved soil in a Kilner jar, to soil cores taken directly from the field. The result is a range of studies which are measuring seemingly the same phenomenon, but the differences and similarities in the hot moment response between studies can only be explained through conjecture. Therefore, the key problem with the current research is the lack of a standardized approach, that would allow robust predictions. The review in chapter 2 was an attempt to rectify this problem.

There was a previous review paper by Kim *et al.* (2012) outlining the impacts of both freeze-thaw cycles and dry-wet cycles on soil emissions. However, it mainly focused on freeze-thaw cycles and carbon dioxide (CO2) emissions. In terms of N2­O hot moments, arguably only 9 studies within the reviews reference list are relevant to temperate mineral soils, as a large proportion of the database is from studies examining tropical forest soils, arctic tundra and rice paddies. Kim *et al.* (2012) did quantify the percentage increase of N2O emissions after rewetting, but they provided no quantifiable predictions in terms of how changes in the key soil variables might impact its size. Therefore the aim of chapter 2 was to provide a summary of the literature investigating hot moments, as well providing the first meta-analysis that could provide a quantitative assessment of the key variables. While it was successful in terms of providing a quantitative starting point, the analysis was only as good as the papers that it could cite, and as previously stated the research is of mixed quality. However, it highlighted some key aspects that need to be considered, namely the need for a standardized approach, and secondly that the degree of wetting and the starting soil water content are seemingly the most important factors, which some studies do not even adequately measure or state (Bergsma *et al.,* 2002; Ruser *et al.,* 2006). The lack of current research will mean that this meta-analysis will need to be revised when there is more data on the key factors. As it was missing *in-situ* plot trials, information on the impact of drought length, and missing experiments that controlled and measured how carbon (C) and nitrogen (N) pools changed throughout the drying and rewetting. This has resulted in more than 30 years of research that still has not provided a clear conclusion on why the microbes are producing this pulse, and which C and N pools they are using.

**6.1.2 How does adjusting these antecedent moisture variables impact the resulting N2O emissions?**

While the literature review and meta-analysis in chapter 2 provided a simple explanation of the relationship between the antecedent moisture variables and the size of the hot moment, an experimental approach was necessary to determine the importance of the soil’s carbon and nitrogen pools, as well as the role of drought length, as this had to be dropped in the meta-analysis due to the lack of recorded observations. It was assumed that drought length would have a similar impact to the WFPS change and WFPS intensity, where increasing its size would increase the size of the hot moment, until eventually this effect would plateau. The results of chapter 4 supported the results of the meta-analysis in terms of WFPS change and intensity, but it revealed that drought length instead had an inverted U shape relationship with N2O hot moments. While this relationship was only observed using one soil type, this brings into question the validity of studies which do not state the drought period, as without knowing this variable you cannot determine the importance of the other antecedent moisture variables. For example, if we were to compare across studies without knowing the drought length, we may conclude that a decrease in the WFPS change results in higher emissions, but this could have been a result of a much longer drought length. Therefore, it is important that future studies not only state the drought length, but also conduct experiments determining the degree of its quadratic effect on the soil they are using.

Chapter 3 showed that a much larger hot moment can occur just through changes in antecedent moisture, without there being significantly more extractable TON, NH4+ or DOC. However, in chapter 4 the soil chemistry results did show an increase in NH4+ concentrations after the soil was dried, and then this pool was seemingly consumed upon rewetting, matching the increase in N2O emissions. The results of this PhD are a microcosm of the wider literature as there is currently not an empirical consensus on the importance of the soil substrate pools. Some studies observe increases in DOC and other soil N substrates, when the soil is dried and rewetted, and these increases correlate with an increase in N2O emissions as seen in chapter 4 (Harrison-Kirk *et al.,* 2013). Other studies like the experiment in chapter 3, see no explanatory change in the soil’s N and C pools (Li *et al.,* 2008). While it is clear from both the meta-analysis in chapter 2, and the broader understanding of N cycles, that increasing labile nitrogen in the soil will increase emissions (Cardenas *et al.,* 2010), it is not clear that this is the main causal mechanism for hot moments, as is commonly hypothesized (Borken and Matzner, 2009).

**6.1.3 What is the mechanism behind the release of these N2O emissions?**

There is not a settled experimental consensus on the mechanism behind these hot moments, it is even unclear which of the nitrogen pathways are favored by the antecedent conditions. With some studies observing denitrification via denitrifiers (Bergstermann *et al.,* 2011), others observing chemo and nitrifier denitrification as the key pathway (Leitner *et al.,* 2017), and in chapter 4 it was concluded that it might not be either of these. What is clear is that the pathway is not the same from experiment to experiment, and so maybe this is secondary to the mechanism itself, in other words the pathway is dependent on the soil species, the resident microbial population and the edaphic factors at the time of emission, but the hot moment does not select one over the other. Chapter 3 and chapter 4 showed that the extreme water potentials do not necessarily change the functional potential of the soil in terms of nitrogen cycling genes from DNA, and the added measurement of messenger RNA associated with the production of N cycling enzymes also did not reveal any significant changes that could explain the emissions. Understanding the reason for hot moments might require a different approach.

Fortunately, there are now the tools available to look more closely at the soil’s biochemical cycles. Researchers looking at the Birch Effect which is a similar phenomenon with carbon dioxide (CO2) emissions, have started using metabolomic profiling (Warren *et al.,* 2014, Warren *et al.,* 2020). New research using these methods coupled with an induced N2O hot moment, provides promising new territory to explore. Recently Brown *et al.* (2021) used untargeted metabolomic and lipidomic profiling in a field trial with two drought treatments that had drought lengths exceeding 24 days. The least severe drought treatment, called the reference treatment, was artificially kept at a higher soil water content before it was rewetted and it produced the largest amount of N2O emissions, indicating a similar relationship with drought length seen in chapter 4. Before the driest soil was rewetted, Brown *et al.* (2021) observed a higher abundance of both triacylglycerol (TAG) compounds and a group of 4 metabolites (sucrose, arabitol, erythritol and 2,4-diaminobutyric acid), which are associated with osmoprotection. Given the lower emissions from the drier plots, this could be indicating the deeper stage of dormancy suggested in chapter 4, which reduces the magnitude of the response to rewetting in terms of N2O emissions.

This study also supported the hypothesis that changes in soil N availability is unlikely to be the driving factor behind hot moments, as the plots which had been fertilized previously, and therefore had higher quantities of nitrate (NO3-) and NH4+, produced similar amounts of N2O. They did observe a consumption of NH4+ post wetting, similar to that seen in chapter 4, which could be indicating the same nitrifying process. However, there were problems with the wetting treatments imposed by Brown *et al.* (2021). The drought treatment never reached the same water content as the reference treatment, so ultimately both the antecedent and the rewetting conditions were different. This made it difficult to determine whether it was the limited rewetting in the drought treatment that meant it had lower higher N2O emissions, or whether it was the severity of the drought. Either way, replication of studies like Brown *et al.* (2021) will help to distinguish if there are two kinds of hot moments, ones that are caused by an increase in soil N and C from the drying and rewetting, and ones that are caused by a specific microbial reaction associated with osmoregulation.

**6.2 Limitations and future research priorities**

**6.2.1 Researching N2O hot moments**

This PhD focussed on researching hot moments at the soil core scale, using genomics, transcriptomics and soil chemistry as explanatory factors. Each of these methods has limitations. Firstly, while the chapter 2 analysed a range of experimental set-ups and soil types, the experiments in chapter 3 and 4 used a single soil type. Therefore, the results of these chapters can only speculate on how changes in WFPS and drought length impact the size of the hot moment for other soil types. So, *prima facie* a repeat of the chapter 4’s design using different soil types is necessary, especially considering how this field of research lacks repeated, standard experimental designs. This should also include soils that have different vegetation cover, as chapter 2 and Priemé & Christensen (2001) show that grassland soils are prone to larger N2O hot moments.

The soil core scale was selected for this PhD as many of the dependent variables needed to be controlled, in order to have the maximum degrees of freedom allocated to determining the effect of antecedent moisture conditions. This leaves important gaps in the research at larger scales, such as at lysimeter scale, where water and gas movement will be different due to the different macro pore structure and depth of the soil. This is a necessary step before hot moments are explored at field scale, which will be needed to predict the relevancy of hot moments in for example, the UK GHG inventory. Current soil N2O models such as DNDC are grounded at this scale (Abalos *et al*., 2016) and while new modelling at the pore scale is being developed at Rothamsted, it was not within the scope of this PhD.

Despite Bergstermann *et al.* (2011) fertilising its treatments, this PhD opted not to fertilise the soil before inducing any of the antecedent treatments. The primary reason being that adding fertiliser to the soil would reduce the differences in any changes in soil C and N pools caused by the antecedent conditions, undermining the statistical power to determine these differences. Another reason was that labile pools favour microbes which can access and use these pools, which might increase the likelihood of one N pathway over another. Considering the objective of this research was to understand the microbial response, this would have obscured how the antecedent conditions select for the pathway. Now that this PhD has conducted research without fertiliser addition, it would be interesting to explore how this affects hot moments. Although, the primary focus should be on trying to determine which C and N pool the microbes are drawing from, as previously mentioned the use of osmotic metabolites is a key hypothesis for the Birch effect, and the standard approach of K2SO4 and KCL extractions conducted in this thesis and the wider literature have proved inadequate at determining the relationship with soil C and N pools and hot moments. Therefore, the use of lipidomic and metabolomic techniques should be employed in future research.

This PhD has summarized and quantified how different soil variables impact the hot moment in the first meta-analysis of this phenomena. This meta-analysis concluded that the WFPS that the soil is wetted to and the WFPS the soil is kept at during the drought have simple linear relationships, although the results come from a limited dataset, therefore to avoid overfitting polynomic regressions were not used. Chapter 4 was designed to map the polynomic relationship between drought length and the WFPS the soil is kept at during the dry phase. Repeated experiments will be necessary so that mean more complex relationships can be determined between the antecedent moisture conditions and the soil’s N2O emissions, similar to that of chapter 4.

However, drawing from the meta-analysis and chapter 4, as well as the results of chapter 2, a visual of the relationship of these variables and the size of the coefficient used to predicate the N2O emissions has been hypothesized in Figure 6.1. The inverted U shape of drought length as already been demonstrated once experimentally, and so this has stayed the same. The WFPS during the dry period has the most complex relationship. Between 0 and 20% WFPS, the soil is very dry likely inducing the second stage of dormancy, however above 20% and between 30 and 40% WFPS you would expect the maximum priming to occur, and then as the soil gets wetter the priming effect would reduce as demonstrated in chapters 3 and 4. Eventually the soil would be so wet during the supposedly pre-dry stage, that the coefficient would be 0, which is predicted in Figure 6.1 as above 90% WFPS.

The relationship is simpler for the amount the soil is rewetted. Chapter 2 indicates a steady increase in emissions the more you wet the soil, but Ruser *et al.* (2006) demonstrate that there is a substantial increase in N2O emissions once the soil becomes anaerobic, and this incorporated into Figure 6.1 by its sigma shape. This effect will plateau as the wetting cannot affectively increase the soil’s anaerobic conditions to the same degree, due to the non-linear nature of the soil’s water potential. For WFPS change, which is the difference in WFPS between the dry and rewetted phase, the meta-analysis suggests a positive correlation, and this is kept but with it plateauing after anaerobicity is reached. Moreover a minimum degree of rewetting is required as suggested by the results of the control treatments from Owens *et al.* (2016). Experiments specifically designed to test these predictions are an important research priority, and they require less resources than the use of metabolomics and lipidomic methods.

***Figure 6.1.*** *Hypothesised relationships between the N2O coefficient and the four key antecedent moisture variables. It assumes all other variables have been held constant.*

**6.2.2 Improving nitrous oxide emission measurements from the Broadbalk field trial**

The experimental protocol for chapter 5 was developed according to the Broadbalk plot design, and previous work within the experimental group (Chadwick *et al.,* 2014; Cardenas *et al.,* 2010). However, the greenhouse gas emission measurements experiment could have been improved in a number of ways, especially when it comes to the gas measurements from static chambers, which have been shown to be highly variable in their accuracy and reliability (Venterea *et al.,* 2009). Charteris *et al.,* (2020) outline three key principles that should be followed when measuring N2O in-situ:

1. Account for spatial variation

2. Account for temporal variability

3. Allocate resources to minimize flux uncertainty

The first two were adhered to with the best of the PhD’s ability, chambers could only be placed on a specific part of the plots, and consistent sampling was attempted but was hindered by unforeseen circumstances (COVID19 and a very wet winter). However, there are two significant changes that would have been possible when the experiment began, that could have reduced the fluxes uncertainty (and possibly the large negative outliers). The first significant change is the addition of 2 more gas headspace samplings (i.e., measuring at 0, 15, 30, and 45 minutes). This would allow linear regressions (including polynomial regressions) to be fitted to every chamber rather than just the two chambers selected for linearity checks. This would allow us to accommodate the variance that comes from the chosen type of regression fitted to the data (Levy *et al.,* 2011). This would have increased the cost of the trial by at least 30%, and this chapter already required helped from Rothamsted’s Institute Strategic Programme ‘Soils to Nutrition’ budget, as well as another £4000 from STARs (North Wyke also had a limited number of gas vial sets). So, it was not possible for this PhD to undertake the additional measurements without redesigning the trial. However, on reflection, it seems that the dropping of the control treatment would have allowed these additional measurements without adding cost, and losing important empirical information regarding the trials objectives. The second change could have been the removal of ambient measurements, while it did allow a comparison between using ambient measurements as the baseline and T0 in each chamber, it was not essential for the experiment’s objectives. This would allow the reallocation of 10 gas vials in total, 4 from the previous linearity checks and 6 from the ambient measurements. So only another 14 gas vials would be needed to accommodate the additional two measurements. While this would increase the reliability and quality of the gas data, it would also have two more benefits.

The first additional benefit is a reduction in the labor required to insert, stack and remove the chambers; this was a major problem throughout the experiment, often relying on the generosity of others. The second being a reduction in the soil sampling time and the number of samples required for analysis. This would allow more of the budget to be spent on greater quality control for the soil samples, while at every time point at least some soil samples were repeated, it would allow a complete duplication of every time point, giving two pseudo-reps for every soil measurement. In summary, the dropping of the control treatment would allow regressions to be fit to every chamber, it would reduce labor time and soil sampling time, and increase the reliability of the soil measurements.

There are other changes which probably were not possible given the resources available that would have improved the trial. Firstly, the chambers were simple white plastic boxes with lids, and considering they were stacked, it would provide more robust data if there was an internal fan to mix to the air. Secondly a collar in the ground that the chamber could latch onto would have allowed a more reliable seal, as the clayey flinty soil made it difficult to dig in the chambers to a depth of 5 cm. The ultimate change would be to have used a continuous analyser, such as the use of portable infrared analyzers, like those used in chapter 3 and 4.

**6.5 Conclusions and the implications of the research**

This PhD has quantified how the soil’s antecedent moisture conditions in a controlled environment effect the size of a hot moment, as well as providing new data on the effects of drought length, which due to its quadratic nature will change how researchers interpret the impact of more extreme droughts on N2O fluxes. It has also conducted the first field trial on Broadbalk that measured all 3 of the key GHG greenhouse gases from soil. Concluding that the retention of soil C in the plots is the most important factor affecting CO2e, with CO2 making up the dominant portion of CO2e from all treatments.

Since Bergstermann *et al.* (2011) inspired this PhD, it is worth revisiting their conclusions in light of this PhD and newer research. Firstly, Bergstermann *et al.* (2011) selected for denitrification with NO3- and glucose addition during rewetting. Because of this they could not determine if other processes would have been selected if just the rewetting occurred. It is clear that anaerobic denitrification might not be the major N pathway instead chemo-denitrification and nitrifier denitrification seem to be likely, given the genomic and transcriptomic results from chapters 3 and 4, and the work of Leitner *et al.* (2017) and Wrage-Mönnig *et al.* (2018). Bergstermann *et al.* (2011) concluded that the pre-dry could have enhanced the C sources, noting a higher DOC concentration just at the beginning of the rewetting in their pre-dry treatment, however the authors also observed that the pre-wet treatment had much higher DOC concentrations just one day into the rewetting (before peak emissions had been reached). These results are still typical of the literature, where changes in soil C and N pools sometimes follow a clear pattern, and in other circumstances there seems to be little correlation with N2O emissions. This PhD concludes that K2SO4 extractions are not appropriate to determine the source and cause of the emissions, and instead more modern methods of metabolomics and lipidomics are required. In summary, the pathway suggested by Bergstermann *et al.* (2011) of anaerobic denitrification from denitrifiers is clearly not always the cause for N2O hot moments, and the enhancement of soil C and N pools due to the drier treatment is also not always observed, as evidenced by this PhD. Looking at the literature investigating the Birch effect, and the inverted U shape of drought length discovered in chapter 4, it seems likely that catabolism of intercellular osmolytes is the source of emissions. Moreover, the work of this PhD suggests that the hot moment response does not select one N pathway over another, the production of N2O is instead selected according to the edaphic conditions.

While the research is not mature enough to be able to adequately predict hot moments in the field, current evidence suggests that this should be the ultimate goal given that hot moments *in-situ* could be increasingly in likelihood due to climate change (Dodd *et al.,* 2021). Moreover, rewetting pulses of N­2O have been recorded as greater than 20% of the annual emissions from a subtilled wheat trial (Kessavalou *et al.,* 1998), and up to 55% of the annual emissions from a wheat trial in Sydney which had unusually large summer rains (Barton *et al.,* 2008), and depending on the field conditions up to 70% of annual emissions were recorded from drying-rewetting and freeze-thaw events from a corn and soybean rotation (Kim *et al.,* 2009), with the drying/rewetting pulses contributing at least 30%.

Unfortunately, predicting the size of hot moments under field conditions cannot be accurately done until there are many more repeated laboratory trials which adjust just one independent variable, while providing information on the other key variables. Then under field conditions, the various confounding variables can be teased apart, allowing a full assessment of the likely magnitude of this phenomenon given the changing intensity of weather patterns in the century to come.

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