DOCTOR OF PHILOSOPHY

Comparison of nitrogen losses associated with sheep excreta deposition on temperate lowland and upland grassland soils

Mancia, Aude

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Aude Mancia

December 2022

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A thesis submitted to Bangor University in candidature for a Doctor of Philosophy (PhD) degree

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Thesis abstract

While food production must increase in response to global population expansion, a reduction of agricultural greenhouse gas (GHG) emissions and nitrogen (N) losses is of paramount importance. One agricultural source responsible for various N losses is ruminant excretal returns in grasslands, which is particularly concerning in countries where grazing systems are an important sector, such as Ireland. Accurate estimation of these losses, including nitrous oxide (N_2O) emissions, ammonia (NH_3) volatilisation, nitric oxide (NO) emissions and nitrates (NO_3^-) leaching are essential to monitor the trends and facilitate agricultural management decisions. However, due to several factors influencing N cycling, estimation of these losses is uncertain and may vary significantly between distinct grazing systems. There is greater uncertainty associated with losses from sheep excreta compared to cattle. Therefore, the main goal of this thesis was to estimate and compare various N losses from sheep excreta (principally urine) deposited on pasture soils representative of two typical sheep grazing systems in Ireland. Specific objectives were i) to establish N_2O emission factors associated with sheep excretal returns (EF_{3PRP}) on two distinct temperate grasslands, ii) to decipher if inherent soil properties could explain differences of N_2O emissions observed *in situ*, iiii) to determine urine N fate on these two pasture soils by a quantification of various N losses and pools, and iv) to assess the effect of sheep urine on microbial communities involved in N cycling. To address these objectives, three experiments were carried out. Firstly, a long-term field experiment was established on a lowland managed grassland and an extensively grazed upland pasture characterised by mineral and acid peat soil, respectively. Static chamber methodology was used to measure N_2O fluxes following sheep urine and dung applied during different seasons. Soil of these pastures were then incubated under denitrifying conditions to quantify N_2O, NO and dinitrogen (N_2) emissions following urine application using the He/O_2 gas flow method. The same soils were incubated for another short-term experiment to assess NH_3 volatilisation, N_2O emissions, NO_3^- leaching, microbial biomass N and nitrification/denitrification gene abundances. The field experiment showed lower N_2O emissions from sheep excreta on the upland pasture, although EF_{3PRP}s were very low on both grasslands, indicating that current estimations in national inventory may be overestimated. The incubation studies showed a different fate of urine N between the two pasture soils. On the peat soil, acid conditions may have inhibited nitrification leading to very low N_2O, NO and N_2 emissions. Urine derived ammonium (NH_4^+) remained at a high level during the incubation

experiment and was subject to leaching and to some NH_3 volatilisation associated with the raise of pH following urine addition, but there was no sign of immobilisation in microbial biomass. On the mineral soil, urine application led to significant losses of N gas emissions and NO_3^- leaching. Within the three weeks following application, the main loss of urine N was through NH_3 volatilisation. Therefore, low N_2O emissions on the lowland were unlikely to be due to inherent soil properties but may have been the results of a combination of low urine N application rate (127-372 kg ha^{-1}), high plant uptake and urinary N loss through NH_3 volatilisation. Sheep urine had no specific effect on nitrifier/denitrifier microbial community and these microbial populations size could not explain the difference in N_2O emissions between the two soils. Assessment of expression genes involved in nitrification/denitrification would be a better indicator of microbial activity. Our results represent a positive message for Irish sheep sector in Ireland given the low N_2O emissions observed, but the assessment of urine N fate in other type of grasslands is required.

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Contents

Acknowledgments

Firstly, I would like to extend a massive thanks to my three supervisors, Dominika Krol, Sinéad Waters and Dave Chadwick. Your support and precious advice helped me so much through my four years of PhD. You always took time to give me constructive and rapid feedback despite your busy schedules, and of course I would not have been able to publish two papers without you all. Dominika, I am glad to have been your first PhD student, and I will always be thankful for these long hours you spent helping me packing so many kg of sheep poo!

I would like to acknowledge the Walsh Scholar research program which funded my research project, as well as EJP Soil from which I received a research grant that allowed me to carry out an experiment at Rothamsted Research in the UK.

A big thank to all the staff and students in Athenry: Kyra, Noel, Michael, Cyril, and all the others, too many to mention! You allowed me to settle well during my first year in Ireland and helped me a lot during my first experiment. Without you I would not have been able to collect that much quantity of sheep urine and dung!

I also would like to thank all the staff and students at Johnstown Castle, also too many to mention! You always were so kind to me, and always took time for me as soon as I was asking for help.

I would like to extend my sincere thanks to the very kind team in Rothamsted Research: Laura, Nadine, Liz and all the others You were very supportive during my stay in Devon during which I faced many issues.

I want to thank all the awesome housemates I had during my four years in Ireland. Kerry, Lorraine, Mansi, thank you for making me laugh so much. And also Sudipto, Konstantin, Meritxell, Shaun, Darragh, Carlotta, Nicola, Niamh, Adele… Thank you for the deep discussions, the nights out, the board games parties! You all made my PhD life run smoother.

Thank to my family for being supportive and caring, even though you still believe that I am becoming a researcher in poo science.

Thank you to all my friends: Sheu, Daminou, Mel, Mathouf, Loulou, Chachlik, Hippo. We only saw each other once a year at best, but every time it filled me with positive energy. And of course, huge big up to my friend Dydy that filled my week ends with so much fun. Ireland would not have been the same without my Dydy. Big up to Bordo, my lockdown buddy. Finally, a big thank you to Guille with who I discover the Irish West Coast with Oni, the best T3 ever!

And last but not least: big up to my cat Mimi Mesca and her calming purr. You're the best.

Abbreviations

AOA - Ammonia oxidising archaea
AOB - Ammonia oxidising bacteria
ANOVA - Analysis of variance
BD - Bulk density
C - Carbon
CEC - Cation exchange capacity
CH_4 - Methane
CO_2 - Carbon dioxide
DEFRA - Department for Environment Food and Rural Affairs
DOC - Dissolved organic carbon
DON - Dissolved organic nitrogen
DW - Dry weight
ECD - Electron capture detector
EF- Emission factor
FID - Flame ionisation detector
GHG - Greenhouse gas
GC - Gas chromatograph
IPCC - Intergovernmental Panel on Climate Change
KCl - Potassium chloride
MBC - Microbial biomass carbon
MBN - Microbial biomass nitrogen
N_2 - Dinitrogen
NH_3 - Ammonia
NH_4^+ - Ammonium
NI - Nitrification inhibitor
NIR National inventory report
NO_2^-- Nitrite
NO_3^-- Nitrate
N_2O - Nitrous oxide
NO - Nitric oxide
NZ – New Zealand
O_2 - Oxygen
OM - Organic Matter
PRP- Pasture, range and paddock
SEM - Standard error of the mean
TC- Total carbon
TN - Total nitrogen
TOC - Total organic carbon
UK - United Kingdom
WFPS - Water-filled pore space

List of Tables

urine (MU), peat soil amended with water control (PC) and peat soil amended with artificial sheep urine (PU). Values represent means (n = 3) ± SEM. For PC, all N_2O values measured for two of the replicates were below the detection limit, hence the average is lower than the cumulative emissions calculated for the third replicate (i.e. 6.2 g ha^{-1}).

Table 6.1 Background characteristics of the two grassland soils. TN and TC are on a dry soil weight basis. Values represent means (n = 4) ± SEM.

Table 6.2 Spearman's rank coefficient of significant correlations ($p<0.05$) between gas emissions (N_2O, NH_3) and soil chemical and molecular variables. NS indicates absence of significant correlations between two variables, + indicates a positive correlation and – a negative correlation.

List of Figures

Chapter 1

Introduction

1.1 Background and rationale

Agriculture of the 21st century is facing the critical challenge of intensifying food production in response to the increase of global population expected to reach 9 billion people by 2050 (United Nations, 2019), while reducing its negative impact on the environment. Agriculture is currently responsible for about 30% of global greenhouse gas (GHG) emissions including carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) (Lynch et al., 2021). Another negative impact of agriculture pertains to its nitrogen (N) losses: about 50 -70% of N inputs are lost from agroecosystems due to their poor N use efficiency (Cassman et al., 2002). These N losses occur in various forms including N_2O, nitric oxide (NO), ammonia (NH_3) emissions and nitrates (NO_3^-) leaching, with consequences on human health and the environment**.** Such important losses are the result of the unbalanced N cycle, characterised by an accumulation of reactive N (Nr) in agroecosystems (Erisman et al., 2013). Agricultural activities are responsible for an input of Nr which is approximately three times higher than the quantity added by natural fixation (Bowles et al., 2018). One of the main causes of this N accumulation in agrosystems is the intensification of N fertiliser use since its development based on the Haber-Bosch process in the beginning of the 20th century (Erisman et al., 2008).

Efforts must be taken by agricultural producers worldwide to mitigate their GHG emissions and N losses. Signatory countries to the UNFCCC *(*United Nations Framework Convention on Climate Change) must estimate and report their GHG emissions in national inventories. Such inventories also include estimations of NH_3 and NO_3^- leaching losses. Estimations and reports are essential to monitor the trends and evaluate if strategies and policies regarding mitigation are efficient. The Intergovernmental Panel on Climate Change (IPCC) has established guidelines and default coefficients to help in the estimation and reporting of GHG emissions and some N losses (IPCC, 2019), as detailed in **chapter 2.** However, countries are encouraged to develop their own estimations, due to the high spatio-temporal variability of N losses.

This project carried out in Ireland focuses on a specific source of agricultural N losses: sheep excretal returns in pastures. Sheep farming is an important agricultural activity worldwide with most sheep farms based on seasonal grazing (Ledgard et al., 2011). However, grazing also represents a source of pollution (Ripoll-Bosch et al., 2013), including N losses from livestock excretal returns in grasslands and from fertilization, as well as methane (CH_4) emissions from enteric fermentation.

In Ireland, where ruminant grazing systems are the dominant agricultural system (CSO, 2016), it is essential to quantify N losses from ruminant excreta deposited in pastures. There are 32,000 sheep flocks in Ireland, and a total of 2.5 million ewes, allowing the country to export almost 75% of its production of sheep meat (Diskin and McHugh, 2017). The sheep sector is characterised by different grazing systems that can be classified into lowland and hill farming, but both are based on a seasonal grazing system (Ledgard et al., 2011). Lowland farming systems contribute to 85% of meat exportation and most intensive farms are characterised particularly by larger flocks (341 - 429 ewes farm^{-1}), higher stocking rates (9.5 – 11.8 ewes ha^{-1}) and greater fertiliser N inputs (85 - 103 kg N ha^{-1}) compared to hill farms. On extensive hill grazing systems, the average number of ewes, livestock loading rate and fertiliser input are 131 ewes farm^{-1}, 2 ewes ha^{-1} and 13 kg N ha^{-1} (O'Brien et al., 2016). Hill farms are particularly localised in the West of the country, and a high proportion of upland grasslands are based on blanket bogs, characterised by peat soil. Due to differences of pasture species, animal diet, soil characteristics and eventually climatic conditions, we would expect differences regarding N losses between lowland and hill farming systems.

There are no or few data quantifying N losses from sheep excreta in Ireland, whereas estimations of N_2O emissions have been refined recently for cattle excreta deposition, with the development of a country-specific emission factor (EF_{3PRP}; proportion of N applied through livestock excreta which is emitted as N_2O) based on the study of Krol et al. (2016). This country-specific EF_{3PRP} (0.86% for combined excreta) was much lower than the 2006 IPCC default value (2%) previously used to calculate N_2O emissions from cattle excreta in the Irish national GHG inventory report, suggesting national emissions from cattle excreta were overestimated. This refined EF_{3PRP} for cattle excreta appears now to be lower than the 2006 IPCC default value of 1% used for sheep in the inventory, which is inconsistent with studies showing higher emissions from cattle excreta compared to sheep (López-Aizpún et al., 2020). This disaggregation between sheep and cattle is likely to be due to difference in urine distribution and soil compaction between the two animal categories (IPCC, 2006), and may also be related to differences in urine chemical composition (López-Aizpún *et al.*, 2020).

1.2 Objectives and hypotheses

The aim of this thesis was to assess various N losses from sheep urine patch deposition on two distinct grassland soils representative of two main types of sheep grazing systems in Ireland.

The first specific objective was to quantify year-round N_2O emissions *in situ* from sheep excreta deposited on pasture soil in order to establish EF_{3PRP} in two types of grasslands (i.e lowland versus upland grassland), and simultaneously explore the effect of excreta type (urine, dung), season (early, mid, late-grazing) as well as pasture type on N_2O emissions. Characteristics of the two studied grasslands are presented on **Table 1.1**. We expected lower EF_{3PRP} compared to the 2006 IPCC default value which is still used in the Irish national GHG inventory report (NIR). We also hypothesised that N_2O emissions would vary significantly between grassland types, seasons of application and the type of excreta.

A second objective was to evaluate if soil inherent properties would lead to significant differences in sheep urine-derived N_2O emissions that were expected *in situ* between the two grasslands.

As a logical follow-up, the third objective was then to compare the fate of urine N between the two pasture soils, by quantifying various N losses and N pools in these soils under the same treatments and conditions, while putting aside the effect of sheep urine composition. We expected that the contrasting soil properties would lead to different fates of urine N in the two soils. Indeed, several studies have showed distinct urine N fate in different soil types (Sørensen et al., 1996; Clough et al., 1999; Decau et al., 2003), because soil properties influence several processes of the N cycle, as detailed in **Chapter 2.**

Finally, another specific objective was to determine the effect of sheep urine on microbial communities involved in N cycling and to assess if this effect would be soil type-dependent. We expected changes in nitrifiers and denitrifiers communities' size following sheep urine application.

Table 1.1. Description of the experimental grasslands.

Grassland type	Localisation	Soil type	Average annual rainfall (mm) and temperature (°C)*	Sward type	Grassland management
Lowland	Mellows Campus, Teagasc Research Centre, Athenry, Co. Galway	Fine loam over limestome bedrock	1340 mm 10.7 °C	Perennial ryegrass (*Lolium perenne* L.)	Rotationnal grazing NPK fertilisation
Upland	Oorid, Recess, Co. Galway	Peat	1193 mm 9.9 °C	Diversity of species	Rough-grazing No fertilisation

*Annual temperature and rainfall are 30 years averages (source: Met Éireann, 2022)).

1.3 Thesis outline

Chapter 3 to 6 are presented in the form of scientific journal articles, whether they have been published or not. **Chapter 3 and 4** have already been published in the journal Science of the Total Environment.

Chapter 2 is a literature review focusing on N losses from ruminant excreta patches. The review firstly presents the N cycle in soils and losses associated, then describes the effect of ruminant excreta on N cycle and N losses, the estimations and report of N losses from excreta patches, and finally the factors influencing these losses.

Chapter 3 is a chapter review focusing on the uncertainties related to the methods used by countries to estimate N_2O emissions from ruminant excretal returns. This chapter also describes some approaches adopted to consider factors of variation of emissions and highlight the challenges associated with such approaches. This chapter is a follow-up of the general literature review (**Chapter 2**), and it is a transition to **Chapter 4** by justifying the need to estimate country-specific

EF_{3PRP}. This chapter has been published as a review paper in the journal Science of the Total Environment.

Chapter 4 is the first experimental chapter based on the long-term field experiment for the establishment of EF_{3PRP}; on two grasslands in Ireland. The field experiment was performed according to manual gas sampling based on static chamber methodology for the determination of N_2O fluxes following sheep excreta application to grassland soil. This chapter has been published in the journal Science of the Total Environment.

Chapter 5 is an experimental chapter based on an incubation experiment carried out to measure N_2O, NO and N_2 from the soil collected from the two grasslands studied in **chapter 4**, following artificial sheep urine application. This experiment used the He/O_2 gas atmosphere exchange method (Cardenas et al., 2003) to allow for direct measurements N_2O, NO, N_2 as well as CO_2 fluxes. This incubation was established in the DENIS (Denitrification system) at Rothamsted Research, Devon, UK thanks to the "Visiting researcher" grant secured from EJP soil funding call application.

Chapter 6 is an experimental chapter based on another incubation experiment with the same soils and the measurement of various N losses and pools following artificial sheep urine application. Manual gas sampling was performed to assess N_2O fluxes from incubated soil cores, whereas NH_3 fluxes were determined with a photoacoustic gas analyser. An assessment of the effect of urine on microbial communities involved in N cycle is undertaken in this chapter by using molecular tools to determine several N cycling gene abundances.

Chapter 7 presents the discussion points arising from the results obtained in the three experiments. This chapter includes a discussion of the fate of urine N in the two pasture soils, the accuracy of our estimations, the implication of our results and finally some recommendations for future research.

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Chapter 2

Literature review

2.1 Introduction

Biogeochemical flows including nitrogen fluxes are one of the planetary boundaries that have been overreached due to anthropogenic perturbations, leading to a substantial risk of negative impact on Earth system (Steffen et al., 2015). The imbalance of nitrogen fluxes is reflected particularly in emissions of N_2O, a potent greenhouse gas (GHG) projected to increase by 30% in 2050 compared to its level in 2000 (Bouwman et al. 2013).

Anthropogenic activities are estimated to contribute 30 - 45% of N_2O emissions, mostly through soil use, agriculture and fossil fuel burning (Myhre et al., 2013). Among agricultural activities, an important source of N_2O is livestock waste management. The diverse sources of livestock waste management contributing to N_2O emissions are the application to land, burning for fuel production, storage in confinements, indirect sources from volatilised ammonia or leached N and the deposition of excreta onto pasture soils by grazing livestock (Oenema et al, 2005).

About 25% of global terrestrial area, excluding Greenland and Antarctica, support managed grazing systems (Asner et al., 2004). Therefore, it is essential to estimate N losses associated with grazing livestock excretal returns, in order to monitor the trends and aid management decisions aiming to mitigate N losses from agriculture.

This literature review describes the natural N cycle occurring in soil and explains the effect of ruminant excreta on soil properties and pathways of N losses. The general methodology used to report N losses from ruminant excreta deposition in grasslands is then presented. Finally, the factors of variations responsible for the spatio-temporal variability of N losses are discussed.

2.2 Nitrogen cycling and losses

Nitrogen (N) is an element essential life and represents 72% of the atmospheric composition. Despite this abundance, its inert form in the atmosphere, the dinitrogen gas N_2, makes it unusable for most living entities (Galloway et al., 2003). However, some organisms including rhizobia bacteria, some symbiotic archaea, and some free-living bacteria, can fix atmospheric N_2, enriching the pool of NH_4^+ in the soil (Cameron and Haynes, 1986). Dinitrogen fixation can also occur through lightning (Schils et al., 2013). Therefore, N_2 fixation leads to the production and enrichment of reactive form of N (Nr) in the soil. Other processes leading directly to the introduction of reactive N (Nr) into the soil are wet and dry deposition (Cameron et al., 2013).

In soils, N is subject to transformation processes, changing from one reactive form of N to another (Erisman et al., 2013). **Fig. 2.1** shows the various transformations of Nr occurring in soils. Some of these Nr forms can be taken up by plants to support their growth and metabolism (Cameron and Haynes, 1986) and can then return to the soil with animal excretion or animal and plant death and decomposition.

Therefore, enrichment of NH_4^+ pool can also occur directly within the soil through degradation of organic N molecules already present in the soil via the process called ammonification (Butterbach-Bahl et al., 2013). Ammonification also refers to the anaerobic respiration of some bacteria and fungi, a reduction of nitrite (NO_2^-) into NH_4^+ which then can be immobilised (assimilatory reduction) or not (dissimilatory reduction) by microorganisms (Stein and Klotz, 2016).

Under circumstances leading to an increase of soil pH (e.g. application of animal urine), NH_4^+ can be volatilised as ammonia (NH_3) (Cameron et al., 2013). This gas has negative impacts on the environment and is harmful to animal and human health through its impact on respiratory tract (Portejoie et al., 2002). The redeposition of NH_3 can lead to acidification and eutrophication of ecosystems and to indirect emission of N_2O (Cameron et al., 2013).

Ammonium NH_4^+, along with other mineral N forms (nitrate NO_3^- and nitrite NO_2^-) also represents a source of N uptake by micro-organisms for organic molecule synthesis, essential for their growth. This process is termed immobilisation. The N molecules that are not used are released as NH_4^+, which is the process of mineralisation. Mineralisation and immobilisation processes occur simultaneously: this is the mineralisation-immobilisation turn-over (Geisseler et al., 2010). However, environmental factors determine the "overall effect", that is whether there is a higher rate of mineralisation (net mineralisation) or immobilisation (net immobilisation) (Cabrera et al., 2005).

An important process occurring under aerobic conditions is nitrification, which is a cascade of oxidation reactions from NH_4^+ to nitrate NO_3^-, with nitrite NO_2^- and hydroxylamine as intermediates (Ward, 2015). During the process of nitrification, NO and N_2O can be emitted (Baggs & Philippot, 2010; Pilegaard, 2013). Nitrification is undertaken by autotrophic micro-organisms among fungi, archaea and bacteria, which are able to use energy from oxidation to fix their carbon source: the ammonium oxidising bacteria/archaea (AOB and AOA, respectively) and the nitrite oxidising bacteria (NOB) (Ward, 2015). However, some nitrifying microorganisms can undertake both nitritation and nitratation: these are comammox microorganisms, such as some bacteria from *Nitrospira* genus (Daims et al., 2015; Stein and Klotz, 2016). Moreover, it has been discovered that

some heterotrophic organisms are also able to oxidise NH_4^+, using organic carbon molecules as energy sources, such as phenol (Duan et al., 2021; Lu et al., 2014). These heterotrophic nitrifiers could play a key role in ecosystems where common autotrophic nitrifiers are inhibited (Ward, 2015).

Anammox is an anaerobic oxidation of ammonium to N_2 using NO_2^- as an electron acceptor instead of oxygen, and with NO and hydrazine as intermediates (Humbert et al. 2010, Stein and Klotz, 2016). Anammox is an important process in marine N cycle but due to the great diversity of anammox bacteria, this process can be found also in soils, under specific conditions (Humbert et al. 2010).

Nitrification produces nitrates (i.e. NO_3^-), which is a substrate for another microbial process: denitrification. Denitrification is a cascade of reactions from NO_3^- to dinitrogen N_2, reduction occurring under anaerobic or oxygen-restricted conditions and undertaken by bacteria, fungi, archaea or other denitrifying eukaryotes possessing denitrification enzymes (Saggar et al., 2013). Dinitrogen is released into the atmosphere and can be fixed again to renew the cycle (Selbie et al., 2015). Nitric oxide (NO) and (N_2O) are two obligate intermediates that can be released to the atmosphere (Saggar et al., 2013). Indeed, many of the denitrifying organisms only possess some of the denitrification enzymes and so perform an incomplete denitrification (Stein and Klotz, 2016). Contrary to the inert gas N_2, NO and N_2O have negative impacts on health and the environment (Erisman et al., 2013). Nitrous oxide is a potent GHG with a warming potential 273 times higher than CO_2 over 100 years (IPCC, 2022), and contributes to ozone layer depletion (Portmann et al., 2012). Nitric oxide can form particulate matter in atmosphere as well as ground level ozone, which are responsible for health problems, particularly cardiovascular and respiratory diseases such as asthma and bronchitis and is also suspected to be involved in some forms of cancers (Sutton et al., 2011).

It is often assumed that N_2O is mostly produced during denitrification and autotrophic nitrification although other processes can also lead to N_2O production (Baggs and Philippot, 2010). Nitrifier denitrification is an oxidation of NH_3 to NO_2^- followed by the reduction of NO_2^- to N_2O. This process is undertaken by ammonia oxidisers under restricted oxygen conditions. Codenitrification is a hybrid reaction, with one of the N atoms in the N_2O molecule coming from a denitrified compound (NO_2/NO) and the other one from a co-substrate (such as hydroxylamine) or a reduced inorganic N compound such as ammonium (Selbie et al., 2015a). Nitrite produced by nitrification

may also react chemically leading to N_2O, which is termed chemodenitrification (Chalk and Smith, 1983).

The NO_3^- produced by nitrification is subject to leaching more than NH_4^+. This is because NH_4^+ is adsorbed by negatively charged soil colloids, while NO_3- is itself negatively charged, and thus not retained in most of the soils (Cameron and Haynes, 1986; Ward, 2015). Presence of NO_3^- and therefore its transportation through soil can lead to indirect N_2O emissions although the fate of leached N requires more research. Leaching can also occur in the form of dissolved organic N (DON) but has been much less studied (Selbie et al., 2015b; van Kessel et al., 2009). The DON molecules can be divided into two categories: the low molecular weight DON including free amino acids and proteins, and the high molecular weight DON composed mainly of humic substances (Jones et al., 2004).

Soil erosion is a transfer of physical material -including some N compounds- by water or wind (Haynes, 1986). This process depends on wind or water flow intensity, topography, plant cover and soil stability (Troeh et al., 1980). In most grasslands, erosion is considered negligible due to plant cover being consistently present (Haynes, 1986).

Leaching and erosion can lead to high NO_3^- concentration in drinking water which is responsible for human health issues (Erisman et al., 2013). Leaching and erosion are also responsible for N enrichment of some aquatic ecosystems, termed eutrophication. Organisms of low trophic level which are adapted to this N enrichment, such as algae or cyanobacteria, proliferate and create hypoxia in the surface water, leading to death of non-tolerant species (Erisman et al., 2013).

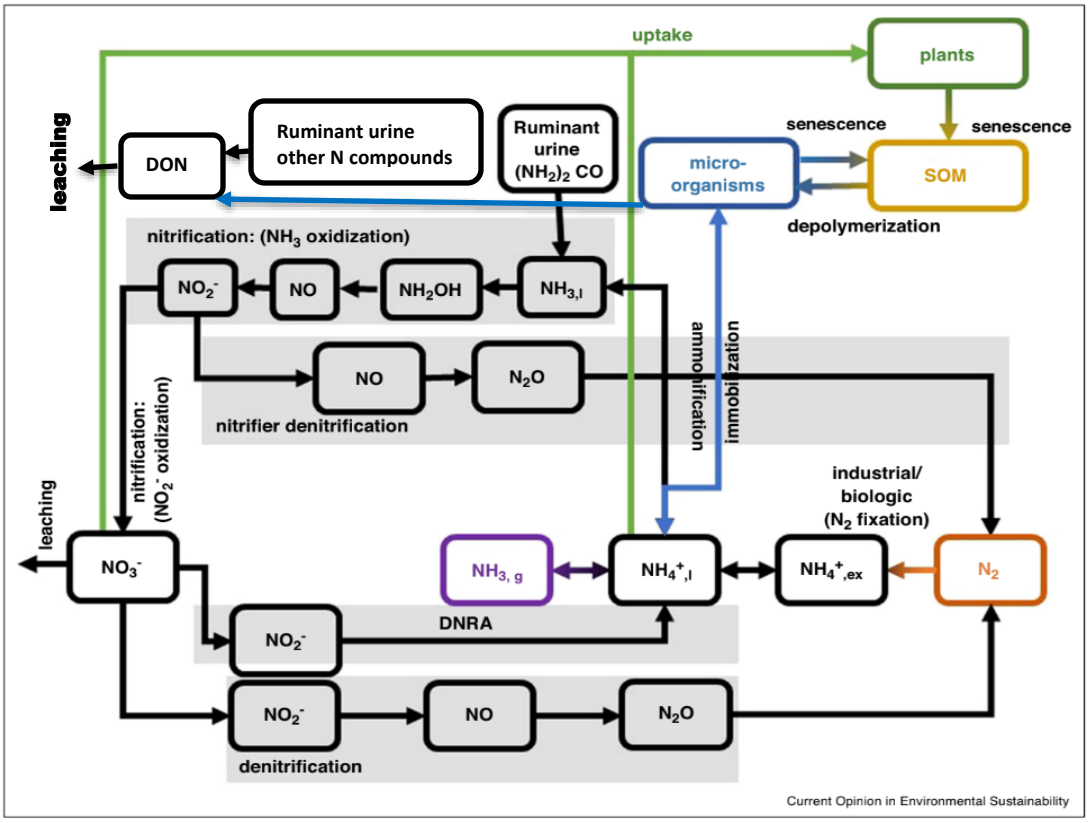

Figure 2.1 Schematic representation of the various processes and losses of the N cycle in soil (adapted from Clough et al. (2020)). DNRA = dissimilatory nitrate reduction to ammonium, NO_3^- = nitrate, NO_2^- = nitrite, NH_4^+ = ammonium, NH_3 = ammonia, NO = nitric oxide, N_2O = nitrous oxide, N_2 = dinitrogen, $(NH_2)_2$ CO = urea, NH_2OH = hydroxylamine, SOM = soil organic matter, DON = Dissolved Organic Nitrogen

2.3 Ruminant excreta: a hotspot for N losses

All these processes of N cycle described earlier occur naturally in soil, but some anthropogenic activities can contribute to the accumulation of Nr in ecosystems, particularly in agricultural soils through the input of external N. Indeed, the transformations of Nr forms into the inert gas N_2 are generally slower than the supply of Nr and the Nr forms are very mobile, leading to losses of N into the ecosystems through NO_3^- leaching, NH_3 volatilisation and N oxides such as N_2O (Erisman et al., 2013).

Excreta patches deposited onto pasture soils by grazing ruminants are hotspots of N_2O emissions and more generally N losses. Ruminants excrete between 75 and 90% of ingested N (Oenema et

al., 2005), leading to high localised N input via excreta, which generally exceeds plant requirements and become available for nitrifiers and denitrifiers (de Klein et al., 2001).

In urine, the N is mostly present in the form of urea which is rapidly hydrolysed into mineral NH_4^+ (Dijkstra et al., 2013, Selbie et al., 2015b). The enzyme involved is urease and is present in every soil (Mobley and Hausinger, 1989). Its activity depends on several factors, including those that influence the micro-organisms which produce the enzyme (Whitehead and Raistrick, 1993). This hydrolysis is associated with an increase in concentration of OH^- ions and therefore of soil pH, thus favoring NH_3 volatilisation (Clough et al., 2020). Other N compounds are present in urine, including purine derivatives, amino acids, creatine, creatinine and NH_3 (Dijkstra et al., 2013), and therefore represent another N source for microorganisms involved in the N cycle.

The NO_3^- produced by nitrification under the urine patch can be accumulated in soil and thus subject to leaching when significant drainage occurs (Di and Cameron, 2002). This process can thus be aided by the water input through urine.

Nitrification and denitrification are also promoted by some conditions created by excreta deposited in soil. Indeed, urine leads to addition of labile C to the soil (Orwin et al., 2010), via the degradation of urine-C or via a solubilisation of soil C due to the increase of pH following urine addition (Monaghan and Barraclough, 1993; Lambie et al., 2012). Urea hydrolysis releases some CO_2 (McLaren and Cameron, 1996) as well as some carbonate HCO_3^- which represent a source of C for autotrophic nitrifiers and may stimulate their growth (Marsh et al., 2005). Release of C can also originate from the soil itself with urine addition causing organic matter (OM) solubilisation (Lambie et al., 2013). Excreta patches can also promote anaerobic conditions which are optimal for denitrification activity (Carter, 2007; Cai et al., 2017). An increase of microbial activity following urine addition can be supported by the quantity of CO_2 produced exceeding the C initially present in urine, suggesting a priming effect on soil C by increased microbial activity (Kool et al., 2006).

Ruminant urine has also been shown to lead to changes in microbial communities. An increase of the size of ammonia oxidising bacteria (AOB) community involved in nitrification (marked by *bacterial amoA* gene) (Di et al., 2014; Pan et al., 2018; Ma et al., 2021), as well as a change of composition in AOB community (Mahmood and Prosser, 2006) has generally been observed in literature. The effect of urine on denitrifier communities is more inconsistent; while Ma et al. (2021) observed no effect of urine on denitrification gene abundances, Jha et al. (2020) observed

an increase of *nosZ I* (marker of N_2O reductase). If the effect of urine on microbial communities is inconsistent throughout studies, it is likely due to its dependence on surrounding conditions, such as soil moisture, that influence microbial functioning (Orwin et al., 2010).

It seems that urine has no specific effect on microbial biomass nitrogen (MBN) (Lovell and Jarvis, 1996; Rooney et al., 2006; Ma et al., 2007), although Wachendorf et al. (2011) found that microbial biomass assimilated up to 17% of urine N applied. As suggested by Lovell and Jarvis (1996), urine addition may increase microbial activity without being associated with an increase of microbial community size. As suggested by the authors, increased activity without an increase of microbial biomass size can reflect a higher active to dormant microbial populations ratio, with activation of some microbial species such as nitrifiers. The effect of dung on microbial community has been less studied, probably because N losses measured from dung are generally lower than those from urine, as highlighted in the studies of Cai and Akiyama (2016) and López-Aizpún et al. (2020). This is mainly due to the N forms present in dung, which are more slowly released and less available for microorganisms (Whitehead, 2000).

Deposited excreta may seem like small, localised patches compared to the pasture area, but after one year post-deposition, they can cover up to 29% of the pasture soil (Selbie et al., 2015b). That means that in areas where grazing systems are an important activity, such as Ireland or New Zealand, excreta deposition of livestock can be a major source of N losses and particularly N_2O emissions, as reflected in the **Fig. 2.2.** In such countries, it is essential to accurately report these emissions in the national GHG inventory reports (NIR)

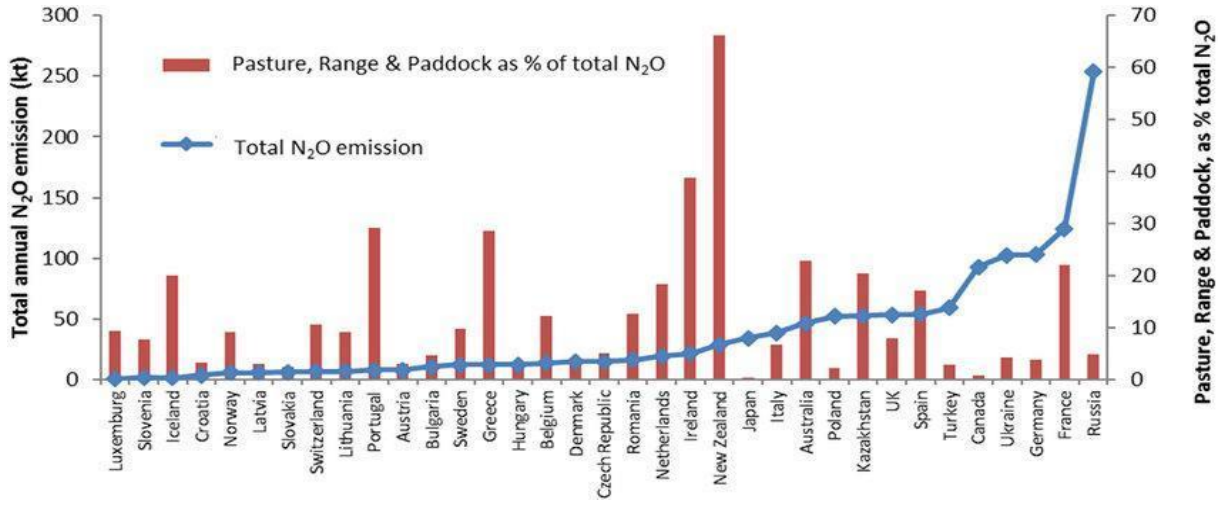

Figure 2.2. Total annual N_2O emissions and contribution of Pasture, Range and Paddock in N_2O emissions for 35 countries (source: Chadwick et al., 2018 according to UNFCCC, 2016).

2.4 Factors of variation

2.4.1 Factors influencing N_2O emissions

As previously explained, N losses from excreta patches are associated with spatio-temporal variability due to an interaction of factors controlling them. Drivers of N_2O emissions have been substantially assessed and the main drivers include N and labile C availability, soil moisture, temperature and soil pH (de Klein et al., 2001; Cameron et al., 2013; Selbie et al., 2015).

2.4.1.1 N availability

Mineral N (i.e. NH_4^+ and NO_3^-) are substrates for nitrification and denitrification involved in N_2O production. Therefore, as observed in the studies of Dai et al. (2013) and Selbie et al. (2014), N_2O emissions are expected to increase with urine N loading rates. However, the effect of N loading rate on N_2O EF_{3PRP} appears to be inconsistent (Singh et al., 2009; Selbie et al., 2014; Marsden et al., 2016) and therefore cannot explain the difference of EF_{3PRP} between cattle and sheep excreta generally observed (Kelliher et al., 2014; Cai and Akiyama, 2016; López-Aizpún *et al.*, 2020).This difference of EF_{3PRP} is more likely to be linked to differences in urine distribution, urine composition, as well as soil compaction between both animal categories (IPCC, 2006; López-Aizpún et al., 2020).

Due to a localised accumulation of N, overlapping of urine N with previous excreta patch or fertiliser is likely to increase N_2O emissions. Indeed, in the study of Maire et al. (2020), N_2O emissions from the combination urine/fertiliser were higher the sum of emissions from each separate treatment (i.e. urine and fertiliser separately), suggesting an interactive effect between both N sources. Lombardi et al. (2022) also observed a synergetic effect with N_2O emissions from overlapped urine and dung patch being higher than the sum of emissions from individual excreta patches. Therefore, we can expect lower N_2O emissions in extensively grazed livestock systems compared to intensive systems due to their lower livestock stocking rates and lower or no synthetic fertilisation and thus lower N inputs.

However, high N rates have also been shown to inhibit nitrification and subsequent N_2O emissions (Clough et al., 2003) but this effect is likely to be regulated by soil properties. To our knowledge, this effect has not been observed with sheep urine.

2.4.1.2 C availability

Clearly, C is essential for microbial growth and therefore external input of C stimulate nitrifiers and denitrifiers activity and subsequent N_2O production (Cameron et al., 2013). The coupling of the N and C cycle leads to the importance of C:N ratio of a material input to soil, which influences the process of N mineralisation/immobilisation and therefore the production of N_2O (Chen et al., 2013). Lower C:N ratio of excreta have been associated with higher N mineralisation and N_2O emissions in several studies (Pelster et al., 2016, Simon et al., 2018; Lombardi et al., 2022).

Carbon cycle is not only coupled with N but with other soil nutrients such as phosphorus (P). In a recent study, O'Neill et al. (2020) showed that C availability was regulating the influence of P on N_2O emissions, with N_2O emissions being reduced in soil with higher P content when C availability was not limited. This suggests that N_2O emissions from grazed grasslands, where C is regularly brought through livestock excretal returns, could be mitigated by soil P status management.

2.4.1.3 Soil water content and aeration status

Soil water content has often been reported as one of the dominant factors influencing N_2O emissions (Smith et al. 2003, van Groenigen et al., 2005, Cameron et al., 2013; Selbie et al., 2015). Indeed, soil moisture determines oxygen availability in soil and an increase of soil moisture creates

anaerobic conditions promoting activity of denitrifiers involved in N_2O production. However, in case of saturated soil conditions, N_2O emissions can be low because of low or absent production of NO_3^- by aerobic nitrifiers (Bell et al., 2015). Absence of oxygen may also be associated with an increase of N_2O reductase activity which reduces N_2O up to N_2 (Lu and Xu, 2014). The major role played by water content on microbial activity leads to the importance of rainfall, which is a proxy of soil water content and aeration status (Schindlbacher et al., 2004; Selbie et al., 2015b; Banerjee et al., 2016). Many studies highlighted the importance of rainfall prior to the excreta deposition because denitrifiers are stimulated before N input and can then use the excretal N more efficiently (Alves et al., 2012; Krol et al., 2016; da Silva Cardoso et al. 2018). Subsequently, soil drainage properties also influence N_2O emissions, which are generally higher in imperfectly drained soils (de Klein et al., 2003: Krol et al., 2016).

Recent studies have highlighted the importance of soil gas diffusivity (Dp/Do), that may be a better predictor of N_2O than soil moisture (Balaine et al., 2016; Owens et al., 2017). This is because Dp/Do better describes the aeration of soil pores by considering the interaction of WFPS with soil bulk density (Clough et al., 2020).

Finally, low soil moisture does not necessarily lead to low N_2O emissions. Bell et al. (2015) observed the highest fluxes after urine deposition in summer which was associated with lower WFPS compared to autumn. The authors suggested that nitrification was the dominant pathway producing N_2O and that nitrification rate was reduced by the high soil moisture conditions in autumn. Moreover, despite similar soil moisture conditions between spring and summer application, higher emissions in summer were likely to be due to higher temperature, which is another influential factor.

2.4.1.4 Temperature

It is well known that temperature regulates microbial and enzymatic activity (Signor and Cerri, 2013). Several studies have shown an increase of N_2O emissions with increasing temperatures (Dobbie and Smith, 2001; Smith et al., 2003; Lu and Xu, 2014). The Q_{10}, which expresses the change of N_2O production rate when temperature increases of 10°C, can reach more than 10, whereas the Q_{10} of other biochemical processes range usually between 2 and 3 (Smith et al., 2003). This is because temperature not only stimulate enzymes activity, but also has an indirect effect on

microbial activity, by favoring activity of anaerobic microorganisms through an increase of anaerobic microsites, thus promoting denitrification (Smith et al., 2003; Butterbach-Bahl et al., 2013). However, this effect of increasing anaerobicity can be associated with complete denitrification and a decrease of N_2O: N_2 ratio (Butterbach-Bahl and Dannenmann, 2011). Finally, the positive effect of temperature occurs if microbial activity is not restrained by substrate availability or soil moisture content (Pilegaard et al., 2013).

2.4.1.5 Soil pH

Soil pH influences several steps of the N cycle. Inhibition of N_2O reductase have been observed under acidic conditions, thus increasing the N_2O: N_2 ratio (Liu and Bakken, 2014). That is why increasing soil pH, through liming for instance, can allow for complete denitrification and reduce the N_2O emissions from soils (Zaman et al., 2007; Žurovec et al., 2021). Acidic soil pH has also been shown to inhibit nitrification (Nicol et al., 2008). Although nitrification was assumed to be very low in acid soils, recent findings showed that acid tolerant AOA were major contributors of nitrification under acidic conditions (Li et al., 2018).

The overall effect of soil pH on N_2O emissions remains unclear (Šimek and Cooper, 2002) most likely due to the interaction of pH with other factors. In a recent meta-analysis, Pan et al. (2022) observed no relation between denitrification rate and soil pH based on the whole dataset, but these two variables were positively correlated under anaerobic conditions.

Nevertheless, the effect of low pH on N cycle is likely to be lifted after urine application which is associated with an increase of pH as reported in most studies (Mahmood and Prosser, 2006; Orwin et al., 2010; Curtin et al., 2020). However, it remains unclear if this short-term change of pH can have an influence on microbial communities in acid soils.

2.4.2 Factors influencing NO emissions

Similar to for N_2O, NO is released during both nitrification and denitrification. Therefore, factors controlling NO emissions are similar to those influencing N_2O emissions, including soil moisture and temperature. In a laboratory experiment, temperature and soil moisture explained about ¾ of variability of NO emissions (Schindlbacher et al., 2004).

Globally, NO emissions emitted from soils are suggested to be low, even after deposition of excreta patches by livestock (Cai et al., 2016, Maljanen et al., 2007). This is probably because NO is a short-lived trace gas (Stark et al., 2002). However, NO emissions from excreta deposition have been much less studied and quantified compared to N_2O emissions and further studies are required to better understand the drivers of NO emissions (Pilegaard, 2013).

2.4.3 Factors influencing NH_3 volatilisation

The main factors regulating NH_3 volatilisation are soil pH, NH_4^+ concentration, soil cation exchange capacity (CEC), soil moisture content and temperature (Cameron et al., 2013). As previously explained, high soil pH conditions lead to the switch from NH_4^+ to NH_3, and the increase of pH following urine application favors NH_3 volatilisation. Soil CEC characterises the ability of clays and organic matter (OM) surface to bind and retain NH_4^+. Therefore, the higher the CEC, the lower will be the quantity of NH_4^+ subject to NH_3 volatilisation (Whitehead, 1993).

Higher temperatures increase transformation of NH_4^+ to NH_3 and the transfer of NH_3 from soil to atmosphere (Flechard et al., 2013). However, Fischer et al. (2016) observed highest NH_3 emissions in spring, associated with the lowest temperatures. The authors suggested that higher volatilisation in spring were due to initial soil moisture increasing urease activity and to the absence of rainfall during the first days post application. Indeed, rainfall has been showed to reduce NH_3 volatilisation after urine application (Saarijärvi et al., 2006), particularly due to N infiltration down from soil surface (Fischer et al., 2016).

Finally, according to the transformation process leading to NH_3 volatilisation, it is obvious that NH_4^+ concentration in soil is an important factor controlling NH_3 emissions and therefore other processes influencing NH_4^+ concentration in soils (nitrification, plant uptake) will influence NH_3 volatilisation (Cameron et al., 2013).

2.4.4 Factors influencing NO_3^- leaching

The quantity of leached NO_3^- is determined by its concentration in the soil, originating from organic matter (OM) mineralisation or external N input, as well as the magnitude of soil drainage (Di and Cameron, 2002). Higher N application through excreta is associated with higher risk of NO_3^- leaching because N supply is more likely to exceed plants requirement at higher N application rate and thus more N become available for nitrification and production of NO_3^-. Di and Cameron

(2007) have observed NO_3^- leaching losses being more than four times higher at an application rate of 1000 kg ha^{-1} compared to the leaching losses at 300 kg ha^{-1}. We can therefore expect lower leaching losses from sheep excreta compared to cattle, due to their difference in N loading rates. Indeed, McDowell et al. (2009) observed greater leaching losses from cattle urine than sheep urine.

In temperate regions, leaching losses have often been shown to be higher in autumn when plant growth is lower and rainfall is generally more important (Stout, 2003; Buckthought et al., 2013). Soil characteristics are also determinant for leaching losses, particularly because they determine soil drainage properties and water content (Cameron et al., 2013). Decau et al. (2003) found N leaching losses up to eight times higher in a free draining soil compared to other soils with moderate draining properties.

Therefore, the several factors influencing various processes of the N cycle and their interactions make it difficult to accurately estimate the N losses from excreta patches deposited onto pasture soils. **Chapter 3** describes specific methods adopted by some countries aiming to consider factors of variations in the estimations of N_2O emissions, including the disaggregation of EF_{3PRP} and the use of models.

2.5 Estimation and reporting of N losses

Countries that are members of the United Nations Framework Convention on Climate Change (UNFCCC)- must report their estimations of GHG emissions in national GHG inventories annually. The IPCC (Intergovernmental Panel on Climate Change) provides methodological guidance to estimate and report GHG emissions. The methodology of estimations is divided into three levels reflecting the accuracy of estimations, as detailed in **chapter 3**. Briefly, Tier 1 and Tier 2 approaches are based on the use of emission factors (EF) and activity data for each polluting source. Tier 1 estimations are calculated with default values of EF provided by the IPCC, while Tier 2 estimations are calculated with country-specific data and therefore are more accurate. Finally, Tier 3 methodology is based on modeling approaches.

In GHG national inventory reports (NIR), the section referring to emissions from agricultural soils include N_2O emissions from livestock excreta deposited in pasture, range and paddock (PRP). The EF_{3PRP} is defined as the proportion of excretal N emitted as N_2O. Activity data include N excretion rate and animal population for each animal category (IPCC, 2019).

IPCC default (Tier 1) EF_{3PRP}s have been refined in 2019, but to our knowledge are not yet adopted in any NIRs. These values (i.e. 0.4 and 0.3% for combined excreta of cattle and sheep, respectively) are more accurate than previous 2006 Tier 1 EF_{3PRP} (i.e. 2 and 1% for cattle and sheep respectively) because they have been established based on a higher number of studies. However, they are still associated with wide range of uncertainties due to spatio-temporal variations of N_2O emissions. This is why the establishment of country-specific EF_{3PRP} is encouraged, in order to decrease uncertainties around the estimations of N_2O emissions. **Chapter 3** of this manuscript assesses the uncertainties associated with the methodology used by various countries in the reporting of N_2O emissions from ruminant excretal returns.

Few countries have developed their Tier 2 EF_{3PRP}, and this is particularly true for sheep excreta which is globally less studied than cattle. In a recent analysis, López-Aizpún et al. (2020) found four times more EF_{3PRP} for cattle urine than sheep urine. The reason why countries are still using Tier 1 methodology for their reporting pertains to the time-consuming process of establishing Tier 2 EF_{3PRP}, based on long-term field experiments.

Other N losses from excreta patch also have their default " fraction loss" provided by the IPCC (IPCC, 2019). The fraction of excretal N lost as NH_3 have been established at 19.7% based on a compilation of studies (Bouwman et al., 2002; Cai and Akiyama, 2016). The fraction lost as NO has been estimated at 0,15% based on the work of Liu et al. (2017). Finally, the fraction of excretal N lost through leaching and runoff (i.e. 24%) has been calculated from two review papers (Di and Cameron, 2002; Cai and Akiyama, 2016) and from about 30 additional studies. There is no disaggregation by animal category for NH_3, NO and leaching fraction losses, which are associated with wide range of uncertainties ([0-29,5%]; [0-14,9%] and [1-73%] for NH_3, NO and leaching fraction loss, respectively).

Efforts are being made to improve the accuracy of N_2O and NH_3 EF from livestock waste management (including N_2O EF_{3PRP}) that could be adopted by inventory compilers using the Tier 1 methodology (van der Weerden et al., 2021). Indeed, one goal of the recent project DATAMAN is to gather and analysed EF_{3PRP}s in order to assess if further disagreggation according to factors of variation are required (Beltran et al., 2021). In an analysis of the DATAMAN database, van der Weerden et al. (2021) have for instance suggested a disaggregation of EF_{3PRP} according to urine/dung N ratio which is influenced by N content in livestock diet.

2.6 Conclusions

Ruminant patches are hotspots for N losses due to their effect on soil properties. It is therefore essential to report these emissions, particularly in countries where pasture-based systems are important. The estimation of these losses is still associated with uncertainties, particularly for sheep excreta which has brought less attention compared to cattle excreta. Uncertainties in estimations are linked to the interaction of several factors influencing these losses, including soil and climatic parameters, as well as factors related to excreta. Therefore, N losses and fate of excretal N are expected to vary between two distinct grazing systems presenting differences in soil type, animal diet and even climatic conditions.

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Chapter 3

Uncertainties in direct N_2O emissions from grazing ruminant excreta (EF_{3PRP}) in national greenhouse gas inventories

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This experimental chapter is based on an article which has been accepted for publication in the journal Science of the Total Environment:

Mancia, A., Chadwick, D. R., Waters, S. M., Krol, D. J., 2022. Uncertainties in direct N_2O emissions from grazing ruminant excreta (EF_{3PRP}) in national greenhouse gas inventories. Science of The Total Environment, 803, 149935.

AM conducted the literature research and prepared the manuscript, DRC, SMW and DK reviewed the manuscript

Abstract

Excreta deposition onto pasture, range and paddocks (PRP) by grazing ruminant constitute a source of nitrous oxide (N_2O), a potent greenhouse gas (GHG). These emissions must be reported in national GHG inventories, and their estimation is based on the application of an emission factor, EF_{3PRP} (proportion of nitrogen (N) deposited to the soil through ruminant excreta, which is emitted as N_2O). Depending on local data available, countries use various EF_{3PRP}s and approaches to estimate N_2O emissions from grazing ruminant excreta. Based on ten case study countries, this review aims to highlight the uncertainties around the methods used to account for these emissions in their national GHG inventories, and to discuss the efforts undertaken for considering factors of variation in the calculation of emissions. Without any local experimental data, the 2006 IPCC default (Tier 1) EF_{3PRP} s are still widely applied although the default values were revised in 2019. Some countries have developed country-specific (Tier 2) EF_{3PRP} based on local field studies. The accuracy of estimation can be improved through the disaggregation of EF_{3PRP} or the application of models; two approaches including factors of variation. While a disaggregation of EF_{3PRP} by excreta type is already well adopted, a disaggregation by other factors such as season of excreta deposition is more difficult to implement. Empirical models are a potential method of considering factors of variation in the establishment of EF_{3PRP}. Disaggregation and modeling require availability of sufficient experimental and activity data, hence why only a few countries have currently adopted such approaches. Replication of field studies under various conditions, combined with meta-analysis of experimental data, can help in the exploration of influencing factors, as long as appropriate metadata is recorded. Overall, despite standard IPCC methodologies for calculating GHG emissions, large uncertainties and differences between individual countries' accounting remain to be addressed.

Key words: Emission factor; Grassland; IPCC methodology; National inventory report; Disaggregation

3.1 Introduction

Nitrous oxide (N_2O) is an important greenhouse gas (GHG) with a warming potential 265 times higher than carbon dioxide (CO_2) over a 100 year period (IPCC, 2014). Through the formation of nitrogen oxides (NO and NO_2), N_2O also contributes to ozone layer depletion (Portmann *et al.*, 2012). Its surface concentration in 2018 was estimated at 331 ppb which is 19% higher than pre-industrial level (Aneja *et al.*, 2019); such an increase leads to negative consequences for human health and the environment (Erisman *et al.*, 2013).

Nitrous oxide is naturally produced in soils, mainly through the microbial processes of nitrification and denitrification influenced by surrounding environmental conditions (Baggs and Philippot, 2010), although other processes may significantly contribute to N_2O production (van Groenigen *et al.*, 2015). The production of N_2O is moderated by nitrogen (N) inputs to soils exceeding plant requirements, availability of labile carbon (C), soil moisture, temperature and pH (de Klein *et al.*, 2001). Some agricultural practices are responsible for large N inputs to the soil, making agriculture the largest emitter of N_2O among anthropogenic activities, with approximately 60% of anthropogenic N_2O produced by this sector (Davidson and Kanter, 2014). Sources of N_2O emissions from agricultural soils include the use of mineral and organic fertilisers, management of organic soils, crop residues and urine and dung deposited by grazing livestock (IPCC, 2006).

Nitrogen returns to soil by grazing livestock are particularly relevant for ruminants which have a low N use efficiency and excrete between 70 and 95% of ingested N (Oenema *et al.*, 2005). Due to these large N inputs combined with a source of labile C and the creation of anaerobic conditions, excreta patches (urine and dung) represent hotspots for N_2O production (Bolan *et al.*, 2004; Saggar *et al.*, 2004; Selbie *et al.*, 2015; Cai *et al.*, 2017). Grasslands cover about 40% of the Earth's land area, excluding Greenland and Antarctic (White *et al.*, 2000). Pasture-based livestock production systems may represent a dominant agricultural activity in some countries such as New Zealand (Ministry for the Environment, 2020), Ireland (CSO, 2016) and the UK (DEFRA, 2019). Therefore, and particularly in those countries, it is imperative to accurately quantify N_2O emissions from excreta deposition of grazing ruminant in national inventories, in order to monitor their trends and help improve agricultural management decisions to aid mitigation.

Parties of Annex I of the United Nations Framework Convention on Climate Change (UNFCCC) must report annually an inventory of their GHGs emitted from each sector, while non-

Annex I countries must submit a biennial update report (BUR). The IPCC has established methodological guidelines to help countries in the reporting of emissions, based on the use of emission factors (EFs) combined with activity data for each sector. EF_{3PRP} is the emission factor related to livestock excreta deposited onto pasture, range and paddock (PRP), defined as the fraction of excreted N deposited in grassland which is emitted as N_2O. The methodology comprises three levels (Tiers) depending on availability of local data. Tier 1 methodology employs default values of EF based on previous studies. Since 2006, default Tier 1 values of EF_{3PRP} were 2 and 1% for cattle and sheep, respectively, however these were revised in 2019. The refined aggregated EF_{3PRP} are 0.4 and 0.3% for cattle and sheep, respectively, and can be further disaggregated by type of excreta (urine and dung) as well as by climate (dry and wet). The 2019 refinement is based on a meta-analysis of 461 EF_{3PRP} values estimated in several studies from 13 countries. The disaggregation of EF_{3PRP} between sheep and cattle adopted in the IPCC 2019 refined guidelines has been justified by a highly significant difference between both animal categories (IPCC, 2019b). In the previous 2006 IPCC guidelines, this difference was explained by less soil compaction and more evenly distributed urination (higher frequency, lower volumes) for grazing sheep compared to cattle (IPCC, 2006). However, there are more complex reasons for this difference, and they are likely to be linked to the relationship between N_2O emissions and N loading rate (Selbie *et al.*, 2014), as well as to urine chemical composition (López-Aizpún *et al.*, 2020).

The 2019 revised values of Tier 1 EF_{3PRP}s are still associated with major variations (i.e. 0-1.4% and 0-1.0% for cattle and sheep, respectively) and this wide range of values highlights the great variability in N_2O emissions from ruminant excreta deposition across the globe. Therefore, adoption of higher tier methodologies to estimate N_2O emissions from grazing excreta is highly encouraged. Firstly, this can be achieved by the adoption of Tier 2 methodology through the development of country-specific EF_{3PRP}. Additionally, the Tier 2 EF_{3PRP} can be further disaggregated to take account of spatio-temporal variability of N_2O emissions, thus improving accuracy of estimations. A first level of disaggregation of emissions by excreta type has already been undertaken by some countries, which have developed Tier 2 EF_{3PRP} based on N_2O flux measurements from ruminant urine and dung separately, on soils and in climates that are typical of the nation (e.g. Ireland (Krol *et al.*, 2016); the UK (Chadwick *et al.*, 2018); New Zealand (van der Weerden *et al.*, 2020)). Finally, the highest Tier 3 methodology is based on the use of models to predict emissions, however to date these have rarely been used for quantifying N_2O from grazing excreta.

Depending on data availability in each country, different accounting methods are used to report N_2O emissions from ruminant livestock deposition in grasslands, and national estimations are uncertain to varying extents. These uncertainties are particularly relevant for emissions from sheep grazing systems, since this animal category has been less studied than cattle (IPCC, 2019b).

Therefore, the aims of this study were to i) highlight the uncertainties linked to the methodology adopted and the EF_{3PRP} used to estimate emissions from ruminant excreta deposition in grasslands, and ii) showcase the importance of disaggregating Tier 2, country-specific EF_{3PRP} in order to improve accounting of national estimations by considering variations linked to factors influencing N_2O emissions.

In order to reach these objectives, a selection of National Inventory Reports (NIRs) submitted to the UNFCCC for the year 2021 were studied (UNFCCC, 2021). Next, ten case study countries were selected, accounting for geographical spread and representing the diversity of approaches used in the reporting of N_2O emissions from ruminant excreta deposition in grasslands. This diversity is characterised by differences in the IPCC methodology used (i.e. Tier 1, 2 or 3), type of EF_{3PRP} used for cattle and for sheep (i.e. Tier 1 or Tier 2, same or different value) and factors of disaggregation of Tier 2 EF_{3PRP}. The second part of this review describes the efforts of disaggregation of EF_{3PRP} undertaken by the case study countries in order to take account of spatio-temporal variations of N_2O emissions and addresses the challenges of such disaggregation.

3.2 Uncertainties linked to methodology used in national reports

3.2.1 Uncertainties relating to Tier 1 EF_{3PRP}

Different values of EF_{3PRP} are used in the NIRs: while some countries use their country-specific values, most of the parties do not have national experimental data and therefore still use the default IPCC EF_{3PRP} (Tier 1) in the 2021 UNFCCC submissions. Although the IPCC guidelines were updated in 2019, including refined combined Tier 1 EF_{3PRP} (i.e. 0.4 and 0.3% for cattle and sheep respectively), changeover in the inventory reporting is slow and therefore Tier 1 EF_{3PRP}s associated with the previous 2006 guidelines are still widely used (i.e. 2 and 1% for cattle and sheep, respectively (IPCC, 2006)). Countries still using IPCC 2006 Tier 1 EF_{3PRP}s for both cattle and sheep include, e.g. Spain, Poland and Brazil.

The revised values are certainly more accurate than the ones provided in the previous set of guidelines, given the greater number of studies used for their derivation. The uncertainty ranges

attest to this difference in accuracy of estimates: 0-1.4% and 0-1% for cattle and sheep, respectively in 2019 guidelines, compared to 0.7-6% and 0.3-3% for cattle and sheep, respectively in the 2006 guidelines. In order to obtain the EF_{3PRP} for cattle, the means of EFs from cattle urine and dung were calculated for wet and dry climates, and then weighted according to a urine: dung excretion N ratio of 0.66: 0.34 (Kelliher et al., 2014). However, due to lack of data for N_2O EF_{3PRP}s for sheep excreta, a mean EF_{3PRP} was derived by combining the values for sheep and cattle. Indeed, the dataset used by the IPCC contained only 35 and 22 values for sheep urine and dung, respectively, compared to 290 and 113 values for cattle urine and dung, respectively (IPCC, 2019b), implying less accuracy for sheep EF_{3PRP} compared to cattle.

Tier1 EF_{3PRP}s defined in the 2006 IPCC guidelines were based on a limited number of studies and have often been shown to overestimate emissions for both cattle and sheep excreta in particular locations (Luo *et al.*, 2013; Tomazi *et al.*, 2015; Krol *et al.*, 2016; Chadwick *et al.*, 2018; Marsden *et al.*, 2018). The 2006 IPCC default values have been derived from studies carried out on lowland managed grasslands on mineral soils and are not necessarily representative of the diversity of grazing systems. Indeed, in countries such as Ireland, New Zealand and the UK, a significant proportion of grazing systems are on uplands, which are characterised by different soil, vegetation, management practices (e.g. approximately 10% of grassland is devoted to rough grazing in Ireland (CSO, 2013) and in the UK (DEFRA, 2019)). Studies such as those of Marsden et al. (2018, 2019) have shown lower N_2O emissions from urine patches in upland systems compared to those in lowlands in the UK, which may justify the use of different EF_{3PRP} for each of these grazing systems, as discussed later in this Review.

An additional source of uncertainty around the use of IPCC Tier 1 EF_{3PRP} may be found in the misuse of these values: Spain for instance applies the same IPCC Tier 1 EF_{3PRP} of 2% (i.e. default value for cattle excreta) for both sheep and cattle.

3.2.2 Differences in approaches to using country-specific EF_{3PRP}

The development of country-specific Tier 2 EF_{3PRP} is a long process requiring field experiments to accumulate experimental data, most of them applying N_2O static chamber methodology (Charteris *et al.*, 2020; de Klein *et al.*, 2020a). Through the implementation of co-ordinated national research programmes, few countries have currently developed Tier 2 EF_{3PRP}. In most cases, these studies were carried out on cattle excreta, leading to a lack of data from sheep excreta for most

countries. Consequently, approaches to report emissions from sheep excreta vary: some countries choose to apply the country-specific EF_{3PRP} established for cattle excreta, while others continue to use the default IPCC 2006 value. These different approaches used by the ten case study countries are summarised in **Table 3.1**.

For example, Ireland developed its own EF_{3PRP} for cattle, calculated as a mean of EFs estimated from N_2O emission measurements following cattle urine and dung deposition in three seasons and on three different soil types in Ireland. The disaggregated Tier 2 EF_{3PRP}s are 1.2% and 0.31% for cattle urine and dung, respectively (Krol *et al.*, 2016). According to the specific excreted N urine: dung ratios calculated for cattle (i.e. depending on diet characteristics), the combined excretal EF_{3PRP} is 0.86%, which is lower than the IPCC 2006 Tier 1 EF_{3PRP} (i.e. 2%) but higher than the IPCC 2019 refined value (i.e. 0.4%). Since no experimental data for sheep excreta has been acquired yet, Ireland still uses the Tier 1 EF_{3PRP} based on the IPCC 2006 guidelines for this animal category (i.e. 1%). This default value for sheep is higher than the Tier 2 excretal EF_{3PRP} for cattle, which infringes the assumption of higher emissions from cattle excreta compared to sheep.

The UK also implemented an extensive research programme for the determination of EF_{3PRP} from cattle urine and dung. One of the field studies involved in this programme included five different experimental sites across the UK, and three seasons of cattle excreta application to soil (Chadwick *et al.*, 2018). A large effort was undertaken to combine the EF_{3PRP}s obtained in this study with other experimental data sets established in the UK to derive new disaggregated Tier 2 values (i.e. 0.63 % and 0.19% for cattle urine and dung, respectively). Depending on the urine N: dung N ratio, the combined UK EF_{3PRP} for cattle ranges from 0.5 to 0.6% (Chadwick *et al.*, 2016), which is lower than the IPCC 2006 Tier 1 values for cattle and higher but close to the IPCC 2019 refined Tier 1 EF_{3PRP} (i.e. 0.4% for cattle). Until the previous GHG inventory (NIR 2020), the UK was applying to sheep excreta the country-specific Tier 2 EF_{3PRP} derived from cattle excreta, in order to be in line with the assumption that emissions from sheep are not greater than those from cattle. Indeed, the IPCC 2006 Tier 1 EF_{3PRP} for sheep (i.e. 1%) is higher than the UK Tier 2 value. Since the IPCC 2019 revised Tier 1 value is lower than the UK Tier 2 EF_{3PRP}, the UK is now applying the Tier 1 IPCC 2019 value to sheep. Recent experimental studies in the UK have focused on emissions from sheep excreta deposition, generating data for a potential establishment of Tier 2 EF_{3PRP} for sheep excreta (Marsden *et al.*, 2017; Marsden *et al.*, 2018; Marsden *et al.*, 2019).

Australia applies the same Tier 2 EF_{3PRP} (i.e. 0.4%) for both cattle and sheep, since similar values of EF_{3PRP} were measured in two field experiments using either dairy cow urine (Galbally *et al.*, 2005) or sheep urine (Galbally *et al.*, 2010). In fact, Galbally et al. (2010) found EF_{3PRP}s of 0.1-0.2% depending on the season of urine addition to the soil. Since the measurement periods were short (approximately one month), the authors assumed to have captured only 50% of N_2O emitted from added urine during these periods, and the measured EF_{3PRP}s were extrapolated from 0.1-0.2% to 0.2-0.4% (Galbally *et al.*, 2010). This is an unusual approach to derive N_2O EF_{3PRP} based on short-term data (as opposed to the full year measurements recommended by IPCC guidelines), as well as to assume that 50% of emissions are captured within a month post-application and then double the value of EF_{3PRP} to be representative of a full year. The same value is also used for cattle and sheep dung, which is based on existing literature suggesting 0.4% is a reasonable mean for dung EF_{3PRP} (Flessa *et al.*, 1996; Oenema *et al.*, 1997; Yamulki *et al.*, 1998). Therefore, the combined excretal Tier 2 EF_{3PRP} is 0.4% for cattle and sheep, which is equal to the Tier 1 value for cattle in the 2019 refined IPCC guidelines. Once again, it is an unusual method to derive a Tier 2 EF_{3PRP} from literature, and besides, which is not local and not recent literature.

Canada developed its Tier 2 EF_{3PRP}s based on two field studies: one carried out in the Western region from beef excreta (Lemke *et al.*, 2012) and one in Eastern region from dairy cow excreta (Rochette *et al.*, 2014). When calculating national N_2O emissions, different values of Tier 2 EF_{3PRP} are used. In the Western Region (four provinces), a single value of 0.04% is used, calculated from EF_{3PRP}s disaggregated by excreta type (i.e. 0.06 and 0% for urine and dung respectively). In the Eastern region (Ontario, Quebec and the four Atlantic provinces), EF_{3PRP}s are province-specific, ranging from 0.48 to 0.61% (i.e. combined excreta), and are calculated from EF_{3PRP}s disaggregated by soil texture class (i.e. 1.09, 0.70 and 0.31% for urine and 0.08, 0.12 and 0.15% for dung applied on fine, medium and coarse soil texture respectively (Rochette *et al.*, 2014)), assuming 75% of N is excreted in urine. In both Eastern and Western regions, the same values of EF_{3PRP} are applied for all livestock categories since no experimental data are available for other grazing animals. These Tier 2 EF_{3PRP}s in both Eastern and Western regions of Canada are lower than the IPCC 2006 default values, but the province-specific Tier 2 EF_{3PRP}s developed in the Eastern region are all higher than the IPCC 2019 refined values (i.e. 0.4 and 0.3% for combined excreta of cattle and sheep respectively). The very low EF_{3PRP} used in the Western prairies compared to the values established in the Eastern Region is justified by the semi-arid conditions in the western provinces of the country, in

line with the observations of Rochette et al. (2008) who found that moisture deficit is a main factor controlling N_2O from agricultural lands in Canada.

New Zealand undertook possibly the most detailed research efforts in the world to establish Tier 2 EF_{3PRP} estimated from a meta-analysis of 139 field studies carried out from the year 2000 to 2017 (van der Weerden *et al.*, 2020). These field studies are representative of the different sheep and cattle grazing systems within the country and provided more than one thousand individual EF_{3PRP} values, allowing a detailed disaggregation of EF_{3PRP}. Indeed, Tier 2 EF_{3PRP}s are disaggregated not only by animal category and excreta type, as suggested in the studies of Hoogendoorn *et al.* (2008) and van der Weerden *et al.* (2011), but also by topography. This third level of disaggregation is based on slope class category, which is a factor highlighted in previous field studies as an important source of variability (Luo *et al.*, 2013). According to excreted N urine: dung ratios calculated from the distribution of excreta N (i.e. across the different slope class categories) presented in van der Weerden *et al.* (2020), Tier 2 EF_{3PRP} for combined excreta are 0.72 and 0.26% for cattle on flat/low and medium/steep slope respectively; and 0.37 and 0.09% for sheep on flat/low and medium/steep slope respectively. These values are all lower than the IPCC 2006 Tier 1 EF_{3PRP}s. However, the Tier 2 EF_{3PRP}s established for flat/low land are higher than the refined IPCC 2019 Tier 1 values (i.e. 0.4 and 0.3% for cattle and sheep respectively).

3.2.3 Access to country-specific activity data for better accuracy of estimations

Total national N_2O emissions from ruminant excreta deposition in grasslands are calculated with a Tier 1 or Tier 2 EF_{3PRP}, multiplied by the quantity of N deposited onto grassland soil. Therefore, activity data in relation to livestock population, N excretion rate (N_{ex}) and proportion of excreta voided at pasture are required. The availability of detailed country-specific activity data will improve national estimates of N_2O emissions.

Annual livestock populations of cattle and sheep in each country can be assessed through national agricultural surveys updated annually, such as the June Agricultural Survey in the UK (DEFRA, 2020) or the Agricultural Production Survey in New Zealand (Statistics New Zealand, 2020). Livestock categories can be more or less detailed in these surveys. The UK has for instance disaggregated the sheep population into subcategories (i.e. ewe, rams and lambs), each of which is associated with its proper Nex. However, this disaggregation has not been undertaken by other countries, using one N_{ex} for the whole sheep category (e.g. Canada, Japan).

The IPCC also provides default values of N_{ex} for each animal category (IPCC, 2019a). Brazil applied default IPCC N_{ex} in its last GHG inventory (i.e. fourth BUR). However, most of the case study countries presented in **Table 3.1** use country-specific N_{ex} for cattle and/or sheep, obtained from national zootechnical literature or calculated according to equations of the Tier 2 approach suggested in the IPCC methodology guidelines (IPCC, 2019a). This calculation is based on N intake and N retention characterised by energy requirement which depends on animal species, stage of growth and level of production. N intake and N retention can be obtained from national data or from specialists' expertise. N intake for cattle and sheep can also be calculated with formulas presented in the IPCC guidelines, if detailed data on diet and crude protein intake are available (IPCC, 2019a). For instance, the UK uses country-specific energy balance equations for the determination of dry matter intake, which is used for the calculation of N intake. Japan uses country-specific equations for the calculations of cattle N_{ex} (Choumei *et al.*, 2006) but applies default IPCC N_{ex} for sheep.

Access to more detailed activity data can allow for the disaggregation of EF_{3PRP} under specific conditions and the adoption of a more accurate method of reporting, such as the approach used in New Zealand. In order to disaggregate EF_{3PRP} by slope class category, New Zealand has determined the proportion of excreta N return on each slope category with a nutrient transfer model (Saggar *et al.*, 2015). Detailed country-specific activity data can also allow for the adoption of Tier 3 method via modeling. Indeed, the USA use Daycent, a process-based model (Del Grosso *et al.*, 2012) to simulate N_2O emissions on non-federal grasslands (whereas N_2O emissions on federal grasslands are still estimated with a Tier 1 approach). The application of such model was possible because of the availability of data on N inputs to grasslands and other additional data (soil types, daily climatic conditions) at a high spatial resolution. The IPCC Tier 3 approach, using modeling for estimating N_2O emissions, offers an advantage compared to Tier 1 and Tier 2 methods, which are often based on EFs estimated from only a few numbers of field studies (except for Ireland, the UK and New Zealand), and therefore cannot fully consider the national diversity of soils and climates. Moreover, modeling also has the advantage of avoiding the time-consuming field studies. However, models need to be parameterised and validated by reliable experimental data, in order to decrease uncertainties around their estimations (Ogle *et al.*, 2020).

Table 3.1 Important characteristics (EF_{3PRP}, disaggregation and activity data) of accounting methods used to report N_2O emissions from cattle and sheep excreta deposition for different case study countries

Country (contribution of grazing livestock to total agricultural N_2O emissions[1])	***Methodological approach***	***Type of EF_{3PRP}***	***Methodology used to develop Tier 2 EF_{3PRP} (references)***	***Factors of disaggregation of Tier 2 EF_{3PRP} [2]***	***Values of EF_{3PRP} [2, 3]***	***Method used for the determination of Nex rate***
Brazil4 (32.6%)	Tier 1	Cattle and sheep: IPCC 2006 Tier 1			Cattle: 2% Sheep: 1%	Cattle and sheep: Tier 1 values
Poland (2.84%)	Tier 1	Cattle and sheep: IPCC 2006 Tier 1			Cattle: 2% Sheep: 1%	Cattle: Tier 2 mass balance approach [5] Sheep: from national report (IUNG, 2014)
Spain (18.3%)	Tier 1	Cattle and sheep: IPCC 2006 Tier 1 (EF_{3PRP} of cattle)			Cattle and sheep: 2%	Cattle and sheep: Tier 2 mass balance approach

Ireland (20.1%)	Tier 2 for cattle Tier 1 for sheep	Cattle: Tier 2 Sheep: IPCC 2006 Tier 1	Field study with cattle urine and dung application to soil at three sites and in three seasons (Krol *et al.*, 2016)	Excreta type	Cattle: 1.2% for urine, 0.31% for dung , 0.86% for combined excreta Sheep: 1%	Cattle: Tier 2 mass balance approach Sheep: from national report (DHPLG, 2017)
Japan (0.38%)	Tier 2 for cattle Tier 1 for sheep	Cattle: Tier 2 Sheep: IPCC 2006 Tier 1	Field study with cattle urine and dung application to soil at one location, five times throughout the year (Mori and Hojito, 2015)	Excreta type	Cattle: 0.68% for urine and 0.02% for dung, 0.46% for combined excreta[6] Sheep: 1%	Cattle: country specific equations (Choumei *et al.*, 2006) Sheep: Tier 1 values
Australia (22.4%)	Tier 2	Cattle and sheep: Tier 2 (same EF_{3PRP} used)	Two field studies in two locations: - cattle urine application to soil at one site in two seasons (Galbally *et al.*, 2005) - sheep urine application to soil at one site in three seasons (Galbally *et al.*, 2010) + Dung EF3PRP based on literature	No disaggregation	Cattle and sheep: 0.4% for combined excreta	Cattle and sheep: Tier 2 mass balance approach
Canada (0.71%)	Tier 2	Cattle and sheep: Tier 2 (same EF_{3PRP} used)	Two field studies in two locations: - in Eastern Region with dairy cow urine and dung	Excreta type, province (for Eastern region only)	Cattle and sheep: *In Western region*	Dairy cow: Tier 2 mass balance approach

			application to soil at two sites and in three seasons (Rochette *et al.*, 2014) - in Western region with beef cattle urine and dung application to soil at two sites and in three seasons (Lemke *et al.*, 2012)		*(four provinces):* 0.06 % for urine, 0% for dung, 0.04% for combined excreta. *In Eastern region (six provinces):* province-specific, ranging from 0.48 to 0.61% (for combined excreta)	Sheep and other cattle categories: Tier 1 default values
New Zealand (49.4%)	Tier 2	Cattle and sheep : Tier 2 (different EF_{3PRP} used)	Meta-analysis of results from a large number of field studies (van der Weerden *et al.*, 2020)	Excreta type, animal category, slope class, adoption of mitigation technology	Cattle: -*on low slope/flat slope*: 0.98% for urine, 0.12% for dung, 0.72% for combined excreta -*on medium/steep slope:* 0.33% for urine, 0.12% for dung, 0.26% for combined excreta - 0.67% for urine *with DCD application*[7] Sheep: -*on low slope/flat slope*: 0.50% for urine, 0.12% for dung, 0.37% for combined	Cattle and sheep: Tier 2 mass balance approach

					excreta *-on medium/steep slope:* 0.08% for urine, 0.12% for dung, 0.09% for combined excreta	
UK (5.39%)	Tier 2 for cattle Tier 1 for sheep	Cattle: Tier 2 Sheep: IPCC 2019 Tier 1	Synthesis of field studies across the country including one study with cattle excreta application to soil at five sites and in three seasons (Chadwick *et al.*, 2018)	Excreta type	Cattle: 0.63 % for urine, 0.19% for dung, 0.48% for combined excreta [6] Sheep: 0.32% for urine, 0.1% for dung [8], 0.25% for combined excreta [6]	Cattle and sheep: Tier 2 mass balance approach
USA (3.62%)	Tier 3 (Day Cent modelling) for non-federal grasslands, Tier 1 for federal grasslands	Cattle and sheep: -IPCC Tier 1 *on federal grasslands* -No EF_{3PRP} used with Tier 3 approach on *non-federal grasslands*			On federal grasslands: Cattle: 2% Sheep: 1%	Cattle: Tier 2 mass balance approach Sheep: data from national reports (ASAE, 1998; USDA, 2008)

[1] The contribution of grazing livestock to total agricultural N_2O emissions is represented here as the proportion of total agricultural N_2O emissions which is attributed to urine and dung deposited by grazing livestock in grassland, according to the national estimations in the 2021 GHG inventory submissions.

[2] The factors of disaggregation and the values of EF_{3PRP} are those given in the NIR 2021 (i.e. BUR4 for Brazil).

[3] Values of IPCC 2006 Tier 1 EF_{3PRP} are for combined excreta since there is no disaggregation by excreta type in the 2006 guidelines.

[4] Brazil is the only Annex II country in this table and has been selected in order to account for geographical spread of case study countries.

[5] The Tier 2 mass balance approach given in the IPCC guidelines is based on N intake and N retention (IPCC, 2019a). This calculation is explained in more detail in the previous section.

[6] Since no combined excretal EF_{3PRP} is given in the NIR of these countries, the combined excretal EF_{3PRP}s are calculated here according to a urine: dung excreted N ratio of 0.66: 0.34 (i.e. same ratio used in the IPCC 2019 refined guidelines).

[7]This EF_{3PRP} is based on the work of Clough *et al.* (2008).

[8]The values of EF_{3PRP} for sheep given in the UK NIR 2021 do not match the 2019 Tier 1 values. The methods of calculation to obtain these values from the IPCC 2019 default values are not detailed in the UK NIR.

3.3 Importance of disaggregation to decrease uncertainties linked to factors of variability

3.3.1 Disaggregation by excreta type and effect of animal diet

Several factors lead to variations of EF_{3PRP} between and within countries (**Table 3.2)** and justify the need for disaggregation.

The first level of disaggregation is the estimation of N_2O emissions from urine and dung separately, which has already been undertaken for the development of Tier 2 EF_{3PRP} in Ireland, the UK, New Zealand, Japan and Canada (**Table 3.1**). This disaggregation is justified by the lower emissions observed from dung compared to urine (van der Weerden *et al.*, 2011; Luo *et al.*, 2013; Luo *et al.*, 2015; Mori and Hojito, 2015; Tomazi *et al.*, 2015), except under some specific conditions such as freeze-thaw events (Wachendorf *et al.*, 2008). However, to date studies have focussed mainly on urine, leading to a lack of data regarding emissions from dung, particularly for sheep. Indeed, in the dataset used to refine the IPCC Tier 1 EF_{3PRP}, out of a total of 461 values only 63 EF_{3PRP} values were derived from dung treatments (IPCC, 2019b). Differences between excreta types are linked to N forms in excreta composition and can also exist within the same type of excreta, for example if animals of the same breed are fed with different diets. Indeed, ruminant's alimentation influences urine composition (Dijkstra *et al.*, 2013 ; Schils *et al.*, 2013; de Klein *et al.*, 2020c). It has been widely shown that lower N intake leads to reduced urinary N in ruminants (Schils *et al.*, 2013; Zhao *et al.*, 2016). Moreover, some forage species have the potential to decrease N_2O emissions through different mechanisms including an increase in the volume of urine (Cheng *et al.*, 2017), or an increase of N partitioning into dung (Carulla *et al.*, 2005), which may reduce urinary N concentration. Some forage species such as plantain also contain particular secondary metabolites with potential nitrification inhibitory properties (Luo *et al.*, 2017; Simon *et al.*, 2019; de Klein *et al.*, 2020c). More research is required to evaluate whether the use of such forages is truly efficient in the reduction of N_2O emissions, and particularly if their inhibitory properties are preserved through the ruminal digestive system (de Klein *et al.*, 2020c). A proven efficacy could lead to the establishment of specific EF_{3PRP} reflecting the adoption of such a mitigation strategy. Consideration of mitigation strategies already appears in the New Zealand 2021 NIR. Indeed, a specific EF_{3PRP} has been established for urine of dairy cattle associated with application of DCD (dicyandiamide), an artificial nitrification inhibitor (NI), to the field (Clough *et al.*, 2008). Artificial

NI such as DCD also represent a potential diet manipulation strategy since reductions of N_2O emissions were observed from urine of cows fed with DCD (Luo *et al.*, 2016; Minet *et al.*, 2016). However, the use of DCD has been suspended since traces were detected in dairy products in New Zealand (MPI, 2013). Consequently, the specific EF_{3PRP} of cattle urine applied with DCD is no longer used in the calculation of N_2O emissions in New Zealand.

The influence of diet on N_2O emissions from ruminant excreta highlights another possible factor of disaggregation of Tier 2 EF_{3PRP}, since two grazing systems in the same country can use different forage resources (e.g. *Lolium perenne* -dominated swards in intensively managed grasslands versus high diversity of forage species in extensive upland grasslands, use of grass or maize silage). In that respect, efforts must be made in the reporting of livestock alimentation in studies of EF_{3PRP} estimation in order to help understand the influence of diet on N_2O emissions (López-Aizpún *et al.*, 2020). However, an important limitation of considering diet in national inventories lies in the collection of activity data regarding livestock's diet on a national scale, which can be difficult to access.

3.3.2 Consideration of seasonal variations

Seasonal variability is another level of disaggregation to consider when developing Tier 2 EF_{3PRP}. This can be achieved by estimating N_2O emissions for several timings of excreta application to soil across the year in order to represent different grazing periods. This is regularly considered in field studies (**Table 3.2**)**.** Temporal variations of N_2O emissions in one given location are linked to changes in climatic conditions such as temperature (Uchida *et al.*, 2011; Cameron *et al.*, 2013; López-Aizpún *et al.*, 2020) and rainfall, a proxy for the soil's water-filled pore space (WFPS) and aeration status (Schindlbacher *et al.*, 2004; Selbie *et al.*, 2015; Banerjee *et al.*, 2016).

The seasonal patterns of N_2O emissions have been explored in the studies for the development of Tier 2 EF_{3PRP} in Ireland and the UK (Krol *et al.*, 2016; Chadwick *et al.*, 2018). The disaggregation by season was not justified in these studies due to the inconsistent effect of seasons throughout the experimental sites. Therefore, Tier 2 EF_{3PRP}s have been calculated as means of values estimated for each season of excreta application to soil. This method of calculation implies equivalent grazing periods (i.e. N_{ex} rates deposited during each grazing periods are equivalent). Indeed, the interaction of climatic parameters with other factors influencing N_2O emissions such as soil characteristics, explains the spatial variability of seasonal pattern of N_2O emissions (van Groenigen *et al.*, 2005).

Consequently, it becomes difficult to disaggregate EF_{3PRP} by seasons on a national scale. This is particularly relevant to temperate climates, compared to tropical regions where seasons are more extreme (i.e. dry and wet seasons). In some tropical regions, the seasonal pattern of N_2O emissions appears more consistent, with higher emissions observed during the wet season compared to dry season (Sordi *et al.*, 2014; Mazzetto *et al.*, 2015; Tully *et al.*, 2017; Cardoso *et al.*, 2019). Another obstacle to a disaggregation of EF_{3PRP} by season is the accessibility of detailed information about grazing management in the country in order to estimate the proportion of N_{ex} deposited during each grazing period.

In its 2019 refinement guidelines, the IPCC suggests a disaggregation of EF_{3PRP} by dry and wet climate in order to take account of soil moisture content, which is regulated by climatic conditions. The division between climates is based on the quantity of precipitation for the tropical regions, and the ratio between precipitation and evapotranspiration for temperate regions (IPCC, 2019b). It is obvious that these definitions are very simplified, given that soil moisture content also depends on soil drainage properties (de Klein *et al.*, 2003), which are not considered in these definitions.

The use of empirical models based on national datasets may be a good way to establish EF_{3PRP} as a function of climatic parameters, while considering other drivers of emissions. This approach has been used for the estimation of the N_2O EF_1 for non-urea inorganic fertilisers in the UK. This EF_1 is disaggregated on a 10 km grid basis and calculated as a function of average annual rainfall and N loading rate determined for each grid square. Therefore, UK EF_1s range from less than 0.5% in the driest zones (<700 mm annual rainfall) with the lowest annual fertiliser application rates (<40 kg/ha) to more than 2.5% in the wettest zones (>1300 mm annual rainfall) with the highest annual fertiliser application rates (>300 kg/ha) (CCC, 2018). Therefore, with this approach, different values of EF_{3PRP} could be calculated throughout the year, depending on the climatic conditions of the grazing period. However, in order to accurately apply such EF_{3PRP}s, appropriate data about grazing management of livestock is required (i.e. distribution of excreta by grazing period). Moreover, this method of calculation requires sufficient datasets of EF_{3PRP}s to increase the accuracy of the empirical model, and climatic data must be available at a high spatial resolution scale.

3.3.3 Consideration of spatial variability

Soil characteristics are spatially variable and have been widely shown to influence N_2O emissions in field and lab incubation studies, particularly through regulating soil moisture content and

subsequent aerobicity (de Klein *et al.*, 2003; Di *et al.*, 2014; Rochette *et al.*, 2014; Harrison-Kirk *et al.*, 2015; Balaine *et al.*, 2016).

Soil drainage properties are a regulator of soil moisture and therefore influence N_2O emissions (de Klein *et al.*, 2003). In Ireland, the exploration of soil drainage properties was undertaken in the study for the development of Tier 2 EF_{3PRP} for cattle urine and dung (Krol *et al.*, 2016) with a measurement of N_2O emissions at three sites representing three common soil drainage classes (well, moderately and poorly drained soils). The study of Krol *et al.* (2016) showed higher EF_{3PRP} on poorly-drained soil. However, since detailed activity data about the proportion of N excreted on each soil class category, as well as the percentage cover of each soil drainage class was not available, the Tier 2 EF_{3PRP} was defined as the average of the values estimated at the three sites. This implies that the grassland areas of each soil drainage class category receive one third of total excreted N, which does not necessarily reflect reality. Detailed information about the quantity of excreta N deposited on each soil drainage category and distribution of soil class categories in the territory are not easily accessible and unavailable in many countries.

Canada and New Zealand are two examples of countries adopting further degrees of disaggregated EF_{3PRP} to account for spatial variability of N_2O emissions associated with soil texture and topography respectively. This has been possible because sufficient activity data are available to combine with measured EF_{3PRP}s in the calculation of total N_2O emissions. Indeed, Canada was able to disaggregate EF_{3PRP} by soil textural class in the Eastern Region of the country (i.e. Quebec, Ontario and the four Atlantic provinces), based on the work of Rochette *et al.* (2014) who observed a significant effect of soil type on $EF_{3PRP,}$ and estimated an EF_{3PRP} from dairy cow urine and dung for each soil texture (fine, medium, coarse). Since information about soil texture proportion was available for each eco-district (i.e. a district with particular ecological characteristics), a weighted EF_{3PRP} was calculated for each eco-district, from the proportion of each soil textural class and the corresponding disaggregated $EF_{3PRP,}$ assuming 75% of N is excreted in urine (Rochette *et al.*, 2014). New Zealand also considered spatial variability in its 2021 NIR through the disaggregation of EF_{3PRP} by slope class categories, based on a meta-analysis of van der Weerden *et al.* (2020). Slope class is indeed a factor controlling N_2O emissions of deposited urine, due to animal behaviour with the creation of camping sites on low slope areas receiving higher excretal inputs (Zhong *et al.*, 2016). In lower areas, water movement also leads to a soil residual nutrients enrichment and increased soil moisture (Luo *et al.*, 2013).

Some studies suggest further disaggregation of EF_{3PRP} by highlighting other parameters responsible for spatial variability of N_2O emissions from ruminant excreta deposited in grasslands. For instance, Marsden et al. (2019) suggested a disaggregation by altitudinal gradient (lowland, semi-improved upland and unimproved moorland) in the UK, since lower emissions have been observed in upland compared to lowland, which may reflect differences in soil characteristics (e.g. low soil pH, high soil organic matter content), grazing management and animal diet (Marsden *et al.*, 2018; Marsden *et al.*, 2019). When using EF_{3PRP} disaggregated by altitudinal gradient, Marsden *et al.* (2019) calculated a reduction of 43% of annual N_2O from grazing sheep in the UK, compared to the current estimation calculated with the Tier 2 EF_{3PRP} established from cattle excreta applied in lowland grazing systems. The authors of this study also suggest an alternative approach for disaggregating sheep EF_{3PRP}, by identifying pastures with low soil pH, and those that are highly anaerobic. These two soil conditions are related to low nitrification rates and consequently low N_2O emissions. However, before setting soil pH as a disaggregation factor, more understanding of its effect on the N cycle processes is required. Indeed, soil pH influences different steps of the N cycle, such as N mineralisation rate (Fu *et al.*, 1987; Cheng *et al.*, 2013), nitrifier communities (Nicol *et al.*, 2008), denitrifiers' enzymes (Liu *et al.*, 2014; Žurovec *et al.*, 2021), and the overall outcome on the balance of N_2O emissions remains unclear (ŠImek and Cooper, 2002). A wider range of soil pH should be assessed in EF_{3PRP} studies in order to help in understanding its effect on N_2O, in interaction with other factors (López-Aizpún *et al.*, 2020).

The nutrient status of soils such as phosphorus (P) level has also been showed to drive to variation in N_2O emissions. In the recent study of O'Neill *et al.* (2020), higher N_2O emissions were observed in soil depleted in P, when C availability was not restricted. The authors suggested that in these conditions, microbial activity was not inhibited by C limitation, and low P availability was leading to a dominance of fungal denitrification, associated with an incomplete denitrification due to a lack of N_2O reductase. Luo *et al.* (2013) also found a relationship between soil P and N_2O emissions but attributed it to a nutrient enrichment in low slope areas, thus enhancing nitrification rate.

Furthermore, soil C content is clearly a regulator of denitrification due to coupled C and N cycles. Inputs of labile C, or processes increasing soil C mineralisation rate (e.g. freeze-thaw event, tilling, fertiliser application) may increase denitrifiers activity and N_2O emissions (Saggar *et al.*, 2013). Nevertheless, increased soil organic matter has been associated with higher N retention, and

consequently less N available for microorganisms (Barrett and Burke, 2000). Therefore, reporting information about soil C and nutrient composition when conducing field studies could help in the establishment of relationship between N_2O emissions and soil chemical composition, along with other influencing factors.

3.3.4 Challenges associated with disaggregation of EF_{3PRP}

Before establishing categories for a factor of disaggregation of EF_{3PRP}, a wide understanding of its effect on N_2O emissions is essential. A way of acquiring such accuracy in disaggregation relies on the gathering and the analysis of field study data, such as the meta-analysis of López-Aizpún *et al.* (2020). As highlighted in that study and in the IPCC 2019 refinement guidelines, the studies for EF_{3PRP} determination are currently from a limited number of locations (mostly in New Zealand) and do not cover a wide range of environmental conditions. Most of the studies analysed in López-Aizpún *et al.* (2020) were carried out in temperate regions, leading to a lack of data from regions with more extreme climatic conditions. In order to assist the understanding of the effect of factors on N_2O emissions, more studies are required with a greater range of soil and climatic conditions.

Clearly, the requirement for multiple field experiments can represent a key constraint to the development of new Tier 2 EF_{3PRP} since the IPCC recommends a 12 months sampling period after N application for the development of Tier 2 EF_{3PRP}. However, in the current revision of N_2O chamber methodology guidelines (Global Research Alliance for Climate Change), a recommendation is made about decreasing the duration of field experiments based on the period taken for which N_2O emissions (and soil mineral N content) induced by N input return to background level (Charteris et al., 2020). Recent work of Vangeli et al. (2020, unpublished data) also suggests that the duration of sampling after excreta application to soil could be reduced, particularly for urine. Shorter experiments would allow more countries to obtain local data for the establishment of Tier 2 EF_{3PRP}.

When carrying out field studies to estimate EF_{3PRP}, it is essential to record a maximum of parameters characterising the grazing systems. Indeed, the current lack of reported metadata restricts the determination of factors influencing N_2O emissions (Buckingham *et al.*, 2014; López-Aizpún *et al.*, 2020). A list of minimum parameters required for reporting of field studies has been proposed in the revision of N_2O static chambers methodology guidelines (de Klein *et al.*, 2020b). Improved reporting of parameters will also facilitate the establishment of relationships between EF_{3PRP} and parameters influencing N_2O emissions through regression modeling and meta-analyses. This is one

objective of the DATAMAN project which is developing a field database linking N_2O emissions and other measured variables (Beltran *et al.*, 2021). Experimental data combined with detailed additional metadata are also essential for the development and the validation of biogeochemical models to simulate emissions at larger scale (de Klein *et al.*, 2020b), such as DNDC (Li *et al.*, 1992) or Daycent, as used in the USA (Del Grosso *et al.*, 2012). Process-based models such as Daycent may estimate very accurately N_2O emissions given their consideration of a greater number of drivers of N_2O emissions compared to empirical models (Yue *et al.*, 2019). Higher level of disaggregation of N_2O EF_{3PRP} or simulation of N_2O emissions by models allow for a better consideration of environmental and/or management factors but requires appropriate and detailed activity data. Indeed, models need large data inputs to decrease the uncertainties around their estimations (Ogle *et al.*, 2020).

3.4 Conclusions and recommendations

Various methods of reporting N_2O emissions from cattle and sheep excreta deposited in grasslands have been adopted by countries in their national GHG inventories, and therefore estimations can be more or less accurate. A first step toward reducing this uncertainty is the development of Tier 2 EF_{3PRP} derived from field studies in a given country. Future field studies should focus on sheep excreta, which have been less studied compared to cattle, leading to a greater source of uncertainties around N_2O emissions from grazing sheep. Moreover, increasing the number of field studies in a wider range of conditions (land management, climatic conditions, soil types, altitudes) would facilitate the disaggregation of EF_{3PRP} and/or the validation of models, in order to more accurately account for spatio-temporal variability of emissions. When conducing field studies, efforts must be undertaken in the reporting of the appropriate metadata such as animal diet (e.g., herbage composition), climatic conditions, soil characteristics (de Klein *et al.*, 2020b; López-Aizpún *et al.*, 2020; Ogle *et al.*, 2020). However, an impediment in the application of disaggregated EF_{3PRP} or the adoption of models is the availability of detailed activity data which can be difficult to access for some countries.

Table 3.2 Experimental N_2O emission factors from sheep urine and/or dung applied to grassland soils, estimated from field studies in various countries.

Country	Reference	Factors of variation assessed in the study	Period of measurement	Range of N loading rate (kg N ha^{-1})	Range of EF_{3PRP} (%)[1]
Brazil (southern)	Tomazi et al. (2015)	- Excreta type - Urine N loading rate	~ 2.5 months	161-403 for urine 13 for dung	From 0.06 to 0.31 *(standard error not reported)*
Brazil (southern)	Savian et al. (2019)	-Grazing management1	~ 1 month	60-75 for dung	From 0.03 to 0.11 *(from dung only; standard error not reported*)
China (Inner Mongolia)	(Ma et al., 2006)	-Excreta type	~ 1 month	180 for urine 90 for dung	From 0.44 to 1.05 (*standard error*

					not reported)
Germany	Hoeft et al. (2012)	-Excreta type	2.5 months	39 for urine 141 for dung	From 0.09 ± *0.11* to 0.48 ± *0.05*
New Zealand	Luo et al. (2013)	-Excreta type -Season of application -Slope class	From 4 to 12 months, depending on the experiment	150-500 for urine 190-330 for dung	From -0.15 to 1.53 (*standard error not reported)*
New Zealand	Hoogendoorn et al. (2016)	-Diet (forage type) -Forage soil type[2] -Season of application	From 3 to 5 months, depending on the experiment	108-304 for urine	From 0.07 ± *0.18* to 3.53 ± *0.63*
United Kingdom	(Marsden et al., 2017)	-Adoption of mitigation strategy	~ 2 months	725 for urine ± *DMPP*	From 0.63 ± *0.10* to 3.87 ± 2.36

		(use of nitrification inhibitor DMPP)			
United Kingdom	Marsden et al. (2018)	-Season of application	12 months	756-111	From 0.03 ± *0.09* to 0.08 ± *0.04* (for urine only)
United Kingdom	(Marsden et al., 2019)	-Season of application	~ 4 or 6 months depending on season	920-1120 for urine/artificial urine	From 0 to 0.01 ± *0.02* [3]

[1] Other treatments may have been assessed in these studies but only the treatments sheep urine, sheep dung, artificial sheep urine and sheep urine + nitrification inhibitor are considered in this table in order to highlight the variation of EF_{3PRP} for sheep excreta deposition due to different influencing factors.

[2] RN = Rotatinious stocking management or RT = traditional rotational grazing management. RT is "based mainly on the greater forage accumulation and the maximum herbage harvested by the animals"; RN is "based on animal behaviour [with an identification of] the optimal sward structure (e.g. sward height) that maximises the animal herbage intake per unit of grazing time" (Savian et al., 2019).

[3] Vegetation cover of pasture is either forage rape or ryegrass clover

3.5 Acknowledgments

This study was supported by the Teagasc Walsh Scholarship Scheme (project code 2018028).

3.6 References

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Chapter 4

Low N_2O emissions associated with sheep excreta deposition in temperate managed lowland grassland and extensively grazed upland grassland

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This experimental chapter is based on an article which has been accepted for publication in the journal Science of the Total Environment:

Mancia, A., Chadwick, D. R., Waters, S. M., Krol, D. J., 2022. Low N_2O emissions associated with sheep excreta deposition in temperate managed lowland grassland and extensively grazed hill pasture. Science of The Total Environment, 850, 158070.

AM, DRC, SMW and DK designed and conceived the experiment; AM conducted the practical work; AM analysed the results and prepared the manuscript, DRC, SMW and DK revised the manuscript.

Abstract

Nitrous oxide (N_2O) is a potent greenhouse gas (GHG) whose emission from soil can be enhanced by ruminant excretal returns in grasslands. The default (Tier 1) emission factors (EF_{3PRP}, i.e. proportion of deposited nitrogen emitted as N_2O) for ruminant excreta deposition are associated with a wide range of uncertainties and the development of country-specific (Tier 2) EF_{3PRP} is encouraged. In Ireland, a Tier 2 EF_{3PRP} has been developed for cattle excreta but no data are available for sheep. The aim of this study was to generate data to contribute to the derivation of a Tier 2 EF_{3PRP} for sheep excreta, while assessing the effect of excreta type, grassland type and season of deposition on N_2O emissions. An experiment was carried out on two sites in the west of Ireland: a managed lowland grassland (LOW) and an extensively grazed upland pasture (UP), characterised by mineral and acid peat soils, respectively. For each season, four treatments were applied to the soil in a fully randomised block design: control (C), sheep urine (U), sheep dung (D), and artificial urine (AU). Nitrous oxide fluxes were assessed over a full year following each application of treatments, using a static chambers methodology. Results showed a brief initial peak following each application of U/AU in LOW but not in UP. Cumulative N_2O emissions were significantly higher from the lowland site. Average EF_{3PRP} for combined excreta was negligible on both sites, thus lower than the 2019 IPCC Tier 1 EF_{3PRP}. Causes of low emissions are likely to depend on site characteristics (e.g. soil acidity in UP) and season of application (i.e. ammonia volatilisation in summer). This study showed very low N_2O emissions from sheep excretal returns in Irish grasslands and highlighted the importance of developing Tier 2, animal-specific EF_{3PRP}. More experimental grasslands should be assessed to confirm these results.

Key words: emission factor; grazing returns, mineral soil; peat soil, nitrogen

4.1 Introduction

Nitrous oxide (N_2O) is an important greenhouse gas (GHG) with a warming potential 273 times higher than CO_2 over a 100 years period (IPCC, 2022). N_2O also contributes to ozone layer depletion (Portmann *et al.*, 2012), and the increase of its concentration in the atmosphere has important consequences on human health and environment (Erisman *et al.*, 2013).

Nitrous oxide is naturally produced in soil, principally through microbial processes of nitrification and denitrification but may also be produced through other pathways such as codenitrification or nitrifier-denitrification (Baggs and Philippot, 2010). Nitrous oxide production is moderated by nitrogen (N) and labile carbon (C) availability in the soil, as well as soil factors such as water content, pH and temperature (de Klein *et al.*, 2001). Some agricultural practices lead to large N inputs to soil and increase the risk of N_2O losses. In that respect, approximately 50% of anthropogenic N_2O stems from agricultural activity (Tian *et al.*, 2020).

Livestock excreta management, and particularly the urine and dung deposited by grazing ruminants, is an important source of agricultural N_2O emissions (Cai and Akiyama, 2017). Indeed, ruminants have a poor N use efficiency and between 70 and 95% of ingested N is excreted through urine and dung (Oenema *et al.*, 2005). Therefore, excreta patches represent a high N input to the soil as well as a source of labile C, and create local anaerobic conditions, which stimulate denitrifiers activity (Bolan *et al.*, 2004; Saggar *et al.*, 2004; Selbie *et al.*, 2015; Cai and Akiyama, 2017). The increase in N_2O fluxes following ruminant excreta application to soil has been widely shown in field studies (Luo *et al.*, 2013; Tomazi *et al.*, 2015; Krol *et al.*, 2016; Chadwick *et al.*, 2018; Marsden *et al.*, 2018), and these emissions must be included in the national GHG inventories.

IPCC has established guidelines to report emissions for every GHG emitting sector. This methodology is based on the use of emission factors (EFs), here defined as the percentage of N applied to the soil which is emitted as N_2O. Regarding N_2O emissions from livestock's excreta deposition in pasture, range and paddock (PRP), default values of EF_{3PRP}, termed Tier 1 EF_{3PRP}, have been established by the IPCC and were refined in 2019, based on a greater number of field studies, and disaggregated by type of excreta (urine/dung) and climate type (dry/wet) (IPCC, 2019). Indeed, higher emissions from urine compared to dung are generally observed in literature (Luo et al., 2015; Mori and Hojito, 2015; Tomazi et al., 2015), linked to different N forms in excreta composition (Krol et al., 2016). However, the refined values (i.e. 0.4 and 0.3% for combined excreta of cattle and sheep, respectively) are still associated with major variations (i.e. 0-1.4% and 0-1% or cattle

and sheep, respectively), due to high spatio-temporal variability of N_2O emissions. Therefore, the development of country-specific EF_{3PRP}s, termed Tier 2 EF_{3PRP}s, is encouraged for greater accuracy of estimation to monitor the trends and assist in agricultural management decisions.

Globally, N_2O emissions from sheep excreta have been much less studied than those from cattle. Mancia *et al.* (2021) explains that in the 2019 IPCC refinement guidelines, only 12% of the EF_{3PRP} values were estimated from sheep excreta, with the remaining majority based on cattle excreta. This leads to higher uncertainties around estimations of N_2O from sheep grazing excretal returns compared to cattle, which justifies the need for more research in this area focusing on sheep.

In Ireland, pasture-based livestock production systems are the main agricultural activity, with approximately 90% of agricultural land devoted to grassland and rough grazing (CSO, 2016). Urine and dung deposited by grazing animals contribute to approximately 20% of agricultural N_2O emissions in the country (Duffy *et al.*, 2021). Ireland has already developed a Tier 2 EF_{3PRP} associated with cattle deposition of urine (1.2%) and dung (0.3%) (Krol *et al.*, 2016). However, no experimental data are available for sheep excreta, and as such IPCC 2006 Tier 1 EF_{3PRP} (i.e. 1%) is still used for sheep grazing excretal returns in the Irish GHG inventory.

There are two main types of sheep production systems in Ireland: the intensively managed lowland sheep system and the extensively managed hill-sheep sector. Differences in N_2O emissions from sheep excreta between various grazing systems have already been shown in the UK (Marsden *et al.*, 2018) and New Zealand (van der Weerden *et al.*, 2011; Luo *et al.*, 2013). New Zealand has even adopted Tier 2 EF_{3PRP}s disaggregated by slope class category to report emissions associated with sheep excreta deposition (van der Weerden *et al.*, 2020). In Ireland, particularly in the west of the country, large areas of extensively managed grassland are on blanket bogs, characterised by acidic organic soils. A recent study carried out in the UK has shown very low N_2O emissions on extensively managed organic soil due to low nitrification rates linked to soil properties such as low pH (Marsden *et al.*, 2019). The difference of N_2O emissions between distinct grazing systems may also be related to difference in urine composition (de Klein *et al.*, 2020c; López-Aizpún *et al.*, 2020). This suggests that emissions might be similarly low on extensively grazed blanket bogs in Ireland, and the currently used Tier 1 EF_{PRP} might substantially overestimate the magnitude of N_2O emissions from Irish sheep systems.

Therefore, the aim of this study was to estimate EF_{3PRP} from sheep excreta deposited onto well managed lowland grassland on mineral soil and extensively grazed blanket bog soil, and to assess the influence of excreta type, grassland type and season of deposition on N_2O emissions.

We hypothesised that the EF_{3PRP} s measured in both grasslands would be lower than those observed for cattle excreta in Irish grasslands, and that emissions from organic acidic soil in rough-grazed pasture would be lower than those on the well managed lowland pasture with mineral soil. We reasonably expected differences in urine composition between both systems similarly to other studies (Marsden *et al.*, 2020), however this was not explored in our study. Moreover, we expected an effect of excreta type with higher emissions from urine compared to dung, as well as an effect of season with higher N_2O emissions measured after excreta deposition in autumn, due to more optimal conditions for denitrification (i.e. lower plant uptake and wetter soils creating anaerobic conditions).

4.2 Materials and methods

4.2.1 Site description and experimental design

The experiment was carried out on two sites with contrasting soils in the west of Ireland: a well-managed lowland grassland on mineral soil at Mellows Campus, Teagasc Research Centre, Athenry, Co. Galway (53°17 'N, 8°46'W) and a rough-grazed-upland pasture characterised by acidic organic soil, in Oorid, Recess, Co. Galway (53°26 'N, 9°36'W). The two sites are designated by LOW and UP, respectively, throughout the article. Annual average daily temperature and annual total rainfall are 10.7°C and 1340 mm in LOW, and 9.9°C and 1193 mm in UP (1981-2010, 30 years average; Met Éireann (2022)). According to the Irish Soil Information System, soil at LOW site is a fine loam over limestone bedrock whereas soil at UP is a peat soil. Soil characteristics of both sites are presented in **Table 4.1.** The grassland in LOW has a sward type dominated by perennial ryegrass (*Lolium perenne L.)*, grazed in a rotational grazing system, and reseeded every five years. This pasture is intensively managed with N fertilisation of approximately 100 kg ha^{-1} $year^{-1}$, as well as phosphorus (P) and potassium (K) fertilisation according to soil requirements, to reach 8 and 150 mg L^{-1} for Morgan's P and K, respectively. The sward type in UP is characterised by a diversity of plant species (including the deergrass *Trichophorum cespitosum* and the heather *Erica tetralix*) and there is no additional management of this pasture other than rough-grazing. No N fertiliser was applied, and sheep were excluded from both experimental sites for a minimum of

four months before the start of the experiment in order to avoid the effect of previous excretal deposition and N fertilizer application on N_2O gas measurement.

On the lowland site (LOW), the experiment consisted of three separate one year measurement periods corresponding to three seasons of treatment application: spring, summer and autumn. The upland site (UP) consisted of two separate one year measurement periods corresponding to summer and autumn application. The spring application in UP was not carried out due to the unprecedented restrictions that were in place due to the COVID-19 pandemic in 2020. Winter season was not included in this experiment since typically high percentage of sheep are housed for winter in Ireland.

At both sites, four treatments were assessed at each season of application: control (C), sheep urine (U), sheep dung (D) and artificial urine (AU).

Each experimental site used a split-plot design with five blocks to take account for spatial variability of N_2O emissions at field scale. Each block was split into sub-blocks, each dedicated to one season of application. Each split sub-block consisted of four plots (associated with a replicate of the treatments. Treatment distribution was fully randomised across each split sub-block. In total, for each season of application, there were five replicates of each treatment (one per block). The experimental site design of LOW (**Fig. S4.1**) and pictures of both sites (**Fig. S4.2**) are presented in **Appendix 1.**

Each plot contained one static chamber for gas sampling to measure N_2O fluxes. Stainless steel static chambers basis (40 x 40 cm) were inserted to about five centimetres depth into the soil, at least 24 h prior to application of treatments in order to minimise soil disturbance which can influence flux measurement (Charteris *et al.*, 2020). Chamber lids were 10 cm high, resulting in a chamber headspace of approximately 16 L. Each chamber received two treatment patches (or nothing for control treatment). In blocks 1, 3 and 5, ten additional treatment patches were created to allow for soil sampling in order to assess mineral N dynamics (ammonium NH_4^+-N and nitrate NO_3^--N). Design of plots are detailed in **Fig. S4.3** of **Appendix 1.**

During the experimental periods, local Met Éireann weather stations recorded daily rainfall, atmospheric pressure and air temperatures (average, min, max). The weather station was ca. 500 m from the experimental site in LOW, whereas the closest station of the upland grassland was ca. 30 km. The latter is located at the same altitude as the experimental grassland site so is likely to record

very similar temperature. However, some differences are possible between the rainfall patterns of the weather station and the grassland site.

Table 4.1 Chemical soil characteristics (0-10 cm) of both experimental grasslands. Values represent averages across all seasons (n = 5 per season). LOW refers to the well-managed lowland perennial ryegrass grassland on mineral soil, and UP refers to the extensively grazed upland pasture on acid peat soil.

Site	LOW	UP
pH	6.3	4.4
Morgan's P (mg L^{-1})	13.1	10.0
Morgan's K (mg L^{-1})	69.1	100.9
OM (%)	10.8	94.3
Total C (%)	4.80	44.1
Total N (%)	0.46	2.04
Bulk density (g cm^{-3})	0.85	0.11
NH_4+-N (kg ha^{-1})	9.2	14.2
NO_3^--N (kg ha^{-1})	5.9	6.5

4.2.2 Treatments preparation and application

The week prior to each application, fresh sheep urine and dung were collected from approximately 15 local ewes (Texel, Suffolk and Belclare breed in LOW, BlackFace Mountain breed in UP) that had been grazing the same vegetation present in the respective experimental grasslands. The protocol was approved by Teagasc Animal Ethics Committee, Bangor University's College of Natural Sciences Ethics Committee and the HPRA (Health Products Regulatory Agency, Irish state agency controlling trials on animals). Ewes were kept in collection pens for a maximum of four hours a day. Collection pens contained a plastic mesh floor retaining dung, and a stainless-steel

tray below to collect urine. Each material type collected from individual animals was pooled and homogenised in one container per material type in order to take account of the variability of excreta materials between individuals. The material was then stored at 4°C prior to analyses. A photograph of the collection pens is presented in **Fig S4** in **Appendix 1**.

At the end of the collection period, the pooled urine was thoroughly mixed and divided into containers to create individual patches. Volume of urine patch was 150 ml in order to represent a typical volume of sheep urination event (Doak, 1952). The same procedure was performed with dung: the pooled dung was divided into individual patches of 150 g to represent a typical quantity of sheep faecal excretion (Haynes and Williams, 1993; Luo *et al.*, 2015). Two days before application, artificial sheep urine (AU) was prepared as described by Lucas and Jones (2006) and stored at 4°C until application. Due to its relatively stable composition, the AU treatment was used to assess more easily the effect of site and season on N_2O emissions. Composition of AU is presented in **Table S4.1** in **Appendix 1.**

Sub-samples of U, AU and D were taken for further analysis at Teagasc, Johnstown Castle, Co. Wexford in order to determine total N (TN), total organic C (TOC) as well as dry matter (DM) for D treatment. Samples were stored at 4°C for a maximum of five days prior to analysis. Composition of treatments is presented in **Table 4.2.**

The day of each application, U, AU patches were carefully poured onto their dedicated areas (see **Fig. S4.1** in **Appendix 1**) inside a circle frame of ø22 cm, to represent a typical sheep urination application rate of 4 L m^{-2} (Haynes and Williams, 1993; Whitehead, 1995). The area within each static chamber basis received two patches in order to increase the magnitude of measured N_2O fluxes. The frame was removed after urine application. Each dung patch of 150 g was spread on a circle of muslin cloth of ø20 cm (Luo *et al.*, 2015) to represent the typical sheep average excretion of 5 kg m^{-2} (Haynes and Williams, 1993; Whitehead, 1995). The muslin cloth underneath the dung patch was used to facilitate the temporary removal of dung patch during soil sampling, allowing the sampling of soil affected by dung degradation, and not sampling through the dung patch itself. The muslin cloth was used only for the soil sampling areas and not in the dung patches deposited within static chambers to avoid any effect of the cloth on N_2O fluxes. At LOW, treatments were applied on the 30/04/2019, 09/07/2019 and 16/10/2019 in spring, summer and autumn respectively. At UP, treatments were applied on the 06/07/2020 and 13/10/2020 for summer and autumn respectively.

Table 4.2. Details of treatment application and chemical properties of sheep urine (U) and dung (D) and artificial urine (AU) for each season of application on both lowland (LOW) and upland grassland (UP). Fresh urine and dung were collected before each application from local ewes grazing the same vegetation present on the experimental site. Artificial urine was prepared according to the recipe of Lucas and Jones (2006). For each season at both sites, 150 mL of U/AU was applied on an area of 375 cm^2 and 150 g of D was applied on an area of 314 cm^2.

Site	**LOW**									**UP**					
Season	**spring**			**summer**			**Autumn**			**summer**			**autumn**		
Treatment	*U*	*AU*	*D*	*U*	*AU*	*D*	*U*	*AU*	*D*	*U*	*AU*	*D*	*U*	*AU*	*D*
DM (%)			17.9			19.5			22.4			14.5			19.9
TN (g N L^{-1} for urine or g N kg^{-1} for dung)	6.47	3.18	9.25	9.31	4.13	6.16	3.86	4.32	6.74	3.3	4.5	3.4	6.2	4.1	3.95
TC (g C L^{-1} for urine or g C kg^{-1} for dung)	6.28	2.96	82.5	13.04	2.85	86.8	2.53	1.80	100.8	4.1	3.4	68.8	4.0	8.1	93.9
N loading rate (kg N ha^{-1})	259	127	445	372	165	294	154	173	322	132	180	110	248	164	95

4.2.3 Gas sampling and analysis for N_2O fluxes measurement

Gas sampling was performed according to the closed static chamber methodology (Charteris *et al.*, 2020). First gas sampling was performed one or two days before each application to assess the background N_2O flux. After treatment application, an intensive sampling frequency was performed for the first five weeks post application, with four samplings the week of application (including one just after application), three samplings per week the second and third week, and two samplings per week the following two weeks, once a week for two other weeks and fortnightly until week 24. After this time, sampling frequency was reduced to once per month until the end of the experimental period. Each sampling event was carried out between 09:00 and 12:00 (except on the application days when the treatments were applied in the morning and the gas samples collected in the afternoon). At this time of the day, fluxes are considered to be representative of the average flux for the day (Alves *et al.*, 2012; Marsden *et al.*, 2017). N_2O sampling followed a prescribed schedule however it was adapted as much as possible to coincide with rainfall to account for rainfall-induced emissions.

For each sampling event, chambers were closed with their lids and gas samples were taken through a rubber septum with a 12 mL syringe fitted with a hypodermic needle at T = 0, 20 and 40 minutes after chamber closure. Gas was then injected into a 7 mL pre-evacuated glass vial with air-tight lid. Five additional ambient air samples were also taken at each sampling event. Samples were then analysed at the Teagasc Research Centre in Johnstown Castle, Co. Wexford. Nitrous oxide concentrations were analysed using gas chromatography (Varian CP 3800 GC, Varian, USA) fitted with an electron capture detector. A linear regression of N_2O concentration over time was used to calculate hourly N_2O fluxes for each sampling event for each treatment (Eq. 1).

$$FN_2O\ (\text{hourly}) = \left(\frac{\delta C}{\delta t}\right) \text{x } \frac{MxP}{RxT} \text{ x } \frac{V}{A} \qquad \textit{(Eq. 1)}$$

Where FN_2O is the hourly flux of N_2O (g $ha^{-1}h^{-1}$), δC is the variation of N_2O concentration during the chamber enclosure period (µL L^{-1}), δt is the enclosure period in hours, M is the molar mass of N in N_2O (28 g mol^{-1}), P is the atmospheric pressure at the time of sampling (Pa), T is the air temperature at the time of sampling (K), R is the ideal gas constant (J K^{-1} mol^{-1}), V is the headspace volume of the closed static chamber (m^3) and A the area covered by the static chamber basis (1.6×10^{-5} m^2). Fluxes were corrected for the area within the chamber bases which was not covered

with treatment. Daily fluxes could then be extrapolated from the hourly fluxes, assuming that the calculated hourly flux is representative of the average flux of the day.

Cumulative N_2O emissions were calculated by linear interpolation of daily fluxes (de Klein *et al.*, 2020a), and EF_{3PRP} for each treatment (except control) were estimated according to Eq. 2:

$$EF_{3PRP}\ (\text{treatment}) = \frac{N_2O(treatment) - N_2O(control)}{Napplied} \times 100\% \quad (Eq.\ 2)$$

Where N_2O (treatment) is the cumulative N_2O emissions from a given treatment calculated for a full experimental period (kg N_2O-N ha^{-1} y^{-1}), N_2O (control) is the cumulative N_2O emissions from the control chamber from the same block, calculated for a full experimental period (kg N_2O-N ha^{-1} y^{-1}), N applied is N loading rate applied through treatment on the first day of the experimental period (kg N ha^{-1} y^{-1}).

4.2.4 Soil sampling and analysis

Initial soil characteristics (**Table 4.1**) were determined at the beginning of each experimental period at both sites by sampling across the field in a W-soil sampling formation.

At each gas sampling event, soil volumetric moisture content (VMC) was measured in each sub-block (n = 3) with a moisture sensor (WET-2, Delta-T Devices, UK). The VMC was used to calculate WFPS at each gas sampling event, according to Eq. 3.

$$WFPS\ (\%) = 100 \times \frac{VMC}{1 - \frac{BD}{PD}} \quad (Eq.\ 3)$$

Where VMC is volumetric moisture content (%), BD is bulk density determined for each season of application, PD is the particle density, assumed to be 2.65 g cm^{-3} for mineral soils, and 1.4 g cm^{-3} for organic soil (Rowell, 1994).

Bulk density (n = 5) was determined at the beginning of each experimental period. Metal rings of 100 cm^3 were inserted into the soil, at 0-5 cm and 5-10 cm depth. Soil cores were oven-dried (105°C) and weighed. Dry soil cores were then sieved (<2mm) in order to remove and weight stones to correct bulk density measurements.

During the experimental periods, soil samples (0-10 cm) were regularly collected from additional patches (blocks 1, 3 and 5) to assess the dynamics of mineral N (NH_4^+ and NO_3^-) in the soil underneath the treatment's patches. Samples were collected once a week during the first month post application and the sampling intensity was then reduced to once a month from week 16 until the

end of the experimental period. A sampling was also performed the week prior to each application to assess background soil mineral N (n = 5, 1 sampling per block). After each sampling, fresh soil samples were sieved (< 4 mm), 20 g was extracted with 2 M KCl (potassium chloride) and analysed for mineral N concentrations with an Aquakem 600 discrete photometric analyser at Teagasc Research Institute, Johnstown Castle, Co. Wexford, Ireland. Subsamples of sieved soil were also used for the determination of gravimetric moisture content (GMC) after a drying at 105 ° C for 24 h. The GMC was used for the calculation of mineral N concentrations in soil.

4.2.5 Statistical analysis

The software R studio version 3.2.5 (R Development Core Team, 2019) was used for statistical analysis. Measured variables were cumulative N_2O emissions and EFs. Linear mixed effect models (LMEM) were applied with site, season, treatment and their interaction as fixed effects and block as random effect. Data was graphically checked for normality and homogeneity of variance assumptions before application of LMEM. If one of these assumptions was not verified, data were log (x+a)-transformed, where a is a constant set as the largest negative value of the dataset to which a small amount (i.e. 0.1) is added to obtain all values strictly positive, as commonly done in literature (de Klein *et al.*, 2020b). In case of significant differences ($p<0.05$), Fisher's Least Significant Difference Test (LSD) was executed to assess pairwise differences between groups.

For the summer period in LOW, we identified one static chamber from control treatment with N_2O fluxes relatively high compared to all other chambers (C and other treatments) for most of the sampling time points. This replicate was identified as an influential observation in our regression analyses by using Cook's distance (Cook, 1977). The Cook's distance of this replicate was > n/4 (where n is the number of observations in the regression analysis), revealing that this chamber produced much more N_2O than other chambers. Therefore, we assumed that this C replicate was not representative of the N_2O fluxes from control, so data from this chamber were removed from the dataset.

4.3 Results

4.3.1 Soil and climatic conditions

Temperature and rainfall data following each application are presented in **Fig. 4.1** (a, b and c) and **Fig. 4.2** (a and b) in LOW and UP, respectively. In LOW, the total rainfall for the first month

post application was 38, 168 and 100 mm in spring, summer and autumn respectively. The average mean air temperature for the first month post application was 10.7, 16.2 and 8.8°C in spring, summer and autumn respectively. Therefore, climatic conditions were the wettest during summer application, and were much colder for spring and autumn application compared to summer. The first month following spring application was also drier and colder compared to the typical month of May in LOW which has a long-term average (LTA) of 75 mm total rainfall and 11.3°C average daily temperature (Met Éireann, 2022). This was also true following autumn application when compared to a typical October month (LTA 129 mm, 10.2°C). However, the first month following summer application was warmer and two times wetter than a typical July month in LOW (LTA 86 mm, 15.5°C).

In UP, the total rainfall for the first month post application was 122 and 212 mm in summer and autumn respectively. The average mean air temperature for the first month post application was 14.1 and 10.3°C in summer and autumn respectively, indicating wetter and colder conditions during the first month post autumn application compared to summer. The first month following both summer and autumn were wetter and colder than the typical months of July (LTA 84 mm and 15.3°C) and October (LTA 145 mm and 11.4°C) at Mace Head, the closest weather station.

Evolution of soil WFPS following each application are presented on **Fig. 4.1** (a, b and c) and **Fig. 4.2** (a and b) in LOW and UP, respectively. The average WFPS for the first week in LOW was 78.7%, 42.7% and 85.3% in spring, summer and autumn respectively. Thus, the autumn application occurred in the wettest soil conditions. In autumn, all the values of WFPS measured during the first 30 days post-application were higher than 75%, and 92% of the measured values were higher than 80%.

The average WFPS for the first week in UP was 98.0 and 92.6% in summer and autumn respectively, thus, indicating slightly wetter conditions during summer application. For summer and autumn, all the values of WFPS measured during the 30 first days post-application were higher than 80%. This suggests very wet soil conditions during both applications in UP, which was visually

noticed with a waterlogged soil in many areas of the experimental site (see **Fig. S4.5** in **Appendix 1**).

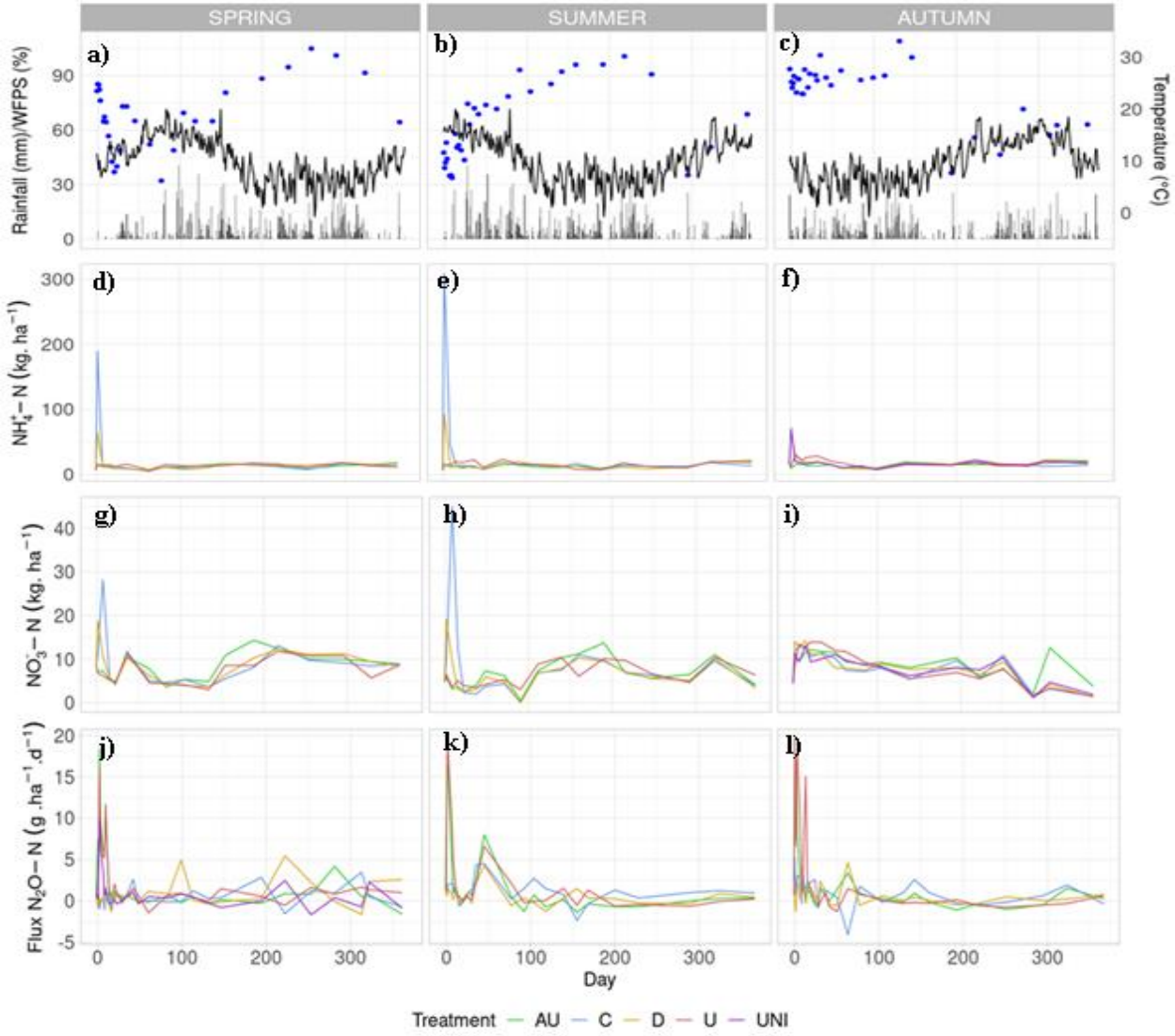

Figure 4.1. The variation in daily rainfall, mean daily air temperature and WFPS (blue dots), soil mineral N (NH_4^+-N and NO_3^-N) and N_2O fluxes during the full year of measurement following treatments application on the perennial ryegrass lowland grassland (LOW) in spring (a, d, g, j), summer (b, e, h, k), and autumn (c, f, i, l). Treatments are control (C), sheep urine (U) and dung (D) and artificial urine (AU). Pooled standard error of the mean (SEM) for NH_4^+-N and NO_3^-N, respectively, was 2.7 and 2.1 kg ha^{-1} in spring, 3.7 and 2.3 kg ha^{-1} in summer, 2.1 and 1.7 kg ha^{-1} in autumn (n = 3). Pooled SEM for N_2O-N fluxes was 1.0 (j), 0.9 (k), and 1.1 g ha^{-1} d^{-1} in spring, summer and autumn respectively (n = 5).

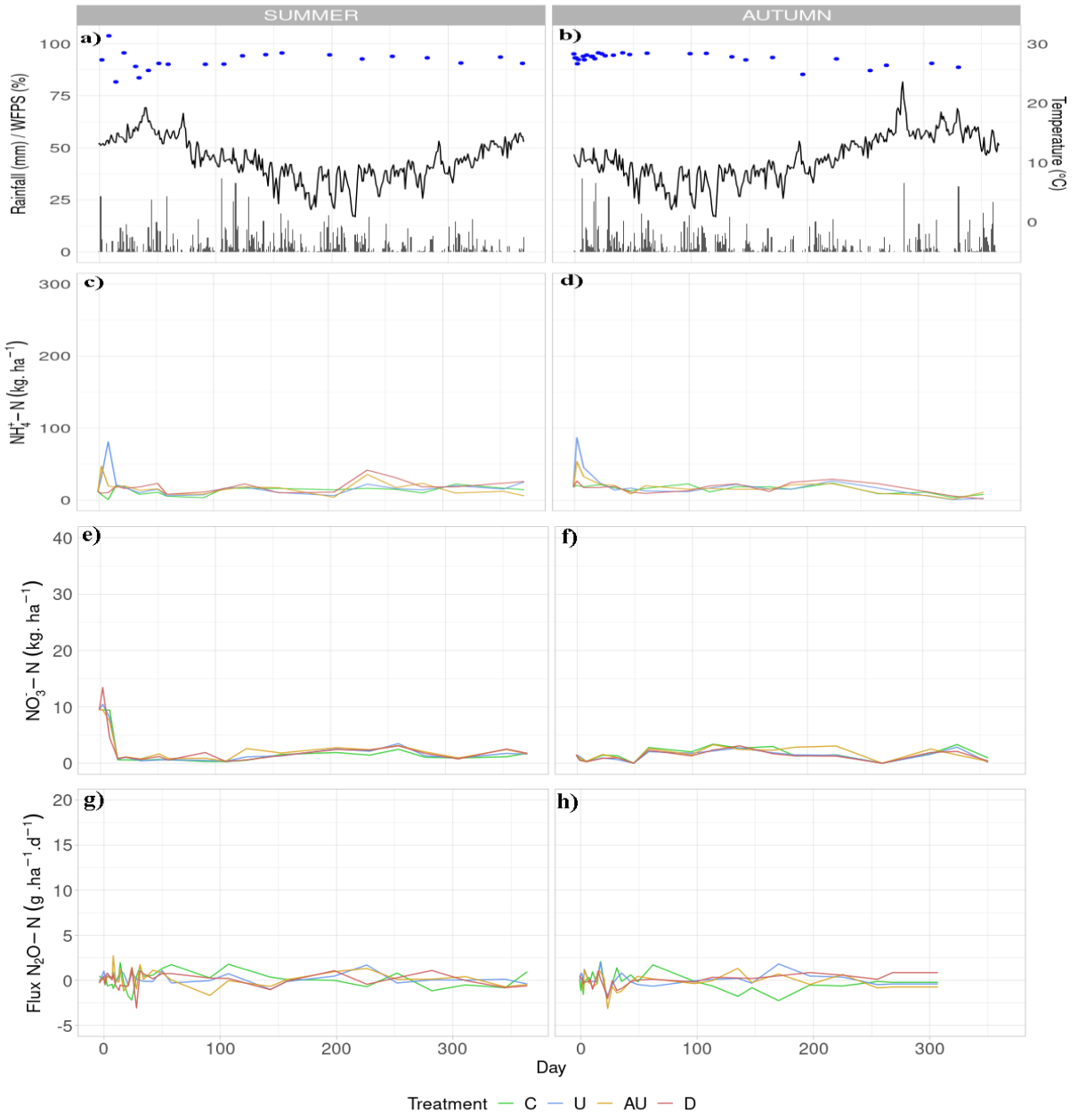

Figure 4.2 The variation in daily rainfall, mean daily air temperature and WFPS (blue dots), and soil mineral N (NH_4^+-N and NO_3^--N) and N_2O fluxes during the full year of measurement following treatment application on the upland pasture (UP) in summer (a, c, e, g) and autumn (b, d, f, h). Treatments are control (C), sheep urine (U) and dung (D) and artificial urine (AU). Pooled SEM for NH_4^+-N and NO_3^--N was respectively 4.8 and 0.6 kg ha^{-1}in summer and 4.3 and 0.3 kg ha^{-1} in autumn (n = 5). Pooled SEM for N_2O fluxes was 0.7 and 0.5 g ha^{-1} d^{-1}in summer and autumn, respectively (n = 3).

4.3.2 Dynamics of soil mineral N

Soil NH_4^+-N and NO_3^- -N concentrations for the full year following each application are shown on **Fig. 4.1** (d, e and f for NH_4^+-N, and g, h and i for NO_3^--N) and **Fig. 4.2** (c and d for NH_4^+-N, e and f for NO_3^--N) for LOW and UP, respectively.

In LOW, the mineral N level from control plots remained consistently low across the three experimental periods despite some variations, with NH_4^+-N ranging from 6.2 to 22.2 kg ha^{-1} and NO_3^- -N ranging from 0.5 to 14.3 kg ha^{-1} across the three seasons. A peak of NH_4^+-N appeared on the first day post-application of U and AU in the three seasons. The NH_4^+-N peak of U treatment was approximately three times higher than the peak from AU treatment in spring and summer, while they were similar in autumn, which is in line with the N loading rates. NH_4^+-N peak from AU were similar for the three seasons (63.7, 92.5 and 63.2 kg ha^{-1} in spring, summer and autumn respectively). For U, NH_4^+-N peak in summer was between 1.5 and 4 times higher than the peaks in spring and autumn, respectively (i.e. 189, 309 and 71.2 kg ha^{-1} in spring, summer and autumn respectively), which is again in line with the urine N loading rates applied at the different seasons. No noticeable increase of NH_4^+-N appeared after D application for any of the three experimental periods. Regarding NO_3^--N level, a noticeable peak appeared in the second week after U application in spring and summer, indicating a nitrification activity. The magnitude of the peak in summer was almost two times higher than in spring (i.e. 28.2 and 45.0 kg ha^{-1}in spring and summer respectively). A small peak was also observed after AU application in spring and summer, with similar magnitude in both seasons (18.8 and 19.2 kg ha^{-1}in spring and summer, respectively). There was no noticeable increase of NO_3^- -N level following autumn application for any of the treatments.

In UP, NH_4^+-N peaks appeared the second week after U application and were similar between both seasons (81.2 and 87.0 kg ha^{-1} for summer and autumn, respectively), which here does not reflect the difference in N application rates between seasons. For AU treatment, NH_4^+-N level reached 47.2 and 53.7 kg ha^{-1} the first week following summer and autumn application, respectively, in line with the similar N loading rates between the two seasons. After the summer application of U, peak soil NH_4^+-N concentration was about four times higher in LOW compared to UP, which can be related to the difference in N loading rates. For the C treatment, the NO_3^--N level was relatively high during the first two weeks post summer application including background level (about 9.5 kg ha^{-1}) compared to the rest of the experimental period (average 1.03 kg ha^{-1}). All treatments followed this same pattern with higher levels during the two first weeks. However, there

were no obvious peak following summer application. After autumn application, despite some variations within the year, levels of NO_3^--N remained low for all treatments (average 1.47 kg ha^{-1}).

4.3.3 Urine and dung chemical composition

Composition of treatments is given in **Table 4.2** for each season of application in both sites. In LOW, N content in urine in summer was two times higher than in autumn, whereas urine N content in spring was intermediate. N content in dung was highest in spring but similar between summer and autumn. Contrary to LOW, urine in UP had higher N content in autumn compared to summer, whereas N content in dung was similar between both seasons. In summer, urine N content was about two times higher in LOW compared to UP whereas in autumn, N content in urine was higher in UP.

The total C content in urine in LOW was also highest in summer (i.e. five and two times higher compared to autumn and spring respectively), whereas the C content in dung was similar between the three seasons. In UP, the C content in urine was similar between both seasons whereas for dung it was higher in autumn. Similar to the N content, the C content in urine was two times higher in LOW compared to UP for summer application whereas in autumn, it was higher in UP. The C content in dung was not very different between both sites for both summer and autumn application, although slightly higher in LOW.

4.3.4 Daily N_2O fluxes

Daily N_2O fluxes following spring, summer and autumn application are shown on **Fig. 4.1** and **Fig. 4.2** (j, k and l) in LOW and UP site, respectively.

In LOW, mean N_2O fluxes from control plots ranged from -4.1 to 5.3 g N_2O-N ha^{-1} d^{-1} across the three experimental periods. The application of U and AU led to the appearance of a peak of N_2O flux within the first three days post-application for each experimental season. The mean N_2O peaks were similar between U and AU treatment, and between the three seasons, reaching 18.4, 17.7 and 16.0 g N_2O-N ha^{-1} d^{-1} for U in spring, summer and autumn respectively, and 16.6, 16.2 and 18.3 g N_2O-N ha^{-1} d^{-1} for AU in spring, summer and autumn respectively. Following summer application, the pattern of N_2O fluxes from AU was slightly different since it showed a double peak. Moreover, in summer, a second peak appeared at day 13 post application of U, reaching 15.1 g N_2O-N ha^{-1} d^{-1}. A second peak also appeared in autumn at day 9 following U and AU application,

reaching 11.5 and 11.7 g N_2O-N ha^{-1} d^{-1} respectively. No N_2O peak was observed within the first days post D application for any of the three seasons. However, two small increases of N_2O flux from D were observed within the year following autumn application, reaching approximately 5 g N_2O-N ha^{-1} d^{-1} at day 99 and 222. All the peaks were brief, and fluxes rapidly decreased. After the initial peaks, fluxes remained globally low, ranging from -4.1 to 8.0 g N_2O-N ha^{-1} d^{-1} across all treatments and seasons, from the third week post application until the end of the experimental periods. However, we observed a noticeable small increase of N_2O fluxes for all treatments at day 45 post spring application following an increase in soil NO_3^--N.

In UP, no peak of N_2O appeared after treatments application for both seasons, and the fluxes remained low for the whole experimental periods, ranging from -3.1 to 2.71 g N_2O-N ha^{-1} d^{-1} after summer and from -5.0 to 2.1 g N_2O-N ha^{-1} d^{-1} after autumn application, across all treatments.

4.3.5 Cumulative N_2O emissions and EFs

Cumulative N_2O emissions for each season and each treatment at both sites are shown in **Table 4.3**. Statistical analyses revealed a significant effect ($p<0.05$) of site and site*season interaction on cumulative N_2O emissions, with lower emissions in UP compared to LOW, lower emissions in summer compared to autumn in LOW and lower emissions in autumn compared to summer in UP. Cumulative N_2O emissions displayed high variability between treatment replicates. In LOW, after U application, cumulative N_2O emissions varied from 0.14 to 0.54 kg N_2O-N ha^{-1} in spring, from -0.11 to 0.51 kg N_2O-N ha^{-1} in summer and from 0.22 to 0.52 kg N_2O-N ha^{-1} in autumn with a relative standard deviation (RSD) of 45, 182 and 34% in spring, summer and autumn respectively. For AU, RSDs were 62, 78 and 113% in spring, summer and autumn respectively and for D, RSDs were 23, 164 and 39% in spring, summer and autumn respectively.

In UP, after U application, cumulative N_2O emissions varied from ~0 to 0.22 kg N_2O-N ha^{-1} in summer and from -0.22 to 0.19 kg N_2O-N ha^{-1} in autumn with a RSD of 89 and 860% in summer and autumn respectively, highlighting very high variability in autumn. For AU and D in both seasons, RSDs were higher than 100%, also showing extreme variability for these treatments.

EF_{3PRPS} for each season and each treatment at both sites are shown in **Table 4.3.** Statistical analyses revealed a significant ($p<0.05$) effect of season only, with higher EF in autumn compared to summer. All average EFs calculated for each treatment at each season and on each site were

lower than 0.04%, except for one average value (dung treatment applied in autumn in UP) reaching 0.09%. However, this treatment was associated with a high variability between replicates, with a SEM of 0.13%. Across all treatments and seasons, the calculated EF_{3PRP}s are -0.01% (±0.03) and -0.03% (±0.03) for LOW and UP respectively, and thus can be considered negligible

Table 4.3 Cumulative N_2O emissions and EF_{3PRP}s calculated with the data obtained from gas sampling undertaken for a full year following treatments application in spring, summer and autumn on the perennial ryegrass lowland managed grassland (LOW; a) and in summer and autumn on the extensively grazed upland pasture (UP; b). Treatments are control (C), sheep urine (U) and dung (D) and artificial urine (AU). Values represent means (n = 5) ± SEM

a)

	Cumulative N_2O emissions (kg N_2O-N ha^{-1})				
		Spring	Summer	Autumn	Average across all seasons
Treatments	C	0.36 ± 0.09	0.22 ± 0.06	0.26 ± 0.09	0.28 ± 0.05
	U	0.34 ± 0.07	0.13 ± 0.10	0.33 ± 0.05	0.26 ± 0.05
	D	0.21 ± 0.03	0.14 ± 0.10	0.43 ± 0.08	0.27 ± 0.05
	AU	0.25 ± 0.07	0.15 ± 0.05	0.31 ± 0.16	0.22 ± 0.06
	EF (%)				
		Spring	Summer	Autumn	Average across all seasons
Treatments	U	-0.01 ± 0.04	-0.02 ± 0.03	0.04 ± 0.06	0.01 ± 0.02
	D	-0.02 ± 0.01	-0.03 ± 0.04	0.04 ± 0.04	-0.01 ± 0.02
	AU	-0.11 ± 0.09	-0.04 ± 0.03	0.03 ± 0.13	-0.04 ± 0.05

b)

	Cumulative N_2O emissions (kg N_2O-N ha^{-1})			
		Summer	Autumn	Average across all seasons
Treatments	C	0.20 ± 0.04	-0.01 ± 0.11	0.10 ± 0.07
	U	0.11 ± 0.04	0.02 ± 0.07	0.06 ± 0.04
	D	0.09 ± 0.06	0.08 ± 0.06	0.08 ± 0.04
	AU	0.04 ± 0.07	0 ± 0.10	0.02 ± 0.06
	EF (%)			
		Summer	Autumn	Average across all seasons
Treatments	U	-0.07 ± 0.03	0.01 ± 0.05	-0.03 ± 0.03
	D	-0.10 ± 0.03	0.09 ± 0.13	-0.01 ± 0.07
	AU	-0.09 ± 0.03	0.01 ± 0.02	-0.04 ± 0.02

4.4 Discussion

4.4.1 Pattern of N_2O emissions following excreta application

4.4.1.1 Lowland grassland

The application of urine and artificial urine on the lowland grassland soil (LOW) led to rapidly occurring peaks of N_2O flux, similar to what was previously observed in studies assessing N_2O emissions after ruminant urine application (Hoogendoorn et al., 2016; Krol et al., 2016; Chadwick et al., 2018; Marsden et al., 2018). This is due to the rapid hydrolysis of urea, which releases readily available N for microorganisms involved in the N cycle (Doak, 1952). Two main microbial processes involved in the production of N_2O in soils are nitrification and denitrification (Baggs and Philippot, 2010). The dominant pathway responsible for N_2O production depends on many factors,

particularly soil aeration status, which is conditioned by soil moisture content and consequently climatic conditions such as rainfall (Selbie et al., 2015; Banerjee et al., 2016) and temperature determining evapotranspiration (López-Aizpún et al., 2020).

Contrary to observations following urine treatments (U and AU), there was no increase in N_2O flux directly after dung application. This is due to N form in dung which is mainly in organic form (Haynes and Williams, 1993), thus less readily available for microbial processes and associated losses to the environment. However, some noticeable increases were observed during the experimental periods and can be linked to climatic events enhancing dung mineralisation. These events are the high rainfall intensity events occurring around day 50 post summer application and around day 200 post autumn application as well as a freeze-thaw effect around day 100 post autumn application (i.e. mean air temperature reaching approximately 0°C). Indeed, freezing can lead to increases in gas exchange between dung patches and the atmosphere, and can lead to cells lysis, releasing N from dung (Wachendorf et al., 2008). These N_2O increases from dung patches were not associated with mineral N increases since N mineralisation probably occurred within the patch itself.

The peaks of N_2O following U application are of similar amplitudes for the three seasons in LOW (16-18.4 g N_2O-N $ha^{-1}d^{-1}$ depending on season). This magnitude of the peak is lower compared to other studies using sheep urine on similar lowland grasslands. For instance, in the UK, after sheep urine application in summer. Marsden et al. (2017) observed maximum N_2O peak more than four times higher (63 g N_2O-N $ha^{-1}d^{-1}$) than the summer maximum peak observed in this study (i.e. 17.7 g N_2O-N $ha^{-1}d^{-1}$). In New Zealand in winter, Hoogendoorn et al. (2016) obtained a maximum flux more than 16 times higher (264 g N_2O-N $ha^{-1}d^{-1}$) than the peak observed in autumn in this study (16 g N_2O-N $ha^{-1}d^{-1}$). This can be related to higher N loading rates used in these studies, as explained in a later section. The initial peaks in our study were also short-lived compared to those in other studies, where the N_2O fluxes following sheep urine application remained elevated for several days. The immediate decrease of peak fluxes in our study were probably due to the rapid depletion of substrates available for microorganisms.

After these initial brief N_2O peaks following U/AU application, the N_2O fluxes remained at low level (lower than 8 g N_2O-N ha^{-1} d^{-1} from third week post application) for the whole experimental periods in LOW, and we even regularly measured negative N_2O fluxes in every treatment and season. Negative fluxes following ruminant urine application have been already observed after

cattle or sheep excreta application in Japan (Mori and Hojito, 2015), Brazil (de Bastos et al., 2020), New Zealand (van der Weerden et al., 2011), Canada (Rochette et al., 2014). In LOW, lowest negative fluxes were measured in autumn for both urine and dung (i.e. -1.41 and -1.62 g ha^{-1} d^{-1} respectively) and are in the same order of magnitude than the minimal fluxes observed by Mori and Hojito (2015) in Japan after cattle excreta application (i.e. -0.72 and -2.64 g ha^{-1} d^{-1} for urine and dung respectively). On a subtropical ryegrass pasture in Brazil, de Bastos et al. (2020) have observed negative fluxes of greater magnitude (i.e. -10.8 g ha^{-1} d^{-1}) after sheep excreta application. In soils, reduction of N_2O up to N_2 represents the only N_2O consumption pathway, but can involve different enzymatic reactions that still need more understanding (van Groenigen et al., 2015). As the negative fluxes following ruminant excreta application have been observed in grasslands characterised by a variety of climates, soil characteristics and vegetation cover, it is difficult to decipher the drivers of N_2O consumption under excreta patches.

These low fluxes led to low cumulative emissions (mean ranging from 0.13 to 0.33 kg N_2O-N ha^{-1} for U, from 0.14 to 0.43 kg N_2O-N ha^{-1} for AU) which were not significantly different than background emissions (i.e. emissions from control). The total emissions on our lowland experimental site were much lower than those observed in other studies on similar grasslands (i.e. lowland with similar plant cover in temperate regions). Indeed, average cumulative N_2O emissions in Marsden et al. (2017) were approximately 5 kg N_2O-N ha^{-1} after only 9 weeks of sheep urine application in summer in the UK. Hoogendoorn et al. (2016) also found total emissions of approximately 5 kg N_2O-N ha^{-1} after 123 days of sheep urine application in winter in New Zealand. Our results in LOW are more similar to those observed in the summer experiment in Hoogendoorn et al. (2016) who calculated that approximately 0.3 kg N_2O-N ha^{-1} of N_2O was emitted after 109 days of sheep application, with an application rate of 280 kg N ha^{-1}. The authors attributed these low emissions to the low soil moisture content leading to low denitrification activity, and a similar explanation could be given in the summer experiment in LOW. Therefore, we could have expected higher emissions after spring and autumn application due to high initial soil moisture conditions, but this was not the case, suggesting that other causes were involved in the low emissions observed in spring and autumn.

4.4.1.2 Upland grassland

In UP, none of the treatments led to a peak of N_2O following application, and this was true in both seasons. This is similar to the results observed in Marsden et al. (2019) where no N_2O peak was observed following sheep urine application on uplands organic soil. The authors attributed the low emissions to the soil properties, e.g, low soil pH, inhibiting the nitrification process. The inhibition of nitrification in this soil can be supported by the absence of build-up of NO_3^--N after treatments application in both summer and autumn. The surprising pattern of NO_3^--N during the first weeks post summer application could let us think that there were a build-up of NO_3^--N, which was actually not the case. The level of NO_3^--N was actually relatively high during the background measurement and the first two weeks post application for all treatments, before decreasing rapidly and remaining steady for the whole year. This pattern is uncommon and consequently difficult to interpret but could be linked to the rainfall pattern before application. Indeed, summer application was preceded by a particular dry period (30 days with total rainfall of 14 mm and average daily rainfall of 0.5 mm), followed by two weeks with more intense precipitation just before the start of the experiment (i.e. 55.4 mm in total with average daily rainfall of 3.5 mm). Therefore, the dry conditions could have caused the death of the less resistant plant species, afterwards the rainfall events would have promoted the release of mineral N from the dead roots into the rhizosphere.

Similarly, to the lowland, we observed negative fluxes on this upland grassland for both seasons and for all treatments, with minimum fluxes recorded in autumn for both U (-1.71 g ha^{-1} d^{-1}) and D (-3.13g $ha^{-1}d^{-1}$). These negative fluxes were of similar amplitude than those on the lowland site and we also observed negative total N_2O at the end of the experimental periods (one or two replicates by treatment in autumn, two replicates for AU and D in summer).

4.4.1.3 Effect of season on N_2O emissions

Statistical analysis showed a significant effect of season on total N_2O emissions in our study, with higher emissions in autumn compared to summer in LOW, whereas in UP the contrary was observed.

In our study in LOW, there was no obvious difference between the initial N_2O peaks following each application of treatments. Therefore, the difference of N_2O emissions between autumn and summer is more likely to be due to the environmental conditions over the full experimental periods,

which were more favourable for N_2O production after autumn application. Indeed, among the three experimental periods in LOW, the one following autumn application was the one with highest average WFPS (81.5%), compared to 67.9 and 64.2% for spring and summer experimental periods respectively. Thus, the higher average soil moisture during the experimental period following autumn application could have promoted more denitrification activity in soil.

In UP, the soil moisture and mean daily temperature averaged over the full experimental periods were similar, with a WFPS of 91.0 and 92.9% for summer and autumn respectively and a mean daily temperature of 10.3 and 10.7°C for summer and autumn respectively, with a Therefore, it is not obvious whether the summer experimental period had conditions more favourable to promote N_2O production.

In Ireland, whereas Maire et al. (2020) observed higher total N_2O emissions after autumn application compared to spring and summer, Krol et al. (2016) found that the effect of seasons on total N_2O emissions was inconsistent throughout the experimental sites. This was also observed in a study carried out in the UK where the effect of application timing varied with experimental sites. The inconsistency of the effect of season on N_2O emissions throughout study sites is due to the interaction of climatic conditions with other parameters such as soil properties, as explained in the review of Mancia et al. (2021).

However, a seasonal effect on EF_{3PRP} over all sites was observed in the studies of Krol et al. (2016) and Chadwick et al. (2018), with higher EF_{3PRP}s after autumn and early-grazing application respectively. As in Krol et al. (2016), we also observed higher EF_{3PRP}s in autumn. However, for the artificial urine treatment, the season of application had a significant effect ($p<0.05$) on total N_2O emissions only, and not on EF_{3PRP}. As artificial urine has a relatively stable composition throughout seasons and sites, this result suggests that the effect of season on EF_{3PRP} is likely to pertain to the composition of excreta which varies with seasons.

These results highlight the complexity to disentangle the seasonal pattern of N_2O emissions.

4.4.2 Causes of low emissions

4.4.2.1 Lowland grassland

Various causes can be responsible for low N_2O emissions following U and AU application in our study and these can vary with site and season.

In LOW, the N loading rates were lower compared to other studies on similar grasslands, which observed higher N_2O emissions. Indeed, in summer, the urine N loading rate was 372 kg N ha^{-1} in LOW, which is two times lower than the N loading rate applied in Marsden et al. (2017) who observed emissions almost 40 times higher for a shorter period of measurement. The autumn urine N loading rate in LOW (i.e. 154 kg N ha^{-1}) was also two times lower than in the winter experiment of Hoogendoorn et al. (2016) (i.e. 304 kg N ha^{-1}). In a recent study, the average sheep urine N loading rate of 838 ± 31 kg N ha^{-1} has been calculated based on a sample of sheep fed from both lowland and upland pastures during different seasons (Marsden et al., 2020). This average is more than two times higher than the highest N loading rate recorded in our study (372 kg N ha^{-1} in summer). In autumn in LOW, the urine N content appeared to be two times lower compared to the average sheep urine N content from a lowland temperate grassland (Marsden et al., 2020). This may be linked to the lower N content of the grass measured in LOW (i.e. < 2.3% for the different cuts, data not shown), compared to these other studies (i.e. 3.5% in Marsden et al. (2017) and from 2.35 to 4.23 across seasons and site in Marsden et al. (2020)), since excreted N is correlated with N intake (de Klein et al., 2020c).

Moreover, highly productive grassland of LOW is probably associated with a high mineral uptake by the plant cover, particularly during grass growth period in spring and summer, and therefore an important competition for available N must exist between plants and micro-organisms in this soil. For the first grass harvests following spring and summer application (occurring one month and two months following the spring and summer application respectively), grass yield and foliar N uptake were indeed higher in the chambers treated with U/AU compared to C chambers (**Table S4.2** in **Appendix 1**).

Other season-specific conditions could have inhibited nitrifiers and/or denitrifiers activity in the lowland soil. One of these conditions is soil moisture content, widely known as a primary factor influencing N_2O production and emissions. The average WFPS for the first week post summer application was 41.4%, thus lower than the 60% threshold generally associated with denitrification (Shelton and Sadeghi, 2000; Anger et al., 2003), whereas after spring and autumn application, initial WFPS were > 80% and could have inhibited nitrification or promoted complete denitrification up to dinitrogen N_2 (Butterbach-Bahl et al., 2013).

Another reason for N loss which may have been significantly involved in summer in LOW is ammonia (NH_3) volatilisation, promoted by relatively high initial air temperature (Freney et al.,

1983) and low intensity rainfall (Sanz-Cobena et al., 2011) during the first days post application (i.e. <1mm rainfall and 16.3°C mean air temperature for the first week post application).

4.4.2.2 Upland grassland

On the acid organic soil in UP, the N_2O emissions were significantly lower than those measured on the mineral soil in LOW, which is in line with previous findings (Clough et al., 1996; Marsden et al., 2019; Wen et al., 2021).

The N loading rates on this pasture were lower than those of the lowland site in LOW and therefore much lower than the average sheep urine N loading rate (i.e. 838 ± 31 kg N ha^{-1}) calculated in Marsden et al. (2020). In Marsden et al. (2020), the average urine N content of sheep from upland temperate grassland in autumn was similar to the urine N content in autumn in UP whereas in summer, the N content in UP was two times lower than the average in Marsden et al. (2020). However, the N loading rate was unlikely to be the main cause of low emissions, since emissions from urine were higher in summer, although the urine N loading rate was two times lower than in autumn.

As an upland grassland, slope effect could also have been involved in low emissions, as highlighted in (van der Weerden et al., 2011). However, the area available to set-up the experiment was flat, to avoid the possible influence of another factor in our analysis.

The low emissions in UP are very likely to be mostly due to soil characteristics rather than N application rate, excreta composition or climatic conditions. Indeed, this peat soil is acidic (pH of 4.4) and low pH can have an effect on mineralisation rate (Cheng et al., 2013), nitrifiers communities (Nicol et al., 2008) and denitrification enzymes (Liu et al., 2014; Žurovec et al., 2021). Similar results were reported by Marsden et al. (2019), who did not observe any build-up of NO_3^--N after sheep urine application whereas N_2O fluxes increased after applying a glucose + NO_3^--N solution, suggesting the onset of denitrification.

In this often waterlogged soil, even during summer, complete denitrification with reduction of N_2O to N_2 could have resulted in low N_2O emissions (Firestone and Davidson, 1989). Although N_2O reductase is sensitive to acidic conditions (Liu et al., 2014), an increase of pH within the urine pH could have temporarily remove this inhibitory effect locally. Moreover, this extensively managed grassland is likely to have a high fungal: microbial ratio (Bardgett and McAlister, 1999), and thus a poor ability to reduce N_2O since most fungi lack N_2O reductase (Shoun et al., 2012). However, fungi could have been inhibited within the urine patch due to the increase of pH (Rousk et

al., 2010) allowing an increase of bacterial activity, including the communities able to reduce N_2O. Consequently, an increase of pH could explain low N_2O emissions at the urine patch level.

4.4.3 Importance of developing country-specific EF_{3PRP}s

Our study highlights the importance of developing country-specific EF_{3PRP}, since most of the urine and dung EFs on both sites were lower than the 2019 IPCC Tier 1 EF (i.e. 0.4 and 0.04% for sheep urine and dung respectively). Indeed, emission from sheep excreta on the two study sites were low, with average values of EF_{3PRP}s (overall seasons and treatments) close to 0. This may explain, along with the high variability of N_2O fluxes between replicates, the absence of significant effect of the applied treatments. Our results suggest that a disaggregation of EF_{3PRP} by excreta type is not required for sheep in Ireland, contrary to what has been observed in New Zealand and in the UK. As it was expected, N_2O emissions from sheep excreta appeared to be much lower than from cattle excreta since Krol et al. (2016) calculated EF_{3PRP} of 1.2 and 0.3% for cattle urine and dung respectively. Therefore, the disaggregation between sheep and cattle which has been adopted in most national GHG inventories is once again supported by the present study.

Average EF_{3PRP}s observed in our study on grasslands are also lower than the country-specific EF_{3PRP} established for sheep excreta in New Zealand and the UK. New Zealand established EF_{3PRP} of 0.5% on flat/low slope and 0.08% on medium/steep slope for sheep urine and 0.12% for dung, based on a meta-analysis of 1217 EF data from a high number of field studies (van der Weerden et al., 2020). The UK EF_{3PRP} are 0.32 and 0.1% for sheep urine and dung respectively. However, field studies from sheep excreta in the UK have showed lower EF_{3PRP}, with values of 0.03 (spring) and 0.08% (autumn) on an extensively managed grassland (Marsden et al., 2018); which is closer to the values observed in our study. Among our EF_{3PRP}s, the averages were negative for all treatments in summer on both grasslands, as well as in spring on the lowland. Negative EF_{3PRP}s are due to emissions from control being higher than emissions from excreta and have been already observed following sheep excreta deposition in the UK on an upland (Marsden et al., 2018), and in New Zealand on both lowland and hill-land (van der Weerden et al., 2011; Luo et al., 2013).

Although the N_2O emissions were already very low on the lowland, we observed a significant effect of site on total N_2O emissions. However, there was no effect of site on EF_{3PRP}, probably due the very low values of EF_{3PRP} measured on both sites. The same results are observed when restricting the analyses to the AU treatment only, suggesting that the difference of excreta composition

between both sites is not enough to explain the significant effect of site on total N_2O emissions, and that this effect is mostly due to environmental conditions (i.e. climate, soil characteristics, plant cover). Our results suggest that a disaggregation by grassland type does not seem to be required in the calculation of total N_2O emissions in the national GHG inventory.

If the EF_{PRP}s from these two sites were used instead of the current default EF applied in the 2022 Irish GHG inventory (0.1%, default Tier 1 EF_{3PRP} from 2006 IPPC Guidelines), the contribution of grazing returns to the total agricultural N_2O would decrease from 20.1 to 17.9%. However, we must be careful with the interpretation of these results, since the EF_{3PRP}s have been established on only two grasslands with only two or three timings for excreta application. In order to validate the negligible EF_{3PRP} observed in our study on both grasslands, more experimental sites are required to represent the diversity of sheep grazing systems in Ireland.

4.5 Conclusions

This field study was the first carried out in Ireland to provide data to underpin the development of a country-specific EF_{3PRP} associated with sheep excreta deposition in grasslands. Our results showed very low N_2O emissions from sheep urine and dung on two grassland types, with negligible EF_{3PRP}s for aggregated excreta for each site over all seasons, thus lower than IPCC 2019 EF_{3PRP} (0.3% for aggregated excreta). This result also supports our hypothesis of lower emissions from sheep excreta compared to cattle. Furthermore, contrary to our expectations, this field study suggests that a disaggregation of EF_{3PRP} by excreta type (i.e. urine or dung) is not required at this time. These low N_2O emissions could be explained by multiple factors including low N loading rates through excreta, combined with high plant N uptake on the lowland, high soil moisture content inhibiting nitrification during treatment application (except in summer on the lowland) and low soil pH on the upland grassland which can inhibit several steps of the N cycle. Deeper understanding of N cycling following urine deposition in these soils would be required to support our hypothesis about the causes of low N_2O emissions. In particular, the measurement of the various N losses (i.e. N_2O, N_2, NO, NH_3, NO_3- leaching) and plant N uptake as well as N immobilisation would be helpful to assess the fate of sheep urine-N in these soils, e.g. using ^{15}N labelled urine to distinguish between native soil N and urine N. Such work would help to decrease the uncertainties around the estimations of the N losses associated with sheep excreta deposition, which are much less studied compared to cattle excreta. These estimations could then be included in an N budget assessment of

various grazing systems in order to compare these various grazing systems based on a more systemic approach.

4.6 Acknowledgments

The authors would like to acknowledge the laboratory and field staff at Teagasc research centres of Athenry, Co. Galway and Johnstown Castle, Co. Wexford for their assistance on this project. This study was financially supported by the Teagasc Walsh Scholarship Scheme (project code 2018028).

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Chapter 5

Effect of soil type on nitrogen oxides (NO, N_2O) and dinitrogen (N_2) emissions following sheep urine application

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Abstract

Ruminant urine deposited onto pasture soils represents hotspots for nitrogen (N) losses including the inert gas dinitrogen (N_2) as well as nitrous oxide (N_2O) and nitric oxide (NO) which have negative impact on the environment. While N_2O emissions from excreta patches have been widely assessed, there is less understanding about NO and N_2 emissions following ruminant urine deposition onto soil. This study aimed to quantify NO, N_2O and N_2 emissions following sheep urine application onto soil under denitrifying conditions, and to assess the effect of soil type on these emissions. A short-term incubation was established with two pasture soils (mineral and peat soil) amended with either artificial sheep urine or water as a control treatment. The helium/oxygen gas flow method was used for direct measurement of NO, N_2O and N_2. We observed an effect of soil type on these N gaseous emissions. Application of sheep urine significantly increased N_2O and NO emissions on the mineral soil, whereas negligible emissions were observed on the peat soil. Some noticeable N_2 emissions were also observed for one replicate on the mineral soil amended with urine. On the peat soil, acidic conditions inhibiting nitrification were likely to be involved in the absence of N gas emissions. On the mineral soil, N gas fluxes pattern were quite different between replicates, which may be due to spatial variability of denitrification activity. Further analysis including assessment of microbial communities would be required to determine the fate of urine on these two distinct pasture soils.

Key words: denitrification; nitrogen gaseous emissions; peat soil; mineral soil; incubation

5.1 Introduction

Ruminant urine deposited onto pasture soils represents hotspots for nitrogen (N) gaseous losses including nitrous oxide (N_2O), a potent greenhouse gas which is also involved in ozone layer depletion (Portmann et al., 2012). Nitrous oxide is mainly produced in soils through the microbial pathways of nitrification under aerobic conditions and denitrification under anaerobic conditions (Butterbach-Bahl et al., 2013). Nitric oxide (NO) is another N gas which is an obligate intermediate of denitrification but is assumed to be mostly produced through nitrification (Bruemmer et al., 2008), as it is a by-product of this pathway (Venterea and Rolston, 2008). This gas is an air pollutant involved in acidic precipitation, global warming, and ozone layer depletion (Kampfl et al., 2007). Therefore, the quantification of emissions of both N_2O and NO from agricultural activities favoring their release is crucial.

The production of N_2O and NO is influenced by a number of factors, including soil moisture, temperature, soil pH, and availability of N and other substrates, which can explain the spatio-temporal variability of emissions (Hénault et al., 2012; Pilegaard, 2013). Whilst N_2O production and emissions associated with ruminant urine have been widely assessed (Krol et al., 2016; Chadwick et al., 2018; Marsden et al., 2019; Mancia et al., 2022), allowing a better understanding of the drivers of N_2O. However, data are scarce about NO emissions from grazing excretal returns and particularly for sheep excreta, as highlighted in the meta-analysis of Cai et al. (2016).

Recent field experiments carried out in Ireland showed low N_2O emissions following deposition of sheep excreta onto two distinct pasture soils (Mancia et al., 2022). In this study, lower emissions were measured on an extensively grazed upland grassland characterised by acid peat soil compared to an intensively managed lowland grassland on mineral soil. These lower emissions on the upland grassland were likely due to soil properties, although this would need to be confirmed with detailed experimentation under controlled conditions, in the absence of plants and under optimal conditions for denitrification. It is not clear if the same results would be obtained regarding NO emissions (i.e. low emissions and effect of soil type). Under denitrifying conditions, we would expect low NO emissions due to low nitrification rate and to the rapid reduction of NO to N_2O (Oertel et al., 2012; Pilegaard, 2013). However, high NO emissions produced by denitrification have been observed under anaerobic conditions (Loick et al., 2016). Moreover, factors controlling the NO: N_2O emission ratio are still not well understood (Pilegaard, 2013).

Under high soil moisture conditions, low N_2O emissions could also be observed by complete denitrification to the inert gas N_2. However, the reduction of N_2O up to N_2 is controlled by an interaction of many factors including soil gas diffusivity which depends on soil texture. Therefore, the N_2O: N_2 ratio could vary a lot between two soil types under the same moisture conditions (Saggar et al., 2013). Hence simultaneous measurements of N_2 emissions, alongside N_2O and NO, would increase our understanding of the fate of urine-N following deposition onto pasture soils. However, the difficulties of quantifying N_2 emissions from soils, due to its high concentration in the atmosphere, has led to limited research measuring this form of N loss.

Dinitrogen (N_2) emissions can be measured with the acetylene inhibition technique to hinder nitrification and N_2O reduction. This is a simple and inexpensive method which has been widely used to measure N_2 loss from soils (Rudaz et al., 1999; Zaman et al., 2007; Owens et al., 2016; Lin et al., 2021). However, this method is associated with many drawbacks (Felber et al., 2012), including an underestimation of denitrification rate (Bollmann and Conrad, 1997; Qin et al., 2012; Saggar et al., 2013). Other studies have used more accurate methods to measure N_2 emissions from soil: the ^{15}N gas flux (Stevens and Laughlin, 1998; Harrison-Kirk et al., 2015; Clagnan et al., 2020) and the He/O_2 atmosphere exchange method (Butterbach-Bahl et al., 2002; Kreutzer et al., 2009; Chen et al., 2015; Cardenas et al., 2017; Clagnan et al., 2020). However, these methods require expensive equipment and expert knowledge (Friedl et al., 2020). The ^{15}N tracer method has the advantage to be applicable both in laboratory and field and can be used to distinguish the sources (nitrification/denitrification) of N_2O (Arah, 1997). The He/O_2 gas flow method allows for a direct measurement of N_2 without any labelled substrate addition to the soil but can be performed only on incubated soil cores, which implies soil disturbance (Friedl et al., 2020). Moreover, it requires sufficient initial flushing and a good air tightness to avoid atmospheric N_2 to enter the soil cores (Groffman et al., 2006). Failure to do so can lead to N_2 data difficult to interpret and consequently not presented in some studies (Ma et al., 2021; Barrat et al., 2022). However, this method is considered as one of the more efficient approaches for direct measurement of N_2 in incubation experiments (Friedl et al., 2020), but the combination of methods is being increasingly adopted for more accuracy of measurements (Malone et al., 1998; Mukumbuta et al., 2018; Well et al., 2019).

The aim of this study was to quantify nitrogen gaseous losses (N_2O, NO, N_2) following sheep urine application to two distinct grassland soils under denitrifying conditions, and to assess the effect of soil type on these emissions.

We expected an effect of soil type on N gaseous emissions (NO, N_2O and N_2) following sheep urine application, with lower emissions on the peat soil due to acidic conditions, particularly because low soil pH can inhibit nitrification and consequently the emission of NO and N_2O which are produced during this process.

5.2 Materials and methods

5.2.1 Experimental design and set up

To assess the effect of sheep urine and soil type on gaseous N emissions (NO, N_2O, N_2), a soil incubation experiment was set-up in the denitrification soil incubation system (DENIS; Cardenas et al., 2003) which uses a continuous flow of helium/oxygen (He/O_2) through soil cores. This system comprised 12 vessels, each containing three soil cores (4.5 cm diameter, 10 cm depth). Soil from two grasslands in the west of Ireland were incubated: a mineral and an acid peat soil (six vessels for each soil type). Two amendments were assessed: water control and artificial sheep urine. Therefore, the experiment comprised four treatments: mineral soil amended with control water (MC), mineral soil amended with artificial sheep urine (MU), peat soil with control water (PC) and peat soil with artificial sheep urine (PU). Each treatment had three replicates, with a random distribution of treatments across vessels. A schematic representation and pictures of the experimental set-up are presented in **Fig. S5.1 and S5.2,** respectively, in **Appendix 2.**

5.2.2 Soil sampling, analyses, and packing

Soils were sampled at two sites in the west of Ireland, representing typical Irish grasslands. The mineral soil was collected on a well-managed lowland grassland at Teagasc Research Centre, Athenry, Co. Galway (53°17 'N, 8°46'W) whereas the acid peat soil was sampled on an extensively managed upland grassland on a commercial sheep farm in Oorid, Recess, Co. Galway (53°26 'N, 9°36'W). Intact soil samples (20 x 20 cm to a depth of 10 cm) were taken using a spade every three meters along a 'W' line across each field, resulting in ten samples that were then sieved (<7 mm) to remove vegetation and stones, homogenised to provide one large sample for each soil type and stored at 4 °C for one week until the set-up of the experiment. Replicate samples (n = 5) of each soil were air-dried and ground for analyses of total N (TN), total carbon (TC), pH and organic matter (OM). The bulk density of each site was also determined in five different locations across each field. Metal rings of 100 cm^3 were inserted into the soil, at 0-5 cm and 5-10 cm depth. Soil

cores were oven-dried (105°C) for 24 hours and weighed. Dry soil cores were then sieved (<2 mm) in order to remove and weigh stones to correct bulk density measurements. Soil was transported to Rothamsted North Wyke (under Defra import licence for foreign soils, licence nº104130/198536-3) and packed into the DENIS vessels (three cores per vessel) to achieve a target bulk density representative of field conditions (0.8 and 0.09 g cm^{-3} for the mineral and peat soil respectively). Soil cores were packed in the vessels 48 h before the start of the experiment to allow for adaptation to experimental conditions. Soils were packed in the cores to a height of 75 mm (3/4 of core's height), and before packing, soils were wetted to reach a WFPS of 85% (considering the volume of amendment) in order to ensure optimal denitrification conditions (Loick et al., 2016). Such high value of soil moisture was also often reached *in situ* during the previous field study carried out on both grasslands (Mancia et al., 2022; **chapter 4**). The temperature of the cabinet was maintained at a constant 15°C. Soils characteristics are detailed in **Table 5.1.**

5.2.3 Application of amendments

Artificial sheep urine was made up according to a recipe derived from the average concentrations of urine compounds from ewes collected on both lowland and upland temperate grasslands, at different seasons (Marsden et al., 2020). This recipe was relevant for our experiment since soils studied here are from upland and lowland grasslands. Urine was stored in cold room (<4°C) until application and subsamples of artificial sheep urine were analysed. Chemical characteristics of artificial urine are presented in **Table 5.2.**

The lid of each DENIS vessel contained three holes fitted with gas tight septa to facilitate application of the urine treatment on the top of each core. On the day of application, syringes fitted with needles were used to delicately apply 6.4 mL of artificial urine or water (to the control treatment) to the appropriate soil cores through the silicon septum. This volume was chosen to represent a typical sheep average urination of 4 L m^{-2} (Haynes and Williams, 1993; Whitehead, 1995), resulting in an application rate of 196 kg N ha^{-1}. Typically, amendments are flushed with He/O_2 gas prior to application to the soil in the DENIS system to remove N_2 gas (Cardenas et al., 2003), however, this was not done in this experiment to avoid any N losses from urine via ammonia volatilisation (Ma et al., 2021).

Table 5.1 Background characteristics of the two grassland soils. TN and TC are expressed on a dry soil weight basis. Values represent means (n = 5) ± SEM.

	Mineral soil from a lowland grassland	Peat soil from an upland grassland
pH	6.3 ± 0.2	4.4 ± 0.1
Organic matter (%)	10.8 ± 0.6	94.7 ± 0.6
TC (%)	5.2 ± 0.3	41.1 ± 0.2
TN (%)	0.5 ± 0.0	2.3 ± 0.1

Table 5.2 Chemical characteristics of artificial urine. Urea-N could not be measured so has been deduced by subtracting total mineral N from TN. Values represent means (n = 3) ± SEM.

TN (g L^{-1})	4.9 ± 0.2
TC (g L^{-1})	5.9 ± 0.2
NH_4^+ -N (mg L^{-1})	95.2 ± 4.0
NO_3^- -N (mg L^{-1})	5.0 ± 0.1
Urea-N (g L^{-1})	4.8

5.2.4 Gas analysis and fluxes calculation

At the start of the experiment, soil cores in the vessels were flushed from the bottom with an He/O_2 (80:20) gas mixture to remove N gases in the soil to minimal levels, allowing the measurements for N gases generated by soil microbial processes to be quantified accurately. The He/O_2 gas mixture was flushed through the soil at a rate of 30 mL min^{-1} for 14 h, and then the flow of gas was redirected to over the soil surface at a rate of 12 mL min^{-1} for the rest of the experiment, i.e. 15

days. Flushing of soil cores started 48 h before the application of treatments to allow for soil adaptation, according to previous studies for which the adaptation period ranged between one to three days (Cardenas et al., 2017; Loick et al., 2017; Ma et al., 2021).

Airflow from each vessel was directed to different gas detectors for N_2O, N_2, NO and CO_2 measurements, with one vessel analysed every eight minutes, resulting in bi-hourly measurements of each vessel. A gas chromatograph (Perkin Elmer Instruments, Beaconsfield, UK) fitted with an electron detector capture (ECD) and a flame ionisation detector (FID) were used to quantify N_2O and CO_2 respectively. A second gas chromatograph equipped with a helium ionisation detector (HID, VICI AG International, Schenkon Switzerland) was used for N_2 concentration determination. Finally, NO concentrations were analysed with a chemiluminescence analyser (Sievers NOA280i, GE Instruments, Colorado, USA). Fluxes of each gas were calculated after correction for the gas flow rate of each vessel recorded daily, and for the surface area of soil cores (three cores of 15.8 cm^2 for each vessel). Fluxes of NO, N_2O and N_2 were expressed in g N. $ha^{-1}d^{-1}$ and fluxes of CO_2 in g C. $ha^{-1}d^{-1}$.

For N_2 fluxes, flushing of the soil cores with He/O_2 was not sufficient to remove all the N_2 gas prior to amendment application. Insufficient flushing affected differently each vessel, leading to N_2 baselines which were variable between vessels. These baselines were estimated for each vessel using flux data for the last 24 h of the experiment when fluxes became constant. An exception was made for vessel 3 which was the only vessel not following a decreasing pattern from the start to the end of the incubation. Since fluxes of vessel 3 were often equal to zero, baseline was set to zero. Baselines were then subtracted from the measured fluxes to represent the amendment-induced fluxes only.

Cumulative emissions were calculated from the area under the curve by linear interpolation, from t = 0 (amendment application) to t = 14.9 days post application (end of the experiment), using the DescTools package (AUC function) in R studio version 3.2.5 (R Development Core Team, 2019).

5.2.5 Parallel incubation for destructive soil sampling and analyses

A parallel soil incubation was set up with the same treatments (i.e PC, PU, MC, MU) to allow for destructive sampling at different time points following amendment application and the assess-

ment of some soil chemical characteristics, as well as abundance of targeted genes of nitrification/denitrification. This parallel incubation was initially set-up simultaneously to the incubation in the DENIS system, but due to logistical issues (i.e samples lost by carrier), it was not possible to analyse soil samples in a timely way. Therefore, the parallel incubation was repeated several weeks after the first incubation under the same experimental conditions. This parallel incubation is presented in **chapter 6.**

5.2.6 Statistical analyses

For the fluxes of each vessel, outliers were removed by using the Rosner test (Rosner, 1975). Cumulative NO and N_2O emissions were compared between treatments with an ANOVA. For N_2O, since no data was available for two of the three replicates from the peat control treatment (i.e. data below the detection limit), it was not possible to calculate cumulative emissions for these two replicates. Conditions of ANOVA were checked with Levene's test (Homogeneity of Variance of residuals) and Shapiro-Wilk test (normality of residuals). If these conditions were not satisfied, data were log transformed prior to ANOVA.

5.3 Results

5.3.1 Nitrogen gas emissions

5.3.1.1 Nitric oxide (NO) emissions

Fluxes of NO are shown in **Fig. 5.1** (mineral soil) and **Fig. 5.2** (peat soil). There was no noticeable NO increase for the treatments PC, PU and MC. However, for treatment MU, an increase of NO fluxes started from around day 5 post application, with an average flux reaching ca. 4 g ha^{-1} d^{-1} on the 11^{th} day post application. When the incubation experiment ended, the flux was still decreasing. According to an estimated second-degree polynomial trend line ($R^2 = 0.81$) fitted to the values measured on the two last sampling days, the NO flux would have reached background levels by ca. day 16, so not long after the end of the experiment.

Statistical analyses showed a significant effect ($p<0.05$) of treatment on cumulative NO emissions, with emissions higher from the mineral soil compared to peat soil, and higher emissions after urine application compared to control in the mineral soil only. For the cores treated with urine, the

proportion of added N emitted as NO was 0.02% from the mineral soil, whereas it was negligible on the peat soil (i.e. <0.01%).

5.3.1.2 Nitrous oxide N_2O emissions

Fluxes of N_2O on the mineral soil are shown on **Fig. 5.1.** There was no noticeable increase of N_2O emissions from the peat soil following either water or urine application, with low fluxes throughout the measurement period. Indeed, >95% of flux data from the PC and PU replicates were below the detection limit, hence these data were not presented in **Fig. 5.2.** On the mineral soil, the application of urine and water both led to an initial N_2O peak within the two first days, with the average fluxes reaching about 130 g ha^{-1} d^{-1} and 145 g ha^{-1} d^{-1} for MC and MU, respectively. The magnitude of the initial peak was quite variable between replicates for both MC and MU (**Fig. S5.2** in **Appendix 2**). For the MU treatment only, another peak of N_2O fluxes starting from around day 5 post application and reached a maximum of ca 140 g ha^{-1} d^{-1} by day 10.

For two replicates of PC, all the values were below the detection limit and therefore cumulative emissions were considered negligible for these two replicates. Statistical analyses showed higher emissions on mineral soil compared to peat soil, and higher emissions following urine application on the mineral soil. Cumulative emissions are shown in **Table 5.3.** For the cores treated with urine, the proportion of added urine-N emitted as N_2O reached 0.77% on the mineral soil, whereas it was negligible on the peat soil (i.e. <0.01%).

5.3.1.3 Dinitrogen (N_2) emissions

Fluxes of N_2 are shown on **Fig. 5.1** and **Fig 5.2**. These fluxes are more difficult to interpret, particularly due to problems of insufficient removal of residual N_2 from the soil profile before the addition of urine or water. This led to different N_2 background concentrations between vessels, and thus different baselines. Moreover, some N_2 entered the vessels with the application of amendments, and the quantity introduced varied from one vessel to another, leading to high variability of initial fluxes between replicates of the same treatment. Consequently, we did not calculate cumulative N_2 emissions, and we also chose to represent fluxes of each replicate instead of the average fluxes per treatment in **Fig 5.1** and **Fig 5.2**.

According to the patterns of N_2 fluxes there was no noticeable apparent increase in N_2 fluxes after water or urine application on the peat soil for any of the replicates. However, on the mineral

soil, some increase was observed for some replicates after water and artificial urine application. Indeed, for MC treatment, one replicate (vessel 2) showed an increase of N_2 flux following the initial peak of N_2O flux, from around day 3 to day 6 post application. A positive linear trend line ($R^2 = 0.80$) fitted to the values from day 3 to day 6 validated this increase of N_2. For the other replicates (vessels 4 and 12), no noticeable increase was observed.

For the MU treatment, it is also likely that urine-induced N_2 was emitted for one replicate (vessel 3) which showed two noticeable increases in apparent N_2 fluxes. Indeed, after low initial fluxes (<1.7 kg N ha^{-1} d^{-1}) during the two first days post application, fluxes increased to a level ca. 15 times greater, and then remained fairly stable at ca. 8.5 kg N ha^{-1} d^{-1} until day 7 when fluxes decreased again, even below the detection limit. Level of fluxes became high again from day 12 post application, and this increase in apparent N_2 fluxes was even higher than the first peak (~1.5 times higher). When compared to the N_2O fluxes, these two increases in N_2 fluxes followed the peaks of N_2O of the MU treatment.

For the two other replicates of MU treatment (vessels 8 and 9), there was an initial increase of N_2 fluxes during the first few hours post application. At this time, it is unlikely that the denitrifiers community would be active enough to produce N_2 at a noticeable rate. This initial increase is more likely to be due to the introduction of N_2 with water and urine amendments.

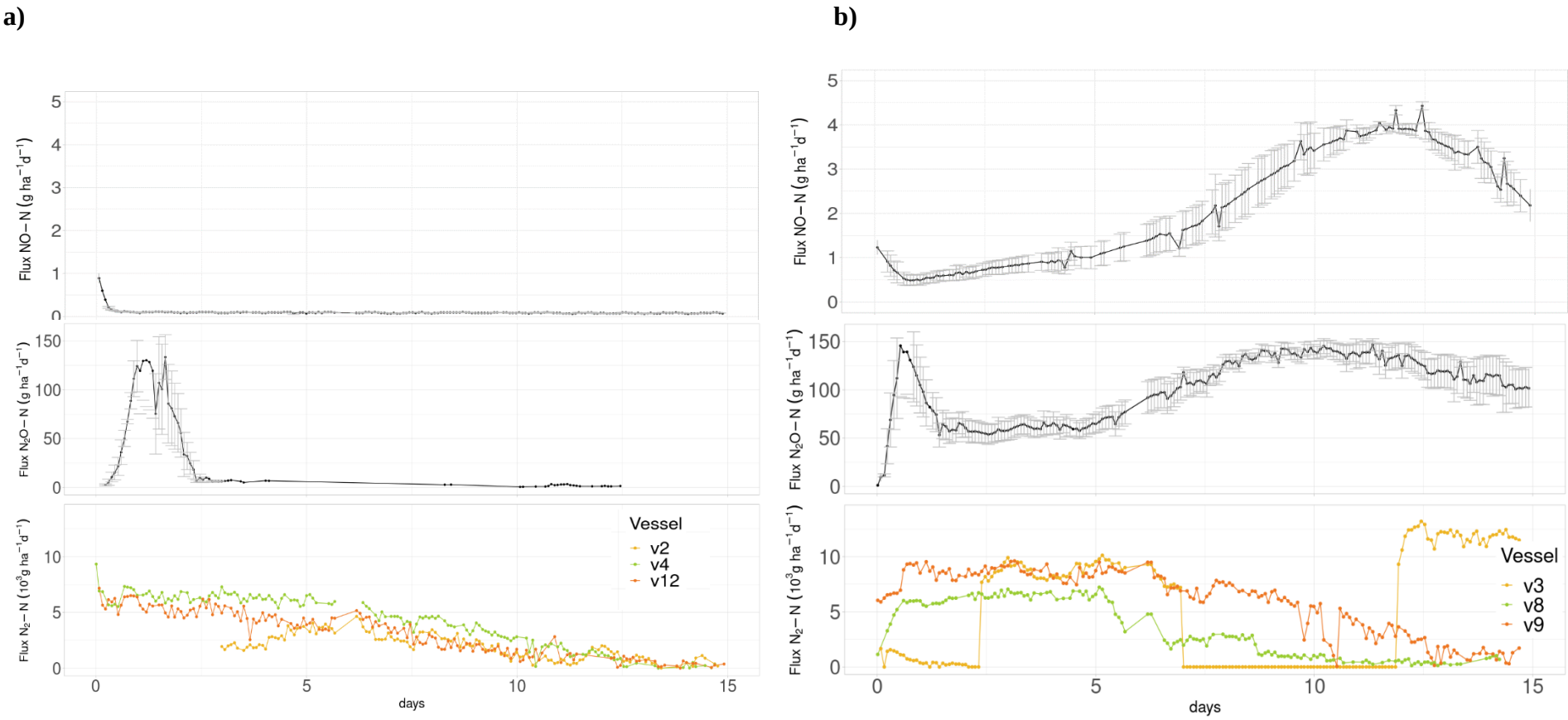

Figure 5.1 Nitric oxide (NO), nitrous oxide (N_2O) and dinitrogen (N_2) fluxes measured from the incubated mineral soil after water (a) and artificial sheep urine (b) application. For NO and N_2O, the average fluxes (n = 3) calculated from the three replicates are represented whereas for N_2 the graph shows the individual fluxes of replicates. Note the different y-axis range for the NO, N_2O and N_2 emissions. Error bars in grey on NO and N_2O fluxes plots represent standard errors to the mean (SEM).

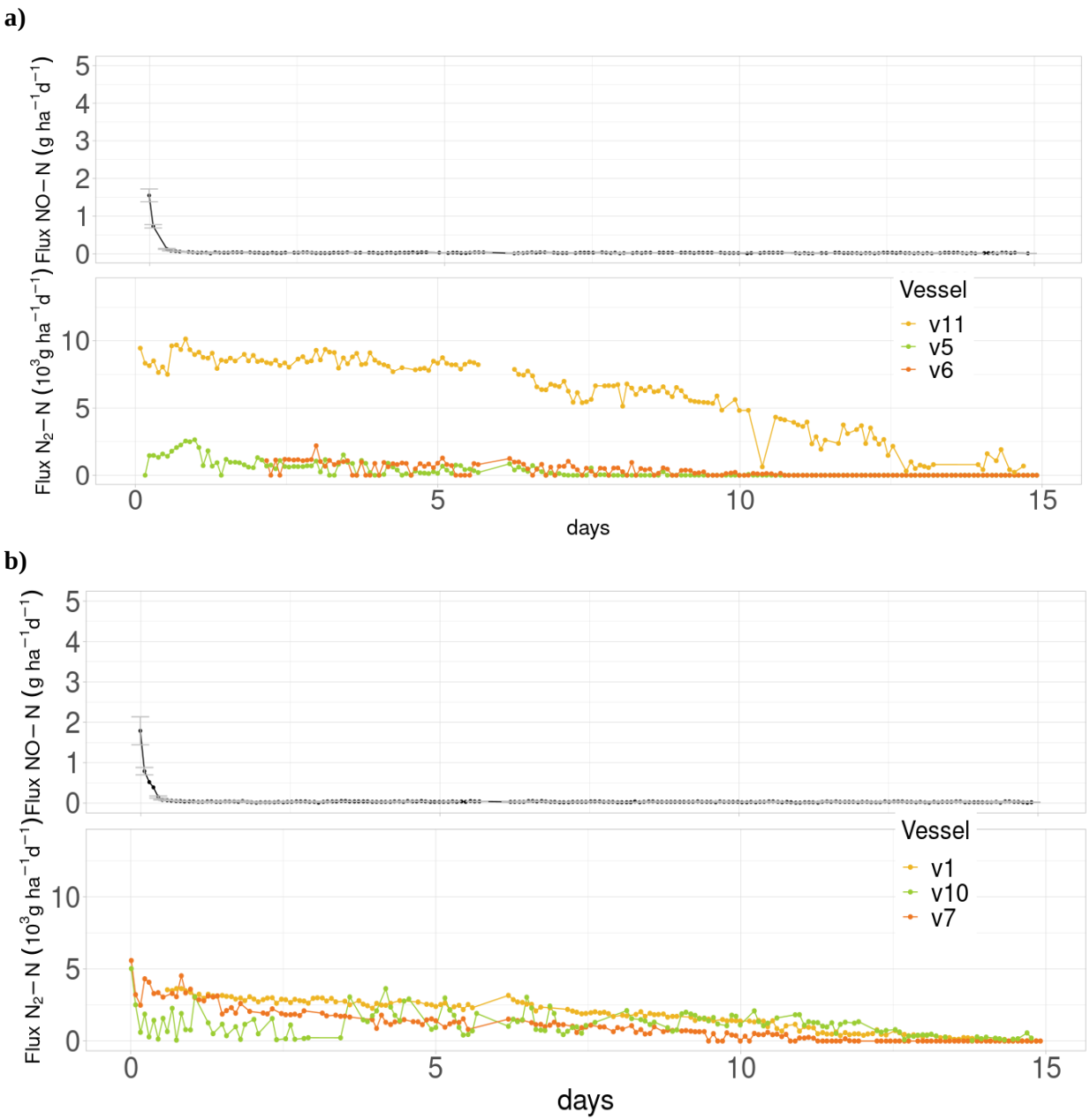

Figure 5.2 Nitric oxide (NO) and dinitrogen (N_2) fluxes measured from the incubated peat soil after water (a) and artificial sheep urine (b) application. For NO, the average fluxes (n = 3) calculated from the three replicates are represented whereas for N_2 the graph shows the individual fluxes of replicates. Graphs of N_2O fluxes are not represented because more than 95% of values were below the detection limit for PC and PU. Note the different y-axis range for the NO and N_2 emissions. Error bars in grey on NO fluxes plots represent SEM.

Table 5.3 Average cumulative nitric oxide (NO) and nitrous oxide (N_2O) emissions calculated two weeks following application of water or artificial sheep urine to the two soil types. Treatments are mineral soil amended with water control (MC), mineral soil amended with artificial sheep urine (MU), peat soil amended with water control (PC) and peat soil amended with artificial sheep urine (PU). Values represent means (n = 3) ± SEM. For PC, all N_2O values measured for two replicates were below the detection limit, hence cumulative emissions were set at 0 for these two replicates.

Treatments	MC	MU	PC	PU
Cumulative NO-N emissions (g ha^{-1})	1.5 ± 0.1	31.3 ± 2.5	0.6 ± 0	0.7 ± 0.1
Cumulative N_2O-N emissions (g ha^{-1})	169.8 ± 37.7	1504.5 ± 14.7	2.08 ± 2.08	2.06 ± 1.06

5.4 Discussion

The results from this study validated our hypothesis of the effect of soil type on gaseous N emissions following urine application. Indeed, we observed lower gaseous N emissions (NO, N_2O and N_2) on the acid peat soil than on the mineral soil. The effect of soil type on N gaseous emissions has been already observed in the study of Clough et al. (1996) which showed lower N_2O on a peat soil compared to a mineral soil.

5.4.1 Negligible N gas fluxes on the peat soil

Similar to the previous field experiment (**chapter 4**; Mancia et al., 2022) there was no noticeable increase of N_2O from the peat soil amended with artificial urine or water. In this incubation study, there was also no noticeable increase of NO and N_2 fluxes. These findings are similar to the study of Zaman et al. (2007) who observed no noticeable changes in N_2O and N_2 emissions on a wetland soil (i.e organic soil) after cow urine application at 200 kg N ha^{-1}. The authors attributed these low emissions to the high soil moisture conditions inhibiting nitrification. Inhibition of nitrification by high soil moisture could have been the case as well in our study on where we held the WFPS at ca, 85%. Unfortunately, we were not able to verify the absence of nitrification with the pattern of

mineral N due to missing samples, as previously explained. However, results from another incubation established on the same soil and under similar conditions and treatments (**chapter 6**) showed no build-up of NO_3^- -N, indicating the absence of nitrification in this peat soil under high soil moisture conditions.

The high soil moisture content is probably not the main cause of the low nitrification activity, which could be also attributed to low soil pH which influences nitrifiers community (Nicol et al., 2008). Other studies have found low emissions under low soil pH both in field (Marsden et al., 2019) and in laboratory (Allen et al., 1996). Although nitrification can still occur in acidic soil, undertaken by AOA over AOB communities, the application of urine leading to high NH_4^+-N concentration would have inhibited AOA growth, and consequently nitrification activity (Di et al., 2014).

Another hypothesis about the cause of low N_2O emissions after urine application on this peat soil would be an inhibition of fungal community by the increase of pH within the urine patch (Rousk et al., 2010), thus allowing the growth of bacterial community including those able to reduce N_2O. However, this hypothesis is not supported by our results as there was no noticeable N_2 production from the peat soil.

Therefore, the fate of urine N in this soil remains unknown and to answer this question other measurements are still required, i.e an assessment of N immobilisation, leaching, and ammonia volatilisation rates. We hypothesise that most of the urine N may have been immobilised in this peat soil characterised by a high C: N ratio. Indeed, soil with higher OM content have been associated with higher N immobilisation (Barrett and Burke, 2000).

Finally, another possible explanation could be the entrapment of N gases in this very wet soil, as suggested for the peat soil in the study of Clough et al. (1996).

5.4.2 Patterns of N gas emissions following urine application on the mineral soil

The application of artificial sheep urine led to an increase of NO, N_2O and N_2 emissions from the mineral soil. The pattern of N_2O fluxes following urine application showed a double peak for N_2O, similar to the incubation experiments of Ma et al. (2021) and Clough et al. (2003) following real and artificial sheep urine application, respectively. The short initial peak also appeared after water application and may be due to the effect of soil wetting pushing out the N_2O already present

in the soil, or to a stimulation of denitrifiers through the effect of soil wetting similar to the pulse of N_2O observed after a rainfall event (Saggar et al., 2013).

The 2nd peak was therefore mainly the result of nitrification and/or denitrification of urinary compounds following the hydrolysis of urea (Chadwick et al., 2018). However, there was no increase of CO_2 emissions associated with the apparition of this second N_2O peak (see **Fig S4** of **Appendix 2**), although the hydrolysis of urea is generally associated with an increase of CO_2 fluxes (Uchida et al., 2008). The pulse of CO_2 emissions occurred immediately after urine application, indicating that urea hydrolysis probably occurred rapidly after urine application. Therefore, the delay of apparition of the second peak of N_2O may be due to a lag phase associated with an activation of the dormant nitrifiers (Webster et al., 2005). Unfortunately, this delay in microbial activity could not be verified with the mineral N patterns of this incubation. However, results from the other incubation experiment on the same soil and under similar conditions (**chapter 6**) indicated a delay of nitrification process, which could thus have been also the case in the present study.

Under high moisture conditions of the incubated soil cores (i.e. ~85% WFPS), we would expect an inhibition of nitrification and therefore denitrification being the dominant process involved in N_2O emissions, with denitrifiers using mainly soil native N. Wachendorf et al. (2008) showed that 65% of N_2O emissions after urine application were derived from soil native N. This can be supported by the NO/N_2O ratios calculated for the mineral soil which are <1.0 (i.e. 0.01 and 0.02 for MC and MU, respectively): according to a commonly used presumption, this would indicate denitrification as the dominant process (Cheng et al., 2004; Medinets et al., 2015). The use of ^{15}N labelled urine could be useful to elucidate the sources of emissions, similar to the study of Carter (2007), Di and Cameron (2008) and Wachendorf et al. (2008).

The pattern of gaseous N fluxes was quite variable between replicates of a same treatment, despite the incubated soil cores being maintained under controlled soil moisture and temperature conditions. The initial N_2O peak for MU was for instance about two times higher for one replicate compared to other replicates **(see Fig S3** in **Appendix 2**). A quantification of mineral N and of nitrification/denitrification genes abundances in the soil cores within the DENIS would have been useful to decipher if by differences in mineral N and microbial community size could explain the differences of N gas patterns between replicates. However, there was no method of taking soil samples from the DENIS vessels without affecting the gaseous N measurements.

This difference of initial peak magnitude between MU replicates could explain why the vessel with the highest initial flux (vessel 3) had some N_2 production whereas no noticeable increase was observed for the other replicates (i.e. the quantity of N_2 produced may not have been detectable). Moreover, the second N_2O peak from vessel 3 of treatment MU decreased quicker than the others, which were just starting their decrease when experiment ended. This may explain why no N_2 was observed at the end of the experiment for the two replicates from treatment MU. Our results highlight the variability of denitrification activity, which has been suggested to follow a log normal distribution due to heterogeneity of soil microsites (Parkin, 1987).

In a similar incubation experiment using the same denitrification system (DENIS), Ma et al. (2021) also observed an effect of urine on N emissions following real sheep urine application on a clay loam soil at 65% WFPS, with a peak of NO emission appearing about 15 days post application, and whose magnitude was about two times higher than in our study. For a similar period of measurement, cumulative NO emissions calculated in Ma et al. (2021) were about three times greater than those in our study for MU treatment, which was probably due to their higher N loading rate (about two times higher). However, the proportion of applied N emitted as NO was similar to our study. Regarding N_2O emissions, the initial and second peaks in Ma et al. (2021) were of similar amplitude to those observed in our study, as well as the cumulative N_2O emissions. Hence Ma et al. (2021) calculated a lower proportion of applied N emitted as N_2O (0.45%) compared to treatment MU in our study (0.77%). In another incubation experiment on a silt loam soil with the same period of measurement and similar N loading rate, total NO emissions following artificial urine application were about 20 times higher compared to our study (Clough et al., 2003). The authors attributed NO emissions to the nitrification process, which was probably more active in their soil at 70% WFPS than in the very wet soil of our study. N_2O emissions were also about four times higher than those measured from MU treatment in our study. The authors observed a much higher proportion of applied N emitted as N gases (i.e. 2.4% for both NO-N and N_2O-N). These higher N emissions observed in Clough et al. (2003) are probably due to soil conditions favouring simultaneous nitrification and denitrification.

The comparison of our results with other incubation experiments on mineral soil highlights the variability of N emissions, which cannot only pertain to N loading rate or soil moisture content.

5.5 Conclusions

This short-term incubation experiment was designed to follow up the observations made from a previous long-term field experiment which showed low N_2O emissions from sheep excreta deposited onto two distinct temperate grasslands swards. The present study revealed that differences in soil type could explain the difference of N_2O emissions following sheep urine deposition between the two grasslands (a lowland grassland on mineral soil and an upland pasture on acid peat soil). The effect of soil type was also seen for other nitrogen gaseous emissions (i.e., NO and N_2). Under conditions favoring denitrification, N gaseous emissions were negligible on the peat soil, which may be attributed to low pH and high soil moisture conditions. On the mineral soil, N_2O emissions appeared after a lag phase and were accompanied with NO and N_2 emissions. Our results on the mineral soil also highlighted the variability of N gas emissions, which is due to intrinsic properties of the soil. To further assess the fate of urine N deposited to these soils, other N transformation pathways should be investigated (i.e. NH_3 volatilisation, microbial immobilisation, and leaching). Analysis of genes and transcripts abundances could be a useful indicator of the effect of urine on microbial communities involved in N cycle.

5.6 Acknowledgments

The authors would like to thank the staff at Rothamsted Research; Devon, UK for their help and for having made available their equipment for this experiment. The authors would like also to acknowledge the laboratory and field staff at Teagasc research centre of Johnstown Castle, Co.Wexford, Ireland and Rothamsted Research, Devon, UK for their assistance on this project. This study was financially supported by the Teagasc Walsh Scholarship Scheme (project code 2018028) and EJP Soil Visiting researcher Grant.

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Chapter 6

Fate of sheep urine N in lowland and upland grassland soils

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Abstract

Ruminant excreta patches deposited onto pasture soils are hotspots for nitrogen (N) losses including nitrous oxide (N_2O), nitrates (NO_3^-) leaching and ammonia (NH_3) volatilisation. However, there are still uncertainties about the magnitude of these losses from ruminant urine patches, which may vary significantly with soil type. Different urine fate between soil types could be explained by distinct nitrifiers/denitrifiers community's size. The aim of this study was to assess the fate of sheep urine N in two distinct pasture soils and to compare the effect of sheep urine on nitrification/denitrification gene abundances in these two soils. A soil incubation experiment was established for 21 days under denitrifying conditions and included four treatments: mineral soil receiving water control (MC), peat soil receiving water control (PC), mineral soil receiving artificial sheep urine (MU), and peat soil receiving artificial sheep urine (PU). Two parallel incubations were established: one for pH and gas fluxes measurements (NH_3, N_2O), the other for destructive sampling to assess mineral N, microbial biomass N and C, abundances of phylogenetic (*ITS, bacterial and archaeal 16S rNRA*), nitrification (*bacterial* and *archaeal amoA*) and denitrification (*nir K/S, nosZ I/II*) genes. Results showed different fates of urine N on the two soils. On the mineral soil, at the end of the incubation, urine N was mostly lost as NH_3 (12.6%) followed by NO_3^- leaching (9.9%), whereas NH_4^+ leaching and N_2O emissions were very low (0.1 and 0.3 % respectively). Low N_2O emissions were probably due to the delay of nitrification after artificial urine application on the mineral soil. On the peat soil, urine N was mainly lost as NH_3 (4.4%) followed by NH_4^+ leaching (1.2%), whereas NO_3^- leaching and N_2O loss were negligible due to the absence of nitrification in this acid soil. There was no specific effect of urine on microbial biomass N (MBN) and on nitrification/denitrification gene abundances. Assessment of gene transcripts would be a better indicator of the effect of sheep urine on microbial activity.

Key words: ammonia; nitrous oxide; leaching; nitrifier; microbial biomass; gene abundance

6.1 Introduction

Ruminant grazing systems represent an important agricultural sector worldwide (Robinson et al., 2011) and consequently it is essential to assess the sources of pollution from this activity to monitor the trends and implement mitigation strategies if needed.

Ruminants have a poor nitrogen (N) use efficiency and therefore urine deposited onto grassland soils represent hotspots for various N losses, mainly ammonia (NH_3) volatilisation, nitrous oxide (N_2O) emissions and nitrate (NO_3^-) leaching (Cai and Akiyama, 2016).

After urine deposition, urea, the main N constituent of urine, is rapidly degraded into ammonium (NH_4^+) which may be subject to NH_3 volatilisation associated with the increase of pH following urea hydrolysis (Selbie et al., 2015). Emissions of NH_3 have negative impacts on human and animal health and on the environment, particularly through eutrophication of ecosystems (Portejoie et al., 2002). Saggar et al. (2004) considered that between 4 and 46% of urine N is volatilised as NH_3. This wide range highlights the uncertainties around estimations of NH_3 emissions from urine patch, due to the influence of several influencing factors including soil pH, moisture, cation exchange capacity (CEC) and temperature (Bolan et al., 2004). Low NH_3 emissions are expected on acidic soil, but an increase of pH within the urine patch could potentially promote NH_3 volatilisation. Leiber-Sauheitl et al. (2015) observed very low NH_3 emissions from urine applied on an acid peat soil, but the authors simulated irrigation after application, which can suppress NH_3 volatilisation (Black et al., 1987; Clough et al., 1996).

Ammonium (NH_4^+) produced by urea hydrolysis can be nitrified and subsequently denitrified, producing N_2O gas, a potent greenhouse gas (GHG) with a warming potential 273 times higher than CO_2 over a 100 years period (IPCC, 2022). Emissions of N_2O from urine patches have been widely assessed both in field and in laboratory experiments to quantify these emissions and decipher the factors influencing them (Cai et al., 2017; López-Aizpún et al., 2020). Soil properties constitute an important driver of N_2O emissions from urine patches (Mancia et al., 2022b), and one feature of soils that can influence the production of N_2O pertains to the microbial community structure and diversity (Braker and Conrad, 2011). Several studies have assessed the relationships between N_2O emissions and nitrification/denitrification gene abundances in urine-amended soils (Di et al., 2014; Pan et al., 2018; Jha et al., 2020; Ma et al., 2021). The authors of a recent study observed a difference in the abundance of the denitrification gene *nosZ I* between two soil types amended with cattle urine, which were associated with significantly different N_2O emissions (Jha

et al., 2020). The authors also observed an increase of denitrification genes after urine application, similar to the results of Di et al. (2014). Some soils which present very low N_2O emissions from urine patches, such as acid peat soil, could be associated with small and inactive nitrifier and /or denitrifier community, as suggested in previous studies observing negligible emissions following urine application on acid organic soils (Marsden et al., 2019; Mancia et al., 2022a).

Urine N which is not volatilised as NH_3 or lost by nitrification/denitrification can be taken up by plant cover, leached or immobilised in microbial biomass. Urine N is assumed to be leached mainly in the form of nitrate (NO_3^-), but other forms have been observed in leachates (Selbie et al., 2015). Clough et al. (1996) have indeed observed NH_4^+ in leachates of both mineral and peat soils after sheep urine application. Monaghan et al. (1989) also observed NH_4^+ leached from a mineral soil, which was not the case in Fraser et al. (1994). The presence of NH_4^+ in leachate is likely to be an indicator of nitrification activity, and thus is an interesting variable to measure in assessment of urine N fate.

The effect of urine on microbial biomass nitrogen (MBN) is still unclear: while several studies observed no specific effect of urine on MBN (Lovell and Jarvis, 1996; Rooney et al., 2006; Ma et al., 2007), Wachendorf et al. (2011) showed an immobilisation of ^{15}N-labeled urine in the soil microbial biomass.

The aim of this study was to assess and compare the short-term fate of urine-N applied to two distinct grassland soils. A second objective was to determine if the abundance of several specific genes involved in nitrification/denitrification would be altered by sheep urine and could explain the difference in N gas emissions (NH_3, N_2O) between soil types.

We hypothesised an effect of soil type on the fate of urine N, with higher total N losses on the mineral soil, due to low N_2O and NH_3 emissions on the acid peat soil. We also hypothesised that urine would influence nitrifier/denitrifier community's size, and that differences in N gas emissions between the two soils could be related to differences in nitrification/denitrification gene abundances.

6.2 Materials and methods

6.2.1 Experimental design and set up

To assess the effect of soil type on the fate of sheep urine N under denitrifying soil conditions, a short-term (22 days) incubation experiment was established. Two contrasting soil types (a mineral soil and an acid peat soil) collected from temperate grasslands were incubated under controlled conditions. Two amendments were assessed: water (control) and artificial sheep urine. Therefore, the experiment comprised four treatments: mineral soil amended with water (MC) or with artificial sheep urine (MU), peat soil amended with water (PC) or with artificial sheep urine (PU).

6.2.2 Soil sampling, analyses and packing

Soils were sampled at two sites in the west of Ireland, representing two typical Irish grasslands. The mineral soil was collected from a well-managed lowland grassland at Teagasc Research Centre, Athenry, Co.Galway (53°17 'N, 8°46'W), whereas the acid peat soil was sampled from an extensively managed upland grassland at a commercial sheep farm in Oorid, Recess, Co. Galway (53°26 'N, 9°36'W).

Spade-squares (20 x 20 cm to a depth of 10 cm) of soil were taken every three meters along a 'W' line across each field. Both soils were sieved (<7 mm) to remove stones and vegetation, homogenised and stored at 4 °C until the set-up of the experiment. Pictures of intact soil cores are presented on **Fig. S6.1** in **Appendix 3.**

Samples of each soil were air-dried and ground for analyses of total N (TN), total carbon (TC), total organic carbon (TOC), pH and organic matter (OM) content. Initial characteristics of each soil are presented in **Table 6.1**. On the day of soil sampling, bulk density of each grassland soil was also determined. Methods of soil analyses have been previously described in **chapter 4**.

Sieved soils were packed into the cores 48 hours before the start of the experiment to allow for adaptation to experimental conditions, to be in line with the previous incubation experiment (**Chapter 5).** Two types of containers were used. For destructive sampling allowing for chemical, microbial and molecular analyses, soils were packed into 50 mL centrifuge tubes (28 mm diameter) whose bottoms have been cut, to a height of 75 mm (3/4 of a core's height). A mesh cloth was attached to the bottom with an elasticated band to hold the soil in place. The same containers were used to pack the soil for the leaching test. A total of 100 tubes packed with soil were needed (50 of

each soil). For gas sampling and pH measurement, 180 cm^3 of soil was packed into 0.5 L Kilner jars (100 cm^2 base area), allowing a headspace of 320 cm^3 when the jar's lid is closed. Four Kilner jars were used per treatment, resulting in 16 jars. Lids were drilled with two holes, each fitted with an airtight septum to facilitate manual gas sampling. These septa also allowed for the introduction of the tubing of a closed-loop system attached to a photoacoustic multi-gas analyser (Innova 1412, AirTech Instruments, Ballerup, Denmark) which was used to make potential NH_3 emissions measurements. A picture of the photoacoustic analyser connected to a Kilner jar is presented on **Fig. S6.2** of **Appendix 3.**

The quantity of soil needed in each core was determined to reach a bulk density representative of field conditions (0.8 and 0.09 g cm^{-3} for mineral and peat soil, respectively). Soils were wetted to reach a final WFPS of 85%, considering the volume of amendment that will be added during application. Such a high value of soil moisture was to ensure denitrification conditions (Loick et al., 2016) and was often observed during the previous field study on both grasslands where these soils have been collected (**Chapter 4**). All the cores were incubated in the dark in a growth room at 15°C.

6.2.3 Amendment preparation and application

Artificial sheep urine was made up according to a recipe derived from the average concentrations of urine compounds collected from ewes on both lowland and upland temperate grasslands, across different seasons (Marsden et al., 2020). This recipe was relevant for our experiment since soils studied here are from upland and lowland grasslands. Artificial sheep urine was stored in cold room (<4°C) for a maximum of five days until application, and subsamples were analysed for TN and TC content (i.e., 3.48 and 3.75 g L^{-1} respectively).

On the day of application, the whole surface area of each soil core (jars and tubes) received an appropriate volume of water (for MC, PC) or artificial urine (for MU, PU). The volume was determined to simulate a typical sheep average urination of 4 L m^{-2} (Haynes and Williams, 1993; Whitehead, 1995), resulting in an application rate of 139 kg N ha^{-1}.

6.2.4 Gas fluxes measurement

Manual gas sampling was carried out using static chamber methodology to assess N_2O emissions (de Klein et al., 2020a). An initial gas sampling was performed before treatment application

to assess the background N_2O flux. After treatment application, manual gas sampling occurred at day 0 post application, then at day 1, day 2, day 6, day 9, day 12, day 15 and day 21. At each sampling event, lids of Kilner jars were closed and gas samples were taken through a rubber septum with a 12 mL syringe fitted with a hypodermic needle at T = 0, 10 and 20 minutes after chamber closure. Gas was then injected into a 7 mL pre-evacuated glass vial with air-tight lid. Five additional ambient air samples were also taken at each sampling event. Samples were then analysed at the Teagasc Research Centre in Johnstown Castle, Co. Wexford, to determine N_2O concentrations using agas chromatograph (Varian CP 3800 GC, Varian, USA) fitted with an electron capture detector. A linear regression of N_2O concentration over time was used to calculate hourly N_2O fluxes for each sampling event for each treatment (Eq. 1).

$$FN_2O\ (\text{hourly}) = \left(\frac{\delta C}{\delta t}\right) \text{x } \frac{MxP}{RxT} \text{ x } \frac{V}{A} \qquad \textit{(Eq. 1)}$$

Where FN_2O is the hourly flux of N_2O (g m^{-2} h^{-1}), δC is the variation of N_2O concentration during the jar enclosure period (g m^{-3}), δt is the enclosure period in hours, M is the molar mass of N in N_2O (28 g mol^{-1}), P is the atmospheric pressure at the time of sampling (Pa), T is the air temperature at the time of sampling (K), R is the ideal gas constant (J K^{-1} mol^{-1}), V is the headspace volume of the closed jar (320 cm^3), and A the area of soil surface in the jar (100 cm^2). Daily fluxes were then extrapolated from the hourly fluxes, assuming that the calculated hourly flux is representative of the average flux of the day.

Cumulative N_2O emissions were calculated by linear interpolation of daily fluxes from day 0 (de Klein et al., 2020b), and N_2O emission factors (EF_{N2O}) were estimated according to (Eq. 2).

$$EF_{N2O,\ urine} = \frac{Cumulative N_2O,urine - Cumulative\ N_2O,water}{Napplied} \text{ x } 100 \quad \textit{(Eq. 2)}$$

Where N_2O (treatment) is the cumulative N_2O emissions from a given treatment calculated for the full experimental period (g N m^{-2}), N applied is N loading rate of urine applied to the soil core (g N m^{-2}).

Potential emissions of NH_3 were assessed with the photoacoustic gas analyser in a closed dynamic mode. Gas sampling was performed prior to treatment application to assess background emissions and following application of amendments at day 0 (i.e a few hours post application), day 1, day 2, day 6, day 8, day 13 and day 21. At each sampling event, lids were closed successively and connected to the gas analyser through the tubing system. Each jar was sampled for five minutes, resulting in five measurements (i.e. one per minute) of NH_3 concentrations per jar's headspace. Lids were opened at the end of the measurement period.

Among the five headspace NH_3 concentrations recorded on each sampling date for each jar, the first and last measurements were excluded, and the three intermediate concentrations were used in a linear regression with time to calculate the hourly NH_3 fluxes for each treatment (Eq. 3).

$$\text{Potential } FNH_3 \text{ (hourly)} = \left(\frac{\delta C}{\delta t}\right) \text{ x } \frac{MxP}{RxT} \text{ x } \frac{V}{A} \qquad \textit{(Eq. 3)}$$

Where FNH_3 is the hourly flux of NH_3 (g N m^{-2} h^{-1}), δC is the variation of NH_3 concentration during the jar enclosure period (g m^{-3}), δt is the enclosure period in hours, M is the molar mass of N in NH_3 (14 g mol^{-1}), P is the atmospheric pressure at the time of sampling (Pa), T is the air temperature at the time of sampling (K), R is the ideal gas constant (J K^{-1} mol^{-1}), V is the headspace volume of the closed jar (0.00032 m^3) and A the area of the soil surface in the jar (0.01 m^2). Hourly fluxes were then extrapolated to daily fluxes in the same way as for the N_2O calculation, and cumulative NH_3 emissions calculated by linear interpolation from day 0. The potential NH_3 EF for each treatment was calculated according to equation (4)

$$\text{Potential } EF_{NH3} = \frac{CumulativeNH3}{Napplied} \text{ X } 100 \qquad \textit{(Eq. 4)}$$

Where cumulative NH_3 is the cumulative NH_3 emissions from a given treatment calculated for the full experimental period (g N m^{-2}), N applied is N loading rate of urine applied on soil core (g N m^{-2}).

6.2.5 Soil pH measurement.

Soil pH was measured on each Kilner jar (n = 4 per treatment) using a surface pH meter (Seven2Go, Mettler Toledo, Switzerland). One measurement was made before application of the treatments, and then post application at day 0, 1, 2, 6, 8 and 13.

6.2.6 Soil destructive sampling and analyses

Soil from the batch of cores dedicated to destructive sampling was collected at different time points allowing analysis of NH_4^+-N, NO_3^--N, dissolved organic carbon (DOC), microbial biomass N (MBN) and C (MBC), gravimetric moisture content (GMC) and microbial analyses.

Initial sampling occurred before treatment application, after the adaptation period, to assess background characteristics of both soils (n = 4 replicates per soil: one core = one replicate). After application, destructive sampling took place at day 1, day 6, day 9, day 13, day 21 post application, with no assessment of MBN/MBC on day 9.

At each destructive sampling, four cores (i.e replicates) of each treatment were sampled. For each core, soil was collected and homogenised. Then from each core, 5 g was used for KCl extraction and measurement of NH_4^+-N, NO_3^- and dissolved organic carbon (DOC), and 10 g for determination of GMC. Mineral N and DOC were analysed with an Aquakem 600 discrete analyser at Teagasc Research Institute, Johnstown Castle, Co. Wexford, Ireland. Another 2 g of homogenised soil core was collected into a 2 mL centrifuge tube and stored at -80ºC for DNA extraction and microbial analyses. Finally, 16 g were used for MBC and MBN determination with the fumigation-extraction method (Voroney *et al.*, 2008). A picture of the fumigation-extraction procedure is presented on **Fig. S6.3** in **Appendix 3.** MBC was calculated as the difference between TOC of fumigated soil and TOC of unfumigated soil, divided by a correction factor of 0.45 (Vance *et al.*, 1987; Joergensen, 1996). MBN was calculated as the difference between TN of fumigated soil and TN of unfumigated soil, divided by a correction factor of 0.54 (Joergensen and Mueller, 1996). We applied the same values of a correction factor on both soil types because the use of varied factors for different soil types is still uncertain. Sparling and Zhu (1993) observed great variability in correction factors with soil type, and no clear relationship.

At the end of the incubation, four intact cores of each treatment were collected, oven dried (105ºC, 24h), grinded (<2 mm) and analysed for TN and TC, determined by Leco TruSpec® Analyser (Leco Corp., St. Joseph, MI).

6.2.7 Final leaching test

A leaching test was performed at the end of the experiment (day 22), on a batch of soil cores in the centrifuge tubes, not used for destructive sampling (n = 3/treatment). The tubes were placed on a rack when performing the leaching test. A graduated plastic container was placed underneath each tube to collect leachate.

This test was carried out by simulating a 2-hour rainfall event. The rainfall intensity was determined to be representative of an intense rainfall for the two areas of Ireland where these soils originated. To reach an intensity of an equivalent 9 mm/h, 4.5 mL of deionised water was poured on the top of each soil core at t = 0, t = 0.5 h, t = 1h and t = 1.5 h. Containers for leachate collection were not removed until the end of the rainfall simulation (t = 2 h). At the end of the test, after checking if leaching had stopped underneath each core, the volume of leachate from each core was recorded and subsamples of leachate were analysed for NH_4^+ and NO_3^-.

6.2.8 DNA extraction and assessment of gene abundances

At each destructive sampling time point, prior to sampling soil for KCl extraction and GMC determination, approximately 2 g of fresh soil was collected from three replicates of each treatment. These soil samples were immediately frozen at -80 °C to avoid DNA degradation until DNA extraction. DNA was extracted (about 0.25g fresh weight soil), after thawing, with a DNeasy PowerSoil Kit® (Qiagen, Ireland) according to the manufacturer's protocol. DNA quality was checked by electrophoresis on a 1% agarose gel using Sub-Cell® GT gel electrophoresis system (Bio-Rad, Hercules, CA). Extracted DNA was then quantified with Qubit dsDNA BR Assay® Kit (Thermo Scientific, Waltham, MA) and normalised with sterile deionised water to a concentration of 1 ng μL^{-1}.

An inhibition test was performed for each sample prior to qPCR assays in order to determine if some inhibitors of the DNA polymerase were present in the soil samples. This was done by spiking each sample with 10^4 copies of plasmid DNA (pGEM®-T). Plasmid copies in each sample were

ples was detected when the plasmid did not amplify to the same extent as the spike in the absence of the samples. Subsequently, inhibition was removed from samples by an additional cleaning step using the OneStep PCR Inhibitor Removal® Kit (Zymo Research, USA) and following the manufacturer's protocol.

Quantitative PCR assays were performed using Biorad CFX384 Touch Real-Time PCR Detection System, targeting the following genes: gene coding for ammonia monooxygenase in archaeal and bacterial nitrifiers (*amoA*), genes of denitrifiers including those coding for nitrites reductases (*nirK, nirS*) and for nitrous oxide reductase (*nosZ* clade I and II) as well as phylogenetic gene markers of total archaeal, bacterial and fungal communities (*16S rRNA archaeal*, *16S rRNA bacterial* and *ITS*, respectively).

PCR reactions consisted of 5 µl of Mastermix TakyonTM Low ROX SYBR® 2X MasterMix blue dTTP (Eurogentec, Ireland), the appropriate concentration of each (forward and reverse) gene primer, 200 µg BSA Bovine Serum Albumin (BSA; Thermo Fisher scientific, Ireland) and 2 µl (2 ng) of extracted DNA. The final reaction volume was 10 µl per well and each sample had three technical replicates. The primers used and their concentration for each targeted gene are shown in **Table S6.1** in **Appendix 3.** Standard curves of each gene were built according to Smith *et al.* (2007) where ten-fold serial dilution was used, from 10^7 to 10^2 genes copies per µl. Details of standard curves, including efficiency, R^2, slope and intercept are presented in **Table S6.2 in Appendix 3.**

6.2.9 Statistical analyses

One way ANOVA was performed to assess effect of treatment on total N_2O-N, total NH_3-N, NH_4^+-N and NO_3^--N leaching losses and on the following variables at each sampling date: gene abundances, pH, MBC, MBN and DOC. One way ANOVA was also performed to assess the effect of time on the following variables for each treatment: gene abundances, MBC and MBN.

Data were previously checked for assumptions of normality and homogeneity of variance. If one of these assumptions was not verified, data were log (x + a)-transformed before conducting ANOVA, where a is a constant set as the largest negative value of the dataset to which a small amount (i.e. 0.1) is added to obtain strictly positive values, as commonly presented in the literature

(de Klein *et al.*, 2020). In case of a significant effect, a post hoc test (Tukey test) was performed to assess pairwise differences, with application of the Bonferroni method to correct the p-value.

If assumptions were not verified after transformation of data, a non-parametric test (Kruskal-Wallis) was carried out followed by Wilcoxon test to assess pairwise comparisons.

Multiple Spearman correlations test was performed to identify relationships between N gas fluxes (N_2O, NH_3), soil variables (pH, NH_4^+-N, NO_3^--N, DOC, MBC, MBN) and the various gene abundances.

The software R v4.0.3 (R- core Team 2020) was used to perform all statistical analyses. All the tests were based on a significance level of $p < 0.05$.

6.3 Results

6.3.1 Soil background properties

Background characteristics of the mineral soil and the acid peat soil are described in **Table 6.1.**

Table 6.1 Background characteristics of the two grassland soils. TN and TC are on a dry soil weight basis. Values represent means (n = 4) ± SEM.

	Mineral soil from a lowland grassland	Peat soil from an upland grassland
pH	6.3 ± 0.2	4.4 ± 0.1
Organic matter (%)	10.8 ± 0.6	94.7 ± 0.6
TC (%)	4.90 ± 0.32	45.8 ± 0.20
TN (%)	0.52 ± 0.02	2.30 ± 0.03
NH_4^+-N (mg kg^{-1} DW soil)	1.39 ± 1.46	99.6 ± 23.1
NO_3^--N (mg kg^{-1} DW soil)	68.8 ± 5.24	0.89 ± 4.95

6.3.2 Soil pH

Changes of pH for each treatment are presented in **Fig 6.1.** Application of water (control) did not have a noticeable effect on pH on the peat soil, with average values ranging from 3.7 to 4.0

during the whole experiment. However, on the mineral soil, application of water increased the pH, from 5.9 before application to 7.3 at the end of the incubation.

Application of urine increased the soil pH of both mineral and peat soils, which showed similar pH at the end of the experiment (7.6 and 8.0 for PU and MU, respectively, at day 13), although both soils had very distinct initial pH (6.1 and 3.7 for MU and PU, respectively). The pattern of pH evolution was also different between MU and PU. For MU, pH quickly reached a value of 8 just after application (day 0), then slightly decreased to 7.4 at day 2, before progressively increasing until day 13. For PU, pH increased more gradually during the two first days reaching 7.4 at day 2 and remained quite constant for the rest of the experiment.

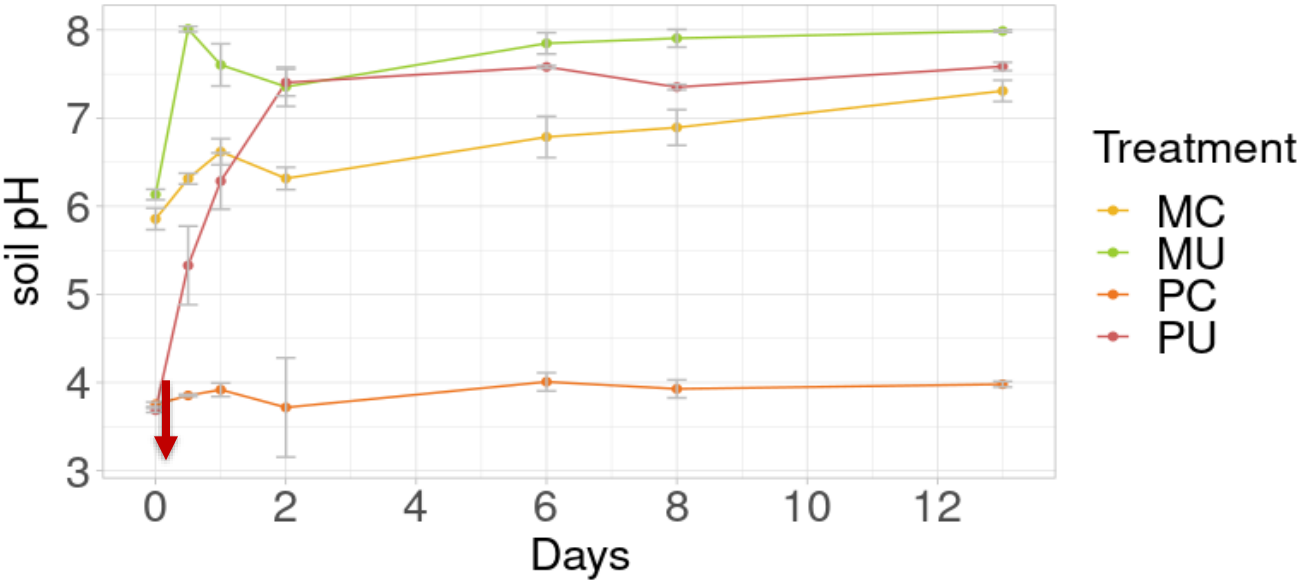


Figure 6.1 Evolution of pH following application (day 0) of artificial sheep urine or water control. Treatments are water control on mineral soil (MC), artificial urine on mineral soil (MU), water control on peat soil (PC), artificial urine on peat soil (PU). Grey vertical bars represent the standard error of the mean (SEM), (n = 4). The red arrow indicates the timing of amendments application.

6.3.3 Evolution of DOC and mineral N

Evolution of DOC is presented in **Fig. 6.2 (a).** Concentration of DOC on peat soil was significantly higher ($p<0.05$) than on mineral soil at each sampling date, being at least five times higher. During the whole experiment, DOC ranged from 2244 to 7714 mg kg DW^{-1} for PC and from 3301

to 7714 mg kg DW^{-1} for PU, whereas it ranged from 32 to 465 mg kg DW^{-1} for MC and from 290 to 465 mg kg DW^{-1} for MU.

On each soil, evolution of DOC followed similar patterns after both artificial urine and water application. However, DOC in MU became significantly higher than in MC at day 1 and day 13 and DOC in PU became significantly higher compared to PC at day 6 and 13.

Application of water led to a noticeable decrease of DOC on the mineral soil: at the end of the experiment, the level of DOC had decreased by about 50% and 37% compared to background level (465 mg kg DW^{-1} soil), for MC and MU respectively.

For both PC and PU, DOC showed a decrease post application followed by an increase from day 6 to day 9. Afterwards, DOC decreased again and remained at low level (i.e. lower than background level) for the rest of the experiment, ranging from 2200 and 3800 mg kg DW^{-1} across both treatments. At day 21, DOC had decreased by about 51 and 57% for PC and PU respectively, compared to background level (i.e. 7714 mg kg DW^{-1}).

Evolution of NH_4^+-N is presented in **Fig. 6.2 (b)**. Background level of NH_4^+-N was much higher on peat soil (100 mg kg DW^{-1}) than on mineral soil (2 mg kg DW^{-1}).

Levels of NH_4^+-N increased following artificial urine application on both mineral and peat soils, although this increase on mineral soil cannot be properly observed on **Fig. 6** due to Y-axis scale. On the mineral soil, the level of NH_4^+-N increased suddenly after urine application and remained high from day 1 to day 13, varying from 110 to 230 mg kg DW^{-1}. On the peat soil the increase following urine application was more gradual and lasted until day 9. From day 9, the level of NH_4^+-N remained high until the end of the experiment, varying from 2521 to 3034 mg kg^{-1} DW.

Contrary to artificial urine application, there was no noticeable increase of NH_4^+-N following water application on both mineral and peat soils, with NH_4^+-N ranging from 0 to 23 mg kg DW^{-1} on mineral soil and from 30 to 229 mg kg DW^{-1} on peat soil.

Evolution of NO_3^--N is presented in **Fig. 6.2 (c)**. Background level of NO_3^--N was much higher on mineral soil (69 mg kg DW^{-1}) than on peat soil (1 mg kg DW^{-1}).

Levels of NO_3^--N showed no noticeable increase on the peat soil, after water or artificial urine application, with concentrations ranging from 6 to 16 mg kg DW^{-1} for both PC and PU. This difference with the low background level cannot be considered as a proper increase when compared to increase of NH_4^+-N following urine application on this soil.

On the mineral soil, there was no increase of NO_3^--N following water application, with concentrations ranging from 27 to 54 mg kg DW^{-1}. However, following urine application, it seems than an increase started at the end of the experiment, with NO_3^--N reaching 116 mg kg DW^{-1} at day 21.

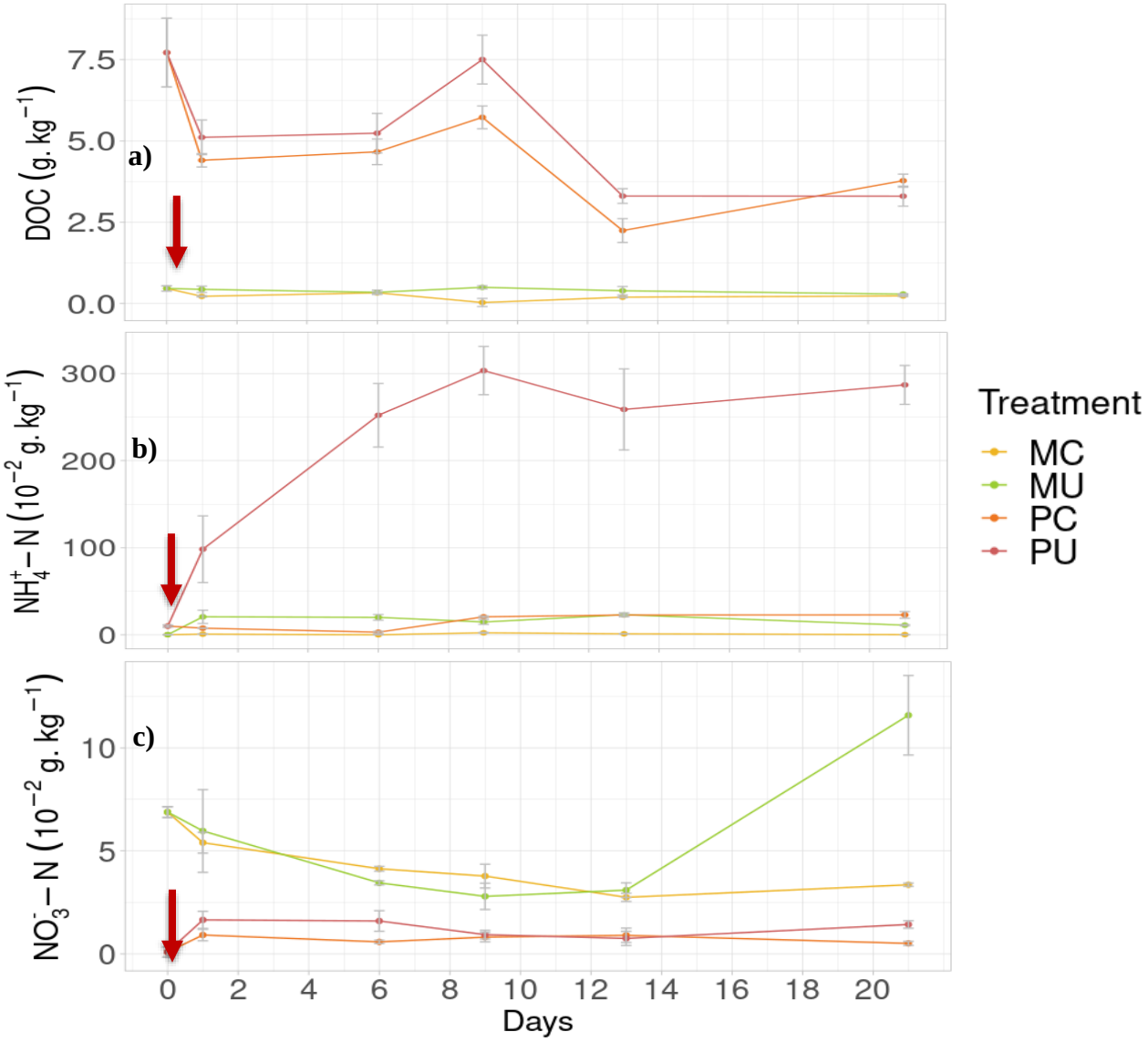


Figure 6.2 Evolution of dissolved organic carbon DOC (a), NH_4^+-N (b) and NO_3^--N (c) following application (day 0) of artificial sheep urine or water control. Treatments are water control on mineral soil (MC), artificial urine on mineral soil (MU), water control on peat soil (PC), artificial urine on peat soil (PU). Grey vertical bars represent the SEM. (n = 4). Units are expressed on a dry weight (DW) soil basis. The red arrows indicate the timing of amendments application.

6.3.4 Potential NH_3 emissions

Patterns of potential NH_3-N emissions for each treatment are shown in **Fig. 6.3 (a)**. No noticeable increase of NH_3 was observed following water application on both mineral and peat soil, with negligible fluxes over the whole measurement period.

However, application of artificial urine led to noticeable increase of NH_3-N. Maximum NH_3-N (g) fluxes occurred on the day of artificial urine application for MU, reaching 0.14 g m^{-2} d^{-1}, and then fluxes decreased gradually and were not returned to background level at this end of the experiment. For PU, emissions of NH_3 increased until day 2 and then remained relatively stable until the end of the experiment, ranging from 0.02 to 0.03 g m^{-2} d^{-1} from day 2 to day 21. Maximum NH_3-N flux for PU was almost five times lower than for MU treatment.

Statistical analyses indicated a significant ($p<0.05$) effect of treatment on total NH_3-N emissions calculated at the end of the experiment (day 21), with higher emissions following urine amendment compared to water control. After application of urine, total NH_3 emissions were significantly ($p<0.05$) higher for MU than PU, and after water application, emissions were higher for MC compared to PC. Average cumulative NH_3-N after the 21 days of incubation was negligible for control treatments (i.e. PC and MC) and was 1.71 ± 0.36 and 0.51 ± 0.05 g m^{-2} for MU and PU respectively. Percentage of urine-N emitted as NH_3-N was 12.6 ± 2.6% and 4.4 ± 0.4% for MU and PU, respectively.

6.3.5 N_2O emissions

Fluxes of N_2O-N are shown in **Fig. 6.3 (b).** There was no noticeable increase of N_2O-N emissions on the peat soil following either water or urine application, with low fluxes throughout the measurement period, ranging from -0.2 to 0.4 mg N m^{-2} d^{-1} for both PC and PU. On the mineral soil, the application of both urine and water led to the apparition of an initial N_2O-N peak within the two first days. The magnitude of the peak was similar between both treatments, reaching 16.1 and 17.0 mg N m^{-2} d^{-1} for MC and MU (equivalent to 161 and 170 g ha^{-1} d^{-1}). From day 12 post application, N_2O fluxes remained low until the end of the experiment for MC. However, for MU, a new increase of N_2O fluxes started at the end of the experiment with N_2O fluxes reaching 2.8 g ha^{-1} d^{-1} at day 21 (equivalent to 29 g ha^{-1} d^{-1}).

Statistical analyses showed significant effect ($p<0.05$) of treatment on cumulative N_2O emissions calculated from day 0 to the end of the experiment (day 21). There was no significant effect

of urine on peat soil. On the mineral soil, total N_2O emissions were significantly higher for MC (80 ± 5 mg N m^{-2}) than MU (40 ± 7 mg N m^{-2}). Total N_2O emissions for the two treatments on mineral soil (MC and MU) were significantly higher than total N_2O for the two treatments on peat soil (PC and PU).

Emission factors N_2O EF calculated at the end of the experiment were - 0,29 ± 0.05% and - 0.01 ± 0,002% for MU and PU, respectively.

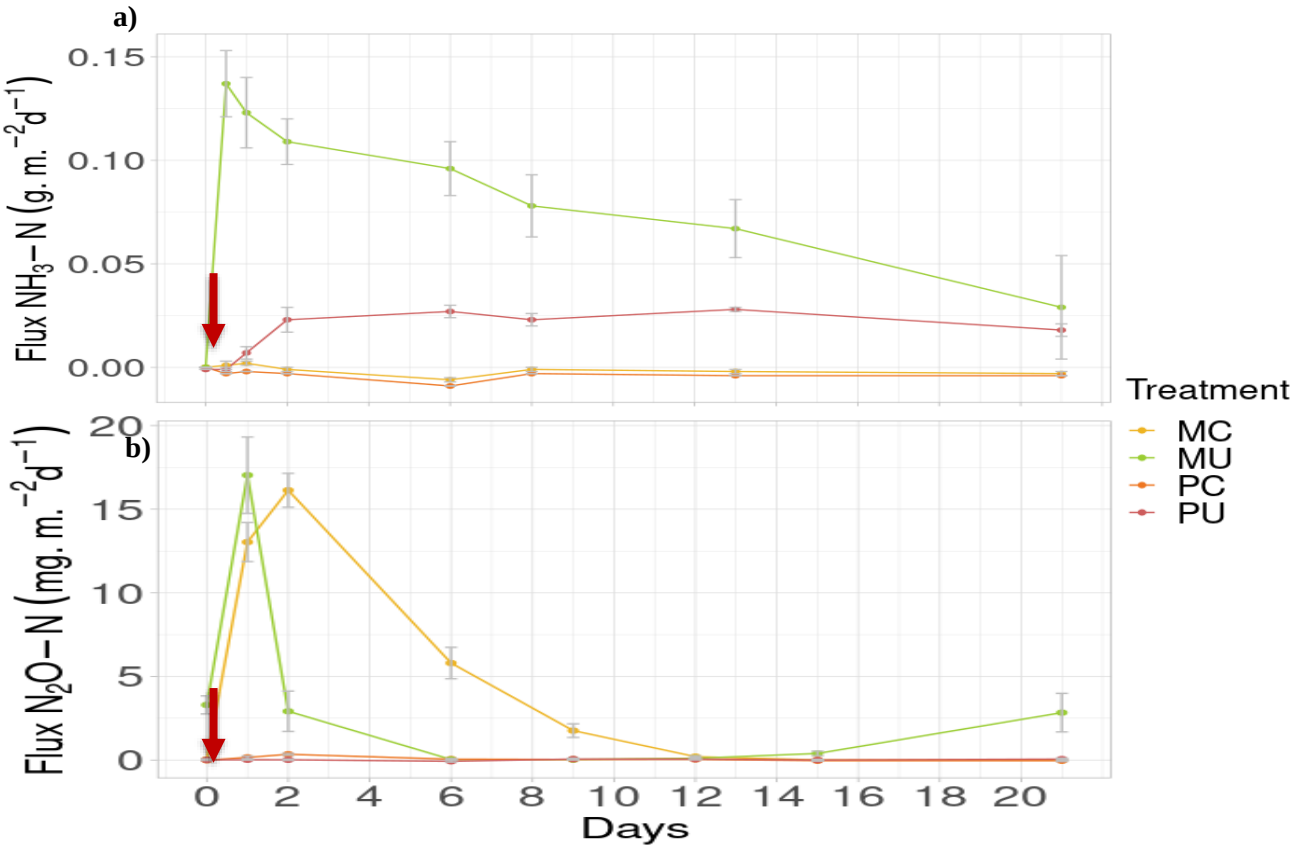


Figure 6.3 Pattern of NH_3-N (a) and N_2O-N (b) following application of artificial sheep urine or water control at day 0. Treatments are water control on mineral soil (MC), artificial urine on mineral soil (MU), water control on peat soil (PC), artificial urine on peat soil (PU). Grey vertical bars represent the SEM (n = 4). The red arrows indicate the timing of amendments application.

6.3.6 Microbial biomass N and C (MBN and MBC)

The MBC and MBN for each treatment at each sampling date are presented in **Fig. 6.4 (a** and **b).** Data of MBC and MBN for day 1 were considered unreliable and were removed from the analysis due to large disparity between replicates, which could have been due to operator error such as recording the wrong soil weights, or a sample mix up.

Values of MBC were significantly ($p<0.05$) higher on the peat soil (PC and PU treatments) than on the mineral soil (MC and MU treatments) for each sampling date. Application of water did not lead to significant change of MBC on both mineral and peat soil, with values averaging 1412 and 7937 mg kg DW^{-1} for MC and PC respectively. Application of urine did not change MBC on mineral soil, with values averaging 1523 mg kg DW^{-1}. However, after urine application on the peat soil, MBC was significantly higher on day 6 compared to background level, with an increase of about 100% compared to background level (i.e 7997 mg kg DW^{-1}). Whereas there was no difference between MC and MU at all sampling date, PU was significantly higher than PC on day 6.

Values of MBN tended to be higher on peat soil compared to mineral soil at each sampling date, even if the difference was not always significant. Application of urine did not lead to significant changes in MBN over time on the two soils, with values averaging 282 and1532 mg kg DW^{-1} for MU and PU respectively. However, application of water control had an effect on MBN. For MC treatment, MBN was significantly higher at day 13 and day 21 respectively, compared to background level (264 mg kg DW^{-1}). For PC treatment, MBN was significantly higher on day 21 compared to other sampling days. A significant effect of treatment was also observed on MBN. Before application of amendment, MBN was higher on peat soil. Afterwards, on day 6, MBN of PU was higher than treatments MC and MU and on day 13, MBN of PU was higher than other treatments. Finally on day 21, MBN of PC was higher than all other treatments.

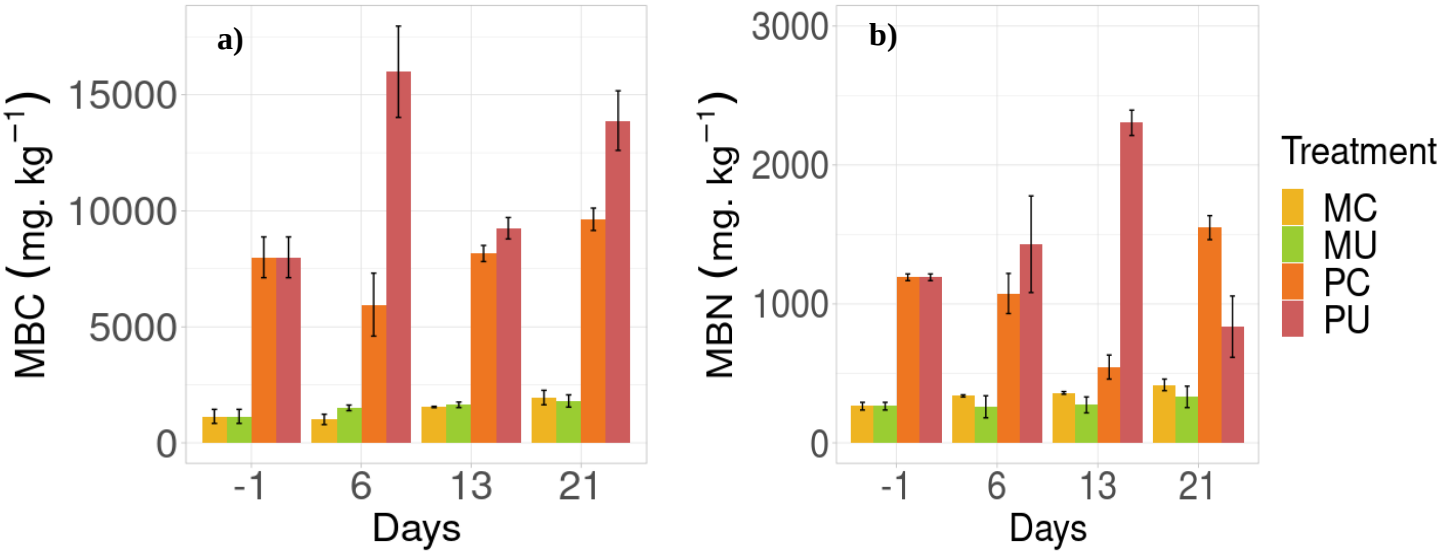


Figure 6.4 Microbial biomass carbon (MBC; a) and nitrogen (MBN; b) following application of artificial sheep urine or water control at day 0. Treatments are water control on mineral soil (MC), artificial urine on mineral soil (MU), water control on peat soil (PC), artificial urine on peat soil (PU). Black vertical bars represent the SEM (n = 4). Units are expressed on a DW soil basis.

6.3.7. Soil N recovery and NO_3^--N leaching

At the end of the incubation experiment (day 22), NH_4^+-N losses were significantly ($p<0.05$) higher in the PU treatment (16.7 ± 3.7 µg cm^{-2})) compared to the MU treatment (1.0 ± 0.1 µg cm^{-2}). There was no significant difference between NH_4^+-N losses of PC and MC (average 1.6 ± 0.8 µg cm^{-2}).

Leached NO_3^- -N losses were similar for both MC and MU treatment (average 74.3 ± 26.8 µg cm^{-2}), and were higher than on peat soil, where NO_3^- -N losses in PU were negligible and significantly lower than losses in PC (average 1.6 ± 0.8 µg cm^{-2}).

This final intense simulated rainfall resulted in 1.20 ± 0.27% and 0.02 ± 0.02% of urinary-N applied leached as NH_4^+-N and NO_3^- -N respectively on the peat soil. On the mineral soil, 0.07 ± 0.01% and 9.87 ± 1.44% of urine-N was leached as NH_4^+-N and NO_3^--N respectively.

At the end of the experiment, TN and TC content had slightly decreased on both soils following water application, compared to background level (i.e. decrease of 0.6% and 1.2% for MC and PC respectively). After urine application, TN had increased by 0.6 and 10.6% on mineral and peat soil

respectively, whereas TC had decreased by 3.3% on mineral soil and increased by 1.6% on peat soil.

6.3.8. Gene abundances

The various gene abundances for each treatment at each sampling day are presented on **Fig. 6.5** (phylogenetic genes), **Fig. 6.6** (nitrification genes) and **Fig. 6.7** (denitrification genes).

Before application, gene abundance was significantly higher ($p<0.05$) on mineral soil compared to peat soil for four of the targeted genes: *bacterial* and *archaeal amoA* (nitrification genes*), nirK* and *nirS* (denitrification genes). The abundance of these genes was significantly higher on mineral soil. However, there was no significant difference of initial abundances of the other genes (*archaeal and bacterial 16S rRNA*, *ITS* and *nosZ I/II)* between the two soils.

Under urine treatment, some gene abundances became higher on peat soil at some sampling days: *nosZ I* at all sampling day except day 13, *archaeal 16S rRNA* at day 1 and 9, *bacterial 16S RNA* at day 13. Only *nirS* gene became higher on mineral soil, at day 9. There were no other differences of gene abundances between soil types under urine treatment.

Under water treatment, some gene abundances became higher on peat soil at some sampling days: *nosZ I* at all sampling days except day 9, *archaeal 16S rRNA* at all sampling days, *bacterial 16S* at day 13 and 21. Other gene abundances became higher on mineral soil: nir*K* at day 1, *nirS* at day 1, day 6 and day 9, *archaeal amoA* at day 1. There were no other differences of gene abundances between soil types under water treatment.

On the peat soil, there was a specific effect of urine only on *archaeal 16S RNA* which became lower under urine treatment compared to water treatment at day 6 only.

On the mineral soil, there was a specific effect of urine on *nirK* gene with lower abundance under urine treatment on day 1.

The application of amendment (water or urine) did not lead to significant change of abundances with time for most of the genes but there were some one-time differences: a higher abundance at day 6 compared to day 13 for bacterial *amoA* and *nirK* for MC treatment, as well as higher *ITS* abundance on day 21 compared to day 6 for MU treatment. Only *nosZ II* gene showed a progressive increase, for PC treatment only.

On **Fig. 6.5, 6.6.** and **6.7.**, it seems that gene abundance of PU treatment at day 21 was the highest for all target genes. However, this tendency was never significant. Indeed, for all genes, PU was never significantly higher than other treatments at day 21, and PU was never significantly higher at day 21 compared to other sampling days.

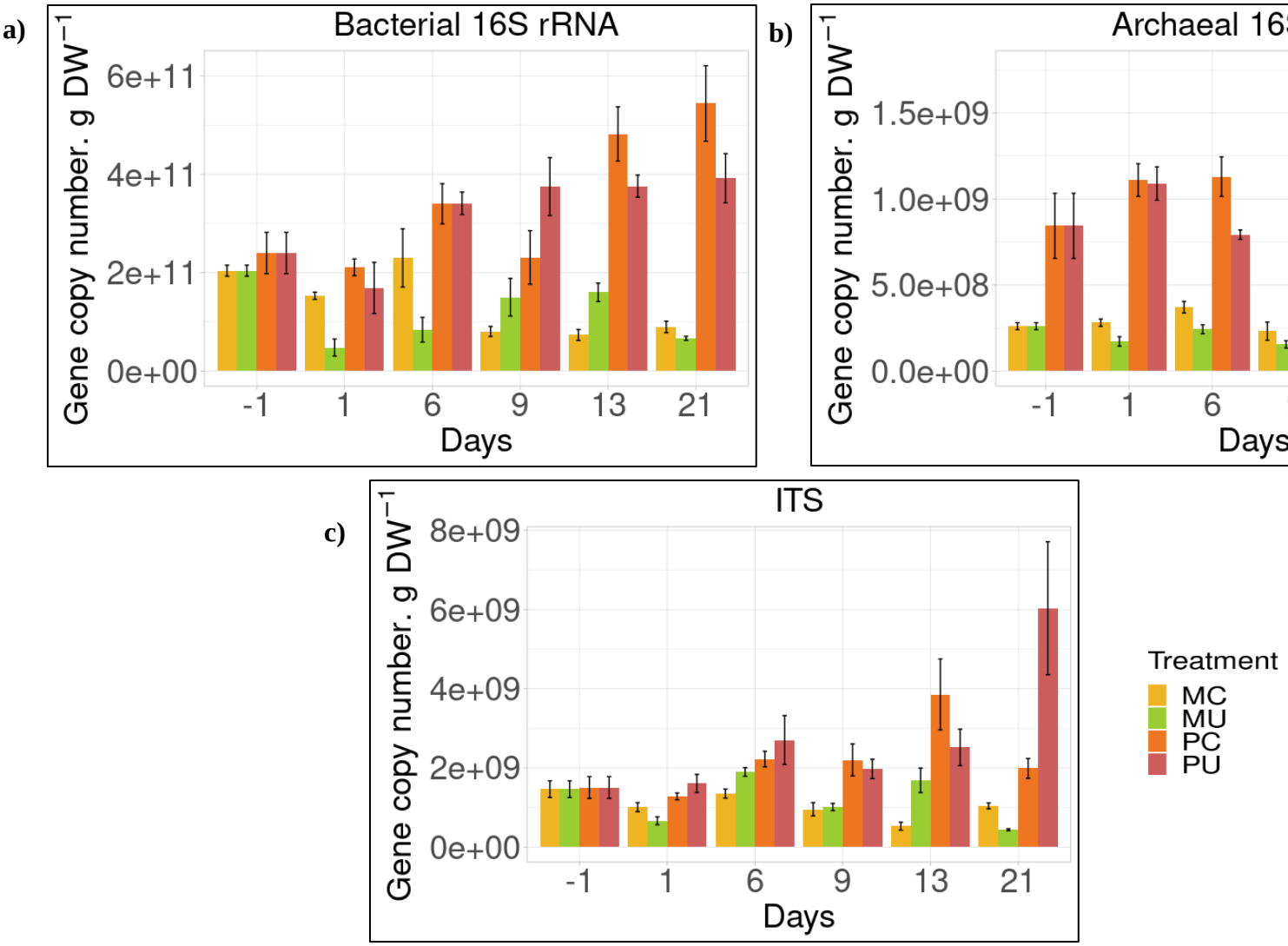


Figure 6.5 Phylogenetic gene abundances of the four treatments at each sampling day. The targeted genes are a) *bacterial 16S RNA*, b) *archaeal 16S RNA* and c*) fungal ITS*. MC = water control on mineral soil, PC = water control on peat soil, MU = urine applied on mineral soil, PU = urine applied on peat soil. Water and urine were applied on day 0. Vertical bars indicate SEM (n = *3*). Note the difference of y-axis scale between graphs. Units are expressed on a DW soil basis.

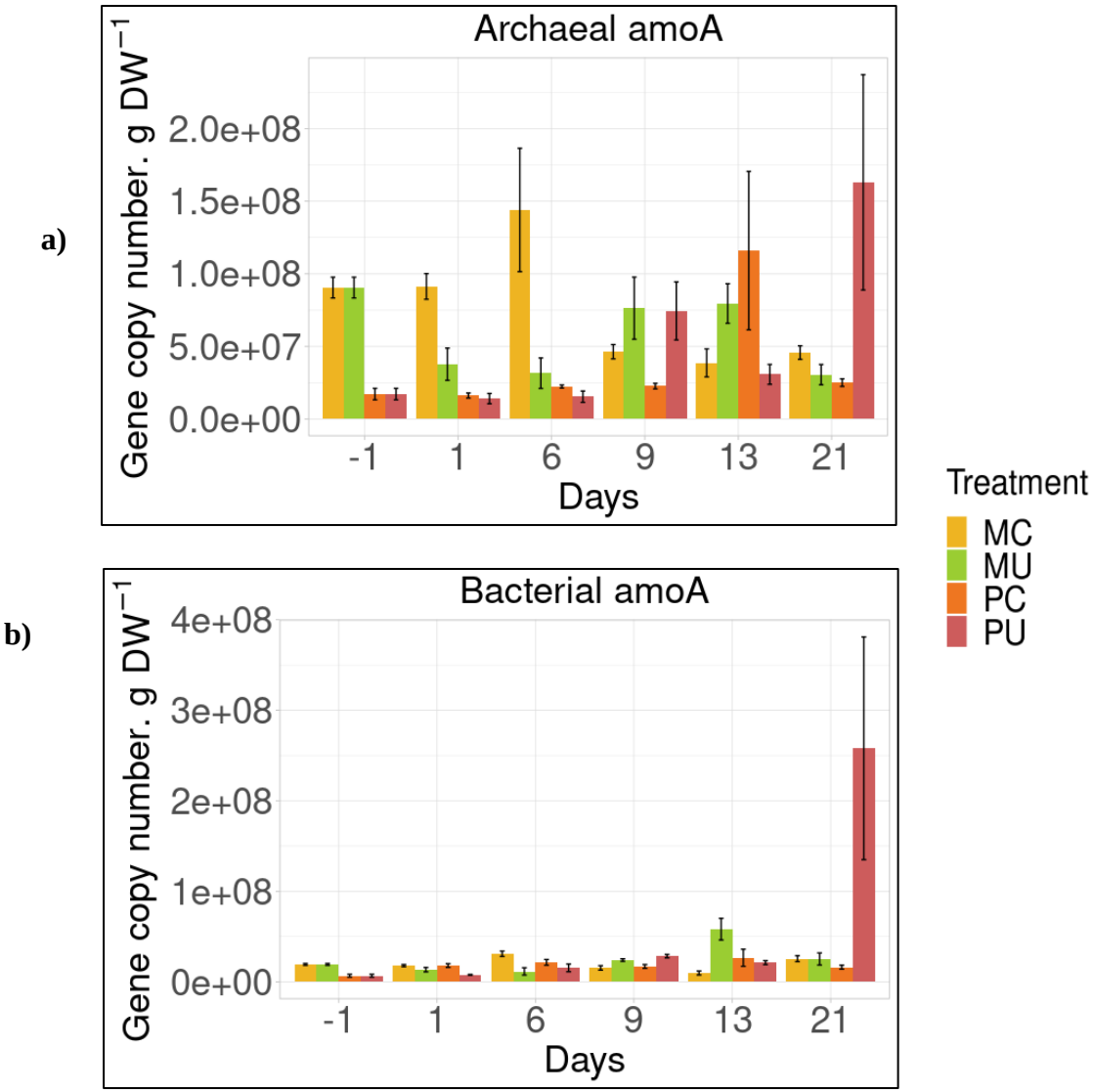


Figure 6.6 Nitrification gene abundances of the four treatments at each sampling day. The targeted genes are a) *bacterial amoA* and b) *archaeal amoA*. MC = water control on mineral soil, PC = water control on peat soil, MU = urine applied on mineral soil, PU = urine applied on peat soil. Water and urine were applied on day 0. Vertical bars indicate SEM (n = *3*). Note the difference of y-axis scale between graphs. Units are expressed on a DW soil basis.

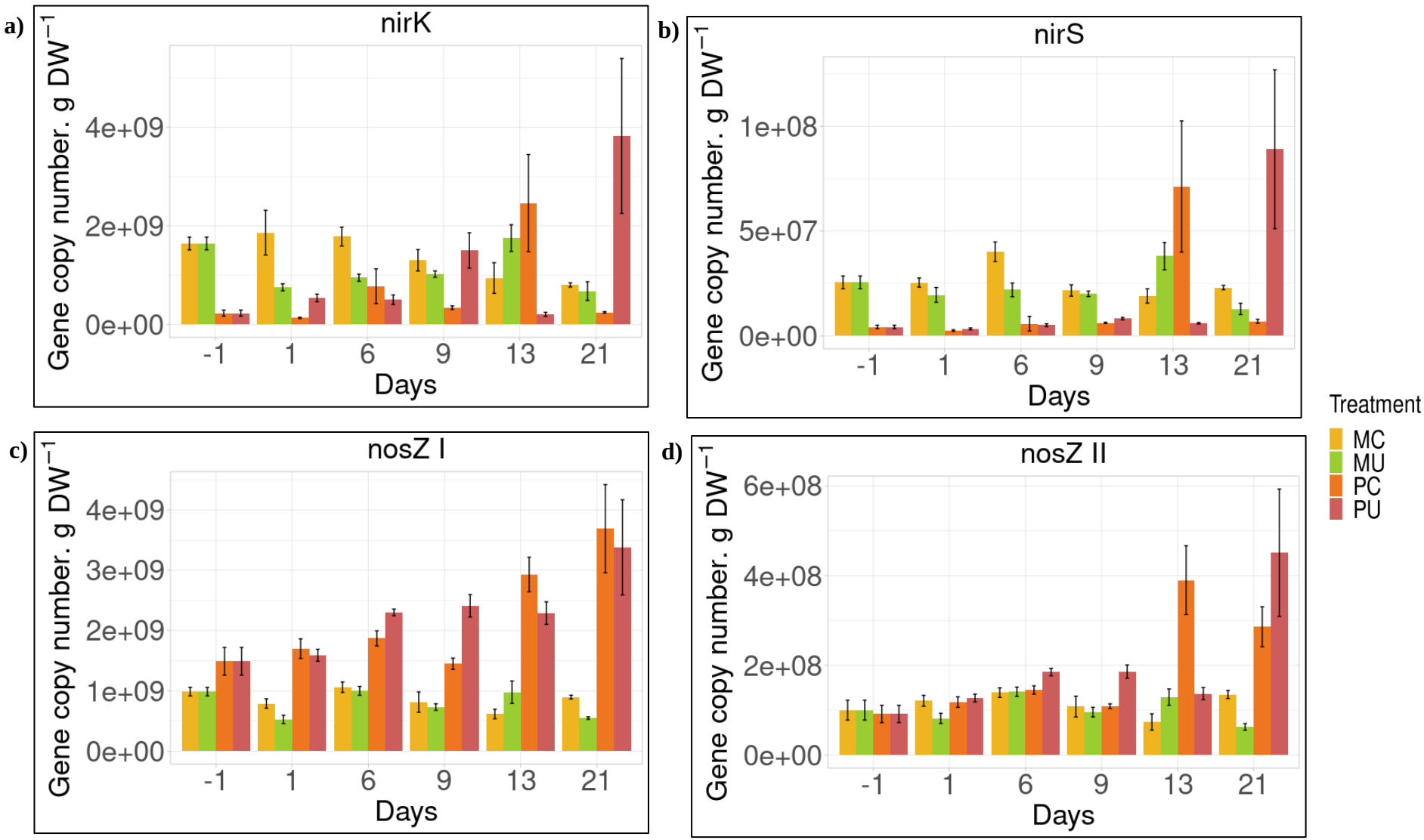


Figure 6.7 Denitrification gene abundances of the four treatments at each sampling day. The targeted genes are a) *nirK*, b) *nirS*, c) *nosZ I* and d) *nosZ II*. MC = water control on mineral soil, PC = water control on peat soil, MU = urine applied on mineral soil, PU = urine applied on peat soil. Water and urine were applied on day 0. Vertical bars indicate SEM (n = *3*). Note the difference of y-axis scale between graphs. Units are expressed on a DW soil basis.

6.3.9 Relationships between N gas emissions, soil variables and gene abundances.

Coefficients (ρ) of significant Spearman's rank correlation ($p<0.05$) between variables are shown in **Table 6.2**. N_2O emissions were significantly correlated with 12 out of the 15 variables analysed, including five gene abundances. The strongest correlation was with soil NO_3^--N ($\rho = 0.51$). NH_3 emissions were significantly correlated with five variables including two gene abundances. The strongest correlation was with soil pH ($\rho = 0.76$).

Table 6.2 Spearman's rank coefficient of significant correlations ($p<0.05$) between gas emissions (N_2O, NH_3) and soil chemical and molecular variables. NS indicates absence of significant correlations between two variables, + indicates a positive correlation and – a negative correlation.

Soil variables	N_2O fluxes	NH_3 fluxes
pH	0.32	0.76
NH_4^+ -N	0.33	0.52
NO_3^- -N	0.51	0.30
DOC	-0.45	NS
MBC	-0.47	NS
MBN	-0.45	NS
GMC	NS	NS
Archaeal amoA	0.27	NS
Bacterial amoA	NS	NS
nirK	0.32	NS
nirS	0.30	0.11
nosZ I	-0.43	NS
nos Z II	NS	NS
ITS	-0.31	NS
Bacterial 16S rRNA	NS	NS
Archaeal 16S rRNA	-0.34	-0.33

6.4 Discussion

6.4.1 Urea hydrolysis

The application of urine led to an increase of pH in both soils, which is generally observed after urine application (Mahmood and Prosser, 2006; Ma *et al.*, 2007; Singh *et al.*, 2009; Curtin *et al.*, 2020). Soil pH on day 13 was similar between MU and PU, and thus the increase after urine application on the peat soil was more pronounced (almost four units) than on mineral soil (about two units). Such increase of pH following urine application is due to urea hydrolysis (Haynes and Williams, 1992; Shand *et al.*, 2000), leading to increased NH_4^+ concentration. However, the increase of pH and NH_4^+-N was slower on the peat soil, which could be due to the enzyme urease being initially inactive in this acid soil. Indeed, optimal pH for urease activity is around 8 (Vlek *et al.*, 1980) and a positive correlation has already been observed between soil pH and urease activity (Fisher *et al.*, 2017). It is possible that the applied urea on peat soil may not have been fully degraded and was instead being immobilised in soil or microbial biomass. Marsh et al. (2005) observed that after 29 days of incubation, only 65% of urea have been hydrolysed on one of the studied soils.

The pH of both urine treatments (MU and PU) remained high until day 14, whereas other studies observed an earlier decrease of pH (Ma *et al.*, 2007; Curtin *et al.*, 2020). The authors associated this decrease with nitrification process and/or ammonia volatilisation. In our study, the delay before the decrease of pH could indicate low nitrification rate or NH_3 volatilisation.

6.4.2 Potential NH_3 volatilisation

There was some NH_3 volatilisation observed following urine application on both mineral and peat soil. Our NH_3 EFs (i.e 12.6 and 4.4% for mineral and peat soil respectively) fitted in the range 4-46% suggested in the review of Saggar *et al.* (2004).

On the mineral soil, NH_3 fluxes increased just after application, which is generally observed in studies (Clough et al., 2003; Singh et al., 2013; Fischer et al., 2016). Indeed, Clough *et al.* (2003) and Singh *et al.* (2013) measured the maximum headspace concentration of NH_3 on the same day of urine application. However, on the peat soil, there was a delay before the apparition of NH_3 volatilisation, which may be related to slower urea hydrolysis and NH_4^+-N increase. The strong positive correlation ($\rho = 0.74$) between NH_3 emissions and pH support this hypothesis.

There was an effect of soil type on NH_3 emissions following urine application, with lower emissions on the peat soil compared to the mineral soil. However, on the peat soil, NH_3 volatilisation was much higher (i.e. up to 0.03 g m^{-2} d^{-1}) compared to Leiber-Sauheitl *et al.* (2015) who observed fluxes lower than 1.2 10^{-7} g m^{-2} d^{-1} following sheep urine application on a peat soil. This huge difference probably pertains to the daily irrigation of soil cores in Leiber-Sauheitl *et al.* (2015), since irrigation is known to suppress NH_3 volatilisation (Cameron et al., 2013).

On the mineral soil, NH_3 emissions were lower compared to other studies. After artificial urine application at 100 kg N ha^{-1}, Clough *et al.* (2003) measured NH_3 emissions reaching 264 g m^{-2} d^{-1}, which is more than a thousand times higher than the peak of NH_3 flux measured on the mineral soil in our study. Possible causes of the lower emissions in our study may include the lower temperature and higher soil moisture content used in our incubation. Under soil saturated conditions, Singh *et al.* (2013) measured a maximum NH_3 flux of 0.99 g m^{-2} day^{-1}, which was seven times higher than the peak from MU in our study. During the first 14 days of incubation, cumulative NH_3 in Singh *et al.* (2013) were 2.5 times higher than cumulative NH_3 from MU in our study, however experimental conditions were different, with higher N loading rate (476 kg ha^{-1}) and higher temperature (20°C). Temperature is indeed an important driver of NH_3 emissions (Freney *et al.*, 1983). These observations highlight the variability of NH_3 emissions which are influenced by several factors among them soil characteristics and environmental conditions (Bolan *et al.*, 2004).

Finally, the difference in NH_3 volatilisation between these studies could also be related to the methodology used: the photoacoustic measurement method in this experiment was based on a short gas sampling time scale (i.e. 5 min per replicate at each sampling event), whereas Singh *et al.* (2013) used a system of acid traps associated with a continuous flushing for several hours to collect all the ammonia produced. The measurement period was also limited in Clough *et al.* (2003), but the authors used another method of measurement based on mass spectrometry. A previous study has already shown lower values of N_2O fluxes obtained with photoacoustic compared to mass spectrometry (Klein *et al.*, 1999). Therefore, our method is more likely to underestimate NH_3 emissions compared to these other methods.

6.4.3 Nitrification

The increase of soil NO_3^--N concentration at the end of the experiment following urine application on mineral soil indicated the possible occurrence of nitrification process. The delay of nitrification could be due to a lag phase, which is a period of activation of dormant nitrifiers and is linked to ammonia oxidisers community structure (Webster *et al.*, 2005).

On the peat soil, no sign of nitrification was observed during the full experiment. Indeed, NH_4^+-N concentration remained high until the end of the experiment and there was no noticeable build-up of NO_3^--N. The absence of nitrification after urine application on an acid organic soil has previously been observed in the field study of Marsden *et al.*, (2019). The authors suggested that a small or functionally inactive population of nitrifiers may have been responsible for that. The pH after urine application on the peat soil increased a lot, reaching approximately 7.5 units, whereas the soil pH under urine patch in Marsden et al. (2019) did not increase beyond 5.5 units. Therefore, the increase of soil pH may have activated the nitrifiers community that were possibly in a lag phase in the timeframe of the experiment. It would have been interesting to see if any increase of NO_3^--N would have occurred beyond 21 days of incubation.

The application of water and urine did not lead to any significant change in ammonia oxidisers community size on both mineral and peat soils. There was no specific effect of urine, although we would have expected some increase of at least AOB community with the increase of pH (Zhang *et al.*, 2017). No change in AOA/AOB abundance was also found in the study of Ma *et al.* (2021), whereas Di *et al.* (2014) observed an increase of AOB and a decrease of AOA following urine application.

Moreover, *bacterial* and *archaeal amoA* gene abundances were not significantly different between the two soils under urine treatment and therefore nitrifiers community size cannot explain the difference of nitrification activity. The low nitrification activity in the peat soil is more likely to be linked to the initial functional inactivity of ammonia oxidisers. The use of RNA-based PCR would be a better indicator of active ammonia oxidising microbial communities compared to DNA-based PCR which is a measurement of both active, dormant and even dead populations (Mahmood and Prosser, 2006).

6.4.4 N_2O emissions

On mineral soil, a peak of N_2O appeared just after application of water or urine, and was of similar amplitude between both treatments (i.e. MC and MU). This initial peak may be due to the effect of soil wetting chasing out the N_2O that would be already present in soil pores or be related by a stimulation of denitrifiers through the increase of WFPS (Sextone et al., 1985). On the mineral soil, a second peak of N_2O emissions started to appear at the end of the experiment. Other studies have observed a second peak after urine application, but its timing of apparition is variable throughout studies. Zaman *et al.* (2007) observed a second peak around day 13 post application whereas in Clough *et al.* (2003), the timing and the magnitude of the second peak varied with urine application rate. For an N loading rate of 100 kg ha^{-1}, which is similar to our study, the authors observed the second peak around day 15 after application. These different patterns of emissions between studies highlight once again the variability of N_2O emissions, which is influenced by a high number of factors.

In our study, it is not clear which was the dominant pathway responsible for the appearance of the second peak on the mineral soil, as it occurred quite simultaneously with the increase of NO_3^--N at the end of the experiment. Different processes could have been responsible for the second peak including nitrification of urine-derived NH_4^+-N, denitrification of urine derived NO_3^--N and nitrification/denitrification of another pool of urine N (e.g. purine derivatives), as suggested in Chadwick et al. (2018). An increase of either nitrification or denitrification gene abundances before the appearance of the second peak could have indicated which pathway was involved, but no significant increase of nitrification and denitrification gene abundances for MU treatment.

At the end of the experiment, total N_2O from MU were lower compared to MC, leading to a negative EF, which is not a common observation in studies. Such results suggest that by the end of the experiment, only a very low proportion of urine-N had been denitrified, probably due to the delay of nitrification. Total N_2O emissions from urine would probably have been much higher if calculated several days later, after the appearance of the second peak for the MU treatment.

On the peat soil, there was no noticeable peak of N_2O following urine application, which is in line with the low nitrification activity observed on this soil. Similarly to the N_2O emissions measured during the previous field study on the same soils (Mancia *et al.*, 2022a), we once again observed some negative fluxes on the peat soil, which is not uncommon after sheep or even cattle excreta application (van der Weerden et al., 2011; Mori and Hojito, 2015; de Bastos et al., 2020).

In our study, urine application did not cause changes in either nitrification or denitrification gene abundances. In comparison, Di *et al.* (2014) observed an increase of bacterial *amoA, nirK and nosZ I* and *II* and a decrease of archaeal *amoA*, while Ma *et al.* (2021) observed an increase of bacterial *amoA* gene only. This inconsistent effect of urine on gene abundances could be attributed to the composition, diversity and functionality of the microbial communities possessing these genes (Singh *et al.*, 2009). For instance, urine could affect differently the growth of two distinct species of nitrifiers, which both possess *amoA* gene, and therefore the effect of urine on nitrification in one given such will depend on the proportion of these two species in the microbial community.

Without any effect of urine on nitrification/denitrification genes, we found a correlation between these genes (except *nosZ II*) and N_2O emissions. Here, N_2O was correlated with many variables, but these correlations reflected the differences of composition between the two soil types, associated with distinct N_2O patterns. Indeed, variables which tended to be high on the peat soil (i.e. with very low N_2O) were negatively correlated with N_2O (e.g. NH_4^+ -N, DOC, *16S Arch*), whereas variables with lower values on the peat soil (e.g. NO_3^-, *nirS*) were positively correlated with N_2O. Simply put, these relationships between soil variables and N_2O emissions are not necessarily causations. In our situation where we compare two soil types, N_2O seems to be predicted mostly by background soil properties, since application of treatments tended to suppress differences in several variables (i.e. pH, gene abundances).

It is likely that some NO emissions occurred from the mineral soil, as NO is produced by the same pathways as N_2O (i.e. nitrification/denitrification). Given the high WFPS, N_2 may also have been produced on this soil. Measurements of NO and N_2 were not performed during this experiment but the previous incubation under similar conditions (**chapter 5**) showed noticeable emissions of NO and N_2 on the mineral soil amended with artificial sheep urine. On the contrary, NO and N_2 emissions are unlikely to have been produced on the peat soil given the absence of nitrification.

6.4.5 Microbial biomass

The comparison of microbial biomass N and C values between studies must be assessed with caution because of the use of different correction factors. Therefore, it is more accurate to compare the effect of treatment and the evolution of MBC/MBN rather than the absolute values. However, we have noticed that on our mineral soil, the range of values of MBN and MBC were consistent

with the results observed on a clay loam soil in a study using very similar correction factors (i.e. 0.35 and 0.48 for MBC and MBN respectively), (Lovell and Jarvis, 1996).

On the peat soil, the range of values of MBC and MBN were much higher than on the mineral soil. This difference must be related to soil type which is an important factor controlling soil microbial biomass and C turnover (Carter *et al.*, 1999). A significant relationship between soil organic content and MBC has also been observed by Inubushi *et al.* (1996).

In our study, urine had no significant effect on MBN on the two soils, whereas water did increase MBN at the end of the experiment. These results could be due to a stimulation of microbial growth by watering and therefore an increase of N immobilisation from native soil N following water application. Indeed, increased soil moisture can multiply the numbers of anaerobic microsites allowing for anaerobic microorganisms to grow. This increase of MBN following water application was not associated with an increase of MBC, probably because of C loss pertaining to an intensification of microbial respiration, supported by the decrease of DOC following water application on both soils. As with water, urine application is also likely to have led to an increase in microbial biomass, particularly with the increase of pH on the peat soil which would have stimulated microbial growth (Aciego Pietri and Brookes, 2008). However, urine had no effect on MBN on the two soils. Instead, urine may have led to a functional activation of the microbial population involved in the N cycle, rather than to a microbial growth. As suggested in Lovell and Jarvis (1996), urine can lead to significant changes in microbial biomass structure and activity without being accompanied by any change in size.

6.4.6 Leaching

Clough *et al.* (1996) found that leaching following urine applied to a peat soil was only in the form of NH_4^+-N, which is similar to our findings. This observation is not surprising since there was no sign of nitrification, and the NH_4^+-N level on peat soil remained high until the end of the experiment, when the leaching test occurred. On mineral soil, leaching of NO_3^--N was not higher after urine application compared to water. The reason for that is probably the rapid uptake of urine-derived NO_3^--N by denitrifiers.

6.4.7 Fate of urine N

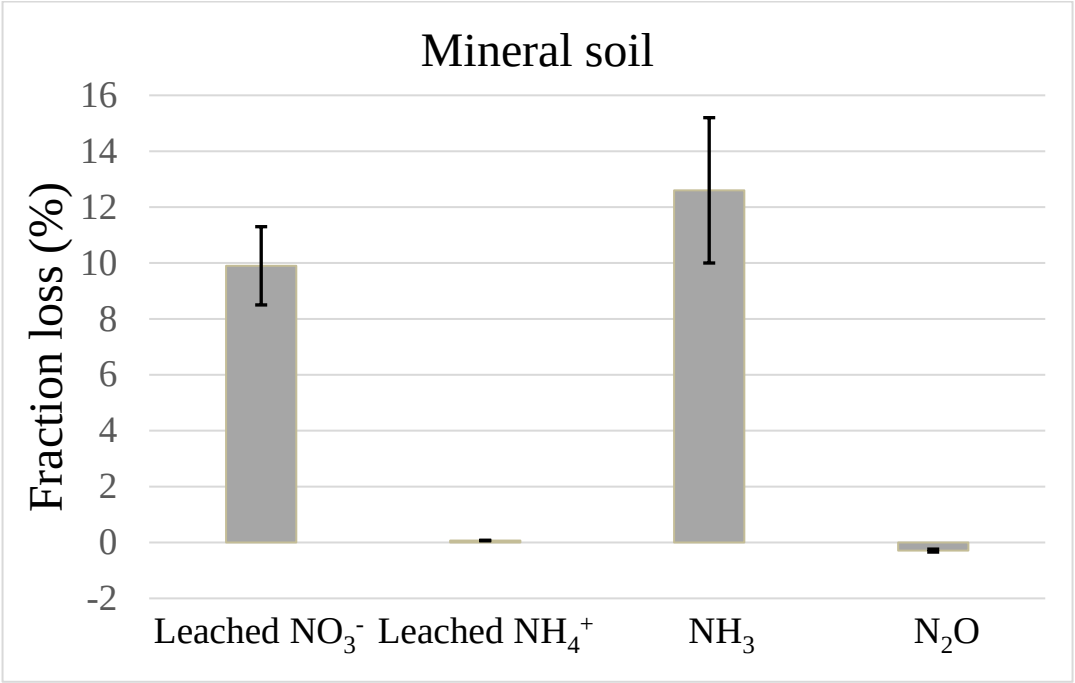
As highlighted on **Fig. 6.8**, the fate of urine N was very different between the two soils, which validate our hypothesis, A more complete urine N balance would include N uptake by plants; measurements of NO and N_2 and leaching of dissolved organic N.

6.5 Conclusions

This study showed different fates of urine N on mineral and acid peat soils. As hypothesised, total N losses (NH_3 and N_2O emissions, mineral N leaching) measured in this study were higher on mineral soil, where NH_3 volatilisation was the main N loss pathway. On this soil, N_2O emissions were low as a result of a delay in the nitrification process, probably due to a lag phase of nitrifiers microbial community. On the acid peat soil, urea hydrolysis also appeared to be slow. However, an important increase of pH following urine application allowed for some NH_3 volatilisation to occur on this acid soil. There was no indication of nitrification on the peat soil, and consequently, negligible N_2O emissions were observed and NH_4^+ was the only form of mineral N being leached. Contrary to our hypothesis, there was no significant increase of N immobilisation in the microbial biomass on the peat soil.

Despite an increase in N_2O emissions following urine application on the mineral soil, we did not observe any specific effect of urine on nitrification/denitrification gene abundances. The difference in N_2O emissions between the two soils could not be explained by variation in microbial communities' size since the application of urine tended to suppress the initial difference in nitrification/denitrification gene abundances. Urine is more likely to have an effect on microbial functional activity and we suggest that RNA-based molecular methods (such as RNA-Seq or metatranscriptomic) could be a better indicator of the effect of urine on soil processes in the two distinct pasture soil

a)



b)

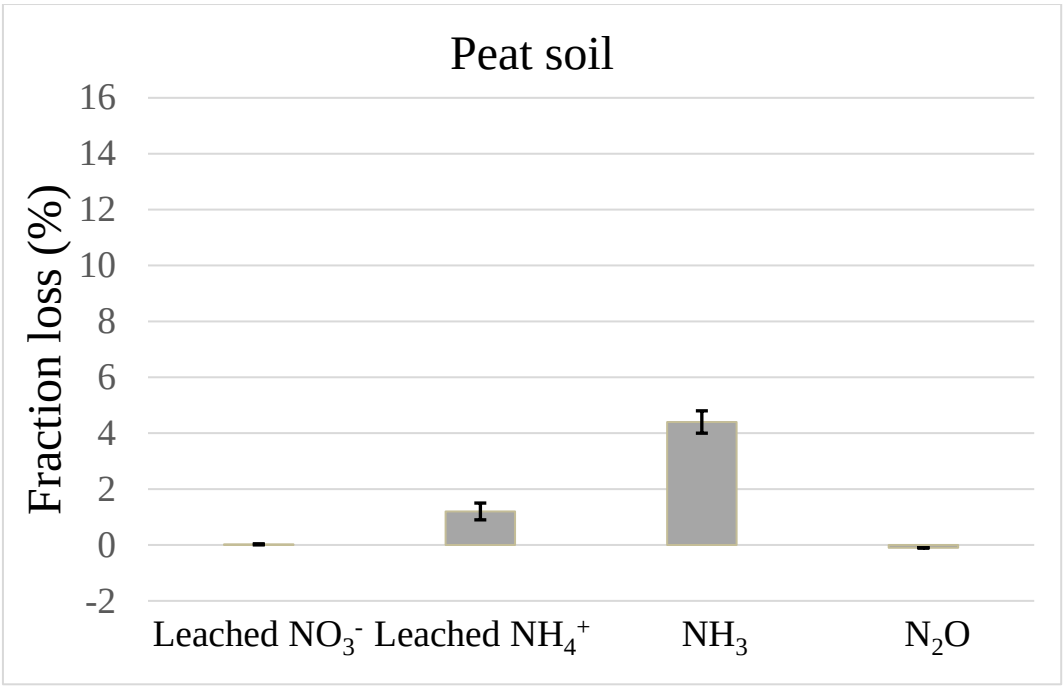


Figure 6.8 Fate of urine-N on the mineral (a) and peat soil (b) after 22 days of incubation. The bar plots show the percentage of urine-N lost through the four N loss pathways measured in this experiment.

6.6 Acknowledgments

The authors would like to acknowledge the laboratory and field staff at Teagasc research centre of Johnstown Castle, Co. Wexford for their assistance on this project. This study was financially supported by the Teagasc Walsh Scholarship Scheme (project code 2018028).

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Chapter 7

General discussion and conclusions

7.1 Project overview and main results

The first objective of this PhD thesis was to generate data towards establishing a country-specific (Tier 2) EF_{3PRP} associated with sheep excreta deposition in pasture, range and paddock (PRP). This research was a logical follow-up of a previous study establishing a Tier 2 EF_{3PRP} for cattle excreta in Ireland (Krol et al., 2016). Estimations of N_2O emissions from ruminant excretal returns were then refined with the adoption of the new Tier 2 EF_{3PRP} for cattle (i.e. 0.86% for combined excreta), while 2006 IPCC Tier 1 EF_{3PRP} (i.e. 1%) was still used for sheep, which is higher than the Tier 2 value for cattle. However, it is now widely recognised that N_2O emissions from cattle excreta are generally higher than for sheep (López-Aizpún et al., 2020), which has led to the need to develop a country-specific EF_{3PRP} for sheep.

In order to establish a Tier 2 EF_{3PRP} for sheep, an experimental design was established to assess key factors that may influence N_2O emissions and to determine if any disaggregation of EF would be required for more accurate estimations. Factors considered were the type of grassland (improved lowland grassland and extensively grazed upland grassland), the season of excreta deposition (early, mid and late season grazing) and the type of excreta deposited (urine and dung). The two grasslands chosen for this study were representative of two main typical sheep grazing systems in Ireland and were characterised by a mineral soil and an acid peat soil, respectively. Our results showed lower N_2O emissions from the urine deposited on the upland grassland, although EF_{3PRP}s were very low on both grasslands. From these observations, hypotheses were raised about the causes of low emissions on these grasslands. Possible causes were related to climatic conditions, excreta characteristics, vegetation cover and soil properties.

The second objective was then to decipher if soil inherent properties could explain differences of N_2O emissions observed *in situ* between the two grasslands, as well as the low N_2O emissions from these pastures. For that, an incubation experiment was established under control conditions (i.e. anaerobic conditions) and in the absence of plants to reduce the number of factors which may interfere in the control of N_2O emissions. During this incubation, N_2O and other N gas emissions (NO, N_2) were measured. Results showed no noticeable emissions on the peat soil, whereas N_2O emissions on the mineral soil were associated with NO and N_2 emissions.

Based on these results, the third objective was to investigate the fate of urine N in these pasture soils, with an additional incubation experiment to assess other N transformation pathways and processes, including NH_3 volatilisation, NO_3^--N leaching and N immobilisation. As expected, we observed different urine N fate between the two soils.

Finally, the fourth specific objective was to assess the effect of sheep urine and soil type on nitrifiers/denitrifiers community population size through analysis of targeted gene abundances. We did not observe any specific effect of urine on nitrification/denitrification gene abundances on the two soils and microbial communities' size could not explain differences in N gas emissions between the two soils.

7.2 Causes of low N_2O emissions on these pasture soils

On the peat soil, the application of urine was not followed by any accumulation of soil NO_3^- and the level of NH_4^+ remained high during the whole incubation experiment, indicating an absence of nitrification in this acid soil. A similar result was observed in the field (**chapter 4**) and by Marsden et al. (2019) following urine deposition to acid upland peat soil. This low nitrification may also be related to the high soil moisture content during both the field and laboratory studies. Indeed, nitrification activity is reduced in low oxygen conditions (Sahrawat, 2008) and the WFPS measured in the peat soil was rarely <75% during these experiments.

Nitrification in acid soils is possible and is suggested to be undertaken by AOA micro-organisms rather than AOB (Lu & Xu, 2014; Li et al., 2018). However, it has been shown that AOA growth can be inhibited by urine application (Di et al., 2014), which would thus prevent nitrification activity in this soil. Moreover, nitrification rates are controlled by the presence of local inhibitors (de Boer & Kowalchuk, 2001). In this upland peat soil, covered by a diversity of plant species, another possible explanation of low nitrification activity could be the presence of biological nitrification inhibitors, released by one or more plant species (de Klein et al., 2022). To our knowledge, none of the species present in this grassland such as the deergrass *Trichophorum cespitosum*, the heather *Erica Tetralix*, *Potentilla anserina*, common cottongrass *Eriophorum angustifolium*, have been studied for potential nitrification inhibition property. However, species of the genus *Potentilla* are rich in tannins (Tomczyk and Latté, 2009). These compounds have already been shown to have an inhibiting effect on nitrification (Baldwin et al., 1983; Adamczyk et al., 2013), although the results are inconsistent throughout studies (Kanerva and Smolander, 2008). Further research is required

on the role and mechanisms of tannins on N cycling, which may depend on the nature of tannins and on soil type (Siniscalchi et al., 2022).

On the mineral soil, for both field and incubation studies, application of urine led to an increase of N losses compared to control treatments (i.e. nothing or water applied). After application of urine *in situ*, a peak of N_2O occurred just after urine application. This initial peak also appeared during the incubation experiments. During the incubation experiments, a second peak appeared (or started to appear) on the second or third week post application. As previously suggested in **chapters 5** and **6,** the initial peak may be due to the effect of wetting on denitrification of residual soil NO_3^- under more anaerobic conditions (Sextone et al., 1985), whereas the second peak is more likely due to the nitrification/denitrification of urine-N compounds (Chadwick et al., 2018). Contrary to the incubation experiments, no second peak appeared after urine application. Therefore, it is possible that the peak observed after each application corresponded to the initial peak due to the effect of soil wetting on microorganisms. If it was the case, it would mean that there was no noticeable peak of N_2O from nitrification/denitrification of urine compounds during the field experiments. The pattern of the initial peak observed just after application supports this hypothesis, since in other field studies N_2O fluxes following sheep urine application are generally increased for a longer period of time (Tomazi et al., 2015; Hoogendoorn et al., 2016; Marsden et al., 2017; Marsden et al., 2018).

From the results of the last incubation experiment, we suggested a delay of nitrification in the mineral soil, which could also have occurred during the field experiment. Unfortunately, soil mineral N data from the first incubation experiment was not available due to loss of samples, and therefore we cannot support our hypothesis based on the mineral N pattern of this experiment. After spring and autumn applications of urine and dung in the field study, nitrification is likely to have been inhibited by the high soil moisture content. From these observations, the mechanism responsible for N_2O emissions during the incubation experiments could be nitrifier-denitrification, effective in low oxygen conditions (Wrage-Mönnig et al., 2018).

7.3 Accuracy of our estimations

We observed low EF_{3PRP}s associated with sheep urine deposition on the two grassland soils, and an effect of grassland type on N_2O emissions. Incubation experiments validated that soil inherent

properties were responsible for the difference of N_2O emissions between lowland and upland grasslands. Incubation experiments also validated that there was no N_2O emitted from the peat soil under high soil moisture conditions. Even if N_2O emissions would have increased in this soil under lower moisture content, this grassland is in a very wet region of Ireland, under high soil moisture content conditions most of the year, indicating that N_2O production and emissions would be negligible for most of the year.

On the mineral soil, incubation experiments showed the potential for this soil to produce significant N_2O under high soil moisture conditions. However, the combination of low sheep urine N loading rate with plant N uptake and other N losses led to low EF *in situ*. The weather conditions during the field experiments were not extreme with average monthly temperatures and monthly total rainfall being similar to the long-term averages of the sites, indicating that N_2O emissions from this site were not measured under atypical circumstances.

Other countries have established Tier 2 N_2O EF_{3PRP} from sheep excreta. As described in **chapter 3**, New Zealand have undertaken the most substantial work to establish its Tier 2 EF_{3PRP} for sheep. These values, disaggregated by slope class and excreta type, are based on a meta-analysis of many field studies (van der Weerden et al., 2020). The calculated EF_{3PRP} for combined excreta were 0.37 and 0.09% for flat/low slope and medium/high slope respectively. The EF_{3PRP} estimated on the two grasslands in our study were thus closer to the New Zealand value on medium/high slope. However, our low values cannot be explained by the effect of slope on N_2O emissions since our two experimental grasslands were on flat areas. Nevertheless, this highlights that EF_{3PRP} from sheep excreta can be very low under specific circumstances. Other studies have observed low EF_{3PRP} from sheep excreta (Hoogendoorn et al., 2016; Marsden et al., 2018; Marsden et al., 2019) and even negative values like in our study (Luo et al., 2013). However, we did not observe any effect of excreta type on N_2O emissions, although higher emissions from sheep urine than sheep dung are generally observed (Kelliher et al., 2014; Luo et al., 2015; Cai and Akimaya, 2016).

Ireland is a country where the sheep sector is an important agricultural activity, and therefore should aim to use the most accurate estimates of N_2O as possible. Before implementing Tier 2 EF_{3PRP} for sheep excreta in the national GHG inventory of Ireland, it appears essential to estimate EF_{3PRP} on other experimental grasslands, with less extreme soil conditions and less productive grassland. Moreover, during the field experiments, even if the N_2O chamber sampling intensity was high during the two first weeks post application, a higher temporal resolution of N_2O chamber

sampling, e.g. through the use of automated chamber measurement, would capture the temporal variability of N_2O emissions more fully (Grace et al., 2020), although the use of automated chambers would limit replication and/or numbers of treatments that could be accommodated. Moreover, N_2O fluxes are also spatially variable, especially after excreta application. This variability of N_2O fluxes is common and its consideration can be improved for instance by increasing the number of replicates across the experimental sites (Charteris et al., 2020).

7.4 Implications of the results

From this project, two articles have already been published. The first one (**chapter 3**; Mancia et al., 2022) is a critical review paper about the uncertainties related to the various approaches adopted worldwide in the reporting of N_2O emissions from ruminant excretal returns. This study showed that efforts can be undertaken in the methodologies of reporting in order to improve the accuracy of estimations in national GHG inventories.

The second article is based on the field study and therefore provides values of EF_{3PRP} associated with sheep excreta deposition in temperate grasslands. However, using negligible EF_{3PRP} in the calculation of emissions would mean a nil contribution of sheep excretal return to N_2O emissions and clearly more experimental values established on other different grasslands are still required to validate these findings. We expect that our EF_{3PRP} could be used for the derivation of a Tier 2 EF_{3PRP} when collated with other similar studies

For the calculation of N_2O emission from sheep excreta in the national inventory, Ireland is still using the 2006 IPCC Tier 1 EF_{3PRP} (i.e. 1%), which is higher than the Tier 2 EF_{3PRP} used for cattle excreta and therefore not consistent. A first step toward more accuracy in the estimations would be for Ireland to adopt the new 2019 IPCC Tier 1 EF_{3PRP} of 0.3% for combined sheep excreta, or at least adopt the same value that is used for cattle.

Even if our EF_{3PRP}s are not used to derive a Tier 2 EF_{3PRP}, they can still be useful for modelling studies or meta-analyses aiming to decipher the drivers of N_2O emissions. Our studies indicated for instance an effect of soil type on N_2O emissions from the sheep urine patch. This effect is likely to be mostly due to the difference in soil pH, which has been shown to be an important driver of EF_{3PRP} in the meta-analysis of López-Aizpún et al. (2020). Soil pH thus appears to be an important input data to consider when modelling N_2O emissions in grasslands.

Data obtained from our three experiments are mainly quantifications of various N losses from sheep urine patch deposited on pasture soils. This type of data can be used in the calculation of N balance studies in order to compare N losses between grazing systems at a bigger scale such as the paddock or the farm system scales, similar to the life cycle assessment study undertaken by O'Brien et al. (2016) on Irish sheep farming systems.

Our results are also important for the agricultural sector in Ireland because they indicate that there is no urgent need to implement mitigation strategies for the abatement of N_2O emissions in the sheep grazing sector. However, low N_2O emissions does not mean low total N losses. We have indeed seen that on the mineral soil, other N losses occurred, including NO_3^--N leaching, NH_3 volatilisation and NO emissions, and these losses have negative impacts on ecosystems, as detailed in **chapter 2**. The sheep sector is also associated with another major GHG, i.e. enteric CH_4, although in Ireland the contribution of sheep to agricultural CH_4 emissions have been estimated at about 5%, compared to >80% for cattle enteric fermentation (Duffy et al. 2022). However, here again estimations for sheep are based on Tier 1 methodology and are thus less accurate than for cattle. Moreover, as for N_2O from excreta patch, it is likely than there are significant differences of CH_4 emissions between lowland and hill farming systems (Fraser et al., 2015).

7.5 Recommendations for future research

We suggested earlier that our low N_2O EF_{3PRP} developed on two grasslands should be validated by making measurements from multiple grassland experiments. While developing EF_{3PRP}, it is important to report a maximum of parameters that characterise each grassland. Such data can then be used for further meta-analysis to assess the drivers of N_2O emission from ruminant excreta patches such as the studies of López-Aizpún et al. (2020) and van der Weerden et al. (2021). Moreover, these additional sheep-grazed pastures could be subject to analyses for the assessment of other N losses and the fate of urine N. Such analysis can be performed on incubated soil similar to the incubation experiments of this PhD but can also be carried out in the field with analyses facilitated by the use of ^{15}N labelled urine. The data from such experiment could thus be implemented into modeling looking to assess the effect of soil characteristics on the various N pathways. Finally, assessment of N fluxes in grazed pastures could also be done on a paddock scale, by scaling up the analysis of urine N fate. In such studies, field scale N_2O fluxes could be measured by Eddy covariance technique, similar to the recent study of Murphy et al. (2022).

There are many possibilities to improve the last incubation experiment and have a better understanding of the fate of urine N in these pasture soils. Firstly, a longer period of incubation would have allowed the observation of the potential second peak of N_2O on the mineral soil and the changes of the various measured variables on a longer timescale. For instance, changes in microbial biomass can occur on longer time frames as observed in Wachendorf and Joergensen (2011). An experimental design including more treatments could be implemented to help understand the factors influencing urine N fate in these soils. Soils cores could be for instance established under lower moisture conditions, to determine if soil anaerobicity is involved in the absence of nitrification on the peat soil. Incubation could also include intact soil cores with the pasture plant cover in order to have a better representation of the fate of urine N and thus more accurate estimations of the N losses. Moreover, real urine could be used instead of artificial urine in the studies of N dynamics since artificial urine is not fully representative of real ones and can lead to differences, e.g. in microbial respiration (Lambie et al., 2013).

The partitioning between the sources (i.e. urine N or native soil N) of the various N losses could be determined by using ^{15}N-labeled urine, both for field and incubation experiments, similar to the studies of Di et al. (2002), Wachendorf et al. (2008) and Dixon et al. (2010). The use of ^{15}N labelled urine could also be used with tracing models such as the study of Rex et al. (2021) which have already contributed to the understanding of N transformations dynamics under ruminant urine patches.

Finally, during the last incubation experiment we only assessed gene abundances through DNA extraction and qPCR. However, the assessment of gene transcripts could give new insights into the effect of soil type and urine application on the expression of both nitrification and denitrification genes, such as the study of Di et al. (2010). The authors observed an increase of bacterial *amoA* gene transcription following urine application whereas archaeal *amoA* decreased. To our knowledge, most of the studies have quantified only genes abundances because RNA abundance is more difficult to assess due to its instability. Nevertheless, it is increasingly suggested than gene expression would represent a better indicator of microbial activity (Mahmood and Prosser, 2006). Another molecular tool that could be adopted is metagenomic sequencing of soil microbiome to help understand the effect of urine on microbial community structure such as the studies of Singh et al, (2009) and Ganasamurthy et al. (2021).

7.6 Conclusions and outlook

This four-year project consisted of a logical sequence of field and incubation experiments aiming to assess the fate of sheep urine N in lowland and upland grassland soils, and to quantify the various N losses from urine patch.

Long term field experiments were carried out to establish N_2O EF_{3PRP} on two grasslands representative of main sheep grazing systems in Ireland in order to generate data that could be used to support the revision of N_2O emission factor from sheep excreta in the Irish GHG inventory. These EF_{3PRP}s measured on both grasslands appeared to be negligible, thus indicating another fate of urine N other than its emission as N_2O.

From these results, two incubation experiments were established under denitrifying conditions to quantify other possible N pools and losses from sheep urine deposited on these pasture soils. During these experiments we observed an effect of soil type on the fate of urine N. Urine deposited on the incubated mineral soil from the lowland grassland led to significant N gas emissions (NH_3, NO, N_2O, and N_2) and NO_3^- leaching, indicating that low N_2O emissions observed in *situ* on the lowland grassland may not be related to inherent soil properties. During the last incubation experiment, within three weeks following urine application, urine N was lost mainly through NH_3 volatilisation (12.6%), followed by NO_3^- leaching (9.9%) and then N_2O emissions (0.3%). On the peat soil from the upland grassland, there was no sign of nitrification, probably due to the acidic conditions inhibiting nitrifiers activity. Therefore, we did not observe noticeable N gas emissions on this soil, except some NH_3 volatilisation associated with the raise of pH following urine addition. On the incubated peat soil, most of the N compounds from hydrolysis of urea remained in the form of NH_4^+. These NH_4^+ions may be either leached, fixed on clay particles, or volatilised as NH_3 with the increase of pH following urine deposition, and could also be taken by plants *in situ.* Within three weeks following urine application on the incubated peat soil, urine N was lost mainly through NH_3 volatilisation (4.4%), followed by NH_4^+ leaching (1.2%) whereas N_2O emissions and NO_3-leaching were negligible.

Our results represent a positive message for the sheep sector in Ireland, since low N_2O emissions were observed from excreta deposited on two grasslands representative of the main sheep grazing systems. However, we observed very different urine N fate between the two grassland soils. Therefore, N_2O emissions from the sheep urine patch could be much higher in another type of grassland, which is why one of our main recommendations is to increase the number of measurements from

contrasting grasslands before implementing a Tier 2 EF_{3PRP} for sheep excreta in Ireland, and to assess the fate of urine N in these other soils. Data from such studies will help to understand the drivers of N pathways in urine amended soils and is essential for models aiming to predict N losses in grasslands.

7.7 References

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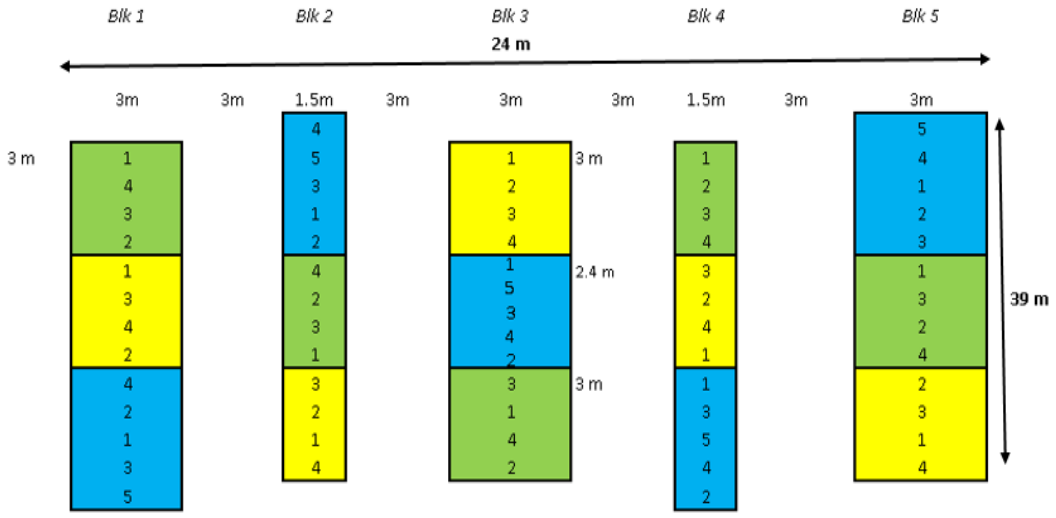
Appendix 1: Supplementary material for Chapter 4

a)



b)

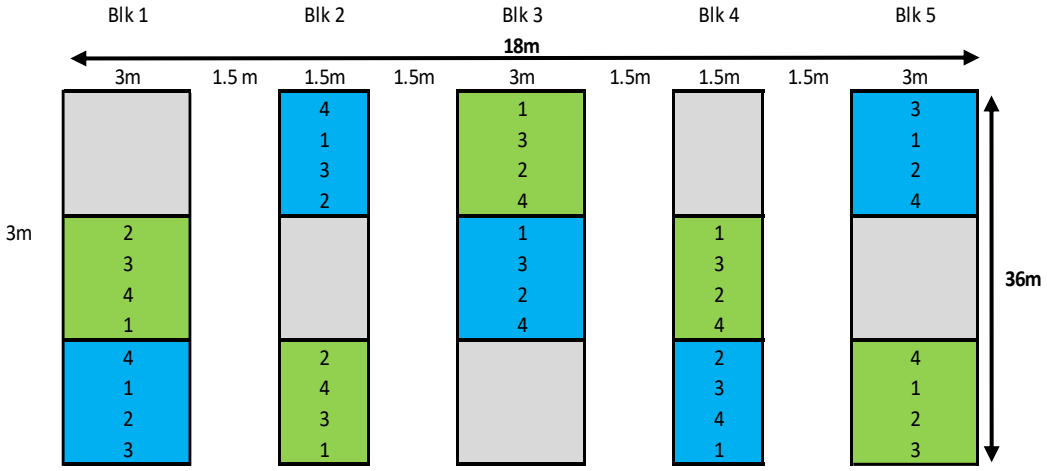
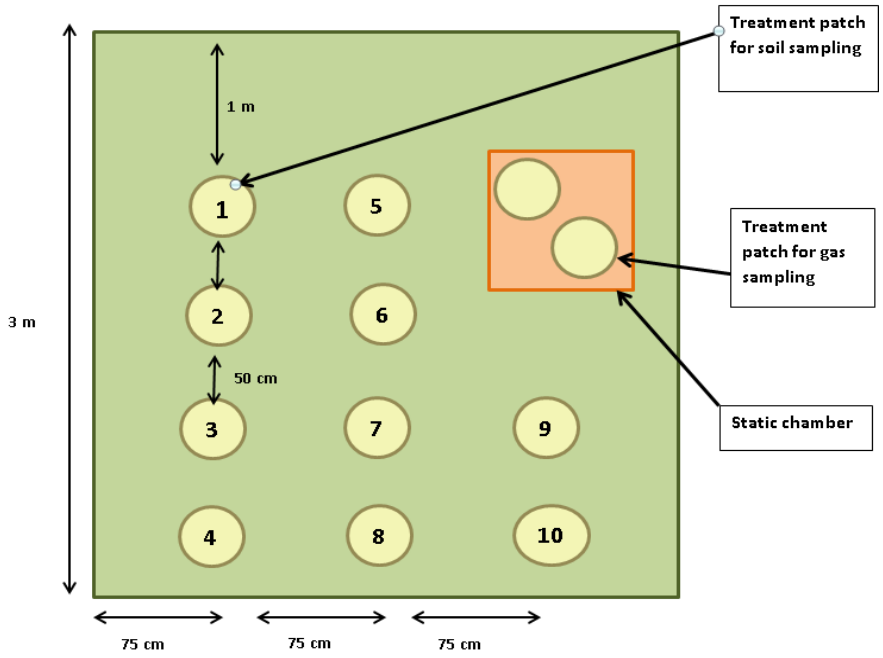


Figure S4.1 Experimental design of the study site on LOW (a) and UP (b)

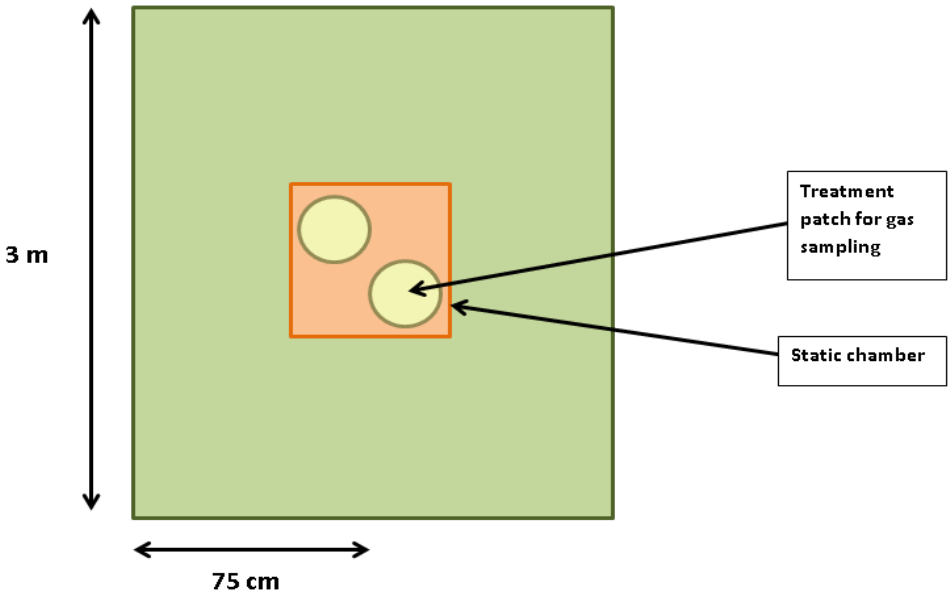
Numbers refer to treatments: 1 = C, 2 = U, 3 = AU, 4 = D, 5 = U+NI. Colours refer to the season of application: yellow = spring, green = summer and blue = autumn. In Blocks 1, 3 and 5, each plot contains one static chamber with two treatment patches for gas sampling and ten additional patches for soil sampling. In Block 2 and 4: each plot contains one chamber with two patches for gas sampling.

Figure S4.2 Pictures of the site: (a) Overall view of the site in Athenry (LOW) (b) sub-block in LOW showing the four plots with the static chambers and additional patches (with flags) for soil sampling (c) general view of the site in Oorid (UP).

a)


Treatment patch for soil sampling
1 m
1
5
Treatment patch for gas sampling
2
6
3 m
50 cm
3
7
9
Static chamber
4
8
10
75 cm
75 cm
75 cm

b)


3 m
Treatment patch for gas sampling
Static chamber
75 cm

c)

Figure S4.3 Experimental plot design**:** a) Design of plots in Blocks 1, 3 and 5. b) Design of plots in Blocks 2 and 4. c) Picture of a plot containing ten additional patches for soil sampling.

Table S4.1 Chemical composition of artificial urine, prepared according to Lucas and Jones, 2006.

Ingredients	% N	g compound /L	g N /L
Urea	46.6	6.4	2.982
Hippuric Acid	7.8	1.85	0.144
Allantoin	35.4	0.6	0.212
Uric Acid	33.3	0.005	0.001
Creatinine	37.1	0.015	0.006
Creatine	32.1	0.85	0.273
Glycine	18.7	0.01	0.002
Hypoxanthine	41.2	0.001	*negligible*
Ammonium Chloride	10.3	0.015	0.002
Potassium bicarbonate		6	
Potassium chloride		3.5	
Sodium sulfate Na_2SO_4		0.4	
Total N (g N L^{-1})	**3.62**		

Figure S4.4 Pens used for collection of sheep urine and dung.

Figure S4.5 Waterlogged soil area on the upland grassland (UP).

Table S4.2 Summary of grass yield (t DM ha^{-1}) and foliar N uptake (kg ha^{-1}) for different harvests following spring and summer treatments application to the managed lowland pasture in LOW. The first harvest following autumn application occurred seven months after treatments application, so we assumed that a grass analysis for this harvest was not accurate. Values represent means ± SEM (n = 3). The presence of an asterisk indicates a significant difference ($p < 0.05$) compared to the control level (i.e. effect of treatment at each harvest). For Spring H3 and Summer H1 (same harvest), there were no effect of season of application on grass yield and N uptake ($p < 0.05$).

Treatments	Spring H1 (28/05/19)	Spring H2 (03/07/19)	Spring H3 (13/09/19)	Summer H1 (13/09/19)
		Grass yield (t DM ha^{-1})		
C	1.21 ± 0.20	1.03 ± 0.38	1.62 ± 0.37	2.02 ± 0.41
U	2.25 ± 0.28*	1.35 ± 0.07	1.80 ± 0.06	3.44 ± 0.41*
AU	1.91 ± 0.35*	1.06 ± 0.15	1.93 ± 0.30	2.24 ± 0.34
D	1.26 ± 0.14	1.04 ± 0.04	2.37 ± 0.50	1.72 ± 0.31
		N uptake (kg N ha^{-1})		
C	22.7 ± 3.94	22.9 ± 8.75	37.5 ± 8.99	47.18 ± 15.2
U	70.4 ± 6.93*	27.6 ± 1.8	40.3 ± 4.23	73.9 ± 42.7*
AU	51.7 ± 11.5*	21.5 ± 3.6	44.7 ± 10.6	44.6 ± 7.05
D	28.2 ± 3.42	24.0 ± 2.41	53.6 ± 9.48	41.5 ± 8.56

Appendix 2: Supplementary material for Chapter 5

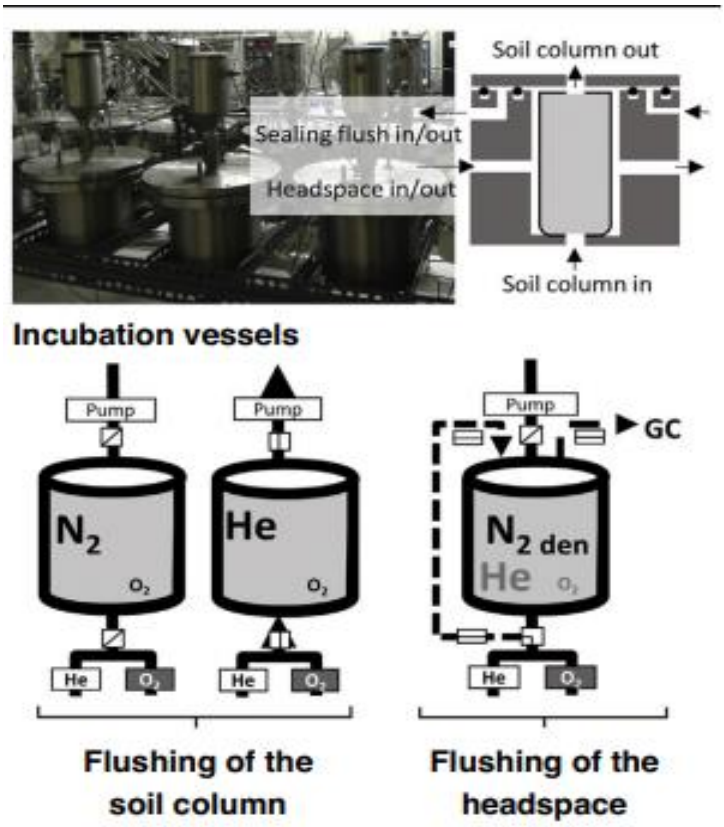
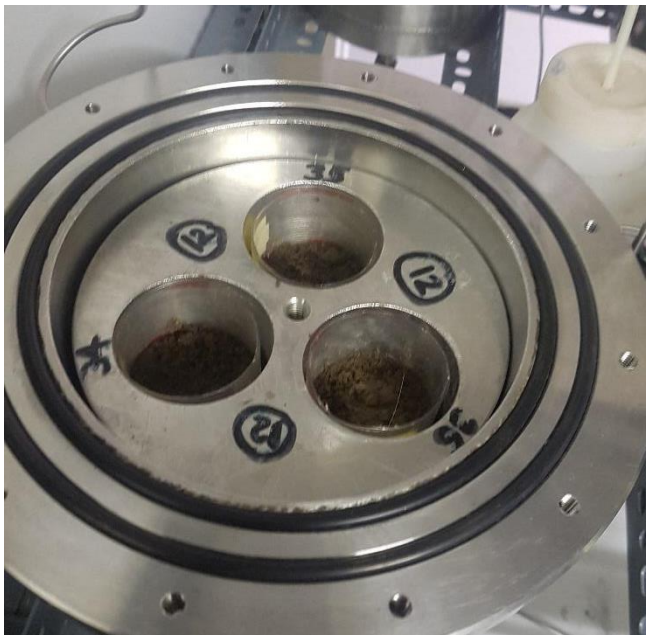


Figure S5.1 Schematic representation of the experimental set-up of the DENIS incubation systems showing the flushing of the vessels with the He/O_2 gas for N_2 sampling and measurement by the gas chromatograph (GC), (source: Friedl and al., 2020).

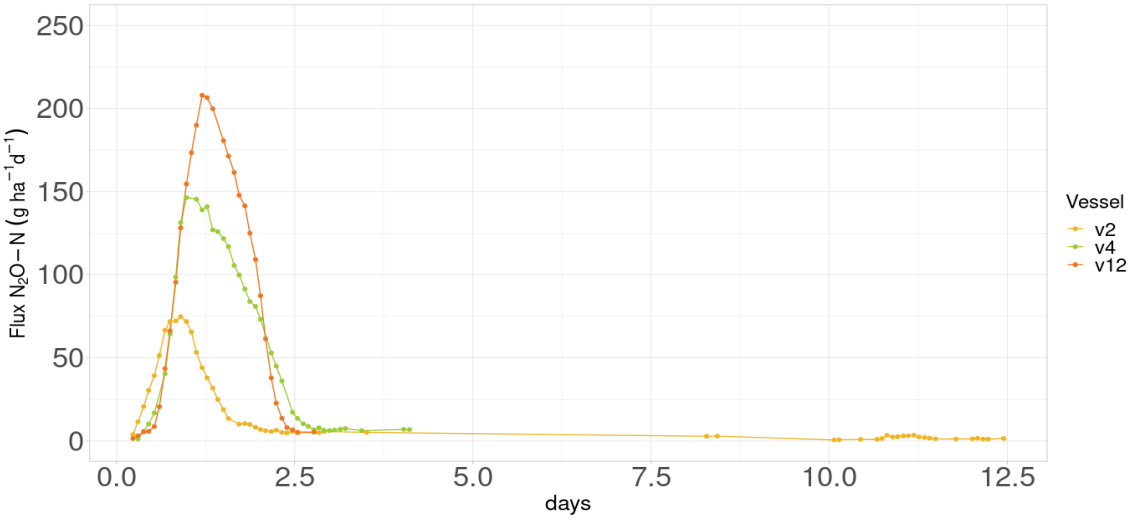
a)



b)

Figure S5.2 Pictures of a) one vessel of the DENIS incubation system containing three soil cores, b) analysers for N gas measurements in the DENIS laboratory

a)



b)

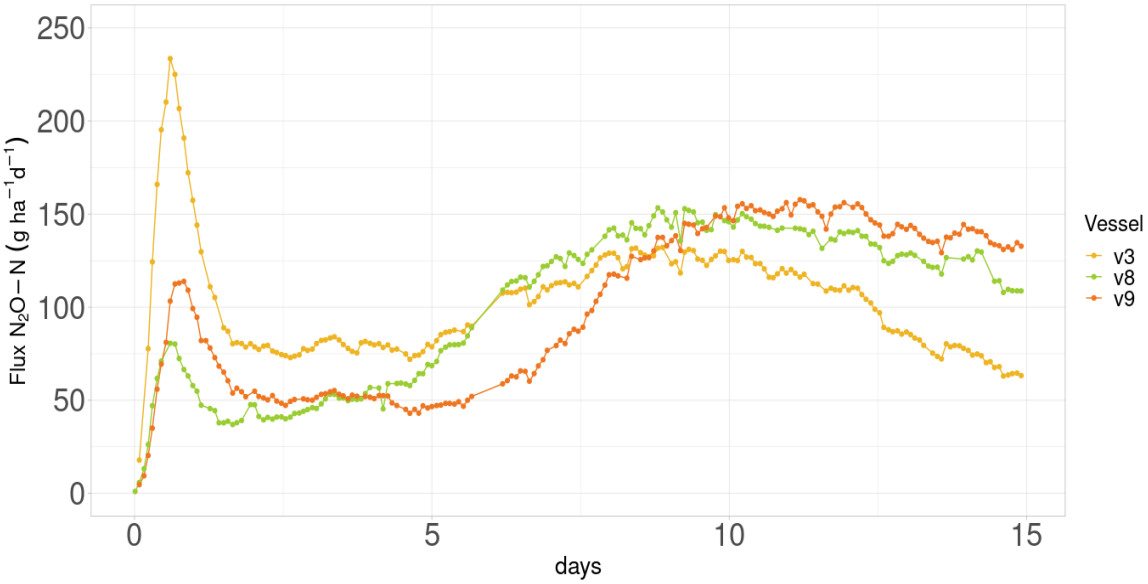


Figure S5.3 Pattern of N_2O fluxes for each replicate of a) MC (mineral soil amended with water) and b) MU (mineral soil amended with sheep artificial urine) treatment.

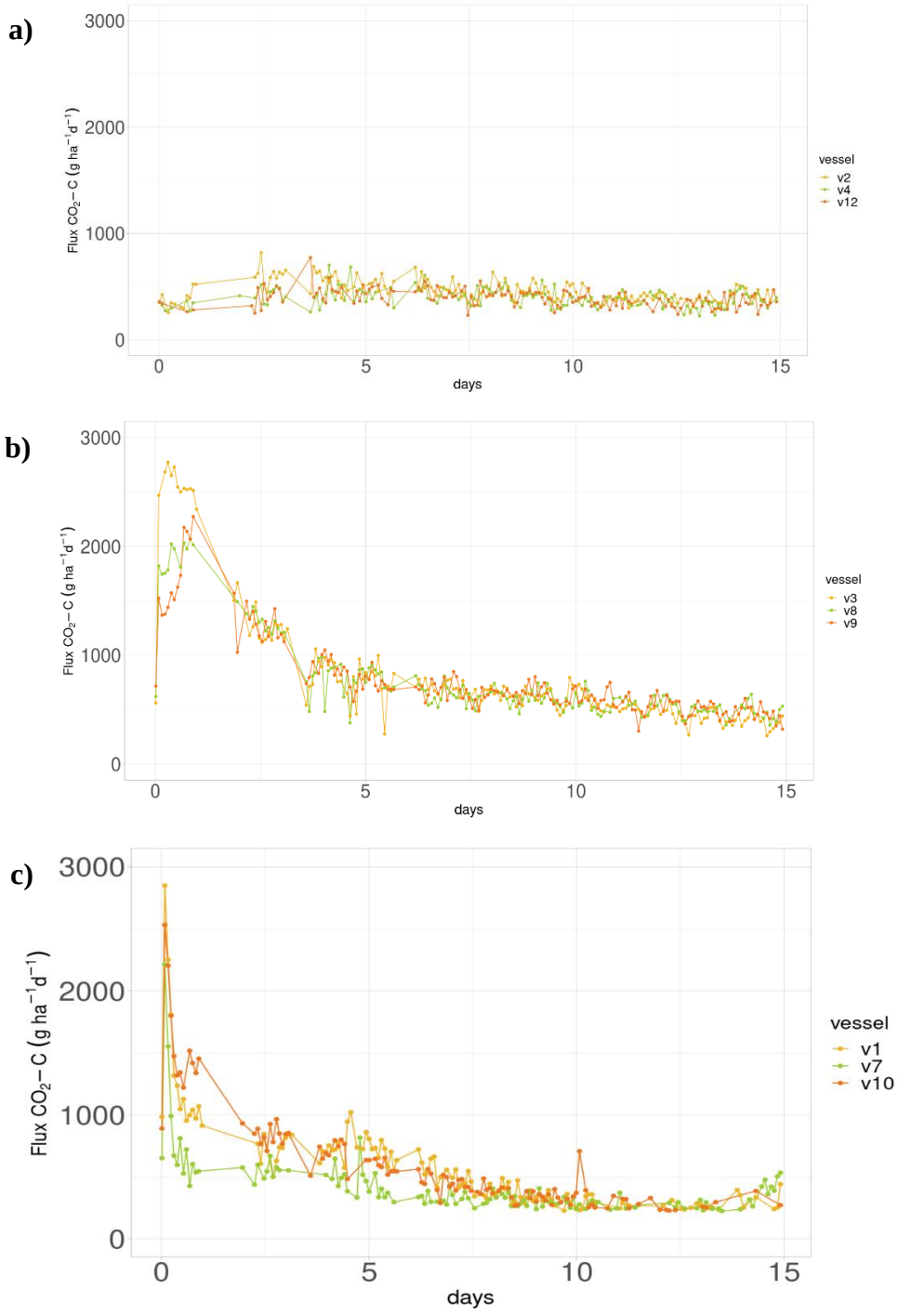


Figure S5.4 Pattern of CO_2 fluxes for each replicate of a) MC (mineral soil amended with water), b) MU (mineral soil amended with artificial sheep urine) and c) PU (peat soil amended with artificial sheep urine). The graph for PC treatment (i.e. peat soil amended with water) is not presented because >95% of values were under the detection limit.

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Appendix 3: Supplementary material for Chapter 6

a)

b)

Figure S6.1 Intact soil cores (20 cm depth) after collection of a) mineral soil from the lowland grassland b) peat soil from the upland grassland

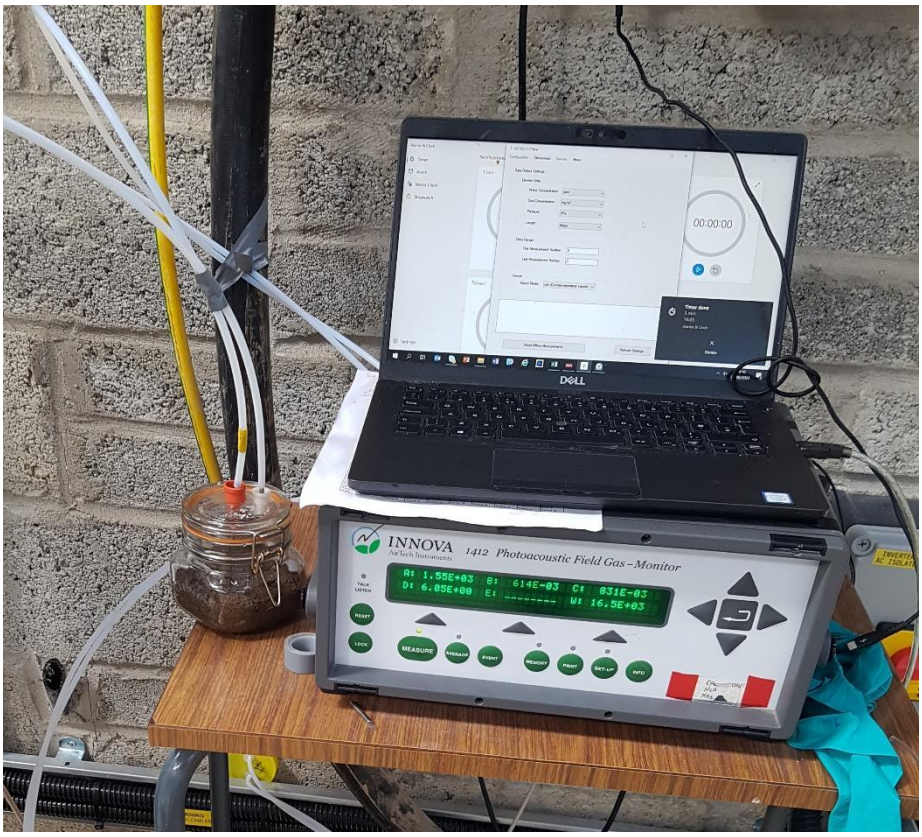

Figure S6.2 Photoacoustic gas analyser connected to a Kilner jar containing incubated soil or the measurement of NH_3 emissions

Figure S6.3 Desiccator connected to a vacuum pump and containing soil samples under chloroform fumigation.

Table S6.1 Primers used for each targeted gene

Gene name	***Primer Name***	Primer concen-tration (µM)	**Sequence (5' - 3')**	**Reference**
16S rRNA (thaumar-chaea)	771F	0.2	ACGGTGAGGGATGAAAGCT	Ochsenreiter 2003
	957R		CGGCGTTGACTCCAATTG	
16S rRNA (bacteria)	341f	1.5	CCTACGGGNGGCWGCAG	klindworth et al., 2013
	785r		GACTACHVGGGTATCTAATCC	
amoA Archaea	crenamoA23f	1	ATGGTCTGGCTWAGACG	Tourna et al., 2008
	crenamoA616r		GCCATCCATCTGTATGTCCA	
amoA Bacteria	Bac amoA1F	1	GGGGTTTCTACTGGTGGT	Rothhauwe et al.,1998
	Bac amoA2R new		CCCCTCBGSAAAVCCTTCTTC	Hornek et al., 2006
ITS	ITS4r	0.2	TCCTCCGCTTATTGATATGC	De Beeck et al., 2014
	ITS86f		GTGAATCATCGAATCTTTGAA	
nirK	nirk1040	0.2	GCC TCG ATC AGR TTR TGG TT	Hallin et al., 2009
	nirk876		ATY GGC GGV CAY GGC GA	
nirS	cd3AF	0.5	GTSAACGTSAAGGARACSGG	Throback et al., 2004; Yergeau et al., 2004
	R3cd		GAS TTC GGR TGS GTC TTG A	Throback et al., 2004
nosZ I	nosZ2F	0.2	WCS YTG TTC MTC GAC AGC CAG	Henry et al., 2006
	nosZ2R		ATG TCG ATC ARC TGU KCR TTY TC	
nosZ II	nosZ II F	1	CTI GGI CCI YTK CAY AC	Jones et al., 2013
	nosZ II R		GCI GAR CAR AAI TCB GTR C	

Table S6.2 Details of q-PCR standard curves

Gene Target	Slope	Efficiency (%)	R2
Bacterial 16S rRNA	-3.327	99.8	0.990
Archaeal 16S rRNA	-3.576	90.4	0.995
ITS fungi	-3.583	90.2	0.994
Archaea amoA	-3.620	88.9	0.990
Bacteria amoA	-3.568	90.7	0.995
nirS	-3.584	90.1	0.990
nirK	-3.493	93.3	0.999
nosZ clade I	-3.547	91.4	0.999
nosZ clade II	-3.641	88.1	0.999

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