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Assessing the impact of long-term soil phosphorus on N-transformation pathways using ^{15}N tracing

R.M. O'Neill [a,b,*], D.J. Krol [a], D. Wall [a], G.J. Lanigan [a], F. Renou-Wilson [b], K.G. Richards [a], A. B. Jansen-Willems [c], C. Müller [b,c,**]

[a] Environmental Research Centre, Teagasc, Johnstown Castle, Co, Wexford, Ireland
[b] University College Dublin, Dublin, Ireland
[c] Institute of Plant Ecology, University Giessen, Germany

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ABSTRACT

A laboratory incubation study was conducted on a temperate grassland soil to quantify the main mineral nitrogen (N) transformation rates and pathways via a ^{15}N tracing approach. Soil samples were taken from a long-term phosphorus (P) trial to investigate the effects on gross N-transformations under high and low phosphorus amendment. The soils were incubated over a 2-week period and treated with ammonium-nitrate (NH_4NO_3) which was applied to the soil both with and without a glucose amendment and labelled with ^{15}N either on the ammonium (NH_4^+) or nitrate (NO_3^-) moiety at 50% atom enrichment. The results showed immobilisation to greatly outweigh mineralisation and that NO_3^- was predominantly produced via heterotrophic nitrification. Individual pathways for NO_3^- production were quantified including oxidation of NH_4^+, recalcitrant and labile organic N. Oxidation of labile organic N to NO_3^-, a newly considered pathway, accounted for between 63 and 83% of total NO_3^- production across the various treatments and P levels. This process was significantly higher in the low-P rather than the high-P soils ($p < 0.05$), highlighting the effect of soil P on the microbial community.

1. Introduction

Nitrogen (N) and phosphorus (P) are essential for biological proliferation and are the dominant rate-limiting nutrients in most natural systems, as such they are major constituents of agrochemical fertilisers (Stark and Richards, 2008; Guignard et al., 2017). Fertilisers were central to the 20th century 'Green Revolution,' which has resulted in about half the world's land converted to grazed grassland or cultivated crops (Kareiva et al., 2007). However, fertiliser application is notoriously wasteful and results in large nutrient losses to the environment (Guignard et al., 2017). Losses of N from the soil give rise to nitrate (NO_3^-) leaching, and gaseous emissions especially those of ammonia (NH_3) and the potent greenhouse gas (GHG) - nitrous oxide (N_2O) with a global warming potential (GWP) 298 times that of CO_2 (IPCC, 2019). Meanwhile losses of P can reduce biodiversity and result in local extinctions via the dominance of a few competitive species. This in turn, influences plant and microbial community structure and function, ultimately reshaping ecosystem services by altering nutrient transfer and cycling (Guignard et al., 2017). Agricultural efforts are particularly focussed to reduce N losses and pollution e.g. via N_2O mitigation efforts, as it is one of the dominant contributing sectors accounting for 60% of total global and 93% of total Irish N_2O emissions (Duffy et al., 2020). Improved grassland ecosystems are a major contributor to N-pollution; comprising approximately 25% of the Earth's terrestrial surface, they are intensively managed for grazing, which results in significant N-inputs via both animal excreta and inorganic fertiliser (Oenema et al., 2005; Saggar et al., 2013). These N-inputs undergo a series of transformations in the soil; the drivers and interactions of which are often unclear. Frequently the stores, fluxes and cycling of N and P have been examined separately, partly because of the relative ease of tracing N cycles compared with P, but these elements are intrinsically linked and need to be considered relative to one another (Guignard et al., 2017). At the organism level, N and P availability are known to underpin photosynthetic processes, cell growth, metabolism and protein synthesis, but how this extrapolates to span multiple organisational levels and scales is much less understood (Chapin et al., 2011). Often the interactions which

* Corresponding author. University College Dublin, Dublin, Ireland.
** Corresponding author. University College Dublin, Dublin, Ireland.
E-mail addresses: rosie.oneill@teagasc.ie (R.M. O'Neill), christoph.mueller@ucd.ie (C. Müller).

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exist between such nutrients, also have a greater impact on their overarching processes than their individual contributions (Davidson and Howarth, 2007).

The mineralisation of organic to inorganic forms of N and P is completed primarily by microorganisms as they metabolise carbon (Spohn and Kuzyakov, 2013). N undergoes many transformations as it passes through the N cycle. The main pathways are those of nitrification and denitrification; where N compounds (NH_4^+, organic N) are oxidised to NO_3^- which can then be reduced via denitrification to gaseous N compounds such as N_2O or to inert di-nitrogen (N_2) (Signor and Cerri, 2013). Transformations from organic to inorganic forms of N via mineralisation make N available to plants and microbes (Signor and Cerri, 2013). Microbes require this N to synthesise the alkaline phosphatase enzymes that release P. As organic N concentrations are higher than those of P, this investment is balanced by N availability and the corresponding gain in P (Guignard et al., 2017). In grassland systems, P cycling is closely linked to mycorrhizal fungi in plant roots that release extracellular phosphatases. If N is applied above a certain threshold, P cycles faster in response to the greater N availability for producing phosphatase enzymes. Higher concentrations of phosphatase enzymes in turn results in faster rates of P removal and increased P limitation (Vitousek et al., 2010).

P is a key limiting nutrient as it is required in very small proportions relative to C and N, and as per Liebig's law of the minimum, any deficiency of it can seriously impede the entire N-cycle (Agren et al., 2012). Grasslands are frequently limited by P via depletion from biomass removal (harvesting), therefore they are frequently amended with P-fertiliser to boost herbage yield (Massey et al., 2016; Sheil et al., 2016). Previous research on the same soils sampled in this experiment demonstrated that soil P had a significant effect on N_2O fluxes with higher emissions at lower P-concentrations indicating that P is an important driver behind N-cycling (O'neill et al., 2020).

Soil C, N, and P cycling maintain a certain degree of homeostasis with one another (Elser et al., 2007; Cleveland and Liptzin, 2007). Soil C, the energy substrate for microbial communities, drives microbial activity and thus nutrient cycling. Low C inputs can mask the roles of various micro-organisms by limiting their activity and restricting their access to other critical nutrients such as N and P (O'neill et al., 2020). Under C-limited conditions they may also metabolise (esp. fungi) less available recalcitrant forms of carbon (Gougoulias et al., 2014). Fungi are particularly well-adapted to accessing resources and are a major contributor to decomposition and nutrient cycling (Boswell et al., 2007). Due to their ability to thrive in a broad range of conditions especially in permanent and undisturbed grassland systems, fungi usually form at least half the microbial biomass in many soils and filamentous fungi often form the dominant component (Boswell et al., 2007). It has been suggested that as fungi, especially arbuscular mycorrhizal fungi (AMF) have a higher C-demand and can be more tightly linked to plant growth than bacteria, they can outcompete many bacterial species for C, particularly those derived from root exudates (Griffiths et al., 2012; Cassman et al., 2016). Whereas bacteria have been reported to be more constrained by nutrient stoichiometry, especially that of mineral nutrients N and P in bacterial carbon use efficiency (CUE) (Nottingham et al., 2018; Keiblinger et al., 2010). As such, changes in available nutrients have the potential to alter the microbial community structure and/or activity pattern, soil productivity and subsequent nutrient cycling processes, all of which influence NUE and GHG emissions (Blagodatskiy et al., 2008; Blagodatsky and Richter, 1998). This emphasises the need for nutrient turnover studies quantifying individual gross transformation rates and drivers in the system.

Since Kirkham and Bartholomew (1954) developed the isotope dilution theory to quantify gross soil N production and consumption rates, many studies have utilised and further developed their initial concept, most notably via the consideration of dilution and enrichment systems that are estimated simultaneously (Booth et al., 2005; Mary et al., 1998). Since some of the major assumptions of the Kirkham and Bartholomew model are oversimplified (e.g. that there is no significant recycling of labelled N into the substrate pool or constant rates) (Jansen-Willems et al., 2016), nowadays the isotope dilution-enrichment approach coupled with source-partitioning is mainly used (Mary et al., 1998; Müller et al., 2004). As research in this area has progressed, recent studies have indicated a growing importance of previously unconsidered pathways such as heterotrophic nitrification (Zhang et al., 2015; Rütting et al., 2010). This study evaluates the effect of soil P and C on N-cycling with a particular focus on pathways involved in nitrification, using a ^{15}N tracing tool that considers all targeted N-transformations and pathways (Müller et al., 2007). The objective of this experiment was to investigate the interactive effect of soil P and C on N-transformations in a temperate grassland soil. As part of this study the *Ntrace* tool (Müller et al., 2007) had to be further developed to explain the data by adding an additional nitrification pathway.

2. Materials and methods

2.1. Site description

This incubation experiment was designed using soil sampled from a long-term ungrazed grassland phosphorus trial established in 1995 at Johnstown Castle, County Wexford, Ireland (52°17′55″N, 6°29′47″W). The trial was established on a grass sward dominated by *Lolium perenne* (perennial ryegrass). Johnstown Castle has a temperate climate with monthly rainfall and temperature averaging 70.7 mm and 6.3 °C for February over the past 30 years (Met.Eireann, 2010). The site is a fine loamy textured soil, classified as a moderately drained brown earth (Sheil et al., 2016). The trial plots received either 0 (P0) or 45 (P45) kg P ha^{-1} $year^{-1}$ as 16% P superphosphate, applied once in February of each year since establishment, with four replicate plots measuring 20 m^2, in a randomised block design. Aboveground plant material was harvested eight times per year to a height of 5–6 cm using a plot harvester. After each harvest, all plots received 40 kg N ha^{-1} as calcium ammonium nitrate (CAN), and potassium was also applied as a muriate of potash (KCl) at a rate of 125 kg K ha^{-1} $year^{-1}$ to compensate for potassium removal (Massey, 2012; Randall et al., 2019). The trial was reseeded in 2016 with *Lolium perenne* (Massey et al., 2016).

2.2. Soil sampling and preparation

Composite soil samples were taken with an auger to a depth of 10 cm randomly in a 'W-shaped' pattern across the P0 and P45 plots in February 2019, before the annual P amendment. Samples were sieved and incubated in glass Weck © jars (750 mL) jars under the conditions of 15 °C and 70% humidity to represent average Irish springtime conditions. The fresh-weight equivalent of 100g oven dried soil was packed into each jar, compacted to a soil bulk density of 1g cm^{-3} and adjusted to 70% water-filled pore space (WFPS) (Haney and Haney, 2010). Soils were treated with ^{15}N-labelled ammonium-nitrate fertiliser with either the NH_4^+ or NO_3^- moiety labelled ($^{15}NH_4NO_3$ or $NH_4^{15}NO_3$) to quantify N-transformations (Müller et al., 2007) as well as a ± glucose addition (shown below). The ^{15}N enrichment was 50 atom % and was applied at field equivalent rate of 40 kg N $ha^{-1}$and the C was applied at a rate of 0.1 mg C g^{-1} to represent a relatively low daily addition rate for plant carbon inputs across a range of soil types (Girkin et al., 2018; Grayston and Campbell, 1996). The experiment was arranged as a randomised block design, with four replicates of each treatment combination (see below) and one set of microcosms (32 jars) prepared for each sampling time (3, 26, 74, 170, and 337 h after application) to allow for destructive sampling.

Treatment Levels:

1. P0 + $^{15}NH_4$-NO_3/NH_4-$^{15}NO_3$ -C (4 reps)
2. P0 + $^{15}NH_4$-NO_3/NH_4-$^{15}NO_3$ +C (4 reps)
3. P45 + $^{15}NH_4$-NO_3/NH_4-$^{15}NO_3$ -C (4 reps)

4. P45 + $^{15}NH_4$-NO_3/NH_4-$^{15}NO_3$ +C (4 reps)

Treatments were applied evenly to the soil surface in the sample jars using a syringe with a side-port-needle. Jars were randomly allocated on a shelf within a climate-controlled chamber (WP series Weisstechnik, walk-in test chamber). Jars were covered with a pierced Parafilm (SigmaAldrich, USA) to prevent moisture loss but allow gaseous exchange. All jars were maintained at 70% WFPS and moisture loss was minimal.

2.3. Soil mineral nitrogen and ^{15}N analyses

Mineral ammonium-N (NH_4^+-N) (mg kg^{-1}) and nitrate-N (NO_3^--N) (mg kg^{-1}) were characterised prior to fertilisation, on each sampling day and at the end of the incubation. The entire contents of the soil jars were extracted with 2M potassium chloride (KCl). Extractions took place on 5 occasions (3, 26, 74, 170, and 337 h) after the addition of treatments. The soil in the jars was transferred to a homogeniser (RW20 Janke & Kunkel IKA-WERV) with 2M KCl and blended for 1.5 min. The filtrates were centrifuged at 2000 rotations per minute (Sigma type 6–16) for 5 min. The supernatant was filtered sequentially through glass fibre filters (GF50 125 and GF50 090) (GF Healthcare Life Sciences Filter Unit Whatmann) and filtrates were stored at 4 °C prior to analysis.

Soil NO_3^- and NH_4^+ concentrations were determined using an automated continuous flow wet chemistry analyser (SEAL AutoAnalyzer 3 HR, UK). ^{15}N enrichment of the NH_4^+ and NO_3^- pools were determined by conversion to N_2O(Stevens and Laughlin, 1994, 1998). Using this method the ratio differences between the normal and enriched N_2O of the headspaces enable the mole fraction of the NO_3 and NH_4 and the fraction proportional to the amount of ^{15}N-labelled N_2O in the headspace to be calculated (Stevens and Laughlin, 1998).

2.4. ^{15}N tracing analysis

The^{15}N tracing analysis tool, $Ntrace_{Basic}$ was used to quantify the gross N-transformation rates. The premise of this basic tool is to consider only the most essential N-transformations needed to explain the dynamics of the measured data. The characteristics and properties of the soil in question are incorporated into *Ntrace*, thus specifying it to our individual dataset (for full characteristics see (O'Neill et al., 2020). To obtain the best fit, a range of model scenarios are tested which includes the complexity of the model (i.e. how many N-transformations are considered) and the kinetics of each N-transformation. The selection of the most suitable setup is guided by the goodness of fit and Akaike's Information Criteria (AIC), and the least number of parameters that can best explain the data to ensure a simple parsimonious model which does not suffer from poor predictive performance due to becoming over-parameterised (Cox et al., 2006). No adequate fit could be obtained using the original model published in Müller et al. (2007), and the performance of the model was only improved after including a new additional pathway of oxidation from labile N (O_{Nlab}) to NO_3^-. All N-transformations were described by zero or first-order kinetics.

Thus, the model presented in this study is an extended version of the $Ntrace_{Basic}$ and considers six pools and fourteen simultaneously occurring N-transformations (Fig. 1). The N pools were; ammonium (NH_4^+), adsorbed ammonium ($NH_{4\,ads}^+$), labile soil organic N (N_{lab}), recalcitrant soil organic N (N_{rec}), nitrate (NO_3^-) and stored nitrate ($NO_{3\,sto}^-$) (see Table 1 in Supplementary Material for full pathway abbreviations). The initial NO_3^- and NH_4^+ pool sizes were determined by extrapolating the first two extraction times back to time zero. The initial $NH_{4\,ads}^+$ and

Table 1
Total N-transformation rates in μg g^{-1} day^{-1}, where; $\mathbf{I_{NH4\_tot}}$ = total immobilisation of NH_4^+ to the labile and recalcitrant N pools, $\mathbf{I_{NO3\_tot}}$ = total immobilisation of NO_3^- to the labile and recalcitrant N pools, $\mathbf{I_{tot}}$ = total immobilised N from NH_4-N and NO_3-N ($\mathbf{I_{NH4\_tot}}$ + $\mathbf{I_{NO3\_tot}}$), $\mathbf{M_{\_tot}}$ = total mineralisation of labile and recalcitrant N to the NH_4^+ pool, $\mathbf{N_{\_tot}}$ = total NH_4-N, labile N and recalcitrant N oxidised to the nitrate pool. Letters denote significant differences based on least significant difference (LSD) between P and C treatments as indicated by the column headings.

Total N-transformation rates(μg g^{-1} day^{-1})	P0 -C	P0 +C	P45 -C	P45 +C
$\boldsymbol{I_{NH4\_tot}}$	2.758^{abc}	2.885^{abc}	2.870^{abc}	3.858^{d}
$\boldsymbol{I_{NO3\_tot}}$	0.058^{abc}	0.136^{abc}	0.272^{abc}	1.804^{d}
$\boldsymbol{I_{\_tot}}$	2.816^{abc}	3.021^{abc}	3.142^{abc}	5.661^{d}
$\boldsymbol{M_{\_tot}}$	0.015^{ac}	0.064^{b}	0.007^{ac}	0.240^{d}
$\boldsymbol{N_{\_tot}}$	3.085^{ab}	2.630^{ab}	3.832^{c}	4.863^{d}

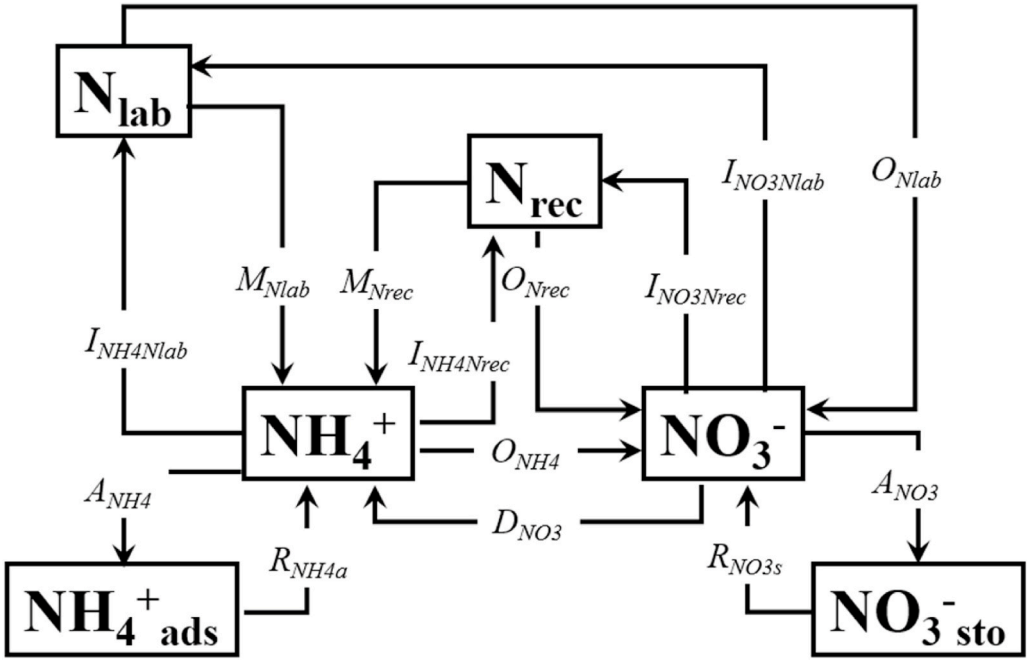

Fig. 1. Conceptual ^{15}N tracing model used for the analysis of gross soil N-transformation rates modified from Müller et al. (2007) (see text and appendix for abbreviations and details).

$NO_{3\,sto}^-$ pool sizes were based on the discrepancy between the added and the initial NH_4^+ and NO_3^- assuming that the initial NH_4^+ and NO_3^- already present in the soil had a ^{15}N content the same as natural abundance (Müller et al., 2004). The initial organic N concentration (N_{rec} and N_{lab}) was based on previous published measurements taken from the site (Table 2 in Supplementary Material) (O'neill et al., 2020).

All modelled N-transformation rates are presented in $\mu g\ g^{-1}\ day^{-1}$. Combined rates were calculated as follows.

$I_{NH4\_tot} = I_{NH4\text{-}Nlab} + I_{NH4\text{-}Nrec}$ (total NH_4^+ immobilisation)

$I_{NO3\_tot} = I_{NO3\text{-}Nlab} + I_{NO3\text{-}Nrec}$ (total NO_3^- immobilisation)

$I_{tot} = I_{NH4\_tot+}I_{NO3\_tot}$ (total immobilisation from NH_4-N and NO_3-N)

$M_{\_tot} = M_{Nlab} + M_{Nrec}$ (total mineralisation)

$N_{\_tot} = O_{NH4} + O_{Nlab} + O_{Nrec}$ (total nitrification)

2.5. Calculations and statistical analyses

Analysis of least significant difference was conducted using Sigma Plot 14.0. to determine significant treatment and P level effects on pathways (Table 1). A simple effect comparison of pathways within treatment was carried out using the GLIMMIX procedure, using the Tukey method as an adjustment for multiple comparisons (SAS version 9.4). Significant differences between total autotrophic and heterotrophic nitrification processes were determined by comparing their confidence intervals, assuming a difference of at least $p < 0.05$ if no overlapping occurred.

3. Results

3.1. Soil N-transformation model performance

The ^{15}N tracing tool $Ntrace_{Basic}$ used in this study was developed to consider the complex internal soil N cycle (Müller et al., 2007). Including the additional pathway added in this study, in total 14 parameters were simultaneously optimised with the aim to identify the parameters common to all steps with the least complex model. Initially the model run was carried out with the original $Ntrace_{Basic}$ design, but this was unable to provide a suitable fit to the measured data. Therefore, the model was extended by an additional heterotrophic nitrification pathway from labile organic N to NO_3^- (O_{Nlab}) which satisfactorily simulated the observed data. In total, five optimised runs were carried out for the P0 -C, four runs for P0 +C, six runs for P45 -C and nine runs for P45 +C. The best fits, as indicated by the lowest AIC for each treatment were run 5, run 4, run 4 and run 9 respectively. There was good agreement between the optimum model fits and the observed data $r^2 = >0.99$ for P0 -C, P0 +C, and P45 -C and an $r^2 > 0.96$ for P45 +C (Fig. 2). The modelled data represents the best fit to our datasets: the measured concentrations of NH_4^+ and NO_3^- and the ^{15}N enrichments of NH_4^+ and NO_3^- pools.

Table 2
Relative rates of heterotrophic and autotrophic nitrification calculated from modelled pathways (Table 1) per treatment. Letters denote significant differences calculated from confidence intervals.

Pathway	Pathway/total nitrate($N_{\_tot}$)	P0 -C	P0 +C	P45 -C	P45 +C
Autotrophic Nitrification	$O_{NH4}/N_{\_tot}$	0.2%[a]	1.1%[b]	1.6%[c]	0.7%[d]
Heterotrophic Nitrification	$O_{Nlab}/N_{\_tot}$	82.9%[e]	81.9%[f]	63%[g]	71%[h]
Heterotrophic Nitrification	$O_{Nrec}/N_{\_tot}$	17% [ij]	17% [ij]	35.5%[k]	28.4%[l]

The $^{15}NH_4$ atom % enrichment was maintained around 45% for the first two days before dropping gradually to its lowest value of around 3% on the last day of sampling, this change in atom % was significant with time ($p = 1.98e\text{-}07$) but not with C, P or their interaction ($p > 0.05$). The ^{15}N present in the $^{15}NO_3^-$ pool had its highest value of only 14% for the first two days and did not change significantly over the duration of the experiment or with treatments ($p > 0.05$). This is attributed to be a result of immediate $^{15}NO_3^-$ dilution due to a larger amount of natural abundance NO_3^- being present in the soil initially (see NO_3^- concentration at time zero). The unchanged enrichment over the duration of the experiment indicates that the NO_3^- pool was not being diluted, hence no NO_3^- at natural or lower abundance as the current enrichment of the NO_3^- entered this pool (Fig. 2: d, h, l, and p).

3.2. N-transformation rates

The combined modelled N-transformation rates are presented in Table 1 (see Table 1 in Supplementary Material for all pathways). These five main N-transformations were all significantly highest in the P45 +C treatment indicating that the interaction between P and C is greater than either on its own. The only significant effect of P, when no C was added, was on $\boldsymbol{N_{\_tot}}$ transformations. Whereas in P0 levels the only significant effect of C was on $\boldsymbol{M_{\_tot}}$ transformations. $\boldsymbol{M_{Nrec}}$ was much higher than $\boldsymbol{M_{Nlab}}$ in all treatments (see Table 1 in Supplementary Material) and showed a significant response to P and C treatments which was greatest in their interaction at P45+C. Whereas $\boldsymbol{M_{Nlab}}$ had no significant response to either treatment. $\boldsymbol{M_{\_tot}}$ rates were much lower than those of $\boldsymbol{I_{tot}}$, indicating that there is net immobilisation occurring in these soils.

$\boldsymbol{I_{tot}}$, $\boldsymbol{I_{NH4\_tot}}$, and $\boldsymbol{I_{NO3\_tot}}$ were significantly higher ($p < 0.05$) in P45 compared to P0 in C supplemented treatments only.

The rate of $\boldsymbol{I_{NH4\_Nrec}}$, (immobilisation of NH_4-N to recalcitrant N) was much higher for all treatments than that of $\boldsymbol{I_{NH4\_Nlab}}$. Higher rates of $\boldsymbol{I_{NH4\_Nlab}}$ were observed in the C-excluded treatments with no significant effect from P addition while $\boldsymbol{I_{NH4\_Nrec}}$ displayed significant differences in terms of C level and a C*P interaction.

$\boldsymbol{I_{NO3\_tot}}$ rates of N-immobilisation were much lower than those from the NH_4^+ pool ($\boldsymbol{I_{NH4\_tot}}$), but conversely the highest rates of $\boldsymbol{I_{NO3\_tot}}$ were observed for N immobilised into the labile pool ($\boldsymbol{I_{NO3\_Nlab}}$) compared to NO_3^- immobilisation into the recalcitrant pool ($\boldsymbol{I_{NO3\_Nrec}}$). There was no significant difference between P0 +C and P45 +C on $\boldsymbol{I_{NO3\_Nrec}}$ but significant differences were observed between P and C and their interaction on all other treatments, indicating that the organisms carrying out this N-transformation could have been saturated at the P45 +C treatment. $\boldsymbol{I_{NO3\_Nlab}}$ on the other hand, showed its greatest significance at the P45 +C treatment showing a P*C interaction but did not show any significances regarding C and P individually.

3.3. Heterotrophic and autotrophic nitrification

The most surprising and interesting result were the ways how NO_3^- was produced. The different nitrification pathways of the various source pools of NH_4^+ (O_{NH4}), labile (O_{Nlab}) and recalcitrant (O_{Nrec}) oxidation to NO_3^- are presented in Table 2. Total heterotrophic nitrification ($O_{Nrec} + O_{Nlab}$)/($O_{Nrec} + O_{Nlab} + O_{NH4}$) accounted for over 98% of all nitrification, with the majority arising from the O_{Nlab} pool. The highest O_{Nlab} rate was obtained in the P0 -C treatment and these rates decreased significantly with increasing P. Conversely, the O_{Nrec} pool nitrification rates were higher at P45 (Table 2). Heterotrophic nitrification from the labile pool showed a significant response to C, while heterotrophic nitrification from the recalcitrant pool only showed a significant response to C in the high P soils. Regarding autotrophic nitrification, the highest rate was at the P45 -C treatment, followed by the P0 +C treatment, and showed a significant response to both P and C treatments.

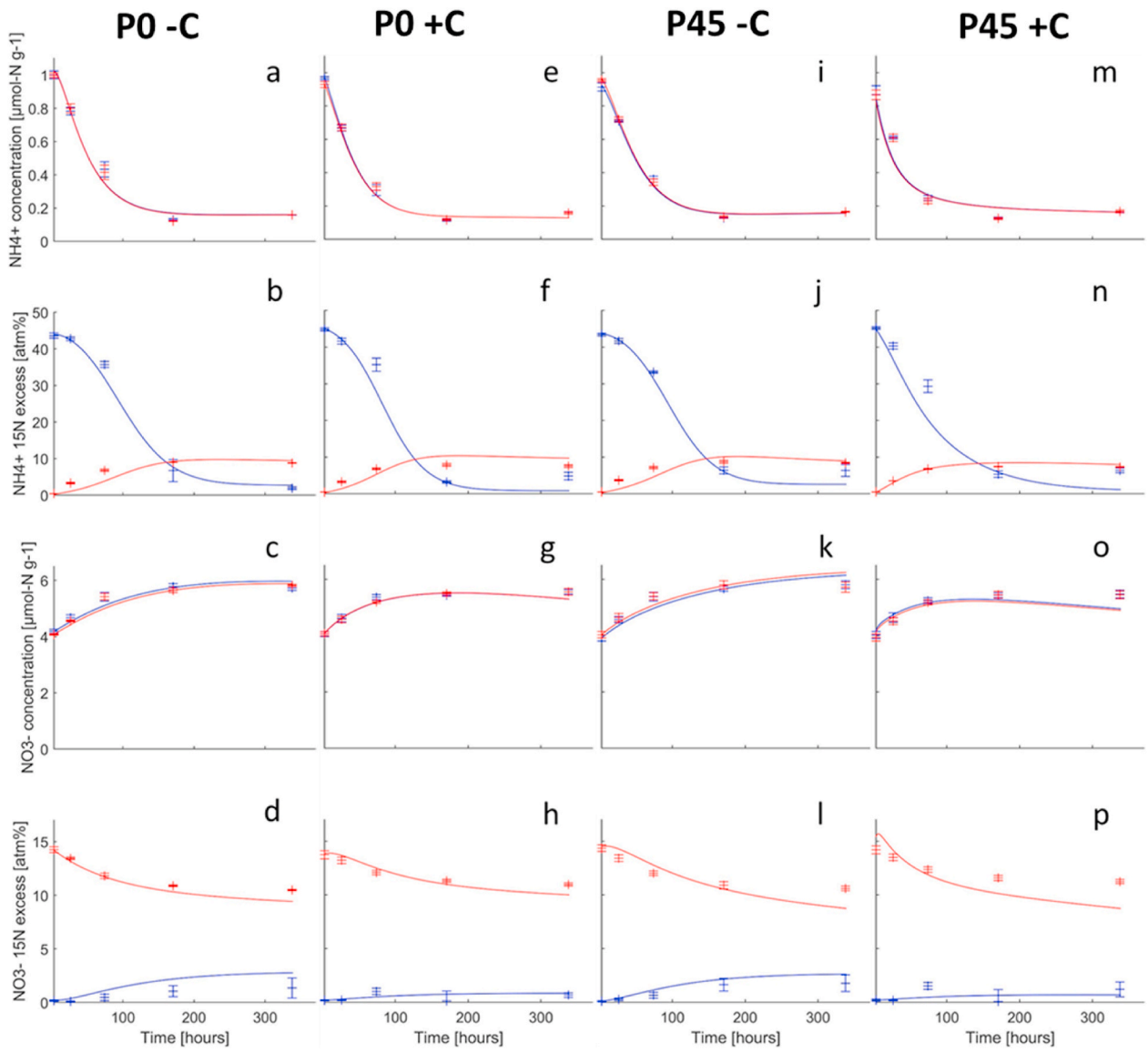

Fig. 2. Observed (data points including standard deviation) and modelled (solid lines) concentrations of $^{15}NH_4$-NO_3 (blue) and NH4-$^{15}NO_3^-$ (red) (a, c, e, g, i, k, m and o) and their respective ^{15}N enrichments (b, d, f, h, j, l, n, and p) for each treatment (P0 -C, P0 +C, P45 -C, and P45 +C). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

We observed that P45 +C stimulated the greatest N-transformation rates of all treatments (Table 1), indicating that the microorganisms involved in N-cycling were likely be co-limited by P and C, which agrees with the findings of Randall et al. (2019) in a previous study carried out on the same soils. Interestingly the production of NO_3^- was governed in all treatments by the oxidation of N_{lab} (heterotrophic nitrification) (Table 2). The O_{Nlab}/O_{N_tot} rates were highest in the P0 -/+C treatments. Although, autotrophic nitrification has been widely accepted to be the dominant form of nitrification in soils (Robertson and Groffman, 2015; Barnard et al., 2005), this research found that heterotrophic nitrification vastly outweighed autotrophic nitrification, being responsible for 98% of all nitrification. This behaviour must be soil dependent because in other studies, using the same ^{15}N-tracing approach, autotrophic nitrification was found to be dominant (Ernfors et al., 2014; Harty et al., 2017; Zhang et al., 2015). Although some ^{15}N tracing studies did find heterotrophic nitrification to be dominant over autotrophic (Müller et al., 2014; Liu et al., 2019), the ^{15}N *trace* tool used in this study is the first to incorporate the pathway of the oxidation of labile organic N directly to NO_3^- (O_{Nlab}) and found it to account for the majority of heterotrophic nitrification. This shows that the main route of NO_3^- production in this soil occurs via the oxidation of labile organic N, which has not been included in *Ntrace* so far because oxidation of N_{rec} as a heterotrophic transformation pathway was sufficient to explain all previously published data (Müller et al., 2014). Using the Kirkham-Bartholomew pool dilution approach, it is not possible to distinguish NO_3^- production into pool-specific pathways and it is likely that this pathway for NO_3^- has been overlooked. Therefore, heterotrophic nitrification via oxidation of recalcitrant but also of labile organic N is most likely more widespread than previously thought.

Autotrophic nitrification has been found to be dominant in studies conducted on C-rich soils in similar geographical regions (Harty et al., 2017; Rex et al., 2018; Murphy et al., 2015). In contrast, comparable studies where autotrophic nitrification was inhibited showed similar levels to the (uninhibited) autotrophic nitrification found in this study (Harty et al., 2017; Ernfors et al., 2014). The rates of heterotrophic nitrification from the recalcitrant pool (O_{Nrec}) were similar to those observed by Ernfors et al. (2014) and Harty et al. (2017). However, all autotrophic nitrification rates (O_{NH4}) were almost 2 orders of magnitude

lower (0.06 μg N g^{-1} soil d^{-1}, compared to the uninhibited rates of 10.1 (Ernfors et al., 2014 and 11.64 μg N g^{-1} soil d^{-1} (Harty et al., 2017)). Heterotrophic nitrification of organic N consumes reductant (in contrast to autotrophic nitrification) and may serve as a strategy to dissipate excess reductant generated during growth under conditions where reduced C sources predominate (Wehrfritz et al., 1993). In addition, autotrophic nitrifiers derive their C from CO_2 or carbonates, rather than from organic matter and therefore may not be responsive to the addition of glucose (Robertson and Kuenen, 1990b). Whereas heterotrophs have the capacity to oxidise NH_4^+ but it is not necessarily coupled to energy conservation, but rather has been linked to re-oxidation of NAD(P)H under; hypoxic conditions in bacteria (Robertson and Kuenen, 1990a), endogenous respiration in fungi (van Goole and Schmidt, 1973), and a form of defence against competing organisms in soil (Robertson and Groffman, 2015; Stein, 2011; Verstraete, 1975). The low NH_4^+ concentrations measured throughout this experiment could be representative of nitrification dominating over denitrification in the site from which these soils were taken, especially as the NO_3^- levels remained very high, which is typical in agricultural systems where nitrification is dominant (Subbarao et al., 2013; Raven et al., 1992).

The variation between these N-transformations highlights that there are different microorganisms responding to the added treatments but also that they have different affinities to the type of N they transform. Recalcitrant N was the dominant form of N being transformed to and from NH_4^+ whereas labile N was the dominant form of N being transformed (albeit at much lower rates) to and from NO_3^-. Immobilisation of NH_4^+ was the fastest N-transformation rate observed in this study. Although such fast adsorption is normally attributed to abiotic immobilisation, it has been demonstrated that biotic processes can often dominate NH_4^+ immobilisation in particular (Fitzhugh et al., 2003). The dominance of biotic immobilisation of NH_4^+ here is attributed to a fast response from the microbial community to the highly labile carbon (glucose) that was added. Comparable incubation studies saw similar results in that net N immobilisation was greater in soils incorporated with glucose than with more complex C compounds such as humic acid and that total C is positively correlated to N immobilisation (Magill and Aber, 2000; Accoe et al., 2004). Other studies have demonstrated that the composition of soil C plays different roles in regulating N cycling, through effects on microbial biomass and community composition (Booth et al., 2005; Barrett et al., 2002). For instance, it has been shown that highly labile compounds increase biotic N immobilisation through stimulating microbial growth and subsequent microbial N demand for metabolism (Cheng et al., 2017). In contrast, soils with high recalcitrant organic compounds can facilitate the incorporation of inorganic N Into soil organic matter (SOM) through abiotic reactions (Chen et al., 2014). This demonstrates that C composition and availability rather than its concentration most likely has a more important effect on whether abiotic or biotic N immobilisation occurs (Barrett et al., 2002; Moritsuka et al., 2004).

All microorganisms need N to assemble necessary cellular constituents. Fungi have been suggested to prefer less mobile NH_4^+ due to their filamentous growth, whereas oppositely, bacteria have a greater utilisation potential for NO_3^- than fungi, especially in the absence of exogenous organic C (Cao et al., 2020). This supports the suggestion by Cao et al. (2020) that fungi are responsible for the fast NH_4^+ immobilisation occurring in this incubation in response to glucose addition (Nottingham et al., 2018). In further support of this theory, the lifespan and functionality of AMF, which comprise a large proportion of the total soil fungal community (Chen et al., 2019), have been shown to be uncoupled from their hosts' plant lifespan, indicating that extraradical hyphae could have survived the disruption to their integrity during the soil sampling and sieving process (Pepe et al., 2018). In light of this, and in addition to AMF being able to outcompete other nitrifiers for NH_4^+ and other nutrients (Storer et al., 2018), fungi could be responsible for the majority of N-transformations occurring in these soils. This theory is in line with research such as Zhu et al. (2015) who showed that heterotrophic nitrification was more dominant than autotrophic nitrification in a subtropical forest soil of China, and that it was dominated by fungi rather than bacteria.

Additionally, the higher rates of heterotrophic nitrification at the P45 compared to the P0 treatments when originating from the recalcitrant pool could be attributed to a greater bacterial population present in high-P soils. This is further supported by findings from the same site, of increased abundance of bacterial phosphatase metabolising (*PhoD)* genes in the high-P soils compared to low-P soils (Randall et al., 2019). When P is added, especially in combination with other nutrients, bacteria can out-compete fungi and become dominant (Nottingham et al., 2018). Bacteria could be responding to a priming effect from the added C and N, where this addition of exogenous substrate accelerates their activity (Hicks et al., 2019). The addition of glucose in alleviating microbial limitations to such an extent that previously inaccessible soil nutrient reserves are mobilised has been observed in another similar Irish grassland soil (Murphy et al., 2015). The soils used in this experiment originate from a long-term, ungrazed, open system, where there has been continuous carbon removal via harvesting and very little carbon return to the plots (Sheil et al., 2016; Massey et al., 2016). Due to this, the microbial community has adapted to a low-energy environment (Randall et al., 2019), specialised in sourcing their carbon requirements from recalcitrant forms. This promotes conditions more sustainable for heterotrophs, which have been found to be superior at mobilising nutrient-poor organic matter (Booth et al., 2005; Paterson et al., 2008). Future experiments should consider adding C substrates across a range of labilities (glucose to glucans to cellulose/lignin) as well as progressing further microcosm studies to include plants that would expect to further strengthen the findings of this research. Although this incubation was conducted in the absence of plants and therefore could not account for plant-soil interactions and associated effects on N-cycling, the similar patterns observed both at a field scale and here reinforce the importance of the relationship between these three critical nutrients and their influence on N cycling.

5. Conclusions

The results of this study have supported the hypothesised P-effect on N-cycling in soils and the varying impact of this between the microbial immobilisation of recalcitrant and labile nutrients. The findings of this experiment reinforce this importance and show a key role from oxidation of labile organic N to NO_3^-. This research identifies that NO_3^- production can occur in at least three clearly identifiable pathways which has implications on management as the turnover of organic N be it labile or recalcitrant can be responsible for NO_3^- build-up in soils. This is further evidence that management must be tailored to individual systems to apply nutrients in their optimum relative abundance which ensures maximum uptake with minimum build-up and loss. It also highlights that if the new O_{Nlab} pathway of NO_3^- production outlined in this research is occurring, despite following a similar pathway from NH_4^+ to organic N and then to NO_3^-, it may not be inhibited by nitrification inhibitors and therefore requires different management options to avoid large N losses via leaching and gaseous emissions. This is particularly important for high organic matter soils such as permanent temperate grasslands, where these various organic N pools with differing metabolic abilities control the rates of N turnover in these soils. This pathway of labile N oxidation to NO_3^- and its potential ability to dominate nitrification under certain conditions aids in the characterisation of soil N cycling. This study provides further insight into the processes governing N losses and particularly highlights the influence of C and P availability on soil N cycling. Further studies investigating microbial communities' responses to their associated drivers and nutrient requirements will provide a clearer perspective and understanding of the N cycle. Determination of gross N dynamics is vital to gain a holistic understanding of nutrient turnover in soils, which is in many parts not fully understood due to the large range of influencing and interacting factors that must be

considered simultaneously. Elucidation of the intricacies and organisation of such processes allow for management efficiency to be maximised and nutrient loss to be reduced.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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