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Linking long-term soil phosphorus management to microbial communities involved in nitrogen reactions

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Abstract

The influence of soil phosphorous (P) content on the N-cycling communities and subsequent effects on N_2O emissions remains unclear. Two laboratory incubation experiments were conducted on soils collected from a long-term (est. 1995) P-addition field trial sampled in summer 2018 and winter 2019. Incubations were treated with a typical field amendment rate of N as well as a C-amendment to stimulate microbial activity. Throughout both incubations, soil subsamples were collected prior to fertiliser amendment and then throughout the incubations, to quantify the abundance of bacteria (16S *rRNA*), fungi (ITS) and *Thaumarcheota* (16S *rRNA*) as well as functional guilds of genes involved in nitrification (bacterial and archaeal *amoA,* and comammox) and denitrification (*nirS, nirK, nosZ* clade I and II) using quantitative PCR (qPCR). We also evaluated the correlations between each gene abundance and the associated N_2O emissions depending on P-treatments. Our results show that long-term P-application influenced N-cycling genes abundance differently. Except for comammox, overall nitrifiers' genes were most abundant in low P while the opposite trend was found for denitrifiers' genes. C and N-amendments strongly influenced the abundance of most genes with changes observed as soon as 24 h after application. ITS was the only gene correlated to N_2O emissions in the low P-soils while microbes were mostly correlated to emissions in high P, suggesting possible changes in the organisms involved in N_2O production depending on soil P-content. This study highlights the importance of long-term P addition on shaping the microbial community function which in turn stimulates a direct impact on the subsequent N emissions.

Keywords Functional genes · Nitrifiers · Denitrifiers · Fungi · qPCR · Phosphorous · Nitrous oxide

Introduction

Nitrogen (N) and phosphorus (P) are limiting nutrients in most natural systems and the major constituents of agrochemical fertilisers (Guignard et al. 2017). Both N and P underpin photosynthetic processes, cell growth, metabolism and protein synthesis (Chapin et al. 2011). However, their natural sources and rates of supply are very different, with N being ubiquitous in the atmosphere, while P is considered a finite resource derived primarily from rock weathering that cannot be readily replenished (Schoumans 2015). Plants require more N per unit biomass than any other nutrient, as such, N-fertiliser is increasingly used in agriculture to meet the food demand from a rapidly growing global population (Stark and Richards 2008). However, N losses resulting from excess N application can lead to environmental issues such as nitrate (NO_3^-) leaching, ammonia (NH_3) and nitrous oxide (N_2O) emissions — the latter of which is a greenhouse gas (GHG) and one of the most potent ozone-depleting agents in the stratosphere (Zhang et al. 2015). Agriculture is responsible for most of these emissions, accounting for 60% globally and over 90% of Irish N_2O (Duffy et al. 2018). In general, agricultural N-inputs via fertilisers and/or animal excreta are extremely inefficient with vast amounts of N

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being lost to the atmosphere or via leaching, and only 17% of added-N finally being consumed by humans (Reay et al. 2012). Due to these losses within agricultural systems, there is often more N fertiliser applied than is required by plants, to compensate for that which will be lost before crop uptake or stored in soil organic matter (SOM) (Sylvester-Bradley 1993).

It is well established that soil biota are drivers of N-cycling and therefore vital to crop nutrient supply, but are concurrently also a major source of atmospheric N_2O emissions (Butterbach-Bahl et al. 2013). The N-cycle can be considered to start and finish with inert di-nitrogen (N_2) gas, which is fixed from the atmosphere and transformed primarily via nitrification; where organic N compounds and/or ammonium (NH_4) are oxidised to NO_3^- which is then reduced during denitrification to gas N compounds such as N_2O or N_2 (Butterbach-Bahl et al. 2013). However, some organisms do not fully complete this cycle. For example, approximately a third of characterised denitrifying bacteria (Philippot et al. 2011) and most fungi have been shown to lack the *nosZ* gene which completes the final reduction of N_2O to N_2, making them a key contributor to soil N_2O emissions (Maeda et al. 2015). As such, N-cycling microbes are potential mitigators to GHG emissions when able to fully complete the reduction of N_2O to N_2 (Hirsch and Mauchline 2015).

Frequently the N and P cycles have been considered separately, partly because of the relative ease of tracing N cycles compared with P and due to the fact that they are frequently linked in stoichiometric feedback loops, their full interactions are often not accounted for (Gruner et al. 2008; Randall et al. 2019; Sterner 1990). At the organism level, this nutrient availability and relative abundance is known to have powerful influences on functional traits and growth rates, but we are only just beginning to understand this at the genomic level (Guignard et al. 2017). The fluxes, feedbacks and availability of N and P fundamentally impact biota at all levels from genes to genomes to ecosystems and ultimately ecosystem processes (Guignard et al. 2017). The way in which these cycles interact with each other, especially at the lowest levels of biological organisation is crucial in order to understand how nutrient management on agricultural soils should be optimised to improve nutrient use efficiency (NUE) and reduce GHG emissions.

The relative availability of soil P to other key nutrients such as C and N, has been found to have contrasting effects on soil N_2O emissions. This depends on whether P application stimulates N uptake by alleviating nutrient limitation, increasing immobilisation and thus reducing N_2O (Mori et al. 2014), or when added-P stimulates respiration further promoting the development of anaerobic conditions, which enhance denitrification and N_2O emissions (Mehnaz et al. 2018; Mori et al., 2013). Whether or not N loss or N uptake occurs can be explained by CNP stoichiometry and nutrient limitation relief to nitrifiers and denitrifiers, which explains why similar studies observe conflicting results depending on the soil's nutrient status. There has been very little research carried out on long-term (> 20 years) P fertilisation regimes (Chen et al. 2019; Korevaar and Geerts 2015) with none relating such P management to N-cycling microbial communities and subsequent N_2O emissions.

As the nutrient requirements of the soil microbial community can vary dramatically between species, changes in the ratios between available nutrients have the potential to alter the microbial community composition and function (Blagodatskiy et al. 2008). The effect of P addition has been widely reported for bacteria and fungi. Mycorrhizal fungi are known to be associated with decreasing P-availability (Antunes et al. 2012; Bolan 1991), while bacteria have been reported to be more constrained by nutrient stoichiometry (Nottingham et al. 2018). However, little is known on how such shifts in nutrient availability may be related to specific changes in the underlying soil N-cycling communities, which is crucial to understand the potential feedback on N_2O emissions. Several studies have reported the dual application of N and P to suppress nitrification (Ning et al. 2021), and to reduce nitrifier abundance (Tang et al. 2016; Wei et al. 2017). Others found that; increasing P-concentration reduced N_2O emissions and was due to reduced N_2O yield by heterotrophic denitrification (Jia et al. 2013), the dual application of C and P increased microbial biomass P (Xu et al. 2020b), and mixed application of N and manure increased *nosZ* clade II abundance and reduced fungal dominance (Xu et al. 2020a). This highlights that the abundance of nutrients relative to one another is often the most important factor in net primary productivity (NPP), stimulating communities which can complete the N-cycle and that such conditions required to achieve this are highly dynamic (Elser et al. 2007; Vitousek et al. 2010).

Here, we studied the effect of P on N-cycling genes' abundance in soils from a long term (approximately 25 years) P-addition field trial. Previous research based on this field trial demonstrated that N_2O emissions from low P soils were nearly 70 times those from high P soils (O'Neill et al. 2020). Another study from this field trial incorporated a ^{15}N isotope label and showed (1) that increasing soil P stimulated N-mineralisation and immobilisation turnover (MIT) and (2) that there was an interactive effect of soil P with soil C and N on N-transformations (O'Neill et al. 2021). Uncertainties remain as to the response of specific microbial communities involved in N-cycling to P-addition. In light of this research gap, our aim was to evaluate the influence of long-term P-addition on the abundance of N-cycling functional genes and whether such effects were associated with the resulting N_2O emissions. To do so, we conducted two incubation experiments to quantify the abundance of

functional N-cycling genes from soils collected from this long-term grassland P-trial. We also determined whether C and N amendments influenced the dynamic of the gene abundance over a period of ten days in low or high P-soils. We related this to the measured N_2O emissions following C- and N-fertiliser addition based on the hypotheses that; (1) The abundance of functional communities involved in N-cycling would differ according to long-term P management, with higher abundance expected in high-P soils, (2) the abundance of N-cycling communities would respond to N-addition, and that this response would differ between P-levels, and (3) the abundance of N-cycling microbial communities would be associated with the N_2O emissions.

Materials and methods

Site description

All soil samples were taken from a long-term, un-grazed, grassland phosphorus trial established on a grass sward dominated by *Lolium perenne* (perennial ryegrass) in 1995 at Johnstown Castle, County Wexford, Ireland (52°17′55″N, 6°29′47″W). Johnstown Castle has a temperate climate with monthly rainfall and temperature averaging 75.45 mm and 10.6 °C over the past 30 years (Met.Eireann 1981–2010). The site is a fine loamy textured soil, classified as a moderately drained brown earth (Sheil et al. 2016). Plots received one of two P application rates, 0 (low P), and 45 (high P) kg P ha^{-1} $year^{-1}$ of 16% superphosphate as a treatment, applied once in February of each year. 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).

Soil sampling and incubation conditions

Soils were collected from a long-term P-trial on two occasions: in August 2018 (incubation 1) and February 2019 (incubation 2). Sample collection and incubations are described in O'Neill et al. (2020) and O'Neill et al. (2021) but briefly, three composite soil samples were collected with an auger to a depth of 10 cm from high P and low P plots arranged in a randomised field block design. Soil samples of 100 g dry weight were incubated in glass Kilner jars under the conditions of 15 °C, 70% humidity to represent mean annual Irish conditions as described in O'Neill et al. (2020) for incubation 1 and O'Neill et al. (2021) for incubation 2. On day 0, prior to gas sampling, both incubations received total N applied at a field equivalent rate of 40 kg N ha^{-1} and glucose (C-source) applied at a rate of 0.1 mg C g^{-1} (Girkin et al. 2018; Grayston and Campbell 1996). N_2O emissions were measured approximately three hours after treatment application on day 0 and continued throughout the course of both incubations on days 0, 1, 2, 3, 4, 7, and 10 and on days 0, 1, 3, 7, and 9 for incubation 1 and 2 respectively. Fluxes were calculated assuming the linear accumulation of the headspace gases and according to the ideal gas law (de Klein and Harvey 2012). During the course of these incubations, subsamples of the soils were collected on the same days as N_2O measurement and stored at − 80 °C to determine the associated abundance of fungi, bacteria, archaea and functional N-cycling genes.

Soil analyses

Exchangeable ammonium-N (NH_4^+-N) (mg kg^{-1}) and nitrate–N (NO_3^--N) (mg kg^{-1}) were determined for both incubations via 2 M potassium chloride (KCl) extraction (Table S5). In incubation 1, mineral N was extracted before treatment application (Day 0), on the day of the N_2O emissions peak (Day 1), and at the end (Day 10) of the incubation as described in O'Neill et al. (2020). In incubation 2, extractions took place on 6 occasions, prior to treatment addition and on days 0, 1, 3, 7, and 9 throughout the incubation as described by (Müller et al. 2014; O'Neill et al. 2021). Physicochemical properties of the site were previously characterised using subsamples collected from within each plot (Table S1) (O'Neill et al. 2020).

DNA extraction and quantitative-PCR

DNA extractions were undertaken on soil subsamples from both incubations: subsamples from incubation 1 were taken prior to fertiliser application (day 0), 24 h (day 1) after treatment application, and on the final day (day 10) of the incubation, subsamples from incubation 2 were taken prior to fertiliser application (Day 0) and then on days 1, 3, 7, 9. Total nucleic acids were extracted from the frozen soil samples using DNeasy Powersoil DNA isolation kit (Qiagen, Ireland) according to the manufacturer's instructions. Briefly, 0.25 g soil was weighed into the bead beating tube and 60-µl solution c1 was added. The mixture was bead- beaten for 15 s at 5 m/s using the Fastprep-24 (MP Biomedicals, Ireland). DNA was eluted in 100-µl elution buffer and frozen at − 80 °C until further analysis. DNA was quantified using the dsDNA BR Assay Kit Qubit fluorometer (Invitrogen Qubit 4, Thermo Fisher Scientific).

To evaluate the abundance of the overall communities and of genes involved in N pathways, quantitative polymerase chain-reaction (qPCR) targeting specific genes or genetic regions was used with the CFX384 Touch™ Real-Time PCR Detection System (Biorad, Ireland). The archaea, bacterial

and fungal communities were targeted via the phylogenetic *16S rRNA* bacterial, *16S rRNA Thaumarchaeota* and *ITS* gene primers, respectively. The functional N-cycling genes targeted were *nirS*, *nirK*, *nosZ* clade I, *nosZ* clade II targeting the denitrification pathway and the *amoA* gene for nitrifying archaea (AOA), bacteria (AOB) and Nitrospira (comammox). Prior to quantifying the genes of interest each sample was tested for inhibitors. Briefly, 1×10^8 gene copies of plasmid DNA (pGEM®-T) were spiked into each sample and the plasmid concentration was quantified using T7F and M13R plasmid specific primers (Table S1). Samples were considered to contain inhibitors when the plasmid did not amplify to the same extent as the spike in the absence of the samples (i.e. plasmid plus water). The addition of bovine serum albumin (BSA) (200 µg/ml) (Biosciences, Ireland) removed the inhibitory effects on the amplification and this was added to each qPCR reaction mixture. Amplifications were performed in 10-µl reaction volumes consisting of 5-µl Takyon™ Low ROX SYBR® 2X MasterMix dTTP Blue Mastermix (Eurogentec, Europe), reverse and forward primers (concentrations in Table S1), 2 ng template DNA and molecular biology grade water. qPCR standards for each molecular target were obtained using a 1 in 10 serial dilution of appropriate standard over an 8 point range from 10^8 to 10^1 gene copied per µl. Standard curve template DNA and the no template control (negative) were amplified in triplicate (and did not amplify above the level of detection), in the same plate as the environmental samples. The efficiency of the qPCR was above 80% for all genes with the majority of runs over 90%. Full details and amplification conditions are provided in the Supplementary information (Table S1 and S2).

Statistical analyses

All analyses were conducted separately for incubation 1 and 2. Two-way Analysis Of Variance (ANOVA) was used to test the effect of P-treatment and time after N- and C-fertilisation on N-cycling gene abundance. If significant effects were detected, a Tukey post-hoc (HSD) test was used to reveal the significance of the differences between class pairs (Blaud et al. 2018). Prior analyses, all data was log transformed, except for N_2O fluxes from incubation 2, which had to be normalised using a box-cox transformation, to satisfy the conditions of the normality and homoscedasticity of the variances. To evaluate the relationships between gene abundance and associated daily N_2O emissions at each P level, we performed separate Pearson correlation tests for each gene and N_2O fluxes within each P level using 'corr. test()' in "psych" package in RStudio. All statistical analyses were performed using R v4.0.3 (R- core Team 2020) and a significance level of $p < 0.05$ was used throughout, significances denoted as; (. $= p < 0.1$, * $= p < 0.05$, ** $= p < 0.01$, *and* *** $= p < 0.001$). All gene copy numbers are presented on a per gram of dry soil basis (Figs. 1, 2, and 3).

Results

Gene abundance

Overall, incubation 1 (Fig. 1(a–c), Fig. 2(h–j), Fig. 3(n–q)), showed a higher average abundance of most genes, across both P-levels, by at least one order of magnitude, compared to incubation 2 (Fig. 1(d–f), Fig. 2(k–m), Fig. 3(r–u)). Total bacteria (*16S rRNA*) however, were largely constrained across both incubations with all copy numbers being in the 10^7 range, with the lowest abundance seen in high P incubation 2 soils (2.21×10^7gene copy number g^{-1}) and the highest observed in high P incubation 1 soils (4.67×10^7 gene copy numbers g^{-1}). Total nitrifier abundance in incubation 2 was lower by one order of magnitude in both P levels than that of incubation 1, whereas total denitrifier abundance was greater by one order of magnitude (Table S7).

Incubation 1: soils collected in August 2018

The effects of P on the abundance of bacteria, crenarchaea and fungi differed. The abundance of crenarchaeal *16S rRNA* was significantly higher in P45, and we found no evidence that this effect varied depending on the time prior or after application of fertiliser (Fig. 1b, Interaction P*Time in Table S8). A similar trend was observed in bacterial *16S rRNA*, but the effect of P-level was only marginally non-significant (Fig. 1a, Table S8). For *ITS,* the effects of P varied depending on the sampling time. Prior to fertilisation at Day 0, *ITS* was more abundant in P45, while after 24 h no significant differences were observed between the two P-levels. At Day 10, we observed the same trend as before fertilisation (Day 0) where high P showed a more abundant *ITS* genes community than low P (Fig. 1c, Table S8). In addition, only *ITS* abundance showed positive correlation with daily N_2O flux in the low P soils, while both *16S rRNA* bacteria and crenarchaea showed a positive correlation with N_2O flux in the high P soils (Table 1).

We found differing trends in nitrifying gene abundance. The abundance of AOB (Fig. 2i) was significantly influenced by long-term P-levels, being more abundant in low P by 30% on average. Overall, comammox abundance was on average 1.5 times higher in high P soils (Fig. 2h), although there was no evidence to suggest this was significant. These results were observed regardless of the time of sampling as we found no evidence of an interaction between P-levels and time after fertiliser application (Fig. 2h, i, Table S8). These effects were not observed for AOA, as this gene abundance was not influenced by P-levels and did not significantly vary

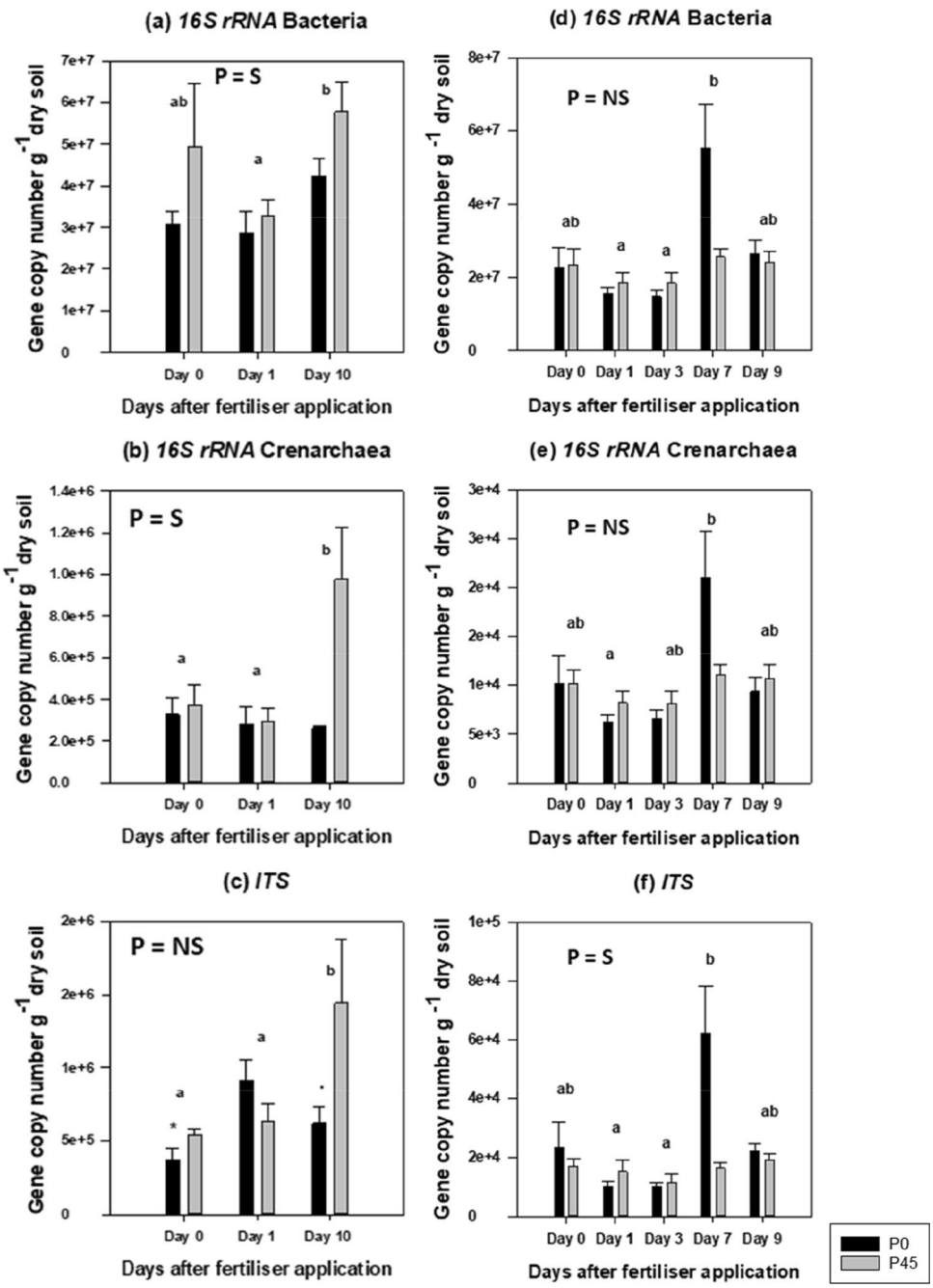

Fig. 1 Phylogenetic Gene Abundance according to the P-level and the time of sampling prior and after fertiliser application from incubation 1 (left (means +/− SEM (n = 6))) and incubation 2 (right (means +/− SEM (n = 8))). Letters denote significant difference in total gene abundance between time points for both P levels and * denote significant interactions between P level and time. Fertiliser was applied on Day 0 after soil sampling, and N_2O peaks were observed on Day 1 for both incubations. Overall significance between total gene abundance and P level shown as P = S, not significant with P level shown as P = NS

over time (Fig. 2j). Out of the nitrifiers, AOA and comammox both were positively correlated with N_2O flux (Table 1).

Except for *nosZ* clade I, all other denitrifier genes were influenced by the P-levels (Fig. 3n–q, Table S8). The abundance of *nirS* and *nosZ* clade II were on average 50% and 43% higher in high P than in low P, respectively (Table S7). We found no evidence that their abundance varied according to the sampling time (Table S8). In contrast, for *nirK*, the effect of the P-levels varied significantly according to sampling time. Prior to fertilisation at Day 0, gene abundance was significantly higher at P45, and such significant differences were not detected at Day 1 or Day 10 (Fig. 3q). In addition, the relative abundance of N_2O-producers *nirS* and *nirK* to the N_2O-reducers *nosZ* clade I and II was influenced by P-levels, being on average 25% higher in high P than low P (Table S7). Out of the denitrifiers, only *nirK* was not positively correlated with N_2O flux. The *nir:nosZ* (total *nirS* and *nirK*: total *nosZ* clade I and II) ratio was significantly higher in high P soils in incubation 1, but did not show any significance with time or N_2O flux (Table S8; Fig S1, Table 1).

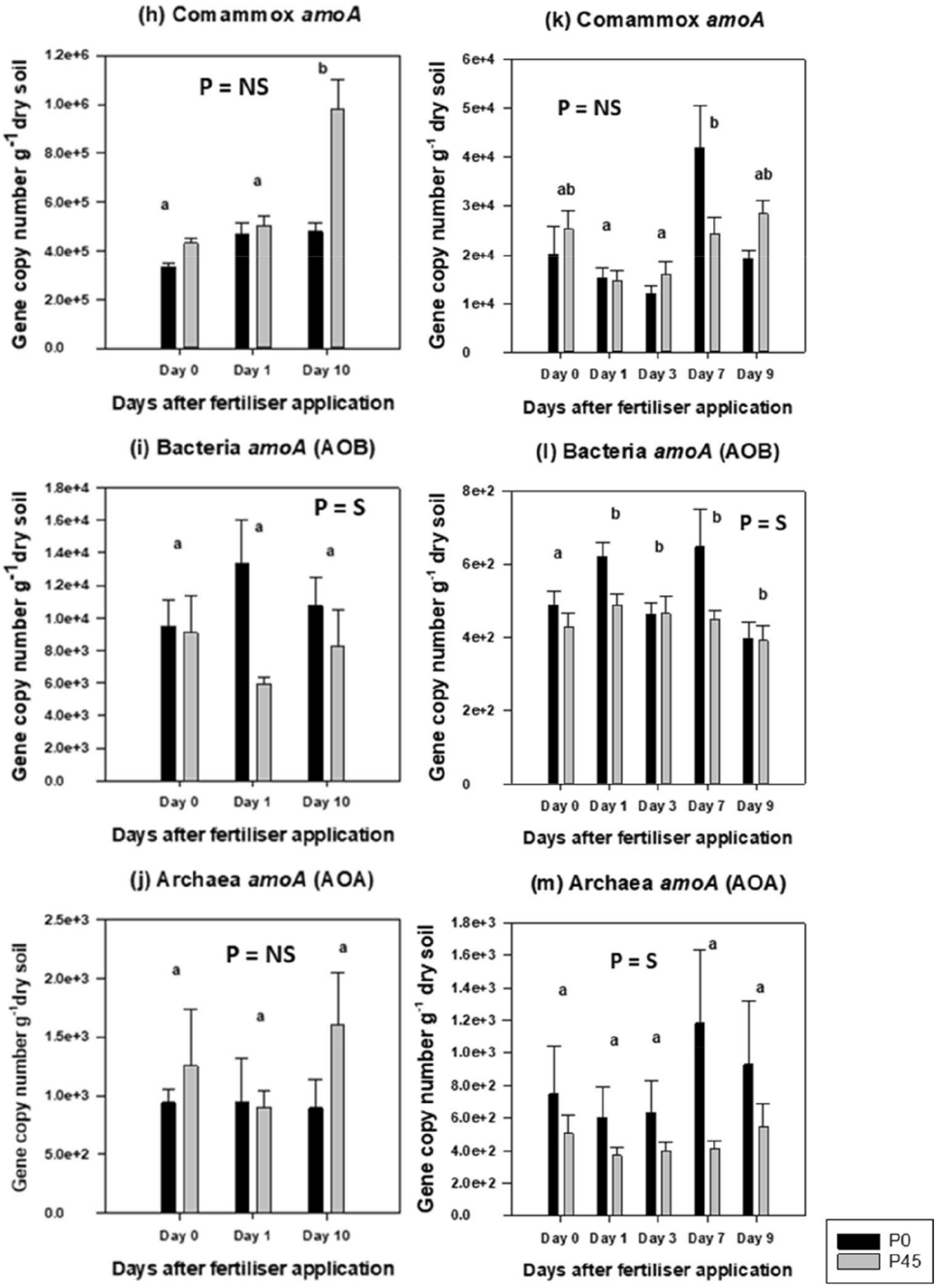

Fig. 2 Nitrifying Genes Abundance according to the P-level and the time of sampling prior and after fertiliser application from incubation 1 (left (means +/− SEM (n = 6))) and incubation 2 (right (means +/− SEM (n = 8))). Letters denote significant difference in total gene abundance between time points for both P levels and * denote significant interactions between P level and time. Fertiliser was applied on Day 0 after soil sampling, and N_2O peaks were observed on Day 1 for both incubations. Overall significance between total gene abundance and P level shown as P = S, not significant with P level shown as P = NS

Incubation 2: soils collected in February 2019

When evaluating bacterial, crenarchaeal and fungal genes, only the abundance of ITS was significantly affected by P level, being consistently higher in low P. We found no evidence that this effect varied depending on the time prior or after application of fertiliser (Fig. 1f, Interaction P*Time in Table S9). No significant effects of P level on *16S rRNA* crenarchaea or bacteria were observed (Fig. 1d, e, Table S9). All structural gene abundance significantly increased over time with *16S rRNA* bacteria showing a marginally significant ($p < 0.1$) peak in low P on Day 7, as well as ITS showing a strongly significant peak ($p < 0.01$) again in low P at this same time. Although *16S rRNA* crenarchaea also shows a visual peak in abundance on Day 7, we found no evidence to suggest this was significant (Fig. 1e). On Day 10, we observed the trends in all phylogenetic gene abundance returning to their prior fertilisation levels (Day 0) (Fig. 1d, e, f). In addition, none showed positive correlation with N_2O flux (Table 1).

Among the nitrifiers, the abundance of both AOA and AOB were significantly influenced by long term P-levels,

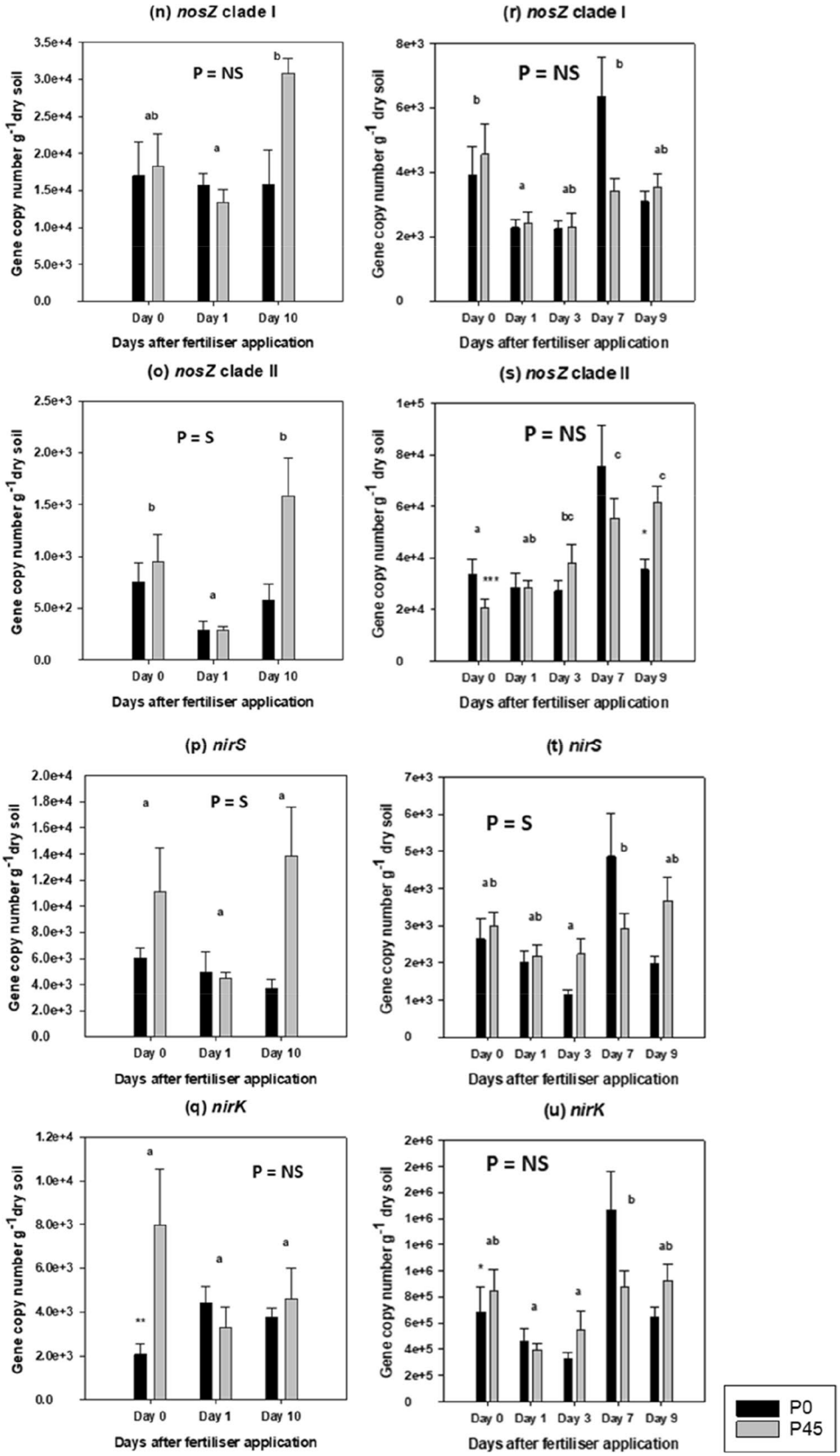

Fig. 3 Denitrifying Genes Abundance according to the P-level and the time of sampling prior and after fertiliser application from Incubation i (left (means +/− SEM (n = 6))) and incubation 2 (right (means +/− SEM (n = 8))). Letters denote significant difference in total gene abundance between time points for both P levels and * denote significant interactions between P level and time). Fertiliser was applied on Day 0 after soil sampling, and N_2O peaks were observed on Day 1 for both incubations. Overall significance between total gene abundance and P level shown as P = S, not significant with P level shown as P = NS

Table 1 Relationship between functional gene abundance and N_2O emissions for Incubation 1 (n = 9) and Incubation 2 (n = 40). This table reports pairwise correlations based on Pearson's test and shows the correlation coefficient (r), the confidence interval (lower and upper r) and the p-value (bold p-values are significant)

Experiment	Gene	Low P level				High P level			
			Confidence interval				Confidence interval		
		r	lower r	upper r	P-value	r	lower r	upper r	P-value
Incubation 1	***16S rRNA* bacteria**	−0.43	−0.85	0.32	0.24	**0.73**	**0.13**	**0.94**	**0.02**
	***16S rRNA* crenarchaea**	−0.09	−0.71	0.61	0.81	**0.74**	**0.14**	**0.94**	**0.02**
	ITS	**0.74**	**0.16**	**0.94**	**0.02**	0.42	−0.34	0.85	0.26
	***nosZ* clade II**	−0.63	−0.91	0.07	0.07	**0.76**	**0.18**	**0.95**	**0.02**
	***nosZ* clade I**	−0.06	−0.7	0.63	0.87	**0.67**	**0.01**	**0.92**	**0.05**
	nirS	−0.06	−0.7	0.63	0.88	**0.75**	**0.18**	**0.94**	**0.02**
	nirK	0.51	−0.23	0.88	0.16	0.21	−0.53	0.77	0.59
	nir:nosZ	0.02	−0.66	0.67	0.97	0.16	−0.57	0.74	0.68
	COMAMMOX	0.23	−0.51	0.77	0.56	**0.66**	**−0.01**	**0.92**	**0.05**
	AOB	0.41	−0.35	0.84	0.28	0.56	−0.16	0.89	0.12
	AOA	0	−0.67	0.66	1	**0.73**	**0.14**	**0.94**	**0.02**
Incubation 2	***16S rRNA* bacteria**	−0.1	−0.4	0.22	0.54	−0.08	−0.38	0.24	0.64
	***16S rRNA* crenarchaea**	−0.01	−0.32	0.3	0.95	0.06	−0.26	0.37	0.71
	ITS	−0.06	−0.36	0.26	0.73	0.03	−0.29	0.34	0.87
	***nosZ* clade II**	−0.14	−0.44	0.18	0.38	**−0.45**	**−0.67**	**−0.17**	**0**
	***nosZ* clade I**	0.03	−0.29	0.34	0.87	0.28	−0.03	0.55	0.08
	nirS	0.02	−0.3	0.33	0.92	0.02	−0.29	0.33	0.88
	nirK	−0.03	−0.34	0.29	0.87	0.02	−0.3	0.33	0.92
	nir:nosZ	0.25	−0.06	0.52	0.12	**0.58**	**0.33**	**0.75**	**0**
	COMAMMOX	−0.07	−0.37	0.25	0.68	0.11	−0.21	0.41	0.49
	AOB	−0.01	−0.32	0.3	0.93	0.07	−0.25	0.37	0.67
	AOA	−0.01	−0.32	0.31	0.97	−0.01	−0.32	0.31	0.97

being consistently higher in low P soils than in high P soils by 46% and 15% respectively (Table S7). This effect was consistent regardless of the time prior or after fertiliser application (Fig. 2l, m Table S9). These effects of P were not observed for comammox, as this gene abundance was not influenced by P-levels nor was there any interaction with time (Fig. 2k; Table S9). There were no significant correlations between any nitrifier gene abundance and N_2O flux (Table 1).

The only denitrifier genes which showed a response to P were *nosZ* clade II and *nirS*. There was a significant interaction between P and time for *nosZ* clade II (Fig. 3s), with gene abundance being significantly higher ($p < 0.001$) in low P soils prior to fertilisation on Day 0 but increased by up to 66% ($p < 0.05$) in high P soils by the end of the incubation on Day 10. The abundance of *nirS* was significantly higher (by on average approximately 10%) in high P and we found no evidence that this trend varied before and after fertiliser application (Fig. 3t, Table S7 & S9). Regarding the relationship between N_2O emissions and genes abundance, only *nosZ* clade II showed a significant correlation to N_2O flux in the high P soils (Table 1). The relative abundance of N_2O-producers *nirS* and *nirK* to the N_2O-reducers *nosZ* clade I and II (*nir:nosZ* ratio) showed a positive correlation with N_2O at high P (Table 1). There was also an interaction between P level and time, with this ratio being highest in high P soils prior to fertilisation, but showed no significant difference between P levels after this time (Table S9; Fig. S1).

Discussion

Relating soil P to N-cycling genes abundance

Overall, except for comammox genes which were not influenced by P-levels, we found reduced nitrifier AOA and AOB abundance in high P soils. In both incubations, AOB was consistently more abundant in low P soils and we also found that AOA was most abundant in low P than high P in incubation 2, with no evidence supporting this effect of P in incubation 1. Similar results were observed in other P and N fertiliser experiments, suggesting that P addition inhibited the growth of AOB and retarded nitrification

(Ning et al. 2021; Tang et al. 2016; Wei et al. 2017). Such decrease in nitrifier abundance under P fertilisation was unexpected, because P-availability stimulates N-mineralisation, thus potentially stimulating ammonia oxidation. However, immobilisation was previously found to greatly outweigh mineralisation in these soils, suggesting that these N-cycling communities are still nutrient-limited despite fertiliser amendment (O'Neill et al. 2021). Due to the un-grazed and harvested management of this field trial, it has been reported to be nutrient limited (Massey 2012; Randall 2016). Under such combined key nutrient limitations of C, N and P, nitrifier abundance would be reduced and may be outcompeted by other soil organisms, such as fungi, which have been reported to dominate heterotrophic nitrification and to be superior at resource acquisition in nutrient poor environments (Boswell et al. 2007; Cassman et al. 2016; Griffiths et al. 2012; Zhu et al. 2015).

In contrast with the nitrifiers response, all denitrification genes (except for *nosZ* clade I) were in general more abundant in high P soils. Such findings suggest that at our site, N was more limiting for nitrifiers while P appeared to be more limiting for denitrifiers. This is in accordance with the drivers of nitrification and denitrification varying, such that denitrification has previously been found to be correlated to P concentration but nitrification was not (White and Reddy 2003). Similarly, other studies found that P addition increased denitrifying gene abundance and suggested this was due to P-induced shifts between soil C:P and N:P ratios which resulted in limited ammonia oxidation but enhanced P-availability for denitrification (Cui et al. 2020; Wei et al. 2017). As P is required in the smallest proportion relative to nutrients such as C and N, it is often the limiting nutrient and can inhibit microbial activity through P-deficiency or saturation due to the subsequent effect on CNP stoichiometric homeostasis (Agren et al. 2012; Chapin et al. 2011; Cleveland and Liptzin 2007).

Although *nosZ* clade II appeared to be sensitive to P-levels, *nosZ* clade I was not responsive. Others have found that available P was an important driver of *nosZ* (Wang et al. 2017). However, as the separation of *nosZ* between *nosZ* clade I and II has only recently been distinguished, uncertainty remains regarding their varying response to soil P and preferred soil conditions and habitat (Hallin et al. 2018; Jones et al. 2013). Despite this, several studies have demonstrated that, in agreement with our results, *nosZ* clade II is more responsive to environmental conditions, including soil properties and fertilisation practices (Domeignoz-Horta et al. 2016; 2018; Xu et al., 2020a; Yoon et al. 2016). Our results further support the hypothesis that, similar to *nirS* and *nirK* harbouring bacteria (Jones et al. 2013; Tang et al. 2016), the two *nosZ* also vary in their niche, with the abundance of *nosZ* clade II harbouring microorganisms possibly being favoured in high P content (Jones et al. 2013; Tang et al. 2016).

N fertiliser application, as part of the incubation studies, strongly influenced the abundance of most genes, regardless of P level, and in some cases such changes were observed as soon as 24 h after application. As N-availability is the main driver of N-cycling, elevated abundance of both nitrifying and denitrifying genes in response to N-application is well documented (Ouyang et al, 2018; Wang et al. 2018). However, such rapid response was unexpected and to our knowledge no other study evaluating gas fluxes and gene abundance simultaneously has recorded such daily changes in abundance. A similar short-term (15 day) incubation study observed that N-addition increased the abundance of the nitrifying bacteria *Nitrococcus*, but decreased that of sulphate reducing bacteria, implying superior completion from the N-cycling *Nitrococcus,* however this study only evaluated genetic abundance at the end of this incubation and not during (Craig et al. 2021). These observed changes in bacterial community composition by Craig et al. (2021) were accompanied by changes in the activity of enzymes involved in C, N, and P cycling which further supports the need to investigate these key nutrients relative to one another with regard to the drivers and mechanisms of such nutrient cycling. Other studies observed changes in the relative abundance of various bacterial communities after as soon as one week (Chowdhury et al. 2022) and after four days in nitrifying communities post N amendment (Szukics et al. 2009). Soils are extremely complex ecosystems, with one gram of soil containing up to 10^9 microbial cells (Gans et al. 2005; Roesch et al. 2007). The rapid response observed in this study is interesting as this indicates an increase in growth from the microbial communities to the added nutrients which implies a reliance on C and N. Further studies are required to identify these organisms and whether the diversity of the community is maintained of if a specific group, such as the oligotrophs, might be dominating.

Interestingly, only ITS (incubation 1), *nirK* (incubation 1 and 2) and *nosZ* clade II (incubation 2), showed a difference in abundance with initial P level prior to fertiliser application, whereas the influence of P level on other genes only became evident post fertiliser addition. This further highlights the different nutrient requirements between the microbes, suggesting that nutrient imbalance may inhibit the ability of certain microbes to further access and utilise available P (Tang et al. 2016; Xu et al. 2020a). For example, *nirK* was initially more abundant in high P soils, but decreased post N-application while this pattern was not observed for *nirS*, which was responding to a relief from N-limitation regardless of P-level. In addition, it is interesting to note that even in the high P treatment, our results show that P levels remained low according to the P classification of the soil index system (Table S1) (Teagasc 2017), possibly further

contributing in shaping the microbial communities and their response to fertilisers. This emphasises the importance of considering 'the law of the minimum' when determining nutrient management, as productivity responds to the resource in shortest supply, even if all others are present in abundance (Davidson and Howarth 2007).

Correlation between N cycling gene abundance and N_2O emissions

Results from previous work on incubation 1 soils found that cumulative N_2O was much higher in low P than in P45 (O'Neill et al. 2020). When evaluating the relationship between gene abundance and the daily emissions, we found that ITS was the only gene whose abundance was positively correlated with N_2O in the low P while in high P soils the emissions were positively correlated to the abundance of *16S rRNA* bacteria, crenarchaea, both *nosZ* clades, *nirS* and AOA. Our results may indicate that the main organisms involved in the production of N_2O may vary depending on soil P, with fungi possibly contributing to N_2O emissions in soils with low P content. Fungi are well-known contributors to N_2O emissions due to their lack of *nosZ* (Laughlin and Stevens 2002; Maeda et al. 2015; Mothapo et al. 2015; Okiobe et al. 2019), as well as being superior competitors to bacteria in nutrient poor environments (e.g. low P soils) (Antunes et al. 2012; Bolan 1991; Nottingham et al. 2018; Shigyo et al. 2019). Interestingly, others have found that the relative role of bacteria and fungi on N_2O emissions may depend on soil conditions (Chen et al. 2015). Experimental approaches using fungal or bacterial inhibitors on soils differing in soil P may confirm or contradict our observations.

In contrast, higher abundance of bacterial denitrification genes (*nirS* and *nosZ* clade II), could potentially indicate that a different N-cycling community is active in the high P soils. In addition, we detected low N_2O production under these conditions. Such functional genes are critical precursors for N_2O, *nosZ* are responsible for its reduction into the environmentally benign N_2 (Jones et al. 2013). Hence, in P45, N_2O may have been converted directly to N_2 resulting in low N_2O emissions. This is further reinforced by the fact that total *nosZ* abundance is highest in the high P soils, being between 1.4 and 2 times that of total *nir* abundance.

In incubation 2, N_2O emissions were much lower than during the first incubation and we found no differences between P-levels. When we evaluated the relationship between genes and N_2O emissions, we found no relationship in low P, but the ratio between total *nir and nosZ* was positively associated with N_2O emissions in high P soils. Despite lower activity in this incubation, N_2O-producing potential is still very high as total *nir* is over 16 times greater than total *nosZ* across both P levels, as well as total denitrifier abundance (*nir* + *nosZ*) being over 20 times that of incubation 1 (Table S7, Fig S1). This further supports our findings in incubation 1 that in high P, the microbial communities may be driving such emissions and possibly completing full denitrification, thus explaining the negligible N_2O observed. Confirming such observations would require measuring N_2 emissions which remains a technically challenging task, with much controversy over the most reliable methodology, due to high probability of background N_2 skewing results (Groffman et al. 2006).

Our results provide an avenue that calls for further exploration with additional experimental approaches to elucidate this effect of P on the relative role of fungi versus other microorganisms on N_2O emissions. Inoculation or fungal/bacterial inhibitory microcosm experiments coupled with an isotopic label has proven to be a reliable indicator that could contribute to evaluate this (Rex et al. 2018; 2019). Further investigation of the effect of P on soil microbial communities would benefit from long-term studies, ideally with field and laboratory experiments carried out together to investigate if controlled conditions reflect those of the ecosystem. This will not only identify the main drivers of N_2O emissions but also provide evidence of how P-levels may be influencing their relative contributions. Ultimately this research suggests that by sustaining a healthy nutrient balance between C, N and P, denitrifier genes which have the capacity to complete the N-cycle are better facilitated (Dendooven and Anderson 1995), which is vital for soil fertility as well as reducing N-emissions.

Interestingly, we found some differences in the responses to added C and N, as well as to the varying P levels, of the functional N-cycling genes between incubation 1 and incubation 2. Total abundance of *nir* and *nosZ* was consistently higher (across both P levels) in incubation 2, while nitrification *amoA* genes; comammox, AOB and AOA were consistently higher in the incubation 1. This contrasting abundance may be reflective of the differences between the total N_2O emissions and time of sampling, inferring that there might be a legacy effect on the community representing the seasonal conditions from which they were taken. This effect may be somewhat linked with the difference of seasonal supply of C exudate from grass roots, which could be reflected by the varying CO_2 emissions observed between these incubations (Table S6). It is known that microbial communities are highly dynamic, often undergoing complete turnovers between seasons (Jung et al. 2012; Luo et al. 2019; Schmidt et al. 2007; Shigyo et al. 2019). Soils collected in winter may have been more severely nutrient limited than the summer soils, such that microbes might be unable to access P due to co-limitation from several nutrients. Wetter, more anaerobic winter conditions might also have facilitated the greater denitrifier gene abundance (Dobbie et al. 1999). Our previous study on soils collected in winter showed that N immobilisation greatly outweighed N mineralisation (O'Neill et al.

2021), further suggesting that microbes were more severely nutrient limited than in soils collected in summer, and may have required a higher fertilisation rate to promote activity.

Conclusions

Long-term P-application influences N-cycling microbial communities and consequently the N_2O emissions evolved. Such effects of P differed according to the functional N-cycling group, with P being more limiting for denitrifiers than nitrifiers. In addition, our results show that only fungal abundance was related to N_2O emissions in low P soils while in high P soils it was rather the bacterial and archaeal N-cycling genes that were related to such emissions, suggesting that P appears to influence the relative role of fungi and other microbes in determining N_2O flux. The addition of N fertiliser exhibited further varying responses of N-cycling genes to P-level which also differed between incubations. This highlights that nutrient limitation masks the effect of P on N-cycling, and that this P effect may fluctuate throughout the seasonal changes in nutrient availability. This research shows the value which lies in maintaining healthy P concentrations relative to other key nutrients in soils as a potential way to mitigate N_2O emissions as well as promoting soil fertility and yield.

Although the effectiveness of using gene abundance as a proxy for N-cycling may be influenced by factors such as; detected genes might originate from lytic/dormant cells, different genes might have different expression and different enzymes might have different functional efficiency (Zhang et al. 2013). However, among all chemical/physical and biological indexes (e.g. pH, mineral N concentrations, water content), gene abundance has been shown to be the best predictor of soil N-cycling rates as this index integrated the information of recent environmental conditions and activity (Petersen et al. 2012). Future investigations could be directed to investigate legacy seasonal aerobic/anaerobic conditions over a range of fertiliser inputs to establish optimum levels of nutrient addition for year-round management of N_2O-reducing communities. Field studies should also supplement laboratory studies which cannot account for the in situ conditions of the field environment (Harty et al. 2017). Furthermore, although fungal hyphae have been shown to survive the disruption to their integrity during the soil sampling and sieving process (Pepe et al. 2018), the microcosm environment is a disrupted soil system which may not accurately represent field conditions and microbial community activity. The preparation of fresh soil as opposed to dry soil has been shown to preserve greater genetic abundance (Blaud et al. 2017). However, while a distinct advantage of incubations is the high level of control they allow, the main shortcomings are the lack of new inputs (Schadel et al. 2020) and due to its isolation from the soil ecosystem the microcosm may not reflect in situ conditions. These lack of inputs can further induce changes in the microbial community such that more oligotrophic microbes might be at an advantage and become more dominant (Schadel et al. 2020).

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Declarations

Competing Interests The authors declare no competing interests.

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