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Biotic and abiotic predictors of potential N_2O emissions from denitrification in Irish grasslands soils: A national-scale field study

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ABSTRACT

Large-scale information regarding nitrous oxide (N_2O) emissions is needed as an evidence base to underpin land use policy and mitigation approaches. However, the highly variable rates of denitrification make the prediction of N_2O emission demanding. Here, we evaluated the role of abiotic and biotic factors on the potential denitrification of Irish soils, in order to identify the key factors regulating potential N_2O emissions at a large scale. To do so, we collected 136 soil samples from 32 sites across Ireland, and characterised the soil physico-chemical properties, the prokaryotic and fungal community composition, the abundance of N-cycling genes and evaluated the soil potential nitrification, denitrification and end product $N_2O/(N_2O + N_2)$. We found large differences in soil potential denitrification between sites (up to 41.5 mg N_2O–N kg^{-1} soil day^{-1}) with most of the emissions released in the form of N_2O rather than N_2. Soils with highest potential nitrification rates also exhibited the highest potential denitrification rates, and similar parameters were linked to both processes. The factors most predictive of soil potential denitrification were soil physico-chemical properties and the prokaryotic community composition. Soil phosphorus content was as important for predicting potential denitrification as was pH and total nitrogen. Soil microbial community structure, rather than denitrifier abundance, was an important predictor of the potential denitrification and the end-product $N_2O/(N_2O + N_2)$. The prokaryotic community composition was more strongly associated with denitrification rates and the resulting end-products than fungal communities. Increased relative abundance of the prokaryotic phyla Actinobacteriota and Crenarchaeota, were positively correlated to complete denitrification. Altogether, these results lay the foundation for a better understanding of the key factors regulating the potential denitrification in soils and identify important properties that enhance prediction of the potential denitrification at larger scales.

1. Introduction

Nitrous oxide (N_2O) is amongst the most important greenhouse gases (GHG), contributing to around 10% of annual global warming, with a global warming potential approximately 265–298 times greater than CO_2 and an average life-time of 116 years (IPCC et al., 2021). It is also of concern with respect to its role in ozone layer depletion (Ravishankara et al., 2009). Currently, atmospheric concentrations of N_2O continue to rise, with N_2O reaching 332 ppb in 2019, an increase of 8 ppb since 2011 (IPCC et al., 2021). At a global scale, the primary human activity responsible for the increased N_2O release into the atmosphere is the intensive use of Nitrogen (N) fertiliser in agricultural lands (Tian et al., 2020). In Ireland, agriculture is the largest contributor to GHG emissions, representing 37.1% of the total emissions, and contributing 92.5% of the N_2O emissions nationwide (Environmental Protection Agency, 2021). It is important to understand the processes leading to the loss of N in a gaseous form, not only as a mechanism to mitigate GHG emissions but also due to the critical role N-cycling plays in the delivery of a range of essential ecosystem services, e.g., plant productivity, nutrient cycling, and soil carbon sequestration (Jones et al., 2014). An enhanced

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understanding of these processes would facilitate the optimisation of fertiliser applications, with resulting reductions of nutrient losses through the air and leaching. In order to inform land management advice aimed at reducing such emissions, it is crucial to identify the key factors influencing the potential of agricultural soils to release N in the form of N_2O.

The major soil processes resulting in N_2O emissions are due to microbial activity via two successional processes called nitrification and denitrification. Both processes are critical steps in the N-cycle, supplying organisms with this rarely bioavailable and growth-limiting nutrient in many environments (Kuypers et al., 2018). The former is the stepwise oxidation of ammonium (NH_4^+) into nitrite (NO_2^-), which is driven by ammonia-oxidizing bacteria (AOB) and archaea (AOA); and the subsequent conversion of nitrite to nitrate (NO_3^-) by nitrite oxidizing bacteria. However, the process can also be undertaken by complete ammonia-oxidizing bacteria (comammox) that are able to sequentially oxidize ammonia to nitrate via nitrite in one single cell (Daims et al., 2015; van Kessel et al., 2015). Ammonia oxidizers can also produce N_2O through the oxidation of hydroxylamine during NO_2^- formation, where the by-product nitric oxide (NO) can then be oxidized to N_2O via NO reductases (Tierling and Kuhlmann, 2018; Wrage-Mönnig et al., 2018). Denitrification, the main process of N_2O formation (Harris et al., 2021; Mosier, 1998), is a facultative anaerobic process resulting in the stepwise reduction of nitrate into either the environmentally harmful gas N_2O or, in case of complete denitrification, into the benign dinitrogen (N_2) gas (Groffman, 2012). The key step of denitrification is the reduction of soluble nitrogen (nitrite) into a gaseous form (NO) catalysed by the nitrite reductase encoded either by the *nirK* or *nirS* genes. The last step of the denitrification is catalysed by the N_2O reductase encoded by the *nosZ* gene, which is the only known sink of N_2O in the biosphere, as it reduces N_2O into N_2 (Hallin et al., 2018).

The capacity to nitrify and denitrify is distinctively distributed among microorganisms, with nitrification being exclusively performed by prokaryotes, while denitrification is present in both prokaryotes and eukaryotes, and carried out by phylogenetically diverse communities within the domains of Archaea, Bacteria and Fungi (Maeda et al., 2015; Philippot et al., 2007). These are modular processes with not all organisms contributing to every step, and thus the soil microbial community structure and abundance are expected to determine N_2O released from soils. Some soil organisms, such as Fungi and Actinobacteria, produce only N_2O because they lack N_2O reductase (Jones et al., 2014; Maeda et al., 2017; Sun et al., 2016; Zhao et al., 2019), while others are only N_2O reducers (Hallin et al., 2018). Further, some organisms such as ammonia oxidizers can also be a source of N_2O emissions under certain edaphic and climatic conditions (Bateman and Baggs, 2005; Khalil et al., 2004). Therefore, the relative proportion of the different denitrifiers and nitrifiers may impact the end-products of soil N-cycling and the resulting potential N_2O emitted. The quantification of functional genes in soils is used as a proxy of the abundances of nitrifiers and denitrifiers but relationships between microbial genes and soil denitrification are not always observed because other key factors interact and influence enzymes activities and the release of N_2O and N_2 in the atmosphere (e.g., Liu et al., 2013).

Denitrification processes are regulated by environmental conditions, including soil physico-chemical properties. Numerous local experiments have demonstrated that denitrification is influenced by edaphic conditions such as soil pH, soil moisture and temperature, soil aeration, water-filled pore space and soil carbon (e.g., Brenzinger et al., 2017; Mehnaz et al., 2019; Norton and Ouyang, 2019; Žurovec et al., 2021). Soil physico-chemical properties may also indirectly influence the N_2O emissions via shaping of soil microbial communities involved in N-processes (Samad et al., 2016). Therefore, interactions between biotic and abiotic soil properties rather than individual factors are expected to influence microbial traits and their activity (Graham et al., 2014), and the identification of key predictors is needed to implement more accurate predictive models.

Despite the known effects of soil physico-chemical properties and implications of microbial activity in denitrification, N_2O emissions remain highly variable and therefore difficult to predict in grasslands (de Klein et al., 2014). More studies integrating biotic and abiotic factors are needed to understand their relative contribution in driving N_2O emissions. One approach to address these aspects is studying natural gradients to understand the key factors influencing N_2O emissions (Almaraz et al., 2020). Using this approach, both the soil physical properties and microbial community composition were identified as the main drivers of potential denitrification in New Zealand (Morales et al., 2015), while Kou et al. (2019) showed that soil physico-chemical conditions such as pH and total organic carbon, were more important than the abundance of functional genes involved in denitrification. These contrasting results highlight that drivers of potential denitrification vary, and that our understanding of the potential of agricultural soils to release N_2O and capacity to extrapolate this to a broader geographical scale, is still limited.

Here, we evaluated the range of potential N_2O emissions from denitrification in Irish grassland soils and evaluated the drivers associated with such processes. The objective was to identify the key factors regulating these processes at a large scale, with the aim of contributing to the broader understanding of the factors with the greatest impacts on N_2O emissions at a global scale. To respond to this objective, we assessed the soil potential denitrification and N_2O emissions in relation to total emissions ($N_2O + N_2$) from soils collected in thirty-two sites across Ireland. We also evaluated the relationships between these processes and climatic conditions, soil physico-chemical properties, nitrifier and denitrifier abundances as well as prokaryotic (bacteria and archaea) and fungal community structure. We additionally evaluated soil potential nitrification as an important driver of N-availability in soils including forms of N that act as the substrate of denitrification.

2. Materials and methods

2.1. Sampling sites

To evaluate soil potential denitrification in Irish grasslands we collected a total of 136 soil samples from 32 sites across the island of Ireland (Fig. 1). The closest sites were within 15 km, while the furthest were at approximately 500 km distance, and sites were chosen to encompass a wide range of geoclimatic variables and soil physico-chemical properties. Climatic conditions for each site were retrieved from WorldClim2 database using a 1 km^2 resolution (Fick and Hijmans, 2017). All samples were collected on mineral soils from managed grasslands, the great majority of which were dominated by ryegrass (*Lolium* sp.). Descriptions of the land management are in Table S1, but briefly, paddocks were grazed by cattle or sheep with stocking rates ranging overall from 1 to 2.9 livestock units per hectare. The most recent fertiliser application was a minimum of ten weeks prior to soil sampling.

2.2. Soil sample collection

Soil samples were collected during the winter period, between mid-November 2019 and early March 2020. Based on previous studies and input from land managers, between three and twelve paddocks were selected for sampling per site in order to incorporate the desired diversity of soil types and a wide range of edaphic properties. Within each paddock, we established a 1 m × 1 m plot from which soil for all subsequent analyses was collected. This approach was used to reduce the spatial variability in prokaryotic and fungal communities, and to better relate soil microbial communities to the soil physico-chemical properties and potential activities.

For each plot, three different soil samples were collected and processed separately to characterise 1) the soil physico-chemical properties, and potential nitrification and denitrification, 2) the bulk density, and 3) the soil microbial communities, including nitrifiers and denitrifiers

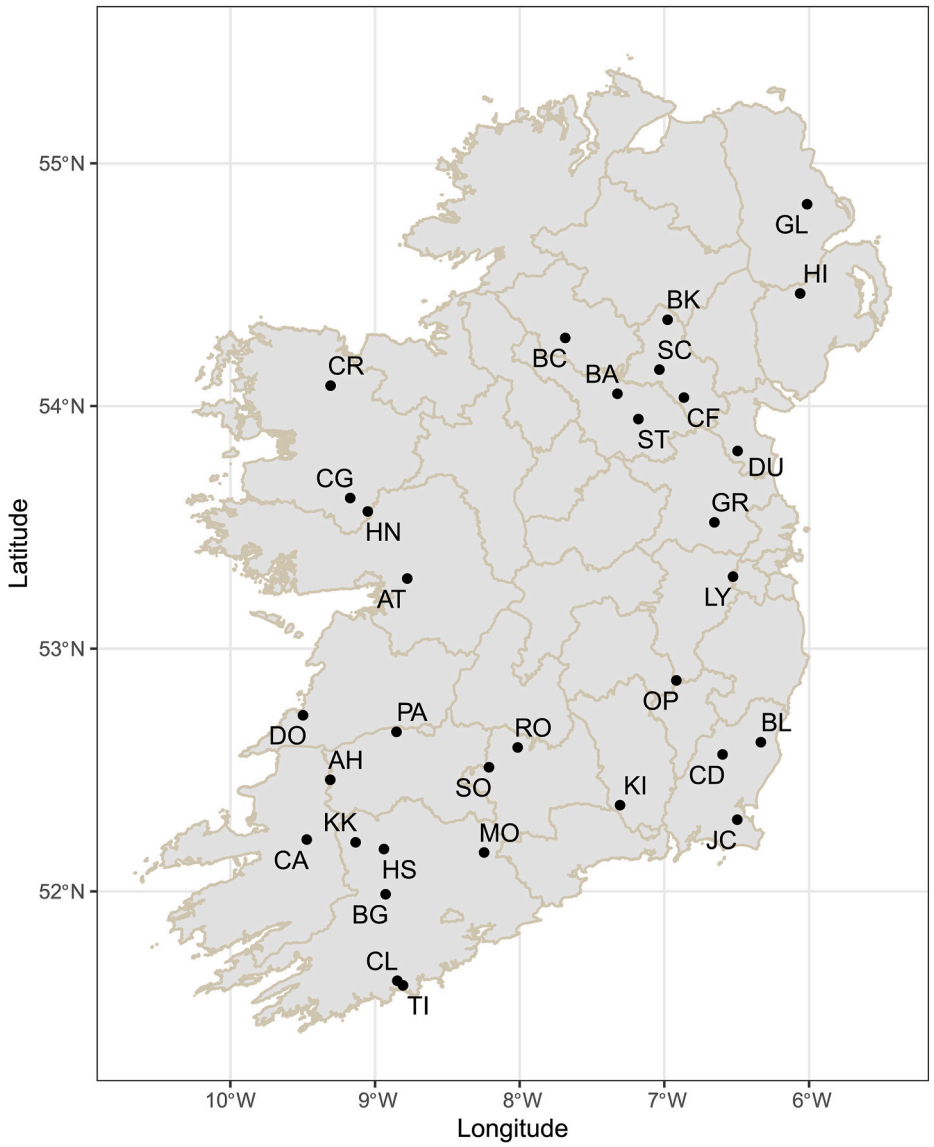

Fig. 1. Map of Ireland showing the location of the sampling sites. Detailed information about the sampling sites can be found in Table S1.

functional gene abundance and overall community composition. For soil physico-chemical characterisation and for both nitrification and denitrification assays, we collected approximately 1 kg of soil from the top 10 cm of the soil surface using an auger. Soil samples were preserved at 4 °C until further processing and analyses were performed. To evaluate bulk density, three samples were taken within the top 10 cm of the soil surface using a 94.28 cm^3 core sampling ring and stored at 4 °C until drying. Finally, to later characterise the soil microbial communities, five soil cores of 10 cm depth were collected from each corner and the centre of the plot and then pooled as one composite sample. This composite soil sample was flash frozen on site using liquid N and then stored at – 80 °C until molecular analyses were performed.

2.3. Soil physico-chemical characterisation

Soil water content and water holding capacity were determined gravimetrically by overnight drying of fresh and water-saturated soils at 105 °C (Brischke and Wegener, 2019). 2*M* KCl was used to extract mineral–N (nitrate–N (NO^{-3}) and ammonium–N (NH^{+4})) from fresh soil (20 g). Extracts were then measured through the Aquakem 600 analyzer (Thermo Scientific, USA). Soil available phosphorus (Mehlich III), phosphorous, potassium and magnesium (Morgans), copper (EDTA), sulphur (Calcium Phosphate), total carbon, inorganic C and total N (LECO Trumac CN, Michigan, USA), pH (1/2.5 v/v soil/water), cation exchange capacity (extracted using buffered Barium Chloride with triethanolamine following Avery and Bascomb laboratory methods, 1974), and particle size (sedimentation) were determined. All analyses were performed on dried and sieved soil (at 30 °C, 2 mm mesh size), except for total carbon and total nitrogen content, analyses were performed on 150 μm ball-milled dried soil. Dry bulk density was determined following the ISO 11272:1998 procedure (Walter et al., 2016). Concisely, soil cores were dried at 105 °C for 48 h and dry weight was recorded before sieving (2 mm sieve), and the dry weight as well as the volume of > 2 mm stones was determined. Bulk density was calculated as the core dry weight divided by the core volume after accounting the weight and volume of stones (Walter et al., 2016).

2.4. Soil potential nitrification

Potential nitrification was evaluated within a maximum of a week of sampling following Drury et al. (2008) procedure. Briefly, 15 g of sieved fresh soil (4 mm mesh) was weighed into a 500 mL schott bottle and 100 mL of freshly prepared NH_4^+ nutrient solution pH 7.2 (1.5 mM NH_4^+ and 1 mM PO_4^{3-}) was added to the soil. Soils were incubated under oxic

conditions at 15 °C and shaken at 130 rpm for 24 h (Wiseshaker SHO-2D, Wisd Lab instruments). Subsamples were taken after 2 h, 4 h, 21 h and 24 h of nutrient addition. Filtrates were analysed in the Aquakem 600 A Photometric analyzer (Thermo Scientific, USA). Nitrification rate for each sample was calculated as described by Drury et al. (2008).

2.5. Soil potential denitrification and N_2O production

Soil potential denitrification was assessed in order to determine the maximum rate of nitrogenous gas released from soils under optimal conditions (i.e. anoxic environment, no nutrient limitations, and optimal moisture content). This was evaluated within a week of sampling by using the acetylene blocking method described by Yoshinari et al. (1977) with some modifications. Briefly, for each sample, two 160 mL glass flasks containing 20 g of previously sieved fresh soil (4 mm mesh) were sealed and pre-incubated at 15 °C overnight. After the pre-incubation period, moisture content was adjusted to 70% water holding capacity and the headspace was flushed with helium to maintain an anoxic environment. For each sample, one flask was injected with acetylene (C_2H_2; 10% v/v of the headspace) to inhibit the reduction of N_2O to N_2, and then be able to calculate the total N-gas formed through denitrification that could potentially be realised as N_2O. Acetylene was injected into the flasks at the start of the incubation (Time 0). The second flask was injected with helium to allow the whole denitrification pathway to occur. Subsequently, a solution of 75 mM KNO_3, 37.5 mM Na-succinate, 25 mM glucose and 75 mM Na-acetate was added to each flask adjusting the respective volumes to reach 70% of soil's water holding capacity. The flasks were incubated for 5 h in the dark at 15 °C and shaken at 130 rpm (Orbital Platform Shaker PSU-10i, Grant Bio VWR). Gas samples were taken every hour after nutrient solution addition by transferring 15 mL gas from the headspace into a 12 mL pre-evacuated gas vial that were then analysed for N_2O concentrations using a 450 Gas Chromatograph with a 63Ni electron capture detector (Bruker, Germany) coupled to a Combi-PAL xt® auto-sampler (CTC Analytics AG, Switzerland). For each flask, the potential N_2O produced through denitrification was estimated as follows:

$$Potential\ N_2O\ (ng\ N_2O-N\ /\ g\ soil\ /\ min) = \left[\frac{\left(\frac{P*h}{R*T}\right)*m}{W}\right]*\frac{28g}{mol}*1000$$

where P is the atmospheric pressure, h is the headspace volume, R is the universal gas constant, T is the temperature, m is the rate of N_2O production per minute and W is soil dry weight. Potential denitrification was estimated as the N_2O produced in the acetylene flask, and the $N_2O/(N_2O + N_2)$ ratio was estimated as the potential of soils to emit N_2O over the total gases and was evaluated as N_2O produced in the helium flask over N_2O produced in the acetylene flask.

2.6. Soil microbial community's characterisation

2.6.1. DNA extractions from soil and amplicon sequencing

DNA was extracted from 0.25 g soil using the DNeasy PowerSoil Kit (Qiagen, Hilden Germany) following the manufacturer's instructions. The yield and quality of DNA extracts were verified by Qubit dsDNA BR Assay kit using an Invitrogen Qubit 3.0 fluorometer (Thermo Fisher Scientific, USA), a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and by running an 1% agarose gel. Extracted DNA was stored at −80 °C until further analysis.

Sequencing libraries were prepared for the V4 region of archaeal and bacterial *16S rRNA* gene using the 515 F and 926 R primers (Parada et al., 2016) and for the fungal ITS2 region, targeted with the 4 R and 86 F primers (De Beeck et al., 2014). The first PCR was performed with the above-mentioned specific primers flanking Illumina overhang adapters sequences in a 25 μL PCR reaction containing 2x KAPA HiFi HotStart ReadyMix (Kapa Biosystems Inc, USA), 1 μM of each primer and 2.5 μL of DNA. For the *16S rRNA* gene, the following conditions were applied: initial denaturation at 94 °C for 3 min, followed by 25 cycles of 94 °C for 45 s, 50 °C for 45 s and 72 °C for 1 min, and a final extension step at 72 °C for 5 min. For the ITS gene, the first PCR was carried out under the following conditions: initial denaturation at 95 °C for 3min, followed by 25 cycles of 95 °C for 30 s, 54 °C for 30 s and 72 °C for 30 s, and a final extension step at 72 °C for 5 min. For both genes, the second PCR was conducted with the Nextera XT index kit (Illumina, San Diego, CA) following manufacturer's instructions. Amplicons were verified on 1% agarose gel and purified using AMPure XP magnetic beads (Beckman Coulter, USA). Concentrated samples and negative controls were pooled in equimolar ratios, diluted to 4 nM and sequenced with a 2 × 250 run on an Illumina MiSeq platform at Teagasc Next Generation DNA sequencing facility (Ireland). Sequencing data was deposited in NCBI under the BioProject accession number PRJNA788893.

Raw DNA sequencing data were processed using DADA2 (Callahan et al., 2016) in R (version 4.0.2; (R Core Team, 2020). Briefly, for 16 S sequences, reads containing ambiguous sequences were removed and based on quality scores, forward and reverse reads were trimmed at 200 and 270 bp, respectively. After denoising using "dada()" function, reads were merged with a minimum overlap of 20 bp and merged reads ranging between 368 and 379 bp were further filtered to remove chimeras using "removeBimeraDenovo()" function. To assign taxonomy, we referred to SILVA SSU r138 database with "IdTaxa()" in the DECIPHER package (Wright, 2016). Likely contaminants were removed with "isContaminant()" in "decontam" package (Davis et al., 2018) using the prevalence method and the negative control. We further removed Amplicon Sequence Variants (ASV) that were not assigned to the domain level and singletons. For the ITS sequences, the sequences were similarly processed, except that for the initial filtering process reads were not trimmed at a defined length (to account for biological variation). After denoising, merged reads with a range between 230 and 413 bp were further filtered to remove chimeras. Taxonomy was assigned using the UNITE v2020 database.

2.6.2. Quantitative PCR amplification (qPCR)

The abundance of microbial communities involved in denitrification and nitrification pathways was estimated by quantitative PCR (qPCR) using SYBR green detection chemistry in a CFX384 touch real-time PCR detection system (BioRad, USA). Marker genes for nitrifiers (*amoA* in archaea, bacteria and comammox), denitrifiers (*nirK* and *nirS*) and N_2O-reducers (*nosZ* clade I and *nosZ* clade II) were quantified as described previously (Fowler et al., 2018; Hallin et al., 2009; Henry et al., 2006; Hornek et al., 2005; Jones et al., 2013; Rotthauwe et al., 1997; Throbäck et al., 2004; Tourna et al., 2008 and details are in Tables S2 and S3). Additionally, abundance of the microbial community was quantified using crenarchaeota, bacterial and fungal specific genes (Table S3). Standards were prepared from a known concentration of a linearized plasmid containing PCR products of the target genes (Duff et al., 2017), which were serially diluted in Tris-EDTA buffer solution for molecular biology (Invitrogen, Thermo Fisher, USA) to create a standard curve between 10^7 – 10^1 copies/μL. All qPCR assays were performed in duplicate for each sample and standards. When possible, DNA of known microorganisms possessing the gene of interest, was included as a positive control. To remove any inhibitory effects on the amplification, BSA was added to each qPCR reaction mixture (Table S2). Reagents, positive controls used and reaction conditions for each gene are shown in Tables S2 and S3. CFX Manager Software was used to calculate gene copy numbers prior to statistical analyses.

2.7. Statistical analyses

All analyses were performed on R (version 4.0.3). For all statistical analyses involving soil physico-chemical properties and gene abundance, we examined the distribution of the data and square root- or log-transformation was applied when appropriate. Pearson tests were used to evaluate the correlation between each predictive variable and either

soil potential nitrification, denitrification, $N_2O/(N_2O + N_2)$ ratio, NH_4 or NO_3. In all analyses, gene abundances were assessed as copies per ng of DNA, and similar trends were obtained when assessed as a proportion of the total 16 S community (data not shown). A Principal Component Analysis was performed to further explore relationships between soil physico-chemical properties, functional genes abundance and the potential denitrification, $N_2O/(N_2O + N_2)$ ratio, NH_4 and NO_3.

For community analyses, normalisation of sequence reads across all samples was not performed because rarefaction curves (using "rarecurve" function in "vegan"; Oksanen et al., 2018) showed that sequence effort was sufficient to reach saturation in all *16S rRNA* (Figs. S1a, 5,453 to 134,011 reads/sample) and ITS (Figs. S1b, 9,625 to 118,915 reads/sample) samples. A soil sample collected in CR containing 2810 ITS reads was excluded from fungal community analyses. To visualize the dissimilarity between either prokaryotic or fungal communities, we used Principal Coordinate Analysis (PCoA), obtained with "wcmdscale()" in "vegan" based on Bray-Curtis distances.

To identify which soil properties were best predicting potential activities, we tested soil variables hypothesised to be important in determining N_2O emissions. We created an individual linear mixed-effect model for each potential activity including "Site" as a random effect to account for the sampling design using "lmer()" ("lme4; Bates et al., 2015) and we visually inspected residuals for normality and homoscedasticity. TOC was excluded from all models because it was highly collinear with TN. Using a multimodel inference and model averaging approach with "dredge()" in "MuMIn" (Barton, 2016), we generated several models containing random subsets of the soil variables, followed by "importance()" and "model.avg()" to obtain the relative importance and the confidence interval of each individual parameter. We refitted the model with only the significant variables to obtain the marginal R^2 using "r.squaredGLMM()". In addition, to evaluate the effect of pH on the ratio $N_2O/(N_2O + N_2)$, we fitted a beta-regression to account for the data ranging between zero and one.

We estimated the bacterial or fungal richness and diversity for each sample using the Chao index with "estimateR()" and Shannon index using "diversity()" in "vegan". To identify significant soil physico-chemical properties that explain variation in the microbial and fungal communities, we performed a stepwise model selection using permutation tests with "ordistep" ("vegan") and then assessed the amount of variation explained by these properties with distance-based redundancy analyses (db-RDA) using "capscale".

To evaluate the effect of the bacterial or fungal community composition on either potential activity or $N_2O/(N_2O + N_2)$ ratio, we fitted individual general additive model using the first two axes of the PCoA as predictors of each potential activity using "ordisurf()" from "vegan". This method takes non-linear relationships into account, provides an estimation of the variation in the response variable that is explained by the community structure and implements the fitted values on the ordination. In addition, we evaluated whether the relative abundance of the microbial and fungal phylum was correlated with either potential activity or $N_2O/(N_2O + N_2)$ ratio using Pearson correlation tests.

To quantify the amount of variation explained by soil physico-chemical properties, functional gene abundance and overall microbial community composition we performed individual variation partitioning using "varpart()" function in "vegan" for each potential activity. For this test, the contribution of the microbial community composition was evaluated by extracting the first two axes of the PCoA. Variables were standardised prior variation partitioning.

3. Results

3.1. Climatic conditions and soil physico-chemical properties

Mean annual temperature across all sites averages 9.45 °C, varying between 7.5 °C and 10.3 °C and mean annual precipitation averages 1053 mm, ranging between 832 mm and 1249 mm (Table S1). All measured soil physico-chemical properties for each site are in Table S4. Briefly, regarding the soil texture, sand content varied between 11.4 ∓ 1.4% and 59.4 ∓ 2.6%, silt between 24.2 ∓ 0.2% and 55.1 ∓ 1.1% and clay between 11.4 ∓ 1.4% and 37.2 ∓ 5.7%. Bulk density ranged between 0.6 ∓ 0.1 g/cm^3 and 1.3 ∓ 0.04 g/cm^3. The range of pH varied between 4.8 and 7.7. Soil chemical properties such as P varied greatly within sites due to the differing fertiliser applications and land management *in situ*. Across all samples, P varied between 10 and 286 mg/L and total N and C varied between 0.25 to 1.25% and 2.34–14.5%, respectively. Potassium and Magnesium varied between 20.0 and 760.0 mg/L and 57 and 375 mg/L, respectively.

3.2. Soil potential nitrification, denitrification and N_2O emissions

Soil potential nitrification averaged 18.6 mg N kg^{-1} dry soil day^{-1} across all soil samples, ranging from 1.9 ± 1.3 mg N kg^{-1} dry soil day^{-1} in CR and 50.2 ± 10.4 mg N kg^{-1} dry soil day^{-1} in SC (Fig. 2a). Soil potential denitrification averaged 22.3 mg N_2O–N kg^{-1} dry soil day^{-1} across all soil samples. A wide difference of potential total denitrification rates was found between sites, ranging from 7.1 ± 0.8 and 7.1 ± 1.3 mg N_2O–N kg^{-1} dry soil day^{-1} in LY and CG, respectively, up to 48.6 ± 16.8 and 42.0 ± 18 mg N_2O–N kg^{-1} dry soil day^{-1} at OP and AT respectively (Fig. 2b). Although the $N_2O/(N_2O + N_2)$ ratios varied between 0.65 ± 0.1 in OP and 0.96 ± 0.04 in TI (Figs. 2c), 75% of the ratios were above 0.71 indicating that most N potential emissions were in the form of N_2O rather than N_2 under the conditions tested. (Fig. 2c).

3.3. Abundance of total and functional microbial communities

Considering the overall microbial communities, total bacteria were the most abundant, ranging from 7.6 × 10^4 and 4.7 × 10^5 gene copy number ng^{-1} DNA (Table S5). Fungal abundance ranged between 1.9 × 10^3 to 1.1 × 10^4 and crenarchaeota abundance ranged between 7.4 × 10^2 and 1.2 × 10^4 gene copy number ng^{-1} DNA (Table S5). The most abundant microbial nitrifiers were the AOA, ranging from 1.7 × 10^3 to 1.6 × 10^4 gene copy number ng^{-1} DNA. The abundance of AOB ranged between 8.6 × 10^2 and 9.7 × 10^3 gene copy number ng^{-1} DNA, while comammox bacteria ranged between 2.2 × 10^2 and 1.6 × 10^3 gene copy number ng^{-1} DNA. Regarding denitrifying microbes, we observed that nitrite reducer abundance with the Cu-containing enzyme encoded by *nirK* gene was higher than the abundance of those with cytochrome *cd*1 nitrite reductase encoded by *nirS* gene, with *nirK* abundance ranging from 1.8 × 10^3 to 1.1 × 10^4 and *nirS* from 1.4 × 10^2 and 1.1 × 10^3 gene copy number ng^{-1} DNA. N_2O reducer abundance varied between 8.2 × 10^2 and 4.5 × 10^3 for *nosZI* and between 6.0 × 10^3 and 7.9 × 10^4 gene copy number ng^{-1} DNA for *nosZII*.

3.4. Soil microbial community composition

We obtained 14,394,872 prokaryotic and 16,194,189 fungal forward reads associated with reverse reads, and after quality control 7,136,044 prokaryotic and 6,398,066 fungal merged reads remained for analyses. Overall, 91.6% of the bacterial and archaeal ASVs were identified to the phylum level. Among the 44 identified phyla, Proteobacteria (28%), Actinobacteriota (15%), Acidobacteriota (14%), Verrumicrobiota (10%), Bacteroidota (8%) and Planctomycetota (6.7%) were the most abundant overall and across all sites (Fig. S2). Other less abundant phyla present in all soil samples were observed: Myxococcota (4%), Nitrospirota (1.7%), Cyanobacteria (1.4%), Crenarcheota (1.4%) Firmicutes (1%) and Bdellovibrionota (0.2%) (Fig. S2). For the fungal community, 98% of the ASVs were identified to the phylum level. A total of 15 phyla were observed, and the most abundant were Mortierellomycota (41.6%), Ascomycota (37.9%), Basidiomycota (13.4%), Glomeromycota (5.6%) and Chytridiomycota (1%) (Fig. S2).

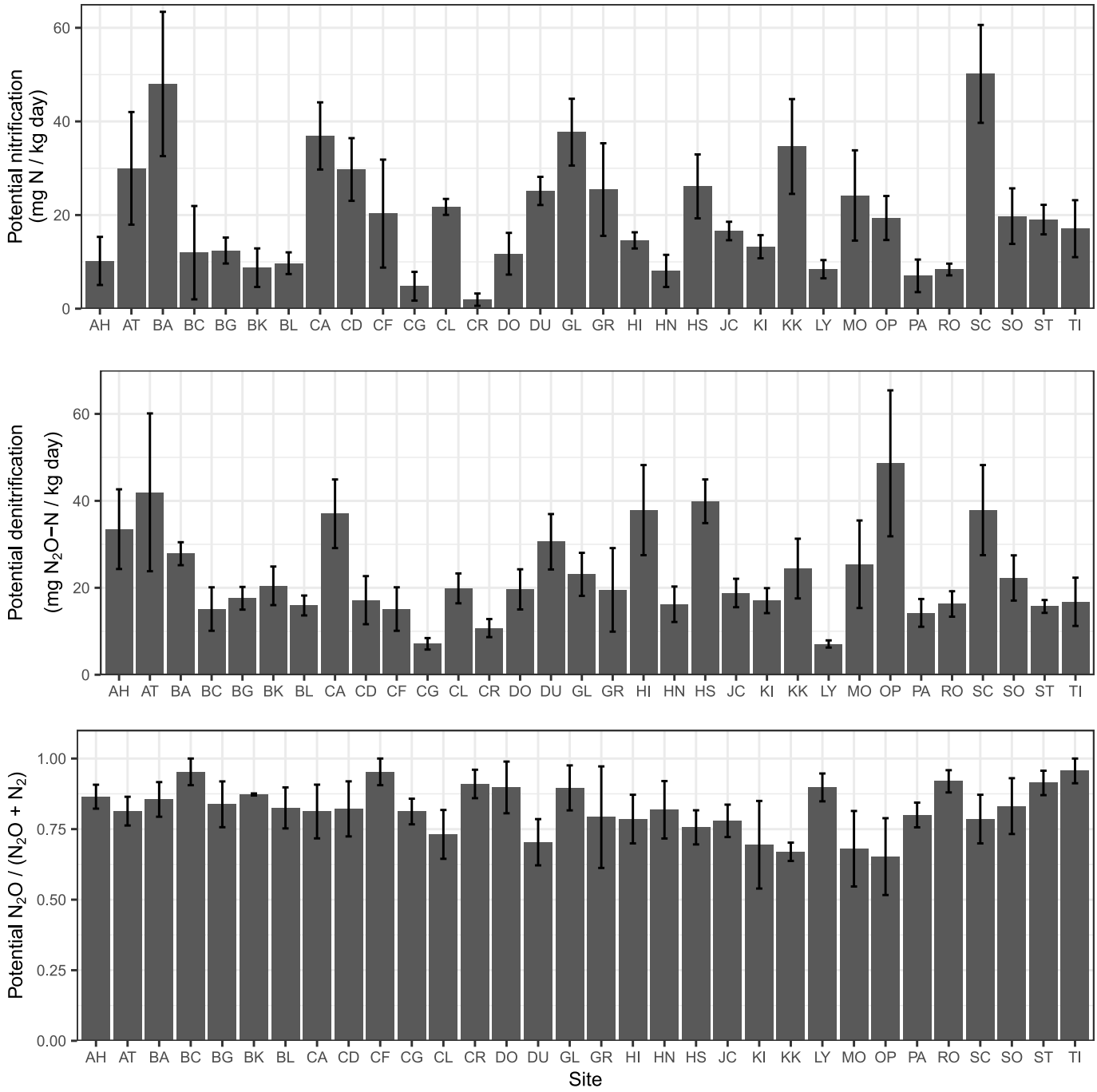

Fig. 2. Potential nitrification (a), denitrification (b) and $N_2O/(N_2O + N_2)$ ratio (c) according to the sampling sites. Bar graph shows the average and error bars represent standard errors. Site descriptions are in Table S1. Numbers of replicates per site are n = 3 for BA, BG, BE, BK, CA, GL, HS, HI, KK, CF, ST, SC; n = 4 for CR, LY, DU, GR, MO, RO, SO; n = 5 for AH, AT, BL, CD, CL, CG, DO, HN KI, OP, PA, TI, n = 12 for JC.

3.5. Abiotic factors associated with potential nitrification, denitrification and N_2O emissions

Soil potential denitrification was highly correlated with soil potential nitrification (Table S6), and similar soil properties were associated with both processes. The best predictors for soil potential nitrification were pH, TN and bulk density with the same importance, followed by K, P and Mg (Table 1) and, when evaluating the best fitting model, all variables were positively correlated with potential nitrification ($R^2 = 0.55$). For potential denitrification, the best fitted model ($R^2 = 0.62$) included pH, TN and P, all presenting the same weights (importance) to the model prediction and positively correlated to potential denitrification (Table 1). Soil pH was the only predictor for $N_2O/(N_2O + N_2)$ ratio (Table 1, $R^2 = 0.28$). To quantify this relationship, we refitted a beta-regression including only soil pH and we observed a negative relationship between pH and $N_2O/(N_2O + N_2)$ ratio, meaning that the increase in soil pH favours the decrease of N_2O emissions in relation to total emissions ($N_2O + N_2$) (beta-regression; z-value = −6.6, pseudo-R^2 = 0.1, p-value < 0.01; Fig. S3).

Neither the mean annual precipitation nor the mean annual temperature were correlated with the potential activities nor $N_2O/(N_2O + N_2)$ ratio (Table S6).

Table 1
Relative importance of soil physicochemical variables for predicting variation in potential nitrification, denitrification and the $N_2O/(N_2O + N_2)$ ratio. Table includes the relative variable importance (corresponds to the sum of "Akaike weights" across models including each variable) and the model-averaged confidence interval for each estimated parameter.

Model	Variables	Relative variable importance	Confidence interval	
			2.5%	97.5%
Potential nitrification	pH	**1**	**0.68**	**1.34**
	TN	**1**	**3.57**	**7.16**
	Bulk density	**1**	**3.69**	**10.94**
	K	**0.82**	**0.13**	**2.01**
	P	**0.78**	**0.09**	**1.75**
	Mg	**0.68**	**0.05**	**0.24**
	clay	0.03	−0.01	0.05
Potential denitrification	pH	**1**	**0.18**	**0.41**
	P	**1**	**0.16**	**0.27**
	TN	**1**	**0.5**	**0.91**
	Bulk density	0.23	−0.41	0.78
	K	0.07	−0.08	0.18
	Mg	0.03	0	0.02
	clay	<0.01	0	0
ratio $N_2O/(N_2O + N_2)$	pH	**1**	**−0.19**	**−0.1**
	Bulk density	0.33	−0.52	0.22
	K	0.31	−0.14	0.06
	clay	0.3	0	0.01
	TN	0.3	−0.12	0.21
	P	0.29	−0.13	0.07
	Mg	0.26	−0.01	0.01

3.6. Biotic factors associated with potential nitrification, denitrification and N_2O emissions

None of the soil physico-chemical properties were strongly associated with the functional gene abundances (Fig. S4), and in return these were weakly associated with the potential activities. The nitrifiers abundance AOA, AOB and comammox were positively correlated with potential nitrification ($r < 0.4$; Table S6). For the denitrifiers, only *nirS* and *nosZ* clade I abundance were positively correlated with the potential denitrification (Table S6). No evidence was found of an association between gene abundances and potential $N_2O/(N_2O + N_2)$ ratio (Table S6).

We then evaluated the influence of the microbial and fungal community richness, diversity and composition on the potential denitrification and on the $N_2O/(N_2O + N_2)$ end-product. The microbial community richness and diversity were positively correlated with the potential denitrification, but such relationships were weak (Coefficient $r < 0.3$; Table S6) and only the microbial diversity was significantly associated with the $N_2O/(N_2O + N_2)$ ratio (Table S6). For the fungal community, only the Shannon diversity index was weakly and negatively correlated to the potential nitrification (Coefficient $r < -0.3$; Table S6). Soil physico-chemical properties including pH, P, TN, Mg, K, bulk density and clay were significantly correlated with soil microbial community's composition (ordistep; p-values < 0.01) and explained 20% and 14% of the variation in the microbial and fungal communities, respectively (Fig. S5).

Both the prokaryotic and fungal community structure influenced both potential activities and the $N_2O/(N_2O + N_2)$ ratio (Fig. 3 and Table 2). The predicted values (splines) follow the same trends as the observed values (symbols; Fig. 3), suggesting that the community composition is a good predictor of both potential activities and the $N_2O/(N_2O + N_2)$ ratio. Such trends are confirmed based on the generalised additive models (Table 2). The deviance explained by the prokaryotic community composition was higher than the fungal community (Table 2). In addition, the fungal community composition only explained 6.7% of the $N_2O/(N_2O + N_2)$ ratio while the prokaryotic community composition explained up to 23.8% (Table 2). We also observed that overall, the communities with higher nitrification levels (Fig. 3a, d) were also communities with higher denitrification levels (Fig. 3b, e) but lower $N_2O/(N_2O + N_2)$ ratio (Fig. 3c, f), suggesting that communities with higher potential nitrification and denitrification release less N_2O as a proportion of total emissions.

We found some significant relationships between the relative abundance of specific phyla and both potential activities and the $N_2O/(N_2O + N_2)$ ratio but all were moderate (below 0.4). Actinobacteriota, Chloroflexi and Crenarchaeota were positively associated with potential nitrification and denitrification (Fig. S6, Fig. S7). Actinobacteriota and Crenarchaeota were both negatively associated with the $N_2O/(N_2O + N_2)$ ratio (Fig. S8), suggesting that an increase in the abundance of these phyla correlates to an increase in N-transformation rates but a decrease in the $N_2O/(N_2O + N_2)$ ratio. The opposite trend was observed with Verrucomicrobiota and Myxococcota, where an increase in their abundance were associated with a decrease of potential activities and an increase in $N_2O/(N_2O + N_2)$ ratio (Fig. S6, Fig. S7, Fig. S8). Regarding the fungal communities, Mortierellomycota and Ascomycota were positively and negatively associated with both potential activities, respectively (Figs. S19 and S10). None of the fungal phyla were correlated to the $N_2O/(N_2O + N_2)$ ratio (Fig. S11).

3.7. Relative contribution of biotic and abiotic factors to soil potential nitrification, denitrification and N_2O emissions

We evaluated the relative contribution of soil physico-chemical properties, functional genes abundance and prokaryotic community composition to the potential activities and $N_2O/(N_2O + N_2)$ ratio. The potential of nitrification and denitrification were mainly explained by the physico-chemical properties of the soil (32% and 29%, respectively) (Fig. 4). An additional 20 and 16% of the variability was explained by the community composition together with the soil physico-chemical properties. On their own the soil functional genes explained between 0 and 3% of the variability of both potential activities. In addition, soil properties together with community structure explained up to 18% of the variation in the $N_2O/(N_2O + N_2)$ ratio, while soil properties alone explained 4% (Fig. 4).

4. Discussion

Reduction of N_2O emissions remains among the global challenges to mitigate climate change. It is therefore crucial to identify the key factors influencing the potential of agricultural soils to release N in the form of N_2O. However, our ability to predict N_2O emissions at large scales remains limited due to the multitude of factors and players involved in the processes leading to the release of N-gases. In this study, we aimed to identify the main factors driving potential N_2O emissions on a national scale, and the relative importance of the biotic and the abiotic factors in this regard. Thirty-two contrasting sites across Ireland resulted in large differences of potential denitrification in managed grasslands soils (up to 41.5 mg N_2O–N kg^{-1} soil day^{-1}). We found that soils with higher potential to nitrify were also soils that had the highest potential to perform denitrification, and similar factors were associated with both processes rates. In addition, most of the potential N denitrified was emitted in the form of N_2O rather than N_2 under the conditions tested, as observed in previous studies (e.g., Laughlin and Stevens, 2002). Nitrification is an important regulator of soil nitrogen availability for plants as N availability increases by converting the less mobile NH_4^+ into a more mobile form NO_3^-, and conversely denitrification results in N gaseous loss from the soil. Consequently, parameters that were associated with high potential nitrification and denitrification may drive a higher N turnover in soils and result in higher emissions from Irish grasslands. While nitrification may also contribute to N_2O emissions (Hooper et al., 1997; Kozlowski et al., 2016), potential N_2O emissions were only measured in conditions favouring denitrification in our study

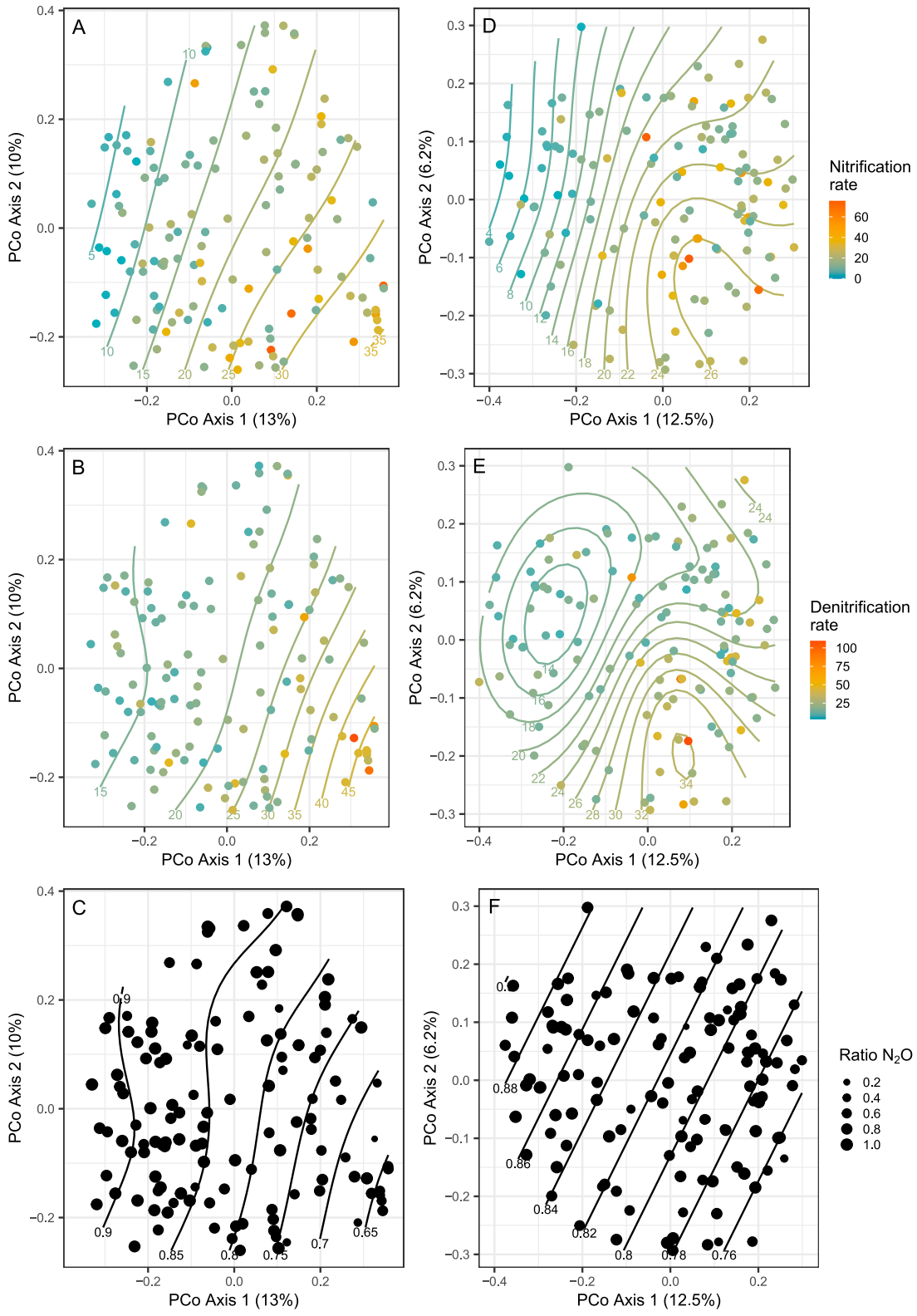

Fig. 3. Principal Coordinate Analysis (PCoA) ordinations showing the prokaryotic (left) and fungal communities (right) and associated potential nitrification (a, d), denitrification (b, e) and $N_2O/(N_2O + N_2)$ ratio (c, f) measured from each soil sample. Each dot represents a community from a soil sample and the colour of the dot shows the level of nitrification (a,d) or denitrification rates (b,e) measured in the soil sample from which the community was characterised (colour varies from blue (lower rates) to red (higher rates) and for potential nitrification rates are expressed as mg N/kg day and for potential denitrification rates as mg N_2O–N/kg day). For the bottom panels, the size of the dots shows the $N_2O/(N_2O + N_2)$ ratio associated with the community. Splines show the expected rates associated with the communities' composition based generalised additive models. Linear relationships are observed when the splines are parallel and non-linear relationships are observed with curved-lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Role of the soil microbial communities in determining the potential nitrification, denitrification and $N_2O/(N_2O + N_2)$ ratio based on generalised additive models.

Soil microbial community	Activity	Deviance explained (%)	P-value
Prokaryotic community	Nitrification	31.6	<0.01
	Denitrification	33.5	<0.01
	$N_2O/(N_2O + N_2)$ product	23.8	<0.01
Fungal community	Nitrification	24.1	<0.01
	Denitrification	19.3	<0.01
	$N_2O/(N_2O + N_2)$ product	6.7	0.01

as it is the predominant N_2O production process in Irish grasslands (Krol et al., 2016).

The potential denitrification was regulated by soil pH, TN and P, but only soil pH influenced the denitrification end-product $N_2O/(N_2O + N_2)$ (Table 1). The important role of soil pH on N_2O emissions has been demonstrated for decades and in numerous studies, either by impeding the assemblage of the N_2O reductase enzyme or via influencing the soil microbial community composition (e.g., Firestone et al., 1980; Samad et al., 2016; Šimek and Cooper, 2002; Liu et al. 2010). Not surprisingly, soil TN was also a relevant predictor of the soil potential denitrification, as it is a substrate for nitrification and denitrification processes, and the concentration of N species in soil influenced N released as N_2O gas (Table S6). Interestingly, soil P content was as important as TN and pH in predicting denitrification rates. Our results show that P may be a key soil property to take into account to predict more accurately potential denitrification in large-scale field studies. Others have reported the potential role of P in denitrification within experimental trials with contrasting results, with either P-addition favouring (Mehnaz et al., 2019; Mehnaz and Dijkstra, 2016) or reducing N_2O emissions (O'Neill et al., 2020). Soil P has also observed to be positively correlated with total nitrification, mineralisation and immobilisation (O'Neill et al., 2021). In our study, we found higher potential denitrification from soils with higher P-levels. The way by which P stimulates denitrification remains unclear, but an overall P-effect on the N-cycle may be due to reducing soil nutrient limitation and thus, influencing the microbial activity (Mehnaz et al., 2019; Mehnaz and Dijkstra, 2016) and/or community structure (Randall et al., 2019). Although no relationship between P and the functional gene abundance was found in our study, such effects remain to be tested using experimental approaches that may provide mechanistic insights into the impact of P on soil microbial communities and resulting N_2O emissions. In addition, others have found that the effects of P on N_2O emissions were dependent on C-substrate availability (O'Neill et al., 2020), but this could not be evaluated in our study as soil carbon content was highly collinear with nitrogen at our sites.

Soil biological properties also contributed to drive the soil potential denitrification and the end-product $N_2O/(N_2O + N_2)$ in Irish grasslands. The prokaryotic community was a better predictor of the potential denitrification than the fungal communities (33.5% vs 19.5%), and the level of prediction of the fungal community composition on the end-product $N_2O/(N_2O + N_2)$ was weak compared to prokaryotic communities (6.7% vs 23.8%). As fungi lack the N_2O reductase converting N_2O into N_2, it was therefore expected that this community would influence the end-product (Maeda et al., 2015). However, our findings are in accordance with experimental studies using bacterial or fungal inhibitors showing that bacteria are more important contributors to denitrification (Maeda et al., 2017), although others have found that the relative role of bacteria and fungi may depend on soil conditions such as pH and soil moisture (Chen et al., 2015). It is interesting to note that several studies have shown the influence of the fungal phylum Glomeromycota, known for their association with plant roots through the mycorrhization process, on N_2O emissions (Gui et al., 2021; Storer et al.,

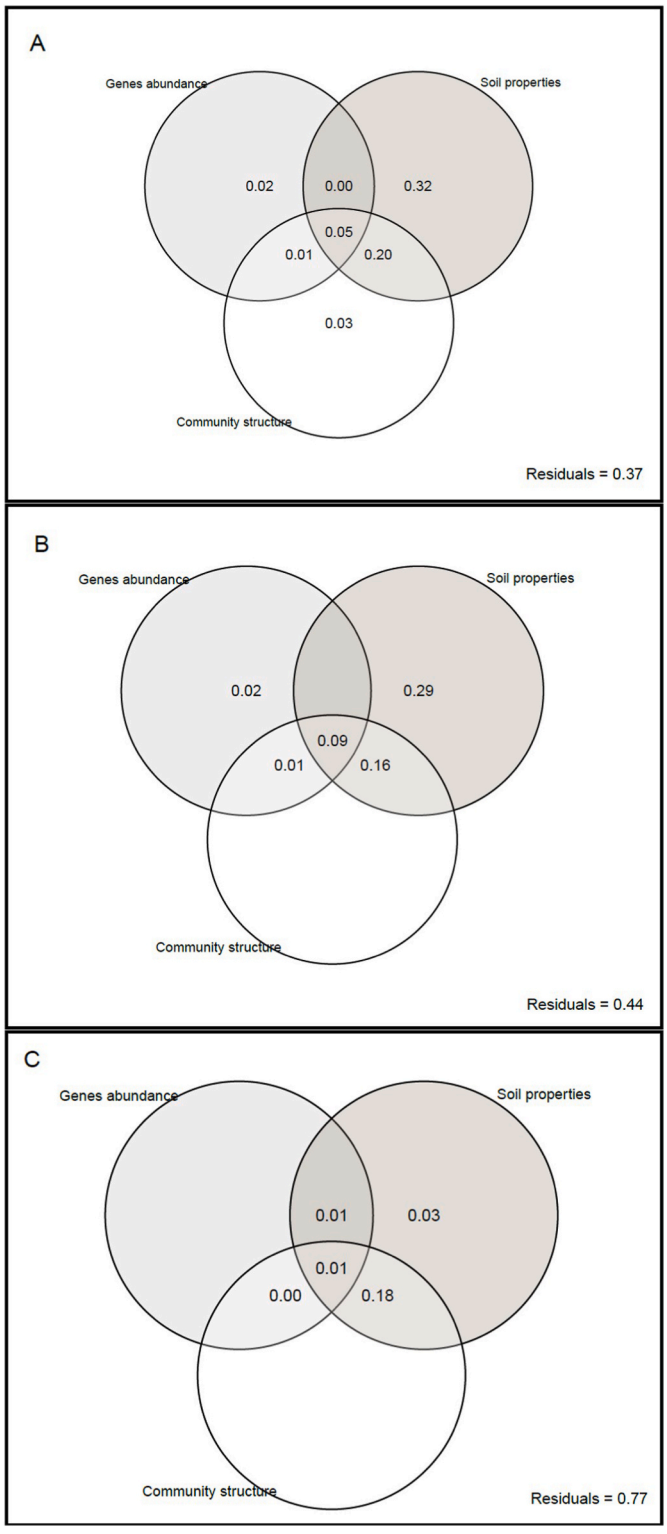

Fig. 4. Relative contribution of soil physico-chemical properties, gene abundance and microbial community structure on the potential nitrification (a), denitrification (b) and $N_2O/(N_2O + N_2)$ ratio (c), based on variation partitioning. Microbial community structure was obtained by extracting the first two axes of the Principal Coordinate Analysis (PCoA). Residuals show the amount of variance that remained unexplained.

2018; Wagg et al., 2014). However, we found no evidence supporting the relationship between the relative abundance of this fungal community and the potential N_2O emissions released from Irish soils.

A noteworthy observation was that the soil prokaryotic communities associated with higher potential nitrification and higher potential denitrification were more likely to favour complete denitrification up to N_2 (Fig. 3). These observations remain correlational in nature as the relationship between communities and N_2O emissions may be in part explained by a cofounding effect of the soil physico-chemical properties. However, we found that the presence and relative abundance of certain prokaryotic phyla showed the same trend, with the increasing abundance of Crenarchaeota and Actinobacteriota also associated with higher nitrification, denitrification and a lower $N_2O/(N_2O + N_2)$. Interestingly, the capacity to nitrify and denitrify is present in members of the Crenarchaeota but Actinobacteriota are not able to perform complete denitrification as *nosZ* genes producing N_2O reductase are absent from their genomes (Hallin et al., 2018). Therefore, the association between Actinobacteriota and $N_2O/(N_2O + N_2)$ remains unclear but our results suggest a role of this bacterial community in overall decreasing N_2O emission relative to N-gases.

The soil microbial community structure, including diversity and composition, were better predictors of the potential denitrification and of the end-product ratio than the functional genes abundance. Only *nirS* and *nosZ* clade I were related to denitrification potential but when evaluated together with community structure and soil physico-chemical properties, their contribution explaining soil denitrification remained below 3%. Interestingly, Domeignoz-Horta et al. (2018) showed that soil properties mainly explained N_2O emissions at low rates while peaks of emissions were mostly explained by denitrifier diversity and abundance. In our study, the lack of variability explained by functional gene abundances relative to other factors is likely due to several aspects. First, denitrification is a facultative process and potential denitrification is related to the size of the enzyme pool at the time of sampling while functional gene abundances reflect the number of microorganisms genetically capable to denitrify. Second, the moderate relationship may also be attributed to denitrification being a modular process with numerous steps, making prediction difficult with each functional gene being involved in one step. For example, *nirK* abundance is more correlated to activity in low pH soils (Bowen et al., 2020) and the great majority of our soil had a pH greater than 5.5, possibly not favouring the expression of this functional gene precursor of the denitrification process. Using transcriptional analysis (mRNA) of functional genes may be more representative of the activity of organisms driving reactions in the N-cycle, and therefore may show a stronger correlation with potential N_2O emissions. However, even with gene transcripts, that is not always the case (Frostegård et al., 2021) and some of the process control (e.g. with *NosZ*) are known to be post-transcriptional. Third, it has been suggested that soil microbial community composition and diversity, including that of functional communities, may be better indicators of the soil potential denitrification by providing additional information that is not accounted for with the functional gene abundance alone, such as interactions between species or niche complementarity (Graham et al., 2016). Further experimental investigation is needed to elucidate the interaction between species interactions and/or niche complementarity and N_2O emissions under controlled conditions. Overall, our results highlight that at larger scales, prokaryotic community structure rather than denitrification gene abundance, may improve prediction of denitrification rates.

Other large-scale studies in New Zealand (Morales et al., 2015) and in China (Kou et al., 2019) assessed potential denitrification using soil incubations. They showed similar effects of the soil physico-chemical properties and soil microbial communities, but also highlighted the determinant role of latitude and climate on the microbial communities and their N_2O emissions. Using similar methods, we found no evidence supporting that climate (Table S6) nor latitude (data not shown) were related to soil potential denitrification. While N_2O measurements are known to be strongly impacted by environmental and climatic conditions *in situ* (e.g., Kiese and Butterbach-Bahl, 2002; Rafique et al., 2011), the lack of an effect observed here may be partly attributed to the fact that potential assays are performed under optimal conditions and therefore not accounting for factors regulating the activity of microorganisms and of the synthesized enzymes. As such it is important to note that potential assay provide mechanistic insights but do not infer actual rates *in situ* (Hazard et al., 2020). However, our findings may also highlight the fact that the relative importance of predictors is scale-dependent but may also vary across regions depending on limiting factors. For example, Kou et al. (2019) that found that mean annual precipitation was a key factor in arid and semi-arid environments but not in mesic sites. In addition, Ireland is characterised by a relatively small total land area and low variability across its territory in terms of climatic conditions, which may also explain the lack of influence on the resulting potential denitrification and N_2O emissions.

In conclusion, we showed that a few key determinants may be considered to improve predictions of N_2O emissions from managed grasslands at large scales, namely soil pH, TN, P, and prokaryotic community structure. While the soil physico-chemical properties were most predictive of N_2O emissions, the addition of prokaryotic community structure data enhanced the level of predictions. We identified certain phyla that were associated with higher denitrification and a lower $N_2O/(N_2O + N_2)$. Soils with higher potential to nitrify were also soils that had the highest potential to perform denitrification, and similar factors were associated with both processes rates. Therefore, considering such factors is important to predict N-transformations at larger scales, and to manage soils in a manner that will reduce gaseous losses of nitrogen from agricultural grasslands.

Authors' contribution

CD, KR, FB, PRP designed the study. CD, AD, PRP and JR collected and processed samples. CD performed the bioinformatics, statistical analyses, and interpreted results. CD wrote the manuscript with help from FB, PRP and MV, and all authors revised this manuscript.

Data accessibility

All data will be archived in Figshare from July 2022, except information regarding farms location and management, at the following link: https://doi.org/10.6084/m9.figshare.19337105.v2. Scripts with the data will be uploaded on bitbucket (https://bitbucket.org/Coline_Dev/IrishDenitrification-MINE/). Raw DNA sequencing data are available on the NCBI database under the accession number BioProject PRJNA788893.

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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2022.108637.

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