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Mineralizable nitrogen and denitrification enzyme activity drive nitrate concentrations in well-drained stony subsoil under lucerne (*Medicago sativa* L.)

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ABSTRACT

Nitrogen (N) inputs to agricultural systems contribute substantially to soil nitrate (NO_3^-) concentrations, which increase NO_3^- leaching and contamination of groundwater. The influence of soil microbes in regulating NO_3^- concentrations in the topsoil are well studied but it is often assumed that microbial regulation of NO_3^- concentrations in the subsoil is negligible. The aim of this study was to test this assumption by determining the relationships between microbial properties and NO_3^- concentrations in both the subsoil and the topsoil. We measured the size of the mineralizable N (N_m) pool, microbial properties (microbial biomass, bacterial richness), nitrifier gene abundance (*amoA* gene copy number), denitrifier gene abundance (*nirK* and *nirS* gene copy number), denitrifier enzyme activity and NO_3^- concentrations in the topsoil and the subsoil in a well-drained stony soil under an established lucerne crop. We used structural equation modelling (SEM) to identify and compare the linkages of microbial properties with NO_3^- concentrations at each depth. In the topsoil, we found higher N_m, gene abundance, denitrification enzyme activity, bacterial richness, and microbial biomass than those in the subsoil, but there were no relationships between these variables and NO_3^- concentrations in the topsoil (the SEM model explained 0.06% of the variability in NO_3^- concentrations). In contrast, in the subsoil, NO_3^- concentrations were strongly correlated with bacterial *amoA* abundance and denitrification enzyme activity, with both variables associated significantly with N_m. We found that bacterial richness was also associated with N_m in the subsoil. Our findings highlight that microbial properties are associated with NO_3^- concentrations in the subsoil (the SEM model explained 82% the variability in NO_3^- concentrations) and this suggest that nitrification and denitrification may contribute to regulating NO_3^- concentrations in the subsoil. Our findings also suggest that denitrification contributes to reducing NO_3^- concentrations in the subsoil. We conclude that studies addressing drivers of NO_3^- leaching need to consider the potential for microbially-mediated attenuation (or an increase) in NO_3^- concentrations throughout the soil profile.

1. Introduction

Soil nitrate (NO_3^-) can be leached from soil, potentially resulting in the contamination of groundwater (Galloway et al., 2008; Lassaletta et al., 2014). This is of particular concern in agricultural systems on well-drained soils, where inputs of urea fertiliser, ruminant excreta, and N-fixing crops contribute to high nitrogen (N) inputs to the soil (Cameron et al., 2013; Carrick et al., 2013). The amount of N that contributes to soil NO_3^- concentrations is regulated mainly by two microbial processes comprising conversion of ammonium (NH_4^+) to NO_3^- via nitrification, and denitrification of NO_3^- into various N gases, with both processes being regulated by the mineralization of organic N

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(Cameron et al., 2013; Saggar et al., 2013). These processes are well studied in the topsoil (0–0.3 m depth) but the contribution of microbial processes to NO_3^- concentrations in the subsoil (>0.3 m depth) is less well studied and is often assumed to be insignificant (Cameron et al., 2013; Hansen et al., 2019). However, there can be substantial N reserves at lower depths (Rumpel and Kögel-Knabner, 2011) and accumulation of NO_3^- through the soil profile in agricultural soils (Ju et al., 2004). This suggests that mineralization of organic N, nitrification and denitrification could contribute significantly to regulating NO_3^- concentrations in the subsoil and leaching losses (Jahangir et al., 2012).

Variations in subsoil NO_3^- concentrations are attributed primarily to leaching from topsoil (Habteselassie et al., 2006; Trolove et al., 2019). However, in the subsoil where root biomass and N uptake are low, the contribution of microbial processes to regulating NO_3^- concentrations may be higher than the contribution in the topsoil. Despite decreases in substrate availability with depth (Spohn et al., 2016) that may contribute to differentiation in the microbial community composition (Stone et al., 2014), there is substantial abundance of nitrifiers and denitrifiers genes (Li et al., 2018; Wang et al., 2017) and microbial adaptations to overcome N limitations (Tian et al., 2017).

The size of the mineralizable N (N_m) pool is a driver for carbon (C) and N availability in the topsoil (Curtin et al., 2012; Mooshammer et al., 2014) and may also play a key role in regulating C and N availability for microbes in the subsoil. Mineralization of organic N is linked to microbial biomass (Mikha et al., 2005; Li et al., 2019), soil water content and temperature (Li et al., 2019). The effects of these variables on nitrification and denitrification and consequently NO_3^- concentrations are not well known for subsoil. Furthermore, soil microbial community composition may change in relation to the availability of substrates (Tardy et al., 2015), so microbial community properties (e.g., bacterial richness) can be expected to be linked to resources (i.e., N_m), particularly in the nutrient depleted conditions in the subsoil (Dopheide et al., 2021). Determining the direction and magnitude of the contribution of N_m to NO_3^- concentrations and its relationship to bacterial richness and microbial biomass in the subsoil is needed to identify management practices that will reduce NO_3^- concentrations and risks of leaching in well-drained agricultural systems.

Most studies that have attempted to describe nitrification and denitrification in the subsoil were conducted in the laboratory under controlled conditions. Artificially increasing the availability of C and NO_3^- to subsoil in incubation experiments increased the potential for denitrification with samples collected at depths to 1 m (Clough et al., 1998) and >6 m (Yeomans et al., 1992). These observations suggest that, in field conditions, the potential for microbial reduction of NO_3^- concentrations may be linked to factors that influence C and N availability.

In this study, our objective was to identify differences in the role of soil microbes in regulating NO_3^- concentrations in the topsoil and the subsoil in field conditions. We selected a single location where lucerne had been grown for two years on a well-drained, stony soil. The two depths for our measurements (topsoil 0–0.1 m and subsoil 0.3–0.5 m) were selected because of contrasting soil environments and root density (Sim et al., 2015). We investigated the extent to which N_m was related to NO_3^- concentration and the relationship of this substrate pool with nitrifier and denitrifier gene abundance and denitrification enzyme activity. To provide further evidence of the role N_m as an indicator of substrate availability in nutrient depleted conditions, we also determined linkages between N_m, microbial biomass and bacterial richness. To interpret the multiple factors affecting NO_3^- concentrations we used structural equation modelling (SEM), which provides a framework to test for the significance of multiple variables driving N transformations (Porre et al., 2016; Feng et al., 2019; Li et al., 2019). We used SEM to distinguish the relationships between the different microbial properties and soil NO_3^- concentrations. We hypothesized that (1) relationships between NO_3^- concentrations, microbial properties and N_m are stronger in the subsoil compared to those in the topsoil, and (2) denitrification enzyme activity and denitrifier gene abundance are positively associated with N_m in the subsoil in field conditions, consistent with observations from incubation studies with artificial increases in substrate availability (Yeomans et al., 1992; Clough et al., 1998; Jahangir et al., 2012).

2. Materials and methods

2.1. Site characteristics and soil sampling

The experimental site was located at the Ashley Dene Research and Development Station, Lincoln, New Zealand (latitude 43° 38.85′ S longitude 172° 20.76′ E, elevation 17 m above sea level). The soil is stony, well drained and classified as a Pallic Firm Brown (FB) (New Zealand classification), Haplic Cambisol (World Reference Base) (Hewitt et al., 2021). Two years after lucerne (Alfalfa, *Medicago sativa* L.) was established, soil monoliths were extracted in autumn (late May) when seasonal plant growth had almost ceased from two adjacent paddocks which were managed for cut-and-carry fodder with no grazing. One paddock received no inputs, and the other was irrigated with water and dairy effluent up to one month prior to sampling. Further details of the characteristics of the field site and irrigation with water and dairy effluent are reported in Laubach et al. (2019) and Graham et al. (2022).

Within a 12 × 30 m rectangular zone in each paddock, six sampling lanes 1.1 m wide and 28 m long were marked, with a 0.55 m buffer zone between each lane, and a 1 m buffer zone around the edge. One sampling point was generated randomly within each lane for the extraction of a soil monolith and the sampling points within each paddock were at least 2.2 m distant from each other. Each soil monolith (32 in total) was extracted by driving a steel tube (0.2 m diameter × 1.7 m length) with a PVC liner vertically into the ground using a commercial drilling rig. This sampling regime was designed to incorporate natural variability to test the role of microbial processes in regulating soil NO_3^- concentrations for the same soil type and environmental conditions.

We collected soil samples for both the topsoil (0–0.1 m) and the subsoil (0.3–0.5 m) with depths selected because of a clear decrease in lucerne root biomass with increasing depth (Sim et al., 2015) and this was confirmed qualitatively during the sampling. All field and laboratory equipment and collection procedures were undertaken under sterilised conditions to avoid cross contamination between samples. The soil samples were homogenised, and all visible roots and stones were removed prior to subsampling for chemical and biological analyses. Subsamples for molecular analyses were frozen immediately in liquid nitrogen and stored at −80 °C until further processing. Subsamples for soil enzymatic assays and chemical analyses were stored at 4 °C for 1 week prior to analysis.

2.2. Measurements of soil properties

To provide context for differences in the soil properties at the two depths, pH and gravimetric water content (W_g) were measured. Soil pH was measured in soil extracts in distilled water (2 soil:5 water mass/volume), and W_g was calculated from the difference in mass between the fresh and oven-dried (105 °C for 24 h) samples.

The concentrations of total soil carbon (C_{total}) and nitrogen (N_{total}) in the soil organic matter (SOM) were measured using a CN analyzer (LECO Corporation, St. Joseph, Michigan, USA). The size of the mineralizable N (N_m) pool was estimated using the standard anaerobic method. Briefly, 10 g of soil was incubated in sealed containers with minimal headspace in anaerobic conditions (fully immersed in water) for 7 days at 40 °C. The difference in ammonium (NH_4^+-N) concentrations before and after incubation for 7 days was assumed to be the N fraction from SOM that is mineralizable (Keeney and Bremner, 1996). The substrate availability for nitrification was estimated from soil NH_4^+-N concentration. Soil NH_4^+-N and NO_3^--N concentrations were measured in extracts with 2 M KCl and a soil:solute ratio of 1:10 mass/volume, using a QuikChem 8500 flow injection analyzer (Lachat Instruments, Loveland, CO, USA).

2.3. Denitrification enzyme activity

Denitrification enzyme activity (D_e) was used as a comparative estimate of the amount of active denitrification enzymes among samples, measured following Chirinda et al. (2011). To determine the activity of actual denitrifier enzymes, while preventing the synthesis of new enzymes (Tiedje, 1994), measurements of N_2O production from soil were made after a short incubation period (6 h) following the application of chloramphenicol (1 g l^{-1}). Briefly, a sample of 10 g of equivalent dry soil was incubated with a 25 ml solution containing KNO_3 (1 mM) and glucose (1 mM), as N and C substrates for denitrification. The soil and solution were placed in a 100 ml hermetically sealed jar with a septum in the lid. Anaerobic conditions were applied by evacuating and flushing the jar three times with helium. Using a syringe, 10 ml of headspace air was removed and replaced with 10 ml of acetylene gas to prevent reduction (full denitrification) of N_2O to N_2. Three replicates for each soil sample were incubated on a rotary shaker at 25 °C and gas samples were removed after 0, 2, 4 and 6 h. N_2O concentrations in the gas samples were measured on a gas chromatograph (SRI-8610, SRI Instruments, Torrance, CA, USA) to determine denitrification enzyme activity from rates of N_2O production.

2.4. Abundance of nitrifiers and denitrifiers genes

DNA was extracted from 250 mg of frozen soil using the Nucleospin™ 96 kit for soil (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol, using a JANUS Automated workstation (Perkin Elmer, Waltham, Massachusetts, USA). DNA extractions were made in triplicate, and a pooled aliquot was quantified using the Quant-iT PicoGreen dsDNA Assay (Invitrogen, Carlsbad, California, USA) and diluted to a target concentration of 1 ng DNA μl^{-1} for qPCR reactions. The abundances of nitrifier and denitrifier genes (gene copy number) were estimated using qPCR targeting genes for nitrification (ammonia monooxygenase *amoA* for bacteria and archaea) and denitrification (*nirS* and *nirK*). For all genes, the qPCR mix was made with 10 µl of SensiFAST™ SYBR Lo-ROX mix (Bioline, Luckenwalde, Germany) and 0.5 µM for each primer in a 20 µl final volume. qPCR runs were undertaken on a MX3000P real-time PCR system (Stratagene, Agilent Technologies Inc., San Diego, California, USA). The primers for the genes are shown in Table S1. The qPCR conditions used for each gene were the same as those from previous studies reported in Table S1. All the qPCR efficiencies were greater than 90%. The gene copy abundance was calculated using the standard curve obtained from a serial dilution of plasmids containing PCR products of each specific gene, ranging from 10^2 to 10^9 gene copies μl^{-1}. The standard was made from a PCR product amplified from a pool of DNA collected. The PCR product was cleaned and prepared using the Pgem-T easy cloning system (Promega, Madison, WI, USA). For the qPCR of standard and samples, the melting curves were analyzed to confirm the single target fragment of the PCR products.

2.5. Bacterial richness and composition and microbial biomass carbon

Microbial biomass and bacterial richness were measured to characterise their relationships with N_m and test whether these relationships are stronger in the subsoil compared to those in the topsoil (hypothesis 1). These variables together with bacterial composition also provide indicators of the differences between the microbial properties in the topsoil and the subsoil.

Microbial biomass was estimated from the amount of C contained in biomass (C_{mb}), measured following the protocol described by Sparling et al. (1990) based on the difference in extracted C between fumigated and non-fumigated samples measured using a CN analyzer.

Bacterial richness and composition were determined using a DNA metabarcoding approach. The bacterial 16s rRNA gene was amplified using the primers F515 and R806 (Caporaso et al., 2011) following the same conditions for amplification and sequencing as those reported in Dopheide et al. (2021).

Demultiplexed forward and reverse DNA sequences were merged and relabelled for each sample using VSEARCH (Rognes et al., 2016). Sequencing adapters and primers were trimmed from the merged sequences using cutadapt (Martin, 2011). Again, using VSEARCH, the trimmed sequences were quality filtered to remove any with >1 maximum expected error, and dereplicated to remove identical sequences. Non-singleton sequences (those represented by at least two identical sequences) were clustered into operational taxonomic units (OTUs) at a sequence identity threshold of 97% and filtered for chimeras, after which OTU abundance was inferred by mapping the trimmed sequences back to the OTU centroid sequences at a sequence identity threshold of 97%. The OTUs were assigned a taxonomic identity using the RDP Naïve Bayesian classifier (Wang et al., 2007).

Extraction blanks, and negative and positive controls, were examined for contamination. Tag jumping (Schnell et al., 2015) was accounted for by regression of contaminant abundances against the maximum of total abundances in all other samples, after which the coefficient estimates for the 90th quantile regression was used to subtract the same number of sequences from the abundances of all OTUs (Makiola et al., 2019). Bacterial richness was then determined for each sample using the R package vegan. For estimation of bacterial composition, the ordinations were estimated using Bray-Curtis distances between OTU abundances per sample converted to proportions to generate the nMDS ordination data (stress = 0.045) using the R package vegan. A PERMANOVA test showed significant differences in bacterial communities with depth. The sequences for these data were deposited in GenBank Sequence Read Archive and can be accessed under BioProject PRJNA781230 (Dopheide et al., 2021).

2.6. Statistical analyses

Differences in the values for the variables between depths were determined by analysis of variance (ANOVA). All statistical analyses were carried out with RStudio using R version 3.5.2 (R Development Core Team, 2018). Coefficients of variability (cv) were used as indicators of variability for each variable at each depth. Relationships between W_g, C_{total} and N_{total} were tested independently for each depth with $n = 32$.

2.7. Structural equation modelling

Structural equation modelling (Lefcheck, 2016) was used to test for direct and indirect relationships between microbial properties and NO_3^- concentrations in the topsoil and the subsoil. The statistical approach was used to test an a priori conceptual model (Fig. S1), whereby causal pathways are defined based on current knowledge of processes in soils, with the pathways arranged mathematically as a set of linear structured equations. The variables were chosen based on relationships between substrates and microbial properties, starting from total resources (N_{total}) and followed by the pool of available substrates (N_m), microbial properties that influence substrate supply (bacterial richness and C_{mb}), and direct microbial properties (gene abundance and D_e) associated with NO_3 concentrations. pH was not included in the analysis because this did not show strong variability among the samples and between depths (Table 1). Other measured variables (W_g, bacterial composition) were used to provide further context for differences in the relationships between the topsoil and the subsoil.

In our model, N_{total} was selected as proxy for total N and C resources because N_{total} was strongly correlated with C_{total} in both the topsoil (R^2 = 0.97) and the subsoil (R^2 = 0.98). We expected that higher N_{total} would be associated with higher N_m and larger microbial biomass (C_{mb}) (St. Luce et al., 2011). We also expected that higher N_{total} would be associated with greater richness because of the greater amounts of resources (Bardgett and van der Putten, 2014). Higher C_{mb} and bacterial richness were expected to be associated with a larger N_m and thus higher

Table 1
Mean ± standard deviation and the coefficient of variability for total soil carbon (C_{total}) and nitrogen (N_{total}) concentrations, carbon to nitrogen ratio (C_{total}:N_{total}), mineralizable nitrogen (N_m) pool, soil gravimetric water content (W_g), pH, nitrate (N-NO_3^-) and ammonium (N-NH_4^+) concentrations, microbial biomass C (C_{mb}), bacterial richness, ammonia oxidizing bacteria (AOB *amoA*) and archaea (AOA *amoA*) gene copy number, copper containing nitrate reductase gene copy number (*nirK*), heme *c* and heme d_1 containing nitrate reductase gene copy number (*nirS*), and denitrification enzyme activity (D_e) for the topsoil and the subsoil. For all variables $n = 32$. The *p* values represent significance from the analyses of variance.

Variable	Topsoil		Subsoil		*p* value
	Mean ± sd	cv (%)	Mean ± sd	cv (%)	
C_{total} (g C kg^{-1} soil)	51.6 ± 4.8	9	21.8 ± 5.9	27	<0.0001
N_{total} (g N kg^{-1} soil)	4.9 ± 0.4	8	1.8 ± 0.5	28	<0.0001
C_{total}:N_{total}	10.5 ± 0.1	1	11.6 ± 0.5	4	<0.0001
N_m (mg kg^{-1} soil)	100.0 ± 18.7	19	9.32 ± 6.0	64	<0.0001
W_g (%)	36.7 ± 2.5	7	12.0 ± 3.3	28	<0.0001
pH	6.0 ± 0.2	3	6.0 ± 0.1	2	0.3
N-NO_3^- (mg kg^{-1} soil)	16.8 ± 6.7	40	1.5 ± 1.4	93	<0.0001
N-NH_4^+ (mg kg^{-1} soil)	2.9 ± 4.0	138	0.9 ± 2.4	267	<0.05
C_{mb} (mg kg^{-1} soil)	658.1 ± 151.9	23	119.5 ± 104.9	88	<0.0001
Bacterial richness	3392.8 ± 599.5	18	2678.5 ± 346.3	13	<0.0001
AOA *amoA* (copies g^{-1} soil)	3.4×10^6 ± 2.0×10^6	59	5.3×10^5 ± 3.2×10^5	60	<0.0001
AOB *amoA* (copies g^{-1} soil)	6.9×10^5 ± 3.4×10^5	49	4.6×10^4 ± 2.2×10^4	113	<0.0001
nirS (copies g^{-1} soil)	1.6×10^7 ± 8.0×10^6	50	2.5×10^6 ± 1.9×10^6	74	<0.0001
nirK (copies g^{-1} soil)	9.8×10^6 ± 4.8×10^6	49	5.3×10^5 ± 3.9×10^5	74	<0.0001
D_e (mg N-N_2O kg^{-1} soil h^{-1})	182.6 ± 67.3	37	7.6 ± 5.5	72	<0.0001

NH_4^+ and NO_3^- concentrations, due to higher enzyme activity and diversity (Puri and Ashman, 1998; Tardy et al., 2015). Further, we expected that higher N_m would supply substrate for nitrification and denitrification, and be associated with higher denitrifier enzyme activity, higher gene abundance for denitrification (*nirK*, *nirS*), and nitrification (bacterial and archaeal *amoA*). Finally, we expected that higher denitrification enzyme activity would be associated with lower NO_3^- concentrations, and that a higher abundance of nitrifiers genes would be associated with higher NO_3^- concentrations (Fig. S1).

We used the piecewise SEM package (Lefcheck, 2016) in R to test our conceptual model against observed data using paddock origin of samples as a random term. The goodness of fit for the model was evaluated using Shipley's test of directed separation, whereby the model was acceptable if Fisher's C was statistically non-significant ($p > 0.05$) (Shipley, 2009).

From initial runs of the model, we found that the archaea *amoA* gene abundance was not associated with N_m and NH_4^+-N concentration was not associated with N_m or archaea *amoA* gene abundance. The model did not explain differences in either, archaea *amoA* gene abudance or NH_4^+-N, so the model was simplified by removing these components. The denitrifying genes *nirK* and *nirS* were combined into one single variable (*nirK* + *nirS*) as they showed the same relationships in the model as those when evaluated independently (data not shown).

Standardized regression coefficients and their significance were used to indicate the strength and direction of the pathways. Conditional (all factors) coefficients of determination were reported for the models for both the topsoil and the subsoil.

3. Results

3.1. Topsoil and subsoil physical and microbial properties

Soil C_{total}, N_{total}, N_m, W_g, C_{mb}, bacterial richness, nitrifier and denitrifier gene abundances and D_e were significantly lower in the subsoil than those in the topsoil ($p < 0.0001$). pH showed no significant difference between depths, with the mean ± standard deviation being 6.0 ± 0.2 (Table 1). Both NO_3^--N and NH_4^+-N concentrations were significantly lower in the subsoil compared with the topsoil.

W_g increased linearly with increasing C_{total} and N_{total} in the topsoil ($p < 0.05$) and the subsoil ($p < 0.001$) (Fig. 1). A higher coefficient of determination was observed for the relationship for the subsoil ($R^2 = 0.5$) compared to the topsoil ($R^2 = 0.2$) (Fig. 1).

Variability in total resources (C_{total} and N_{total}), soil substrates (N_m, NO_3^--N and NH_4^+-N concentrations), W_g, and microbial properties (gene abundance, denitrification enzyme activity, microbial biomass C, bacterial richness) were generally higher in the topsoil compared to the subsoil (Table 1). Non-metric MDS ordinations showed very clear evidence of differences in bacterial community composition in the topsoil and the subsoil ($F_1 = 0.63149$, $p < 0.001$) (Fig. S2).

3.2. Relationships between microbial properties, mineralizable N, and NO_3^--N concentrations in the topsoil and the subsoil

The model explained 82% of the variation in NO_3^--N concentrations for the subsoil, but only 6% of the variation for the topsoil (Fig. 2). For the topsoil, there were significant relationships between N_{total} and N_m, and between N_{total} and C_{mb} (Fig. 2a). In the topsoil, although there were no significant relationships between the driving variables and NO_3^--N, there were relationships between C_{mb} and denitrifier gene abundance, denitrifier gene abundance and denitrification enzyme activity, and N_m and denitrification enzyme activity. The variables measured did not explain any of the variation in bacterial richness or nitrifier gene abundance in the topsoil.

The significant effects of N_{total} on N_m and C_{mb} in the topsoil were also detected in the subsoil (Fig. 2b). In the subsoil, there was a significant relationship between bacterial richness and N_m, and denitrifier gene abundance and nitrifier gene abundance, but no significant link was observed between the denitrifier gene abundance and denitrifier enzyme activity. However, NO_3^--N concentrations were positively associated with both N_m and nitrifier gene abundance, and negatively associated with denitrification enzyme activity (Fig. 2b).

4. Discussion

Using the natural variability in N_m and microbial properties at the field site with the same soil type, we were able to apply the SEM model to identify the associations and quantify differences in the relationships between NO_3^- concentrations, microbial properties, and substrate concentrations for both the topsoil and the subsoil. Our findings showed that bacterial richness and biomass, denitrifier and nitrifier gene abundance, D_e, and N_m were lower in the subsoil compared to the topsoil. This is consistent with observations of declining microbial biomass, gene abundance, diversity and soil resources with increasing depth for a range of ecosystems (Eilers et al., 2012; Hsiao et al., 2018; Stone et al., 2014). The contrasting environments in the topsoil and the subsoil hosted bacterial communities with different composition, consistent with previous reports suggesting that decreasing substrate availability associated with depth is the main driver for differences in community composition (Zhang et al., 2017; Dopheide et al., 2021). The fact that a significant relationship was observed between richness and N_m in the subsoil suggests that the number of bacterial species may influence the size of N_m pool, likely via increased amounts of enzymes engaged in mineralization of organic N (Tian et al., 2017; Uksa et al., 2015). Alternatively, this positive relationship may also have resulted

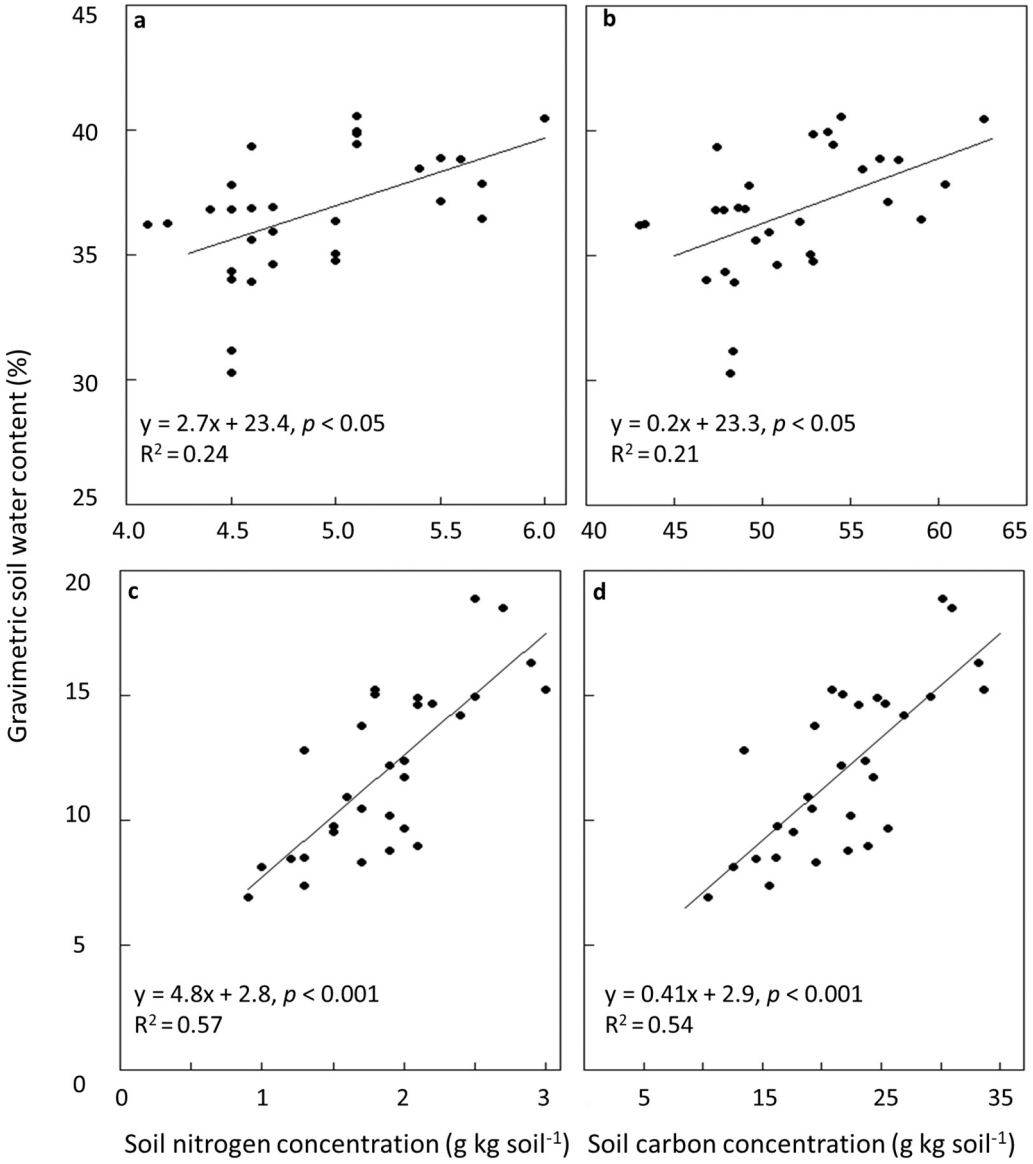

Fig. 1. Gravimetric soil water content (%) in relation to total nitrogen and carbon concentrations in (a, b) the topsoil (depth 0–0.1 m) and (c, d) the subsoil (depth 0.3–0.5 m). The lines shown are linear fits to the data.

from an increasing number of bacterial species with strong contribution to changes in the N_m pool. The low density and high variability of roots (source of N_m) in the stony soil (Sim et al., 2015) may also have contributed to a wider variability in N_m-rich spots in the subsoil, as reflected in the higher coefficient of variability for N_m in the subsoil compared to the topsoil. The findings further suggest that the substrate concentrations are likely to exert a selection pressure for microbes, potentially affecting the number of species (bacterial richness), in agreement with previous observations showing that microbial community composition is strongly related to the size of the substrate pool in nutrient depleted conditions in the subsoil (Dopheide et al., 2021). Future studies are needed to determine how bacterial composition relates to N_m and how changes in composition are functionally related to mineralization of organic N in the subsoil.

Our findings suggest that NO_3^- concentrations can be strongly driven by microbial processes in a well-drained subsoil. NO_3^- concentrations in the subsoil were positively related to bacterial nitrifier abundance and negatively related to denitrification enzyme activity, with both being regulated by N_m. Our findings confirm our second hypothesis of consistency with incubation studies showing that the potential for denitrification and nitrification in subsoil is limited by substate availability (Clough et al., 1998; Swensen and Bakken, 1998; Chen et al., 2018). Furthermore, we provide evidence that, in conditions of low substrate availability in the subsoil, denitrification and nitrification are likely to be regulated by the supply of substrates from mineralization of organic N.

An assumption in this study is that N_m represents the potential supply of C, NH_4^+ and NO_3^- derived from SOM through mineralization (Booth et al., 2005; Kuntz et al., 2016; Curtin et al., 2017). Despite the expected low rates of mineralization of organic N in the subsoil (Jones et al., 2018; Tian et al., 2017) the fact that two independent indicators of denitrification (gene abundance and denitrification enzyme activity) and nitrifier gene abundance were positively linked to N_m support our assumption that N_m, along with C and N supply, contribute to regulating denitrification and nitrification in the subsoil. This observation is consistent with previous reports showing that C availability (measured as water extractable C) is a strong driver for denitrification in the subsoil below the rooting zone (Jahangir et al., 2012; Chen et al., 2018; Yuan et al., 2019).

The variability in water content may also have contributed to the

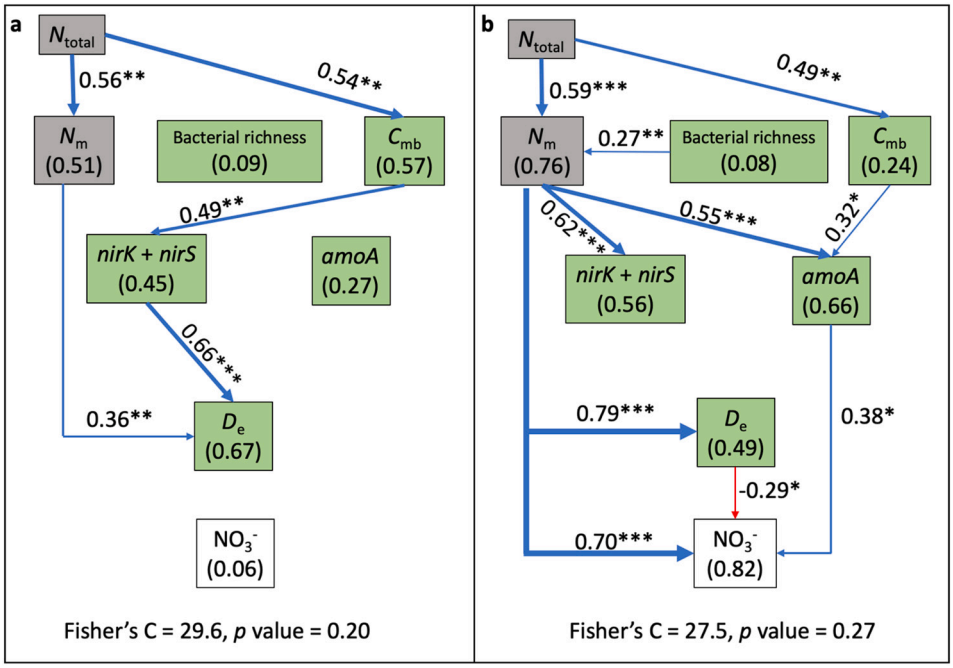

Fig. 2. Structural equation modelling (SEM) to indicate relationships of soil biological and biochemical variables and substrates driving nitrate (NO_3^-) concentrations for (a) the topsoil (depth 0–0.1 m) and (b) the subsoil (depth 0.3–0.5 m). Blue arrows indicate positive relationships and red arrows indicate negative relationships. Numbers adjacent to the arrows are standardized path coefficients (regression coefficients), with the thickness of the lines indicating the relative sizes of the coefficients. *p* values are shown where * ≤ 0.05; ** ≤ 0.01; and *** ≤ 0.001. The conditional coefficient of determination for each variable is shown in parentheses. The symbols are total soil nitrogen concentration ($N_{total,}$), mineralizable N ($N_{m,}$), microbial biomass carbon (C_{mb}), bacterial ammonia oxidizing gene copy number (*amoA*), nitrate reductase gene copy number (*nirK* + *nirS*), and denitrification enzyme activity (D_e). Grey boxes represent substrates and green boxes represent microbial properties.

strong linkages between N_m and denitrifier gene abundance and denitrification enzyme activity. We observed a strong linear relationship between W_g and indicators of SOM (N_{total}, C_{total}) in the subsoil, and the model showed that N_m was linked to N_{total} contained in SOM. Increased SOM amounts could be associated with an increase in water retention (Rawls et al., 2003) and this could contribute to an increase in anaerobic conditions that promote denitrification (Clough et al., 2005). This interpretation is consistent with the lack of an expected relationship between the abundance of denitrifier genes and denitrification enzyme activity in the subsoil. The lack of a relationship between denitrifier gene abundance and denitrification enzyme activity can be explained by the variability in anaerobic conditions in the subsoil associated with variability in water content. Microorganisms harboring denitrifier genes are widespread among heterotrophic microorganisms, but their denitrification activity is triggered in anaerobic conditions (Philippot et al., 2007). We argue that in the relatively substrate depleted conditions in the subsoil compared with the topsoil, SOM hotspots supply substrates and may retain more water, providing microsite reductive conditions that enhance denitrification (Loick et al., 2017), leading to increased reduction of NO_3^-. This suggests that water content may be a strong driver of denitrifier enzyme activity in the subsoil.

The lack of linkages between NO_3^- and soil properties was observed in the topsoil, despite the much higher N_m, microbial biomass, bacterial richness, denitrifier enzyme activity, and abundance of nitrifiers and denitrifiers compared to the subsoil. The lack of a relationship between bacterial richness and N_m in the topsoil could be explained by the higher bacterial richness that may have led to higher redundancy that decoupled richness from function compared to the subsoil (Bardgett and van der Putten, 2014; Louca et al., 2018). It is likely that frequent N inputs from agricultural management and nitrogen fixation, and N uptake associated with high root density in the topsoil (Sim et al., 2015) would have contributed to decoupling of NO_3^- concentration from N_m and indicators of nitrification and denitrification (Abalos et al., 2019), compared with low N inputs, fixation and uptake in the subsoil.

Our findings provide new insights demonstrating that, contrary to the assumption that variations in subsoil NO_3^- concentrations are driven primarily by leaching from the topsoil (Cameron et al., 2013; Hansen et al., 2019), microbially-driven processes also contribute significantly to regulating NO_3^- concentrations in the subsoil in field conditions. Consistent with previous studies suggesting the need to include depth as a factor in estimating nutrient cycling (Yost and Hartemink, 2020), our findings suggest that, to reduce uncertainties in modelling and predicting nitrate leaching losses, studies need to consider the potential for microbially-mediated attenuation (or increases) in NO_3^- concentrations throughout the soil profile.

5. Conclusions

We found differences in relationships between substrate availability and microbial drivers of NO_3^- concentrations in the subsoil and the topsoil under lucerne in a well-drained, stony soil. Soil NO_3^- concentrations in the topsoil were not related to the mineralizable N pool, nitrifier and denitrifier gene abundance or denitrification enzyme activity. In contrast, in the subsoil, NO_3^- concentrations were related, both directly and indirectly, to these variables. Our findings suggest that microbially-driven processes contribute significantly to NO_3^- concentrations in the subsoil. Although substrate concentrations, denitrifier gene abundance and denitrification enzyme activity in the subsoil are much lower than those in the topsoil, enhanced subsoil microbial denitrification could lead to a significant effect on reducing total NO_3^- content because of the much larger volume of soil, and lead to reduced NO_3^- leaching. In well-drained subsoils, management practices to increase denitrification activity should focus on increasing the size of the mineralizable N pool and carbon availability. However, increasing subsoil denitrification may also increase N_2O emissions and this needs to be considered as a trade-off effect in future field experiments.

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.apsoil.2022.104499.

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