Interactions of fertilisation and crop productivity on soil nitrogen cycle microbiome and gas emissions

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Abstract. Fertilised soils are a significant source of nitrous oxide (N_2O), a highly active greenhouse gas and stratospheric ozone depleter. Nitrogen (N) fertilisers, while boosting crop yield, also lead to N_2O emissions into the atmosphere, impacting global warming. We investigated relationships between mineral N fertilisation rates and additional manure amendment with different crop types through the analysis of abundances of N cycle functional genes, soil N_2O and N_2 emissions, nitrogen use efficiency (NUE), soil physicochemical analysis and biomass production. Our study indicates that N_2O emissions are predominantly dependent on the mineral N fertilisation rate and enhance with increased mineral N fertilisation rate. Higher N_2O emissions were attained with the application of manure in comparison to mineral fertilisation. Manure amendment also increased the number of N cycle genes that are significant in the variations of N_2O. Contrary to our hypothesis, there was no significant influence of crop type on soil N_2O emissions. The study indicated that N_2O emissions were mainly related to nitrification in the soil. Quantification of nitrogen cycle functional genes also showed the potential role of denitrification, comammox and DNRA processes as a source of N_2O. Our study did not find soil moisture to be significantly linked to N_2O emissions. Results of the study provide evidence that for wheat, a fertilisation rate of 80 kg N ha^{-1} is closest to the optimal rate for balancing biomass yield, N_2O emissions, and achieving high NUE. Sorghum showed a good potential for cultivation in temperate climate, as it showed similar biomass yield compared to the other crop types and fertilisation rates, but maintained low N_2O emissions and N losses on mineral N fertilisation rate of 80 kg N ha^{-1}.

1 Introduction

The rising demand for agricultural commodities and the management of agroecosystems are important factors contributing to global environmental problems. Increasing crop yield while reducing pollution from agricultural production is crucial (Abdalla *et al.*, 2019; Tilman *et al.*, 2011). Global food demand projections suggest a 50% increase in agricultural production by 2050 (compared to 2012) to feed the fast-growing human population (FAO, 2017). Enhancing agricultural production involves actions such as expanding agricultural land, applying more fertilisers, and using water resources and fertilisers more effectively (Tian *et al.*, 2021). In today's agricultural practises, the applied N with fertilisation is often excessive for plant needs (Robertson and Vitousek *et al.*, 2009; Zhou *et al.*, 2016). About half of the applied N to the fields is not taken up by crops (Coskun *et al.*, 2017); which may lead to N loss in the surrounding environment. Main soil N loss mechanisms include denitrification, ammonia oxidation, N leaching, erosion of soil and ammonia (NH_3) volatilisation (Thomson *et al.*, 2012). This results in adverse ecological impacts, such as eutrophication of aquatic ecosystems and increased gaseous emissions of N into the atmosphere (Cameron *et al.*, 2013; Liu *et al.*, 2017; Whetton *et al.*, 2022).

Fertilised soils are a significant source of nitrous oxide (N_2O), contributing to the greenhouse effect and ozone depletion (Ravishankara *et al.*, 2009; Shcherbak *et al.*, 2014). N_2O has 273 times higher global warming potential than carbon dioxide (CO_2) over a 100-year timescale (IPCC, 2021). Even without adding N fertiliser in the current season or year, background N_2O emissions (BNEs) may still occur. BNEs are caused by different N sources, including residual N in the soil from previous years' N application, deposition from the atmosphere, biological N_2 fixation and mineralised N from plant residues (Gu *et al.*, 2007; Kim *et al.*, 2013, Abdalla *et al.*, 2022).

The key microbial processes leading to soil N loss are nitrification and denitrification (Thomson *et al.*, 2012). In agriculture, N fertilisers added to the soil can be lost due to these processes (Saud *et al.*, 2022). Nitrification was traditionally viewed as a two-step process carried out by separate functional groups of microorganisms, oxidising ammonium (NH_4^+) sequentially to nitrite (NO_2^-) and nitrate (NO_3^-) under aerobic conditions (Kuypers *et al.*, 2018; Koch *et al.*, 2019; Nardi *et al.*, 2020). However, in 2015, a significant advancement in our understanding of nitrification occurred with the discovery that a single microorganism, through the comammox (complete ammonia oxidation) process, can perform both nitrification steps (Daims *et al.*, 2015; Van Kessel *et al.*, 2015). Nitrification can reduce N availability for plant uptake by up to 50%, primarily due to NO_3^- leaching and N_2O emissions (Beeckman *et al.*, 2018). Synthetic fertilizers containing NH_3 offer an immediate substrate for ammonia oxidizers, thus accelerating the nitrification process (Ayiti & Babalola, 2022). Also, fertilizers that raise soil pH can significantly enhance the nitrification rate, as increasing soil pH from 4.8 to 6.7 can boost nitrification rates by 30 times (DeForest & Otuya, 2020).

Denitrification is a microbially-catalysed process under oxygen-limited condition responsible for transforming NO_3^- sequentially to gaseous forms of N: nitric oxide, N_2O and atmospheric N (Philippot *et al.*, 2007; Zaman *et al.*, 2012). The input of N fertilisers affects the soil's mineral N pool by providing larger amounts of available N for nitrification and denitrification processes, contributing to N_2O emissions (Engel *et al.*, 2010). Dissimilatory nitrate reduction to ammonium (DNRA) supplies NH_4^+ to the soil, conserves bioavailable N and prevents the leaching of NO_3^- (Bai *et al.*, 2020; Pandey *et al.*, 2020). DNRA competes with denitrification in NO_3^--reducing processes (Putz *et al.*, 2018). Similarly to denitrification and nitrification processes, DNRA can also be a source of N_2O, although the quantities are modest (Rütting *et al.*, 2011; Stremińska *et al.*, 2012; Zaman *et al.*, 2012). Carbon to nitrogen ratio (C/N) and C/NO_3^- are recognised as the main environmental factors controlling, which nitrate-reducing process is favoured as DNRA and denitrifying microbes compete for NO_3^- and carbon sources (Bai et al., 2020). DNRA is dominant in the presence of a high C/N ratio and low NO_3^- availability, while the denitrification process favours a low ratio of C/N and C/ NO_3^- (Bai et al., 2020; Pandey et al., 2020).

These processes are mediated by different functional marker genes, including archaeal, bacterial and comammox *amoA* genes for nitrification, *nrfA* genes for DNRA and *nosZ* clade I and II, *nirK*, *nirS* genes for denitrification (Zaman *et al*., 2012; Hu *et al., 2015;* Zhang *et al.*, 2021).

C3 photosynthesis, a dominant pathway among plants and found in wheat and barley, uses the Calvin-Benson pathway, while an alternative, the Hatch-Slack pathway, is used by C4 plants like sorghum and maize (Hibberd and Quick, 2002; Ehleringer and Cerling, 2002; Ehleringer, 1979; Ledvinka, 2022). In C3 plants, water loss through transpiration during CO_2 uptake is a risk in hot and water-limited conditions (Joshi *et al*, 2022; Stevens *et al*., 2022). However, C4 plants, with higher water use efficiency and greater tolerance to hot and dry environments, make the cultivation of sorghum and other drought-tolerant plants likely to expand in regions affected by droughts (Anderson *et al*., 2020). Due to climate change, sorghum, as a resilient plant, is considered a novel crop for temperate Europe (Schaffasz *et al*., 2019). Only a limited number of studies have compared N_2O emissions between different crop species. Abdalla *et al*. (2022) found that crop type has significant effect ($p<0.05$) on the BNE values from soil. Furthermore, Bouwman *et al*. (2002) also found that crop type has a significant influence on N_2O emissions. However, study including 372 sites showed that cover crops did not have significant ($p>0.05$) effect on direct N_2O emissions (Abdalla *et al*., 2019).

Previous studies on long-term fertilisation experiments have mostly focused on fertilisation’s yield effects and changes in soil organic matter (Cvetkov and Tajnšek, *et al*., 2009; Hijbeek *et al*., 2017; Káš *et al*., 2010; Spiegel *et al*., 2010; Tajnšek *et al*., 2013). Improved management of arable soils holds significant potential for mitigating greenhouse gas emissions, as agroecosystems contribute ca 66% of total anthropogenic N_2O emissions (Davidson and Kanter, 2014; Paustian *et al*., 2016; Shen *et al*., 2021). Efficient mitigation of N loss requires a comprehensive understanding of microbial processes related to N_2O emissions in agricultural soils (Davidson and Kanter, 2014; Shen *et al*., 2021).

The general objectives of the study were to evaluate temporal patterns of gaseous N loss, link N-cycle processes with abundances of functional N cycle genes in arable soil, and evaluate the performance of different crops (including novel crop in Northern Europe) in terms of biomass production and N_2O emissions under mineral and organic fertilisation. The following hypotheses were tested: (1) crop type significantly affects N_2O emissions; (2) nitrification is the primary pathway of soil N_2O production due to aerobic conditions; (3) in arable soil, low soil moisture results in reduced N_2O losses; (4) amendment of manure fertiliser increases soil N_2O emissions and affects the abundances of functional N cycle genes.

2 Material and methods

2.1 Field experiment description

The field study was conducted on the International Organic Nitrogen Long-term Fertilisation Experiment (IOSDV; Internationaler Organischer Stickstoff Dauerdüngungs Versuch) experimental field. The experimental site is located near Tartu, southern Estonia, Northern Europe (58°22’30” N, 26°39’48” E). The experiment was set up as a three-field crop rotation experiment in 1989 to investigate the long-term effects of mineral and organic fertilisation on crop responses of various crops and soil properties.

In 2022, the average temperature in the area was −2.0 °C in winter, 4.6 °C in spring, 18.1 °C in summer and 7.2 °C in autumn. The mean annual precipitation was 531 mm (Republic of Estonia Environment Agency, 2023) in 2022. A climate diagram for the area during the study period is in Figure S1 in the Supplementary materials.

The soil type is *Stagnic Luvisol* combined with *Fragic Glossic Retisol* (IUSS WG WRB 2015). The thickness of the humus layer is 27-32 cm. Soil texture by FAO classification is sandy loam: 57.86% sand (>0.063 mm), 33.58% silt (0.063–0.002 mm) and 8.55% clay (<0.002 mm). Soil bulk density was in range of 1.5 to 1.6 g cm^{-3} with slightly lower values for manure treatment plots. The average pH levels in spring 2022 were 5.4 for barley plots, 5.3 for wheat plots, 5.6 for sorghum plots without manure amendment, and 6.2 for sorghum plots with manure amendment.

The experiment was organised into 12 plots in a systematic block design (Figure 1) with three sampling spots per plot. Every plot was 50 m^2 in size. The crop species studied were spring barley (cultivar "Elmeri"), sorghum (*Sorghum bicolor x Sorghum sudanense,* cultivar "SUSU"), and spring wheat (cultivar "Mistral"). Initially, the crop rotation was potato–spring wheat–spring barley (Astover *et al*., 2016). In 2019, potato was replaced with sorghum-sudangras hybrid.

The fertiliser treatment consisted of mineral N fertilisation and mineral fertilisation with farmyard manure amendment. All fertilisation treatments are applied continuously from year 1989, when the experimental site was established. Three mineral N fertiliser treatment rates were studied: 0, 80 and 160 kg N ha^{-1}. The farmyard manure rate added to the sorghum plots was 40 t ha^{-1} of manure (231.2 kg N ha^{-1}). The mineral fertiliser applied was ammonium nitrate (NH_4NO_3) and organic fertiliser was farmyard manure. The farmyard manure was cattle dung with straw bedding, freely fermented before use 6-8 months in heap. The chemical properties (C, N, P, K) of manure added in 2022 and during the last ten years are presented in Table S1 in the Supplementary materials. The farmyard manure treatment with mineral fertilisation was applied only to sorghum. Manure treatment is amended with solid farmyard manure (40 t ha^{-1}) in every third year before sorghum/potato. The main management activities and timing in the field are displayed in Table S2 in Supplementary materials.

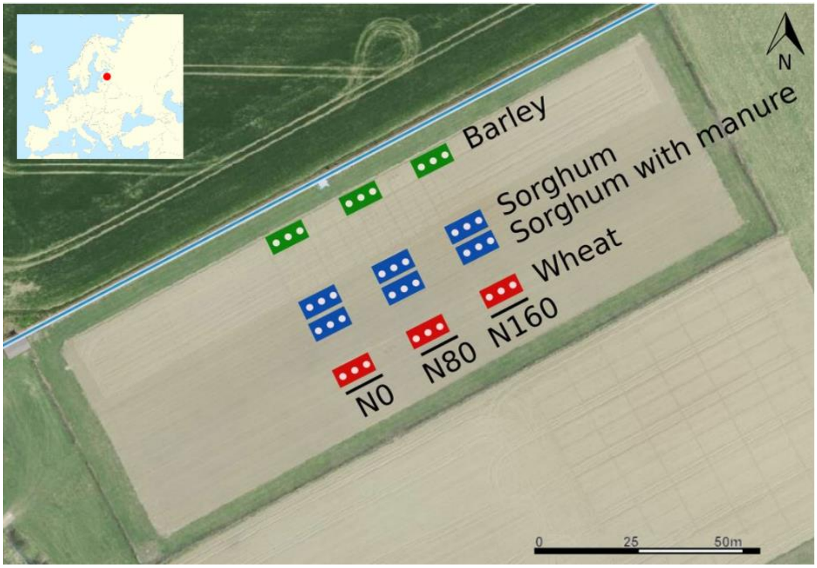

Figure 1: Satellite view of the study site with study plots (from Maa-amet). Each plot constituted of three sampling spots indicated as white dots. N0 – 0 kg N ha^{-1}, N80 – 80 kg N ha^{-1}, N160 – 160 kg N ha^{-1} as mineral fertiliser.

2.2 Gas sampling for N_2O flux analyses

The field study was conducted during the growing season from April 2022 to October 2022. Sampling took place on 15 different dates, starting on April 27th and ending on October 12th (every week until the end of June and then twice a month until the end of September). Gas samples for N_2O flux analysis were collected on all fifteen fieldwork days. N_2O gas sampling was carried out using the static chamber method (Hutchinson and Livingston, 1993). Polyvinyl chloride chambers (Ø 50 cm, volume 65 L) were placed on top of the collars during the gas sampling. Chamber extensions were used for some treatments of sorghum on four occasions as the chambers alone were too small to accommodate the growing crops. Pre-vacuumed 50 ml glass vials were used for gas sampling. Gas samples were collected at 20 minutes intervals for one hour (0, 20, 40, 60 min). The concentration of N_2O in the collected air was measured in the Biogeochemical Cycling Research Laboratory in the Department of Geography, University of Tartu, with the gas chromatograph Shimadzu GC-2014 (Kyoto, Japan), equipped with electron capture and flame ionisation detectors (Poole, 2015).

2.3. Soil sampling and physicochemical analyses

Soils were sampled for chemical and microbiological analyses six times (April 27th, May 9th, June 2nd, July 7th, September 2nd, October 12th). Soil sampling was conducted after gas sampling. Soil samples were collected close to collars with a soil probe from the top 10 cm of the soil. Three auger samples from each point (both bulk and rhizosphere soil were sampled) were collected for one composite sample for chemical and microbiological analyses. All in all, 216 samples were collected for chemical analyses and 144 samples for microbial analyses. Until chemical and microbiological analyses, samples were stored at +4 ˚C and −20 ˚C, respectively. In addition to soil sampling, soil temperature (°C) at a 10 cm depth was measured with a temperature logger (Comet Systems Ltd., Rožnov pod Radhoštem, Czech Republic) and soil moisture (m^3/m^3) was recorded using water content reflectometers (model CS615, Campbell Scientific Inc., Logan, UT, USA). The soil samples were analysed for total carbon (C_{tot}), total nitrogen (N_{tot}), nitrate-nitrogen (NO_3^--N), and ammonium-nitrogen (NH_4^+-N) concentrations in the Soil Science and Agrochemistry Laboratory of Estonian University of Life Sciences. N_{tot} and C_{tot} analyses were done by Dumas method (International Organization for Standardization, 1998) with dry combustion on a VarioMAX CNS elemental analyser (ELEMENTAR, Elementar Analysensysteme GmbH, Langenselbold, Germany). NO_3^--N analyses were done according to EPA (United States Environmental Protection Agency) method 9056: determination of inorganic anions by ion chromatography. NH_4^+-N analyses were done according to Thermo Fisher Application Note 141 (AU204: Determination of Inorganic Cations and Ammonium in Environmental Waters Using a Compact Ion Chromatography System) using ion chromatography. Soil pH was measured using a glass-electrode pH meter in a water solution of 1:2.5. Total phosphorus (P) and potassium (K) concentration in manure were determined through acid digestion using a sulfuric acid solution (van Reeuwijk, 2002).

The hot-water extractable C (HWEOC) represents the readily mineralising C fraction and was determined on dry soil samples by a modified method of Haynes and Francis (1993) in two steps. In the first step the soil was shaken with deionized water at room temperature for 1 h. After that the soil suspension put into the thermostat at 80 °C for 16 h. The mixture was centrifuged for 10 min at 8000 rpm and filtered through a 0.45-µm membrane filter (25-mm diameter, nylon, Agilent®). The HWEOC concentration was determined from the extracts by the VarioMaX CNS analyzer (ELEMENTAR, Elementar Analysensysteme GmbH, Langenselbold, Germany).

2.4 Total biomass

The total biomass (above- and below-ground) was measured at the maturity phase on the harvest day of each crop (Supplementary Table S2). The above-ground biomass was cut from the ground level in a 0.2 m^2 area near each collar. The belowground biomass samples were taken with a soil auger (Ø 34 cm). Frasier *et al.* (2016) provides a more detailed description of the method used for below-ground biomass measurement. The sampling depth extended to the plowing depth, where most of the roots are found, up to a depth of 18 cm. Samples were stored at +4 °C until the roots were washed on a sieve (mesh size 0.5 mm).

Dry matter yield was determined after drying the biomass (including roots) at 70 °C to constant weight. The straw and grains were separated before weighing as air dry. The biomass (straw, grain, roots) were milled and the N_{tot} content was determined by the Dumas method with dry combustion on a VarioMAX CNS elemental analyser (ELEMENTAR, Elementar Analysensysteme GmbH, Langenselbold, Germany).

2.5 Soil microbial analyses

2.5.1 DNA extraction

DNA was extracted from 0.25 g of soil samples using the DNeasy® PowerSoil® Pro Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The difference from the instruction was the homogenisation of samples with homogeniser, Precellys 24 (Bertin Technologies, Montaigne-le-Bretonneux, France), for 20 s at the rate of 5000 rpm. The concentration and quality of the extracted DNA were evaluated with an Infinite 200 M spectrophotometer (Tecan AG, Männedorf, Switzerland). The extracted DNA was stored in a freezer at −20 °C.

2.5.2 Quantification of gene copies using qPCR

Quantification of the 16S rRNA genes of bacteria and archaea, along with the quantification of nitrification (bacterial, archaeal, and comammox *amoA*), denitrification (*nirS*, *nirK*, *nosZI*, and *nosZII*) and DNRA (*nrfA*) genes was done using quantitative polymerase chain reaction (qPCR). qPCR reactions were performed by The Rotor-Gene Q thermocycler (Qiagen). The reaction mixture of 10 µL consisted of extracted DNA (1µL), gene-specific forward and reverse primers, Maxima SYBR Green Master mix reagent (5 µL; Thermo Fisher Scientific, Waltham, MA, USA) and distilled water. Each sample was amplified two times. All of the qPCR assays included two DNA-free negative control samples. Details on thermal cycling conditions and used primers are added in Table S3 in Supplementary Materials. The Rotor-Gene® Q software v. 2.0.2 (Qiagen) and LinRegPCR v. 2020.2. were used to assess the qPCR results. The amount of gene copies was calculated using standard curve ranges, and results were presented in gene copies per gram of dry matter (copies/g dw). Espenberg *et al*. (2018) provides a more detailed description of the used qPCR methodology.

2.6 Statistical analyses and modelling

Statistical software programs Statistica (v. 7.1) and R (v. 4.0.4) were used for statistical analyses and visualising the data. Principal component analysis (PCA) were conducted on soil physicochemical parameters and microbiological data (abundance of functional marker genes) with the “FactoMineR” (Lê *et al.*, 2008) and “factoextra” (Kassambara *et al.*, 2020) packages in the software R. Analysis of variance (ANOVA) with post-hoc Tukey HSD test was used (cumulative N_2O emission values are meeting the assumptions of parametric test) to find statistically significant differences between different fertilisation rates, use of manure and crop types.

Spearman’s rank correlation coefficient measured the association between N_2O and N_2 emissions, gene abundances and environmental factors. Random Forest classification analysis was conducted using Boruta v. 8.0 (Kursa and Rudnicki, 2010) to identify the gene parameters that best predicted N_2O fluxes.

Nitrogen use efficiency (NUE, kg DM kg^{-1} N^{-1}) was calculated as the biomass production per unit of N applied (Pandey *et al*., 2001; Supplementary Methodology S1). The N_2 emissions were estimated from the measured N_2O emissions using the N_2:N_2O ratio, which was calculated as proposed in the DAYCENT model (Parton *et al*., 2001), with the equations described in Del Grosso *et al*. (2000) (Supplementary Methodology S2), where the ratio N_2:N_2O is a function of the content of NO_3^- in the soil, CO_2 emissions, and water-filled pore space (WFPS). The change of soil N content (kg N ha^{-1}) was calculated according to Sainju (2017), as the difference between the initial and final soil total N contents (Supplementary Methodology S3). N losses are calculated by substracting N outputs and change of soil N content from N inputs (Sainju, 2017; Escuer-Gatius *et al*., 2022; Supplementary Methodology S4).

Linear mixed-effects model (LMM) was used to investigate differences in N_2O emissions, cumulative N_2 emissions and gene parameters between different crop types and fertilisation rates using the R package "nlme." For N_2O emissions and gene parameters, spatial (different fertilisation rate) and temporal (sampling dates) effects were used as random effects. For N_2 emissions, spatial effect (different fertilisation rate) was used as a random effect. The Kruskal–Wallis test and the post-hoc Tukey HSD test were used to compare the N_2O, cumulative N_2 emission and gene parameter (not meeting the assumptions of parametric test) values between different crop types and fertilisation rates. Due to limited number of observations in the case of total dry weight biomass, N content in biomass, LMM was not possible to apply for statistical differences between different fertilisation rates and crop types.

3 Results

3.1 Soil physicochemical characteristics and biomass production

The NH_4^+-N content in soil decreased on most of the plots at the beginning of the study period, while NO_3^--N content was increasing (Supplementary Figure S2). Fertilised plots had higher soil N_{tot}, C_{tot}, NO_3^--N and NH_4^+-N content compared to non-fertilised plots according to the principal component analysis (PCA) (Figure 2; Supplementary Figure S2 and Figure S3). For sorghum without manure amendment plots (Figure 2C), NO_3^--N and NH_4^+-N contents were more different from each other compared to sorghum with manure amendment plots, where NO^-_3-N and NH_4^+-N contents were relatively similar (Figure 2D). HWEOC concentrations were higher in sorghum plots with farmyard manure amendment compared to sorghum plots without manure amendment.

Soil moisture ranged from 0.02 m^3/m^3 to 0.32 m^3/m^3 with an average of 0.23 m^3/m^3 over the study period (Supplementary Figure S4). There were no significant correlations between soil moisture and N_2O emissions (Supplementary Table S4). Soil moisture was not significantly linked to gene copy numbers across all crop types, except *nirS*. A climate diagram for the area during the study period is presented in Figure S1 in the Supplementary materials.

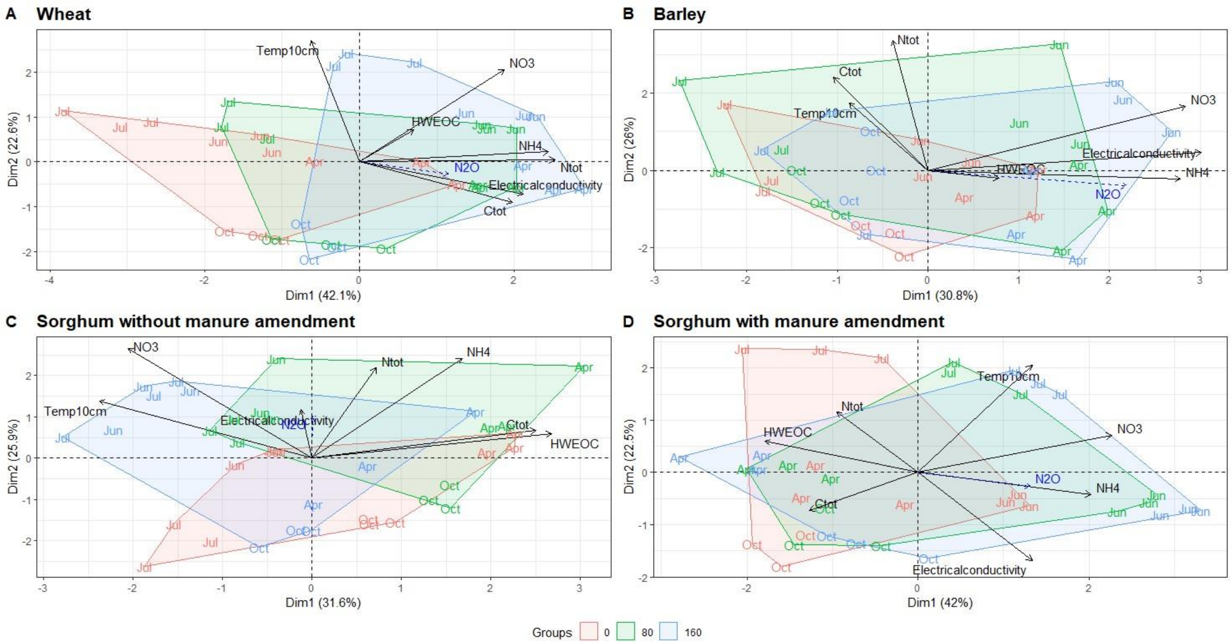

Figure 2: Principal components analysis (PCA) ordination plots demonstrate the grouping of fertilisation rates according to physicochemical parameters for different crop type. N_2O is added as a supplementary variable. The month indicates the sampling time. Abbreviations: Ctot – total carbon content of soil; Ntot – total nitrogen content of soil; HWEOC – hot-water extractable organic carbon.

The total dry biomass of barley ranged between 2.6 to 6.4 t ha^{-1}, and wheat between 4.6 to 8.5 t ha^{-1} depending on the mineral N fertilisation rate (Figure 3). For sorghum without manure amendment, the total dry biomass varied between 2.3 and 7.1 t ha^{-1}, and for sorghum with manure amendment, the total dry biomass varied between 8.2 and 11.7 t ha^{-1}.

The biomass production was higher per unit area of crop growth with higher fertiliser input (Figure 3A). Total biomass was significantly positively correlated with N_{tot} ($p<0.01$), C_{tot} ($p<0.05$) and NO_3^--N ($p<0.001$) levels in soil (Supplementary Table S5). Also, higher N fertilisation rate caused an increase in N content in the crop biomass (Figure 3B).

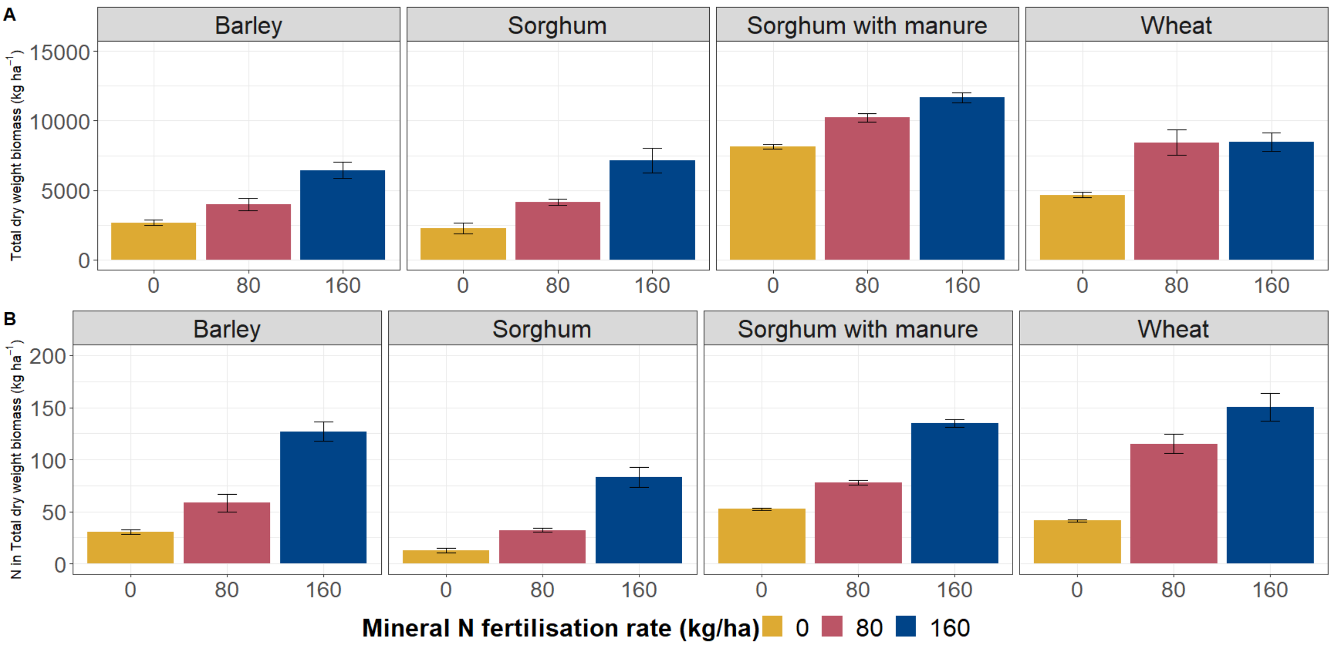

Figure 3: Total dry weight biomass (aboveground + belowground) produced per unit area according to crop types and fertilisation rates. Error bars show standard errors.

The highest values of nitrogen use efficiency (NUE) were obtained from wheat plots, and the lowest from sorghum plots. The average NUE of wheat plots at fertilisation rate 80 was 0.84, and at rate 160, it was 0.64. For sorghum plots with manure amendment, NUE at mineral N fertilisation rate 0 was 0.15, at rate 80 was 0.16, and at rate 160 was 0.25. For sorghum plots without manure amendment, the average NUE at fertilisation rate 80 was 0.12, and at rate 160, it was 0.25. The NUE for barley plots at fertilisation rate 80 was 0.35, and at fertilisation rate 160, it was 0.45. The highest estimated N losses occurred on sorghum plots with manure amendment (Supplementary Table S6). In general, wheat plots at different fertilisation rates lost more N compared to sorghum plots without manure amendment. The lowest estimated N losses occurred on barley plots.

3.2 Nitrogen cycle genes

The abundances of N cycling genes on plots with different fertilisation rates and crop species show different patterns throughout the study period (Supplementary Figures S5, S6, S7, and S8). The principal component analysis (PCA) of the N cycle genes abundances showed differences between sites with different fertilisation rates (Figure 4). There were greater differences in gene abundances between three different mineral N fertilisation rates in sorghum plots compared to barley and wheat plots (Figure 4). For sorghum without manure amendment (Figure 4C), archaeal 16S rRNA and *nosZII* gene abundances were highest for fertilisation rate 80 compared to rate 0 ja 160 ($p<0.001$), but for sorghum with manure amendment (Figure 4D), the highest archaeal 16S rRNA and *nosZII* gene abundances were for fertilisation rate 160 compared to rate 0 ($p<0.001$) and 80 ($p<0.05$). For all sorghum plots, comammox *amoA* gene abundance was highest on non-fertilised plots. However, fertilised wheat and barley plots had higher comammox *amoA* gene abundance compared to non-fertilised plots.

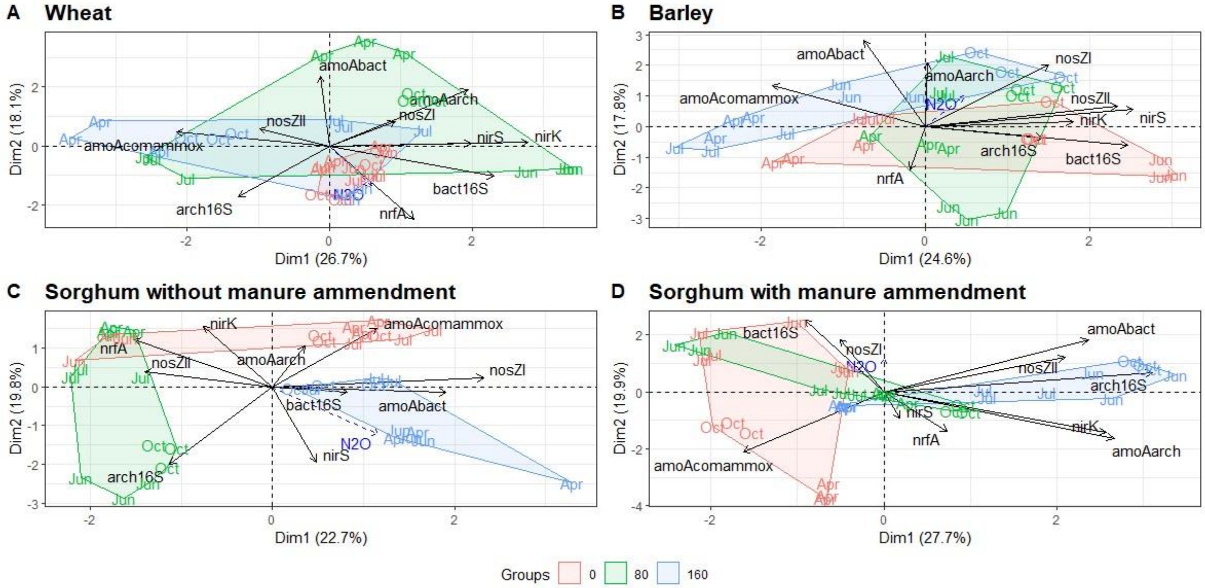

Figure 4: Principal components analysis (PCA) ordination plots demonstrate the grouping of fertilisation rates according to functional marker genes abundances for different crop type. N_2O is added as a supplementary variable. The month shows the sampling time. Abbreviations: bact16S – bacterial 16S rRNA gene; arch16S – archaeal 16S rRNA gene; amoAbact – bacterial *amoA* gene; amoAarch – archaeal *amoA* gene; amoAcomammox – comammox *amoA* gene.

3.3 N_2O emissions

The N_2O emissions over the course of the study period show that different fertilisation rates influence N_2O emissions, and the highest N_2O emissions tend to be emitted from the highest N fertiliser treatment (160 kg N ha^{-1}) (Figure 5). N_2O emissions among all crop species tended to be higher during the first part of the study period (spring and early summer). Taken together, the highest average N_2O emissions for barley plots were measured in the middle of May, for sorghum plots without and with manure in the middle of June, and for wheat plots at the beginning of June.

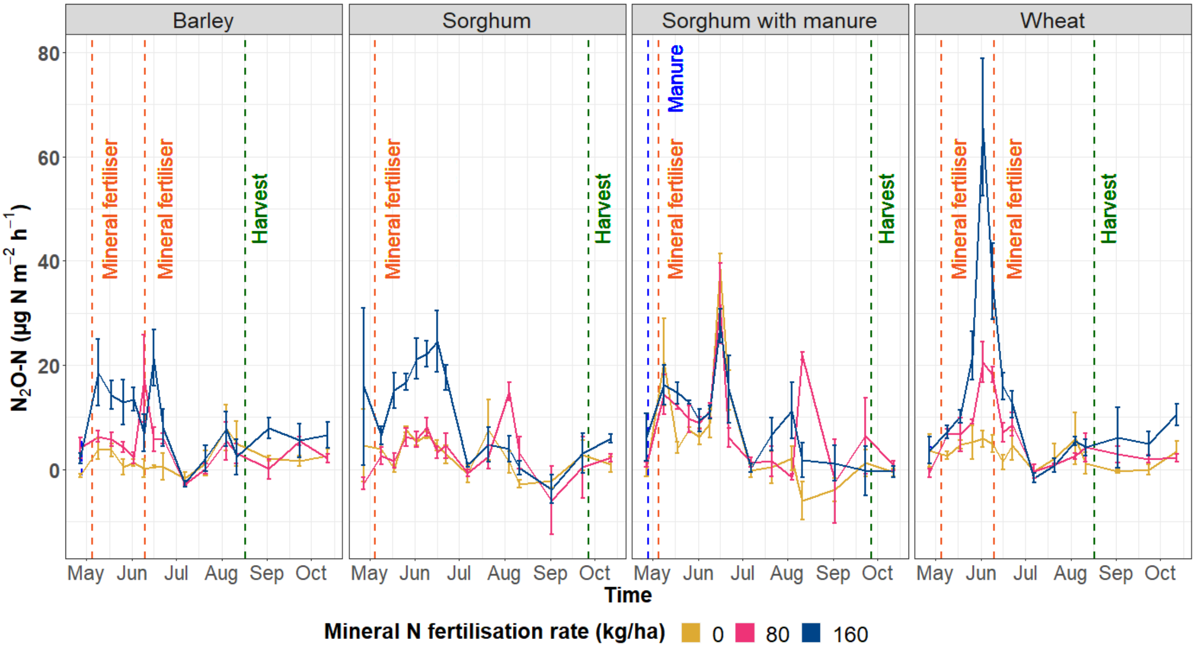

Figure 5: N_2O emissions (µg N m^{-2} h^{-1}) according to crop types and fertilisation rates during the study period.

Throughout the study period, cumulative N_2O and N_2 emissions were highest in plots with the highest fertilization rate, expect sorghum plots with manure amendment (Figure 6A, B). For wheat and barley plots, there is a clear pattern of increasing N_2O emissions with increasing fertilisation rates.

For barley plots, cumulative N_2O emissions did not significantly differ between fertilisation rates 0 and 80 (Figure 6A). However, N_2O emissions on barley plots were significantly higher at fertilisation rate 160 than at rate 0 and 80 ($p<0.05$). Similarly, for wheat plots, cumulative N_2O emissions were also significantly higher at fertilisation rate 160 compared to rates 0 ($p<0.05$) and 80 ($p<0.05$); however, fertilisation rates 0 and 80 did not significantly differ from each other. For plots with sorghum without manure, cumulative N_2O emissions at fertilisation rate 160 were significantly higher compared to fertilisation rates 0 ($p<0.05$) and 80 ($p<0.05$). For sorghum with manure plots, cumulative N_2O emissions at fertilisation rate 160 was significantly different compared to fertilisation rate 0 ($p<0.05$) and 80 ($p<0.05$).

For barley plots, the cumulative N_2 emissions were significantly higher at fertilisation rate 160 compared to rates 0 ($p<0.05$) and 80 ($p<0.05$) (Figure 6B). For wheat, sorghum with and without manure plots, cumulative N_2 emissions emitted from all three fertilisation rates did not significantly differ from each other.

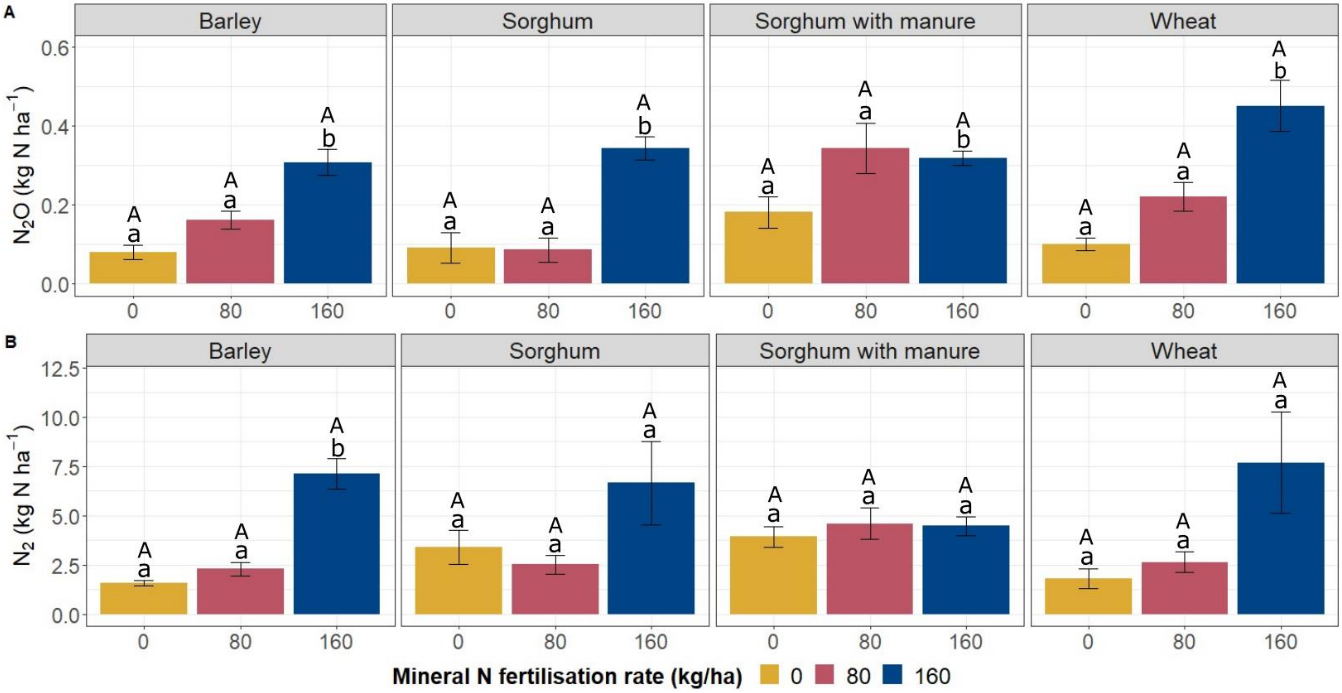

Figure 6: Cumulative N_2O and N_2 emissions according to crop types and fertilisation rates. Error bars show standard errors. Letters above the boxes indicate statistically significant differences at significance level $p<0.05$ according to a post-hoc Tukey HSD test. Lowercase letters indicate comparisons within crop types. Uppercase letters indicate comparisons of the same fertilisation rate over all crop types.

3.4 Relationships between environmental and genetic parameters and N emissions

Mineral N fertilisation rate ($p<0.001$) and manure amendment ($p<0.01$) significantly influenced N_2O emissions (Table 1). Crop type did not significantly influence N_2O emissions. The mineral N fertilisation effect on N_2O emissions ($\omega^2 = 0.528$) was larger compared to the effects of crop type ($\omega^2 = 0.021$) and manure amendment ($\omega^2 = 0.121$).

Table 1: Results of ANOVA testing the effects of crop type, mineral N fertilisation rate and manure amendment on cumulative N_2O fluxes. Significance is indicated as *** – 0.001; ** – 0.01; * – 0.05; ns – not significant.

	Df	**F value**	**Pr (>F)**
Crop type	2	0.957	0.39544
Mineral N fertilisation rate	2	23.995	5.97×10^{-7} ***
Manure amendment	1	11.020	0.00237 **
Residuals	30		

Random Forest classification analysis for the N_2O emissions from wheat plots considered bacterial *amoA*, archaeal *amoA*, *nosZI* and *nosZII* genes relevant (Figure 7). For barley plots, bacterial *amoA*, comammox *amoA*, bacterial 16S rRNA, *nirK*, *nirS* and *nosZII* were deemed as important genes in the variations of N_2O emissions. For sorghum without manure amendment plots, bacterial *amoA*, comammox *amoA*, archaeal 16S rRNA and *nirK* genes were considered important for the N_2O emissions. For sorghum with manure amendment plots, archaeal *amoA*, bacterial *amoA*, comammox *amoA*, *nirK*, *nirS*, *nosZII*, *nosZI* and *nrfA* genes were considered important for the N_2O emissions.

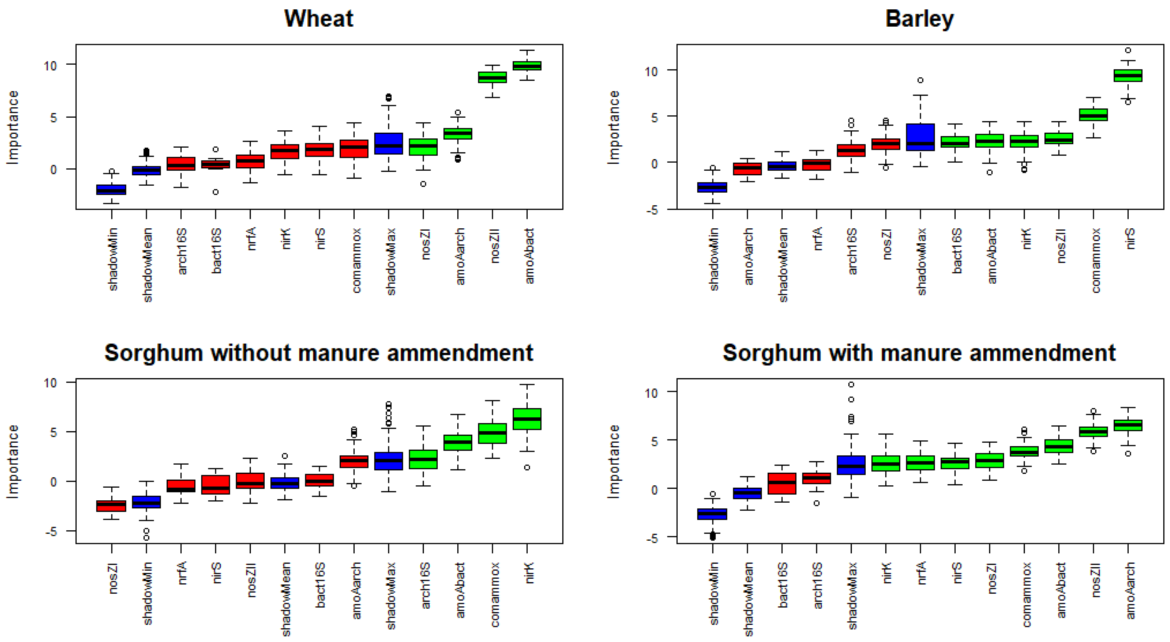

Figure 7: Results of feature selection in predicting the genes that are important in the variations of N_2O emissions. Important factors are indicated in green, unimportant factors in red, and shadow variables (the random shadow copies of features (noise) will be created to test the feature against those copies to determine if it is better than the noise, and therefore significant) in blue. Abbreviations: bact16S – bacterial 16S rRNA gene; arch16S – archaeal 16S rRNA gene; amoAbact – bacterial *amoA* gene; amoAarch – archaeal *amoA* gene; amoAcomammox – comammox *amoA* gene.

The relationships between gene abundances and N_2O emissions showed that the ratio of *amoA*/*nir* was in a significant positive correlation with N_2O emissions ($\rho=0.20$; $p<0.001$). Furthermore, the ratio of *nosZ*/*nir* was also significantly positively correlated with N_2O emissions ($\rho=0.21$; $p<0.001$). *nirS* genes were positively correlated with N_2O emissions over all crops species plots ($\rho=0.19$; $p<0.05$). N_2O emissions from barley plots had also a strong positive correlation with *nirS* gene abundance ($\rho=0.58$; $p<0.001$). On wheat plots, *nosZII* genes were negatively correlated with N_2O emissions ($\rho=-0.46$; $p<0.01$). The correlation matrix is provided as Table S7 in Supplementaryf materials.

The relationship between N_2 emissions and *nrfA* genes showed that N_2 emissions were negatively correlated to *nrfA* genes over all crop types ($\rho=-0.39$; $p<0.05$). *nosZII* genes were positively correlated with N_2 emissions on plots with wheat ($\rho=0.85$; $p<0.01$).

4 Discussion

Mineral N fertilisation positively influenced biomass increase in all three crop types (Figure 3A), with similar findings observed in other IOSDV experiments by Csitári *et al.* (2021) and Tajnšek *et al.* (2013). The results also showed a significant positive correlation between biomass production and soil NO_3^{-}–N, C_{tot} and N_{tot} content, explaining higher biomass production in fertilised soil, as N limitation is the most influencial factor constraining crop growth (Mengel and Kirkby, 2001). Furthermore, increasing mineral N fertilisation led to higher N accumulation in the biomass (Figure 3). The higher N content in biomass can be explained by applying N at rates that exceed crop needs for optimal yield, leading to an increase in crop protein content (Serret *et al.*, 2008; Mengel and Kirkby, 2001).

The sorghum plots without fertilisation yielded 2.3 t ha^{-1}, while those with only manure amendment produced an additional 5.9 t ha^{-1} of total dry biomass (Figure 3A), consistent with the results from Spiegel *et al.* (2010). N_{tot} and C_{tot} were also higher on sorghum plots with manure amendment compared to plots with only mineral fertilisation (Figure S3), which can explain higher biomass production. The positive effect of manure amendment could be attributed to increased availability of nutrients. Meta-analysis by Hijbeek *et al.* (2017), covering 20 long-term experiments (including the IOSDV experimental site used in our study) in Europe, reported that organic input does not necessarily guarantee increased crop yields. Although Hjibeek *et al.* (2017) also found that in specific cases, like spring sown cereals and sandy soils, the use of organic inputs led to an increase in crop yield.

In various ecosystems, N cycle genes have been linked to N_2O emissions (Butterbach-Bahl *et al.*, 2013; Espenberg *et al.*, 2018; Harter *et al.*, 2014). The significant positive correlation between the ratio of *amoA*/*nir* and N_2O emissions ($\rho = 0.20$, $p<0.001$) in our study indicates that nitrification potential was higher than denitrification potential and thereby N_2O emissions were mainly related to nitrification in the soil. Previous studies have also used the ratio of *amoA* to *nir* genes to study N-cycle processes (Kazmi *et al.*, 2023; Tang *et al.*, 2018; Zhu *et al.*, 2018). Additionally, an initial decrease in NH_4^+-N content in soil was observed, suggesting NH_4^+ consumption (nitrification) and mineral N uptake by plants (Supplementary Figure S2). A simultaneous increase in NO_3^--N accompanied by a decrease in NH_4^+-N was recorded, likely resulting from the nitrification production process.

nirS genes exhibited a positive correlation with N_2O emissions across all crops species plots ($\rho=0.19$; $p<0.05$), suggesting that while nitrification is predominant, denitrification is also evident. This finding aligns with results from several other agricultural studies, which also reported a significant positive correlation between *nirS* genes and N_2O emissions (Castellano-Hinojosa *et al.*, 2020; Cui *et al.*, 2016). Additionally, the ratio of *nosZ* to *nir* genes *(nosZ/nir)* was positively correlated with N_2O emissions ($\rho=0.21$, $p<0.001$). It highlights the importance of complete denitrifiers that have a capacity to convert N_2O to N_2. Since N_2O emissions are increasing with a high abundance of the *nosZ* gene, this positive correlation may be also related to N_2O emissions being emitted from nitrification.

For all plots, one or more functional marker genes related to nitrification and denitrification were identified as important in the variations of N_2O emissions (Figure 7), emphasizing the significance of both processes in N_2O emissions. Comammox was also recognized as an imporant process in N_2O emissions, except in wheat plots, indicating its potential important role. Additionally, Li *et al*. (2019) demonstrated an order of magnitude higher abundance of comammox *Nitrospira* clade A compared to ammonia-oxidizing archaea and ammonia-oxidizing bacteria in fertilised agricultural soil. More functional marker genes show significance in the variations of N_2O with manure compared to other treatments (Figure 7), indicating that a greater number of N cycle processes are relevant in plots with manure. Additionally, *nosZI, nosZII* and *nirS* genes were identified as important in the variations of N_2O emissions for sorghum with manure amendment, but not for only mineral fertiliser sorghum plots, which indicates significance of denitrification in these plots. Previous studies also suggest a higher denitrification potential from manure treatment, highlighting the importance of denitrifying microorganisms in manure-fertilised plots (Clark *et al*., 2012; Wan *et al*., 2023). The increased denitrification rate in manure-amended plots may be due to improved soil water retention promoting denitrification and increased availability of labile C content, which is the energy source for denitrifiers (Lazcano *et al*., 2021; Rayne and Aula, 2020). Our results also support higher labile C content in plots with manure amendment (Figure 2). Furthermore, sorghum with manure plots were the only plots where the *nrfA* gene was identified as an important gene in N_2O emissions, suggesting that manure amendment is likely enhancing the rate of DNRA process.

The negative relationship between *nrfA* gene and N_2 emissions is suggesting that the DNRA process is not contributing to N_2 emissions. The DNRA process, which is mediated by *nrfA* gene, is beneficial as it supplies NH_4^+ to the soil and conserves bioavailable N (Bai *et al*., 2020; Pandey *et al*., 2020). In addition, significant positive correlation between *nosZII* genes and N_2 emissions on plots with wheat indicates that there is likely a potential production of N_2 due to high abundance of *nosZII* genes that reduce N_2O into inert N_2. It is also supported by negative correlation between *nosZII* genes and N_2O emissions ($\rho = -0.46$; $p < 0.01$) on wheat plots. It indicates *nosZII* genes role in reducing N_2O emissions (Graf *et al*., 2014). Jones *et al*. (2014) demonstrated that the abundance and phylogenetic diversity of *nosZII* community is an important factor driving the soil's N_2O sink capacity.

Agricultural soils typically act as a source of N_2O (Davidson and Kanter, 2014), as shown in this study. The three mineral N fertilisation rates investigated influenced N_2O emissions, with N_2O emissions increasing with higher mineral N application rate for all three crop species (Figure 5, 6A). This can be attributed to higher available N levels with increased fertilisation rates for processes contributing to N_2O emissions (Engel *et al*., 2010), as N_2O emissions showed a strong positive correlation with both NO_3^--N and NH_4^+-N levels in soil. Prior studies have also highlighted a positive relationship between soil N_2O emissions and mineral N content (Sosulski *et al*., 2014; Yao *et al*., 2009; Yuan *et al*., 2022). Furthermore, among the investigated factors, the mineral N fertilisation rate was the primary determinant of cumulative N_2O emissions (Table 1), indicating that soil N_2O emissions are mainly linked to the excess N added with mineral fertiliser in the cropping system (Supplementary Table S6).

Our study found that crop type did not significantly affect cumulative N_2O emissions, while the effects of mineral N fertilisation rate and manure amendment on cumulative N_2O emissions were significant (Table 1). It suggests that N_2O emissions from soil are more closely related to the excess N in the cropping system than the crop type. Study including 372 sites also showed that cover crops did not have significant ($p>0.05$) effect on N_2O emissions (Abdalla *et al*., 2019). However, some studies have shown a significant effect of crop type on N_2O emissions (Bouwman *et al*., 2002; Kaiser and Ruser, 2000). Manure amendment significantly impacted N_2O emissions (Table 1). Additionally, mineral fertiliser plus manure amendment showed higher soil N_2O emissions compared to mineral fertiliser alone for sorghum. This can be attributed to the overall higher mineral input of N into the cropping system in mineral fertiliser plus manure plots compared to mineral fertiliser-only plots (231.2 kg N ha^{-1} was added extra), enhancing N_2O production. In addition to providing nitrifiable N compounds, manure incorporation improves soil conditions for nitrification and denitrification by increasing moisture and adding C to the soil (Chadwick *et al*., 2000). While the increase in moisture with manure was not detectable from our study, it may be explained by the slow evolution of soil properties over previous years in the 33-year-long fertilisation experiment. Moreover, manure can enhance the activity of soil microbes, oxygen consumption, and the development of anaerobic zones in the soil, favouring denitrification (Akiyama and Tsuruta, 2003).

Soil microbial processes leading to N_2O production are influenced by soil water content, as it directly affects oxygen availability for nitrification and denitrification processes. The recorded lowest soil moisture contents for barley and wheat plots on 7th of July (Supplementary Figure S4) likely explain the lowest N_2O emissions on that date (Figure 5). Previous studies on N_2O emissions and soil moisture dynamics have reported similar trend (Yamulki *et al*., 1995; Yuan *et al*., 2022). Additionally, Thapa *et al*. (2017) reported a reduction in N_2O emissions from wheat fields, which could be due to soil salinity interfering with nitrification and denitrification processes (Dang *et al*., 2016). Dry soils may lead to microorganisms experiencing cell dehydration and increased soil salinity, hindering soil microbial activity and, therefore, the production of gaseous N emissions (Haj-Amor *et al*., 2022; Schimel *et al*., 2018). Although our study did not find significant correlations between soil moisture and N_2O emissions or most of the functional marker genes.

Considering climate changes and population growth, N_2O management should be aligned with the future need to increase crop yield and sustain rapidly increasing human population. Biomass production increased with fertilisation rate (Figure 3), except for wheat plots. In our study, the biomass production on wheat plots between fertilisation rates 80 kg N ha^{-1} and 160 kg N ha^{-1} had very similar biomass values. However, long-term fertilisation experiments (IOSDV) by Káš *et al*. (2010) achieved highest wheat yields from N fertilisation rate of 160 kg N ha^{-1}. Our study shows increasing N_2O emissions at higher fertilisation rate on wheat plots (Figure 6A), indicating potential overfertilisation and suggesting fertilisation rate at 80 kg N ha^{-1} as the optimal fertilisation rate. In addition, the highest nitrogen use efficiency (NUE) was observed at fertilisation rate 80 kg N ha^{-1} for wheat (NUE = 0.84), indicating a balance between low N_2O emissions and high yield. In India, Chaturvedi *et al*. (2006) conducted similar fertilisation experiments with N fertilisation rates of 0, 25, 50, 75, 100, and 125 kg N ha^{-1}, and identified the highest N input rate as optimal.

On fertilisation rate of 160 kg N ha^{-1}, N_2O emissions significantly increased compared to lower rates, but this rate also results in higher total dry biomass (Figure 3; Figure 6). The fertilisation rate of 80 kg N ha^{-1} for sorghum plots with only mineral N fertiliser amendment appears optimal with low N_2O emissions and N losses (Supplementary Table S6). However, in sorghum plots without manure amendment, NUE values are low (160 kg N ha^{-1} NUE = 0.25; 80 kg N ha^{-1} NUE = 0.12).

5 Conclusions

The results of our study (part of the 33 year old IOSDV experiment) showed that the mineral N fertilisation rate was the dominant factor determining cumulative N_2O emissions. The study observed an increase in N_2O emissions with an elevated mineral N fertilisation rate, attributed to higher NO_3^--N and NH_4^+-N levels in fertilised soil. Higher N_2O emissions were measured during spring and early summer when mineral N fertilisers and farmyard manure was applied. These findings supported our hypothesis of higher N_2O emissions on sorghum plots under mineral fertiliser plus manure treatment compared to only mineral fertiliser treatment. Additionally, the number of N-cycle genes that are significant in the variations of N_2O emissions also increased with manure amendment. Contrary to our hypothesis, crop type did not have significant effect on N_2O emissions in this study.

N_2O emissions were mostly caused by nitrification with potential contribution from denitrification, comammox and DNRA processes. Plots with manure amendment exhibited a greater impact of N-cycle microbial processes on N_2O emissions, compared to plots with other crop types. Soil moisture showed no correlation with N_2O emissions and most of the functional marker gene abundances. Nonetheless, the lowest N_2O emissions and functional marker gene abundances were recorded during periods of low soil moisture, suggesting a decrease in N_2O under such conditions.

For wheat, a high NUE value and low N_2O emissions, coupled with relatively high crop yield, suggest that a fertilisation rate of 80 kg N ha^{-1} is optimal. Similarly, in sorghum plots with only mineral N fertiliser amendment, a fertilisation rate of 80 kg N ha^{-1} resulted in low N_2O emissions and N losses considering comparable biomass production with other crop types.

Author contributions. ME, AA, and ÜM designed the experiment and developed the methodology. LK and JEG carried out the fieldwork. LK analysed the results, performed data visualization, and wrote the original manuscript. JEG and ME participated in data analyses and assisted with paper editing. All authors were involved in revising the paper for submission and contributed to its improvement.

Competing interests. The contact author has declared that none of the authors has any competing interests.

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