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 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

15   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. Manure amendment also increased the number of N cycle genes that are significant in the change of N_2O. Contrary to our hypothesis, there was no significant influence of crop type on soil N_2O emissions. The study indicated dominance of nitrification over denitrification in the soil. Microbial analyses also

20   showed the potential role of 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 potential for cultivation in temperate climate, as sorghum maintained low N_2O emissions and N losses on mineral N fertilisation rate of 80 kg N ha^{-1}.

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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

30   (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). The yield of main crops like maise, rice, wheat, and soy are projected to decline globally in present agricultural regions due to climate system changes (Asseng *et al.*, 2015; Liu *et al.*, 2016; Ostberg

35   *et al.*, 2018). By the 2080s, developing countries, often experiencing temperatures near or above crop tolerance levels, are expected to see a 10-25% decrease in agricultural productivity due to global warming. In contrast, developed countries, many with lower mean temperatures, are predicted to experience a 6% decrease to an 8% increase in productivity (Mahato *et al.*, 2014). 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

40   carbon dioxide (CO_2) over a 100-year timescale (IPCC, 2021).

Nitrogen (N) fertilisers enhance plant productivity as plants use N to produce proteins for cell construction, a building block for DNA, and a significant component of chlorophyll (photosynthesis) (Andrews and Lea, 2013; Kaur *et al.*, 2017). However, 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

45   the surrounding environment. 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). 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.*,

50   2022).

The key microbial processes leading to soil N loss are nitrification and denitrification (Thomson *et al.*, 2012). In agriculture, 70% of N fertilisers added to the soil are 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*

55   *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). Denitrification is a microbially-catalysed process under oxygen-limited condition responsible for

60   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) contributes NH_4^+ to the soil for biological production as a N fertiliser, conserving bioavailable N in the soil and preventing the leaching of NO_3^- (Bai *et al.*, 2020; Pandey *et al.*, 2020). DNRA

65   competes with denitrification in NO_3^--reducing processes, both requiring NO_3^- (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). These processes are mediated by different functional marker genes, including archaeal, bacterial and comammox *amoA* genes for nitrification, *nrfA* genes for DNRA and *nosZ* clad I and II, *nirK, nirS* genes for denitrification (Zaman *et al*., 2012; Hu *et al., 2015;* Zhang *et al.,* 2021).

70   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 maise (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-

75   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).

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

80   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 mechanistic process understanding based on abundances of functional N cycle genes in arable mineral soil, and evaluate the performance of

85   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 mineral soil, low soil moisture decreases N_2O losses; (4) amendment of manure fertiliser increases soil N_2O emissions and affects the soil microbial community; (5) sorghum (*Sorghum bicolor x Sorghum sudanense*) is a prospective crop to cultivate in temperate

90   climate.

2 Material and methods

2.1 Field experiment description

The field study was conducted on the IOSDV (Internationaler Organischer Stickstoff Dauerdüngungs Versuch, International

95   Organic Nitrogen Long-term Fertilisation Experiment) Tartu experimental field. 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 types and soil properties. Initially, the crop rotation was potato–spring wheat–spring barley (Astover *et al*., 2016). In 2019, potato was replaced with sorghum-sudangras hybrid. The experimental site is located in Tartu, southern Estonia, Northern Europe

(58°22'30" N, 26°39'48" E). In 2022, the average temperature in the area was −2.0 °C in winter, 4.6 °C in spring, 18.1 °C

100   in summer and 7.2 °C in autumn. The mean annual precipitation was 531 mm (Republic of Estonia Environment Agency, 2023) in 2022. The soil type is *Stagnic Luvisol* combined with *Fragic Glossic Retisol* (IUSS WG WRB 2015). The soil has a sandy loam texture and the thickness of the humus layer is 27-32 cm.

The experiment was organised into 12 plots in a systematic block design (Figure 1) with three replications. Every plot was 50 m^2 in size. The crop species studied were spring barley (cultivar "Elmeri"), sorghum (*Sorghum bicolor x Sorghum*

105   *sudanense,* cultivar "SUSU"), and spring wheat (cultivar "Mistral"). The fertiliser treatment consisted of mineral N fertilisation and mineral fertilisation with farmyard manure amendment. Three mineral N fertiliser treatment rates were studied: 0, 80 and 160 kg N ha^{-1}). The mineral fertiliser applied was ammonium nitrate (NH$_4$NO$_3$). The farmyard manure rate added to the plots with manure amendment was 40 t ha^{-1} of manure (231.2 kg N ha^{-1}). The main management activities and timing in the field are displayed in Table S1 in Supplementary materials.
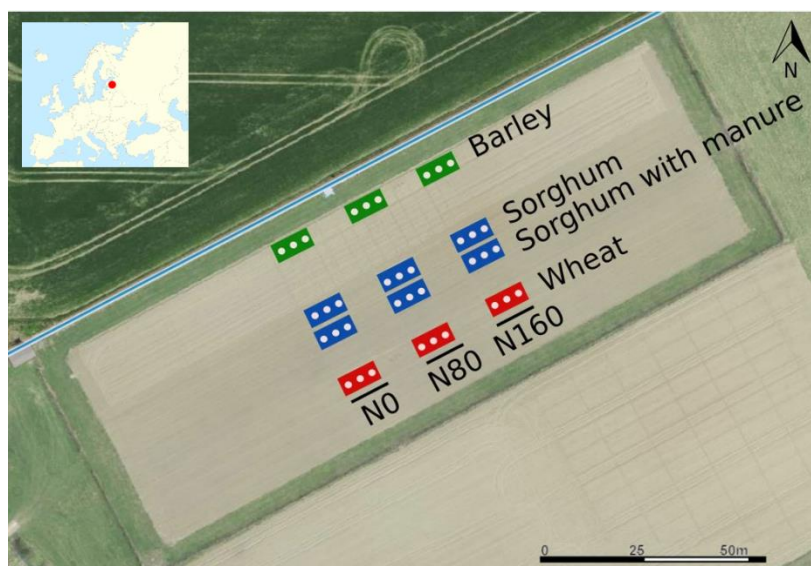
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Figure 1: Satellite view of the study site with study plots (from Maa-amet). Each plot constituted of three sampling spots indicated as white dots. N0 – 0 kg N ha^{-1}, N80 – 80 kg N ha^{-1}, N160 – 160 kg N ha^{-1} as mineral fertiliser.

115   **2.2 Gas sampling for N$_2$O 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$_2$O flux analysis were collected on all fifteen fieldwork days. N$_2$O gas sampling was carried out using the static chamber method (Hutchinson and Livingston, 1993). Polyvinyl chloride chambers

120   (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 low/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,
125   Japan), equipped with electron capture and flame ionisation detectors.

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
130   probe from the top 10 cm of the soil. Until chemical and microbiological analyses, samples were stored at +4 ˚C and −20 ˚C, respectively. In addition to soil sampling, temperature (°C) at a 10 cm depth, moisture (m^3/m^3), and electrical conductivity (dS/m) of soil were measured. 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 with dry combustion on a VarioMAX CNS
135   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.
140   The hot-water extractable C (HWEOC) represents the readily mineralising carbon (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
145   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. 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
150   cm). Frasier et al. (2018) 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.

155 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

160 **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, 165 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 170 quantitative polymerase chain reaction (qPCR). qPCR reactions were performed by The Rotor-Gene Q thermocycler (Qiagen). The reaction mixture of 10 ml consisted of extracted DNA (1 ml), gene-specific forward and reverse primers, Maxima SYBR Green Master mix reagent (5 ml; 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 S2 in Supplementary Materials. The Rotor-Gene® Q 175 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

180 Statistical software programs Statistica (v. 7.1) and R (v. 4.0.4) were used for statistical analysis 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 to find statistically significant differences between different fertilisation rates, use of manure and crop types. Spearman's rank correlation

185 coefficient measured the association between N_2O emissions and gene abundances and environmental factors. Random forest 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 yield produced per unit of N applied (Pandey et al., 2001; Supplementary Methodology S2). The N_2 emissions were estimated from the measured N_2O emissions using the

190 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 S1), 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 et al., 2017;

195 Escuer-Gatius et al., 2022; Supplementary Methodology S4)

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 in the

200 soil was increasing (Supplementary Figure S5). 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). 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

205 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 S6). There were no significant correlations between soil moisture and N_2O emissions. Over all crop types, soil moisture was not significantly linked to gene copy numbers, except *nirS* (Supplementary Table S6).
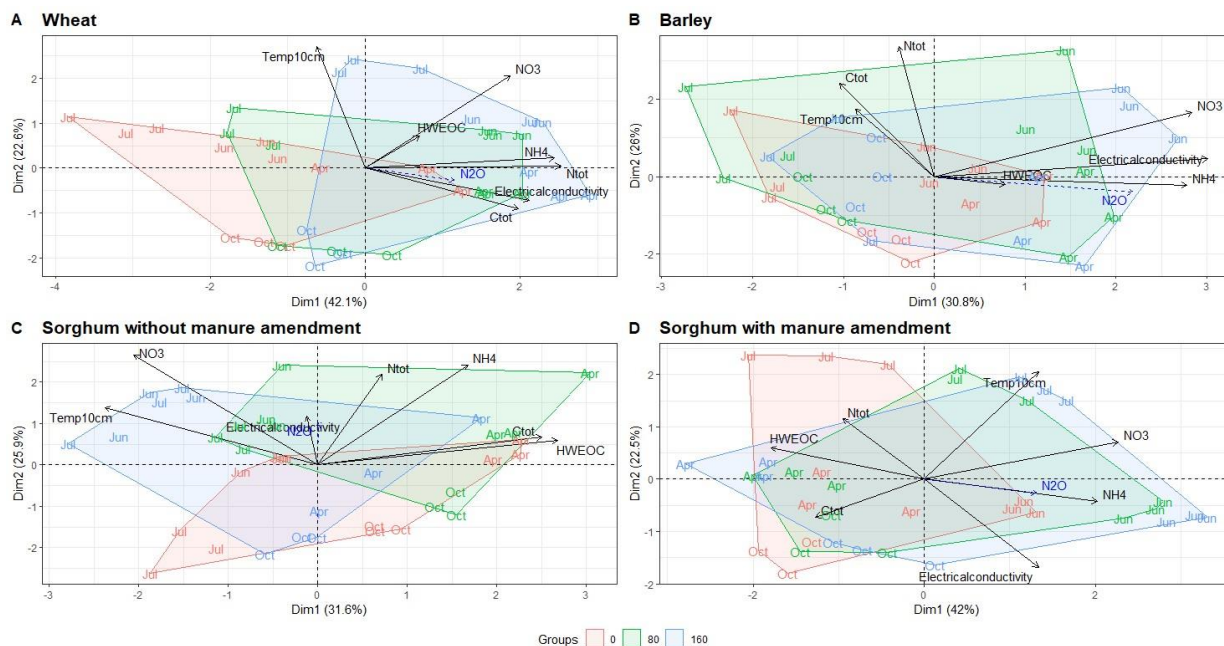
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Figure 2: Principal components analysis (PCA) ordination plots demonstrate the grouping of fertilisation rates according to physicochemical parameters for different crop type. N_2O is added as a supplementary variable. The month indicates the sampling time. Abbreviations: Ctot – total carbon content of soil; Ntot – total nitrogen content of soil; HWEOC – hot-water extractable organic carbon.

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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
220 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 S4). Also, higher N fertilisation rate increased N concentrations in the crop biomass (Figure 3B).
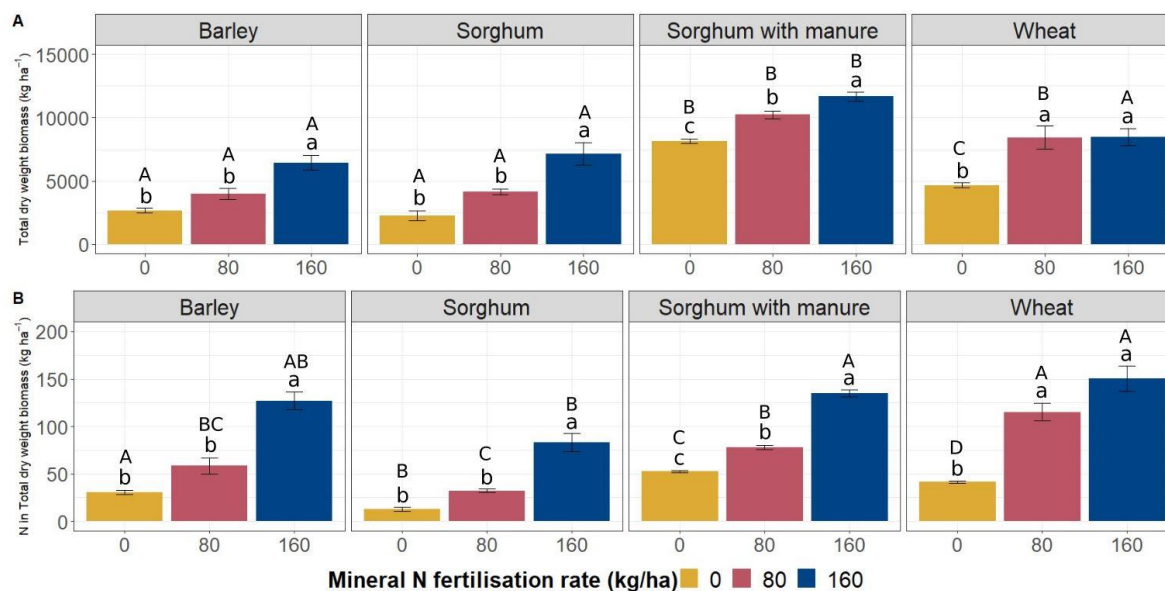
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Figure 3: Total dry weight biomass (aboveground + belowground) produced per unit area according to crop types and
225 fertilisation rates. Error bars show standard errors. Letters above the boxes indicate statistically significant differences at the
significance level $p < 0.05$. Lowercase letter indicate comparisons inside crop type, while uppercase letters indicate
comparisons of the same fertilisation rate over all crop types.

The highest values of nitrogen use efficiency (NUE) were obtained from wheat plots, and the lowest from sorghum plots.
230 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 S3). In general, wheat plots at different fertilisation rates
235 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 S1, S2, S3, and S4). The principal component analysis (PCA) of the N
240 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 (Figure 4) in all sorghum plots compared
to barley and wheat plots. For sorghum without manure amendment (Figure 4C), archaeal 16S rRNA and *nosZII* gene
abundances were highest for fertilisation rate 80, but for sorghum with manure amendment (Figure 4D), the highest archaeal

245 16S rRNA and *nosZII* gene abundances were for fertilisation rate 160. 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.
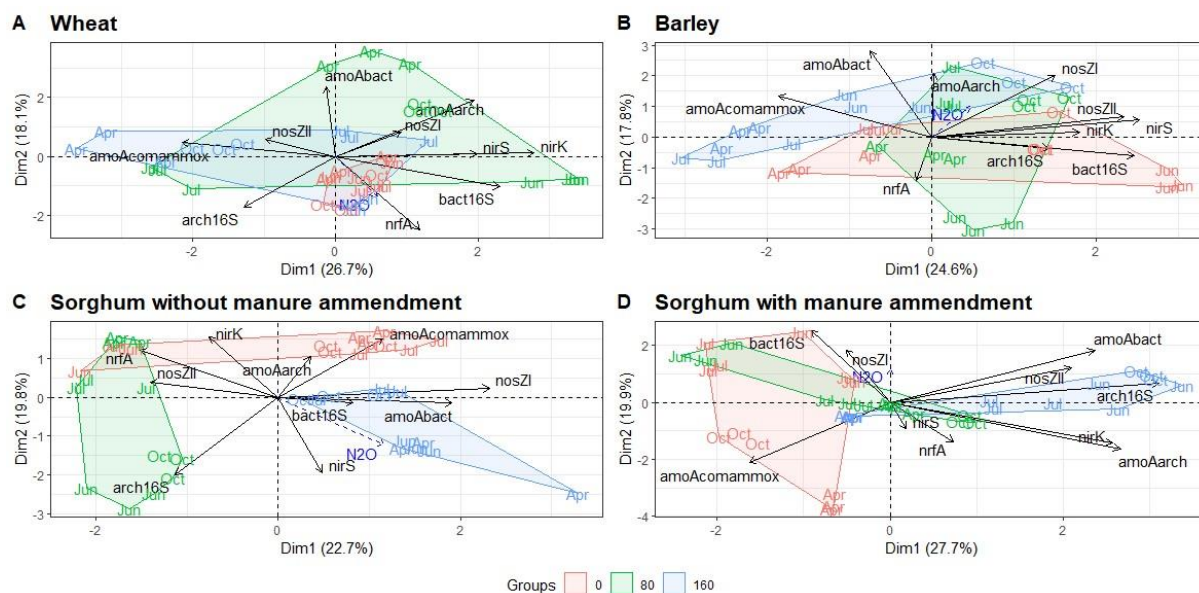

Figure 4: Principal components analysis (PCA) ordination plots demonstrate the grouping of fertilisation rates according to
250 functional marker genes abundances for different crop type. N_2O is added as a supplementary variable. The month shows the sampling time. Abbreviations: bact16S – bacterial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

3.3 N2O emissions

255 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.
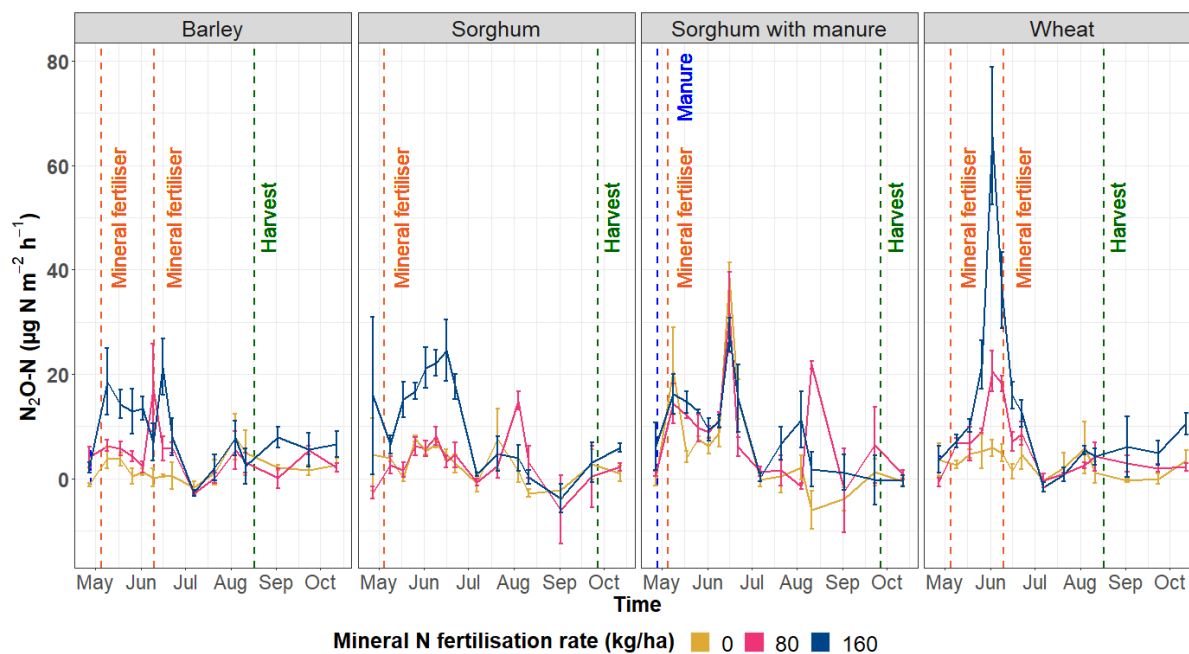
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Figure 5: N_2O emissions (μg N m^{-2} h^{-1}) according to crop types and fertilisation rates during the study period.

Cumulative N_2O and N_2 emissions over the study period show that plots with barley, wheat and sorghum without manure
265   have the highest emissions emitted from the highest fertilisation rate (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
270   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 from the three
different fertilisation rates did not differ significantly from each other.

For barley plots, the cumulative N_2 emissions were significantly higher at fertilisation rate 160 compared to rates 0 ($p<0.05$)
275   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.
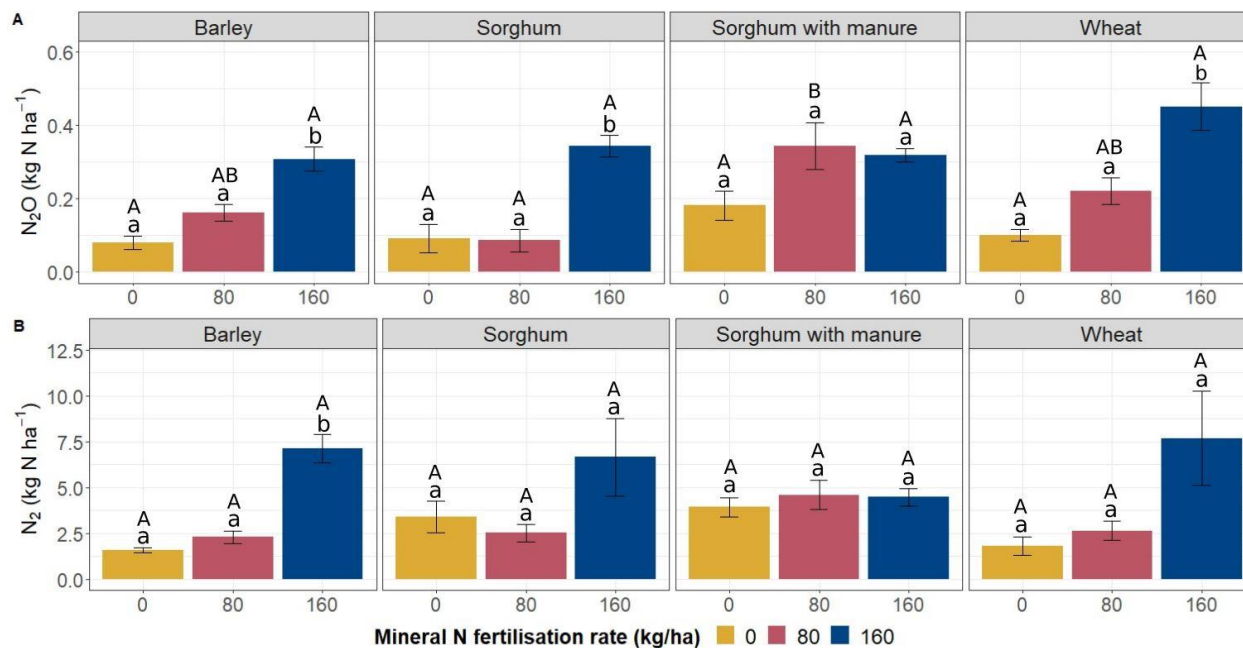
Figure 6: Cumulative N_2O and N_2 emissions according to crop types and fertilisation rates. Error bars show standard errors.
Letters above the boxes indicate statistically significant differences at significance level $p < 0.05$ according to a post-hoc
Tukey HSD test. Lowercase letters indicate comparisons 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 N2O 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 effect of mineral N fertilisation rate on N_2O emissions (ω^2 =
0.528) was larger compared to effects of crop type (ω^2 = 0.021) and manure amendment (ω^2 = 0.121).

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Table 1: Results of ANOVA and effect size (ω^2) 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	Sum of Squares	Mean Square	F value	Pr (>F)	ω^2
Crop type	2	0.350	0.175	0.957	0.39544	0.021
Mineral N fertilisation rate	2	8.772	4.386	23.995	5.97×10^{-7} ***	0.528
Manure amendment	1	2.014	2.014	11.020	0.00237 **	0.121
Residuals	30	5.483	0.183			

Feature selection algorithm for the N_2O emissions from wheat plots considered bacterial *amoA*, archaeal *amoA*, *nosZI* and

300 *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 change 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* genees were considered important for the N_2O emissions.

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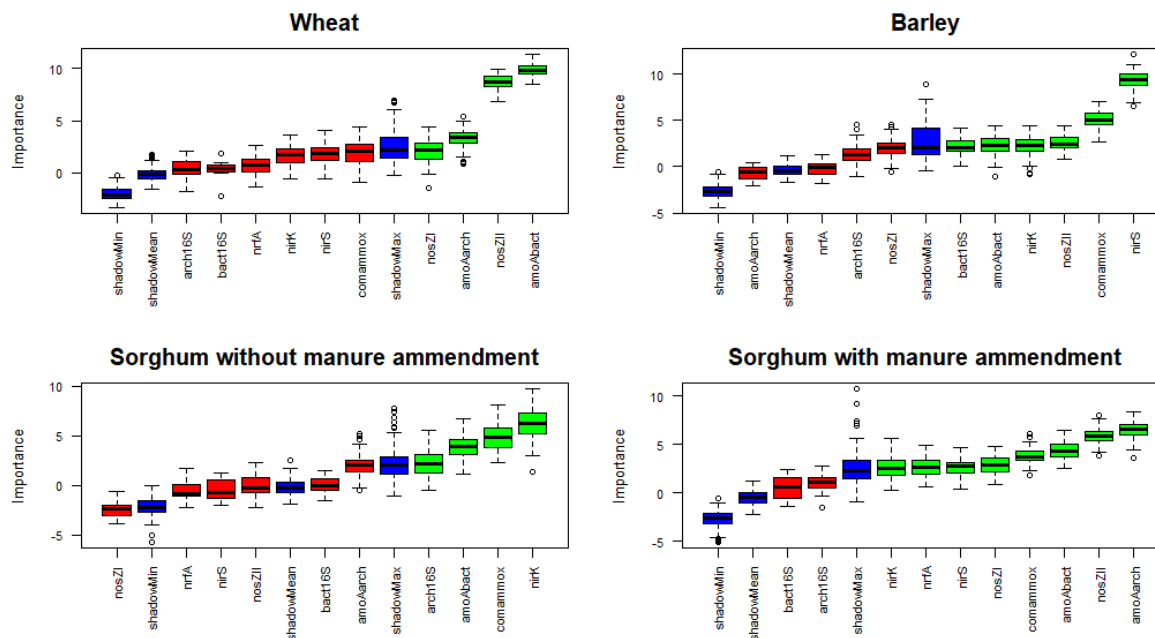
Figure 7: Results of feature selection in predicting the genes that are important in the change of N_2O emissions. Important factors are indicated in green, unimportant factors in red, and shadow variables (the random shadow copies of features (noise) will be created to test the feature against those 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 ($\rho=0.21$; $p<0.001$). Archaeal 16S rRNA genes were positively correlated with N_2O emissions over all crop species plots ($\rho=0.18$; $p<0.05$). Bacterial *amoA* genes were negatively correlated to N_2O emissions on wheat plots ($\rho=-0.40$; $p<0.05$). In addition, comammox *amoA* genes were negatively correlated with N_2O emissions on plots with sorghum without manure ($\rho=-0.47$; $p<0.01$). *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 a strong positive correlation with *nirS* gene abundance ($\rho=0.58$; $p<0.001$). *nosZII* genes were positively correlated with N_2O emissions on plots with sorghum with manure amendment ($\rho=0.41$; $p<0.05$). 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 S5 in Supplementary materials.

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

330 significant positive correlation between biomass and soil NO_3^--N 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). Applying N at higher rates than necessary for optimal yield can increase 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

335 5.9 t ha^{-1} of total dry biomass (Figure 3A), consistent with the results from Spiegel *et al*. (2010). The positive effect of manure amendment could be attributed to increased availability of macro- and micronutrients. The beneficial impact of manure was evident even at higher mineral N fertilisation rates. However, 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.

340 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 (ρ= 0.20, p<0.001) indicates the dominance of nitrification over denitrification in N_2O-producing processes. 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 S5). A simultaneous increase in NO_3^--N accompanied by a decrease in NH_4^+-N was recorded,

345 likely resulting from the nitrification production process.

nirS genes exhibit a positive correlation with N_2O emissions across all crops species plots (ρ=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*/*nir* is significantly positively correlated

350 with N_2O emissions (ρ=0.21, p<0.001). This correlation may be attributed to N_2O being emitted from other sources, such as nitrification.

For all plots, one or more functional marker genes related to nitrification and denitrification were identified as important in the change 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.

355 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 change 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 for sorghum with manure amendment, but not for only mineral fertiliser sorghum plots,

360   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.
      Our results also support higher labile C content in plots with manure amendment (Figure 2). Furthermore, sorghum with

365   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.

      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

370   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

375   (Supplementary Table S3).

      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. However, previous
      studies have shown a significant effect of crop type on N_2O emissions (Bouwman *et al*., 2002; Kaiser and Ruser, 2000).

380   Manure amendment significantly impacted N_2O emissions (Table 1). Additionally, soil N_2O emissions were higher under
      mineral fertiliser plus manure amendment than with 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

385   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 enhances the activity of soil microbes, oxygen consumption, and the development of anaerobic zones in the soil,
      favouring denitrification (Akiyama and Tsuruta, 2003).

      Sorghum plots with manure amendment exhibited high N_2O emissions across all fertilisation rates, with emissions increasing

390   slower than linearly with the fertilisation rate (Figure 6A). Conversely, N_2O emissions for sorghum plots without manure
      amendment increased exponentially with the rising mineral N fertilisation rate. This pattern aligns with findings from other
      studies that observed N_2O emissions responding exponentially to increasing fertilisation rates (Grant *et al*., 2006; Ni *et al*.,
      2021; Shcherbak *et al*., 2014; Walter *et al*., 2015). The potential explanation is that N input may be surpassing crop needs, as

N$_2$O emissions often grow exponentially when the applied N exceeds the necessary amount for crops (Van Groenigen *et al.*,
395   2010; Snyder *et al.*, 2009). This could be attributed to the high NO$_3^-$-N availability in the soil for all crop types in our study,
leading to NO$_3^-$-N accumulation when crop needs are exceeded (Legg and Meisinger, 1982). However, the results suggest
that N$_2$O emissions have a positive linear response to fertilisation rates for barley and wheat plots (Figure 6A). Field studies
by Gregorich *et al.* (2005) and Halvorson *et al.* (2008) reported that N$_2$O emissions tend to show linear growth with
increasing N fertilisation rates when N input is less or matches with the amount needed for maximum yield.

400   Higher N$_2$O emissions were recorded in the initial phase of the study period when fertilisers were applied in spring and early
summer (Figure 5). The addition of both NO$_3^-$ and NH$_4^+$ contributed available N to the cropping system, promoting
microbial activity in N$_2$O-producing processes and subsequently increasing N$_2$O emissions. The increase in N$_2$O emissions
might also be affected by soil ploughing, which enhances the mineralisation of soil organic N and crop residues, releasing
plant-available nutrients (e.g., P, S, N) and thereby increasing substrate availability for microbial processes generating
405   gaseous N (Lal *et al.*, 2007).

Soil microbial processes leading to N$_2$O production are influenced by soil water content, indirectly affecting oxygen
availability for nitrification and denitrification processes. The recorded lowest soil moisture contents for barley and wheat
plots on 7th of July (Supplementary Figure S6) likely explain the lowest N$_2$O emissions on that date (Figure 5). Previous
studies on N$_2$O emissions and soil moisture dynamics have reported similar trend (Yamulki *et al.*, 1995; Yuan *et al.*, 2022).
410   At low soil water content, nitric oxide (NO) is found to be the main soil gaseous N emission instead of N$_2$ and N$_2$O
(Davidson, 1991; Medinets *et al.*, 2015). 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. Although our study did
not find significant correlations between soil moisture and N$_2$O emissions or most of the functional marker genes.

Considering climate changes and population growth, N$_2$O management should align with crop yield. Biomass increased with
415   fertilisation rate (Figure 3), except for wheat plots, where plots with fertilisation rates at 80 kg N ha^{-1} and 160 kg N ha^{-1} had
very similar biomass values. Long-term fertilisation experiments (IOSDV) by Káš *et al.* (2010) achieved highest wheat
yields from N fertilisation rate 160 kg N ha^{-1}, but our study shows increasing N$_2$O emissions at higher fertilisation rates
(Figure 6A), suggesting potential overfertilisation. 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$_2$O emissions and high yield. In India,
420   Chaturvedi et al. (2006) conducted 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 160 kg N ha^{-1}, N$_2$O emissions significantly increase compared to lower rates, but this rate also results in
significantly higher total dry biomass (Figure 3; Figure 6). The fertilisation rate 80 kg N ha^{-1} appears optimal with low N$_2$O
emissions and N losses (Supplementary Table S3). However, in sorghum plots without manure amendment, NUE values are
425   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

430   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 significant in the change of N_2O also increased with manure amendment. Barley and wheat plots exhibited a positive linear response to fertilisation rates,

435   while N_2O emissions from sorghum plots without farmyard manure amendment responded rapidly to the highest mineral N fertilisation rate. Contrary to our hypothesis, crop type did not have significant effect on N_2O emissions in this study.

Nitrification dominated over denitrification in N_2O production in mineral arable soil, with potential contributions from 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

440   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 80 kg N ha^{-1} is optimal. Similarly, in sorghum plots with only mineral N fertiliser amendment, a fertilisation rate 80 kg N ha^{-1} resulted in low N_2O emissions and N losses, positioning sorghum as a potential crop for Northern Europe.

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