The potential nitrous oxide emissions in a permanent grassland chronosequence

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Swedish University of Agricultural Sciences, SLU
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Abstract

As a part of a larger project (Belowground biodiversity of grasslands – conservation needs and potential to promote sustainable agriculture), this study aims to investigate potential nitrous oxide (N_2O) emissions and microbial gene abundances needed for the production (*nirS* and *nirK*) and reduction (*nosZI* and *nosZII*) of nitrous oxide in soil of permanent grasslands of various age. The grasslands have at different times been converted to permanent grasslands and are of interest because of the possibility to shed more light about the passage of time's affects on the soil microbial communities and their activity. Three farms around Uppsala that manage permanent grasslands of various ages were selected and 24 soil samples were taken at dept 0 – 10 cm. These regions have similar backgrounds in cultivation, but they differ in soil type and some soil parameters. The prediction was that the permanent grasslands with the highest age have the lowest production of nitrous oxide gas, because of the lack of the availability of nutrient N over time in permanent grasslands due to no fertilizers being added and what is present will be in high demand by plants and microorganisms. In addition, the gene abundances for *nirS* and *nirK* are expected to become lower with age along with the lower production of nitrous oxide. The results however, thought not significant, showed an increase in N_2O production with age, that possibly could be linked to significantly increasing organic matter content in soil over time. Instead a difference in N2O production was found between regions. Neither the age or the region of the permanent grasslands had a significant effect on the dynamics of different gene abundances, but the genes *nirS* and *nirK* showed a slight decrease with age and the opposite was found for the gene *nosZII*'s abundance.

Keywords: nitrous oxide emission, permanent grasslands, denitrification, nitrous, green house gas, agriculture

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1. Introduction

Belowground biodiversity of grasslands – conservation needs and potential to promote sustainable agriculture is a project with the aim to determine the timescale for when biodiversity and chemical characteristics are re-established in grasslands converted from arable land. This is studied both above- and belowground. Often research has focused on the biodiversity above ground, but the belowground diversity and its need for preservation and protection is not as studied. This study focuses on the belowground microcomunity of bacteria in permanent grasslands and specifically the bacteria related to the denitrification pathway and emission of nitrous oxide (N_2O). The agricultural sector stands for a larger part of the emission of N_2O, which does not only weaken the ozone layer, but also is a green house gas (GHG) approximately 310 times more effective than carbon dioxide (CO_2) (*Lustgasutsläpp - Sveriges miljömål* n.d.). It is therefor important to be aware of how the fluxes of N_2O gas changes with land use and if the time since conversion from arable land has an impact on that. One study focusing grasslands in mountain areas, looked at how the land use affected the emission of non-CO_2 GHG, found that abandoned grasslands had reduced N_2O emissions compared to meadow and pastures and was likely to work as a sink for GHG, with managed grasslands having slightly higher emissions, but still lower than intensely managed croplands and grasslands (Harris et al. 2018). Semi-natural grasslands also give many services such as water supply, carbon storage, pollination, biological control and cultural values, as well as having high biodiversity (Bengtsson et al. 2019). The possible GHG emissions has to be weighted against the positive services of the grasslands. The aim of this study was to get further understanding of the potential activity of bacteria related to denitrification in permanent grasslands in a chronosequence since convertion and if the amount of nitrous oxide gas emission differs over time. The prediction was that the N_2O emissions would become lower with higher age since convertion, because of the limited nitrogen (N) input when the land no longer is cultivated. The abundance of genes part of the transformation of nitrite (NO_2^-) to nitrous oxide, *nirS* and *nirK* was expected to decrease as an explanation to the decreased emissions, when nutrient availability changed over time.

1.1 Background

There has been a study resembling this one about the nitrous oxide efflux during grassland restoration (Scott et al. 2019). They studied the age chronosequence of 35 years in comparison with actively cultivated field and a native prairie. Results from laboratory measurements of N_2O revealed that nitrous oxide efflux decreased exponentially with increasing restoration age. Most likely due to the increasing demand of nitrogen (N) from microorganisms and plants and an increased organic matter content. It was also discovered that the oldest restoration had 50 x lower N_2O efflux than that of the native prairie.

When biodiversity is lost in soil the multifunctionality can also be lost, leading to a change in transformation of nitrogen and by that the N_2O emissions. Highly simplified communities in soils have shown an increase in N_2O emission (Wagg et al. 2014). What could help grasslands in their recovery of biodiversity is the surrounding habitats. Belowground trophic groups respond well to permanent habitats such as forests and grassland, as a source of material for recolonization (Le Provost et al. 2021).

1.2 Theory

1.2.1 Nitrogen cycle

The nitrogen (N) is an essential nutrient for living organisms and depending on its form in soil it will be more or less accessible for these organisms. Microorganisms in the soil play a part in transforming nitrogen and are part of processes like: nitrification, denitrification and nitrogen fixation. There are many reactions and corresponding enzymes that catalyse the chemical reactions of nitrogen compounds in the soil. In this study one part of the nitrogen cycle is more important to have a better understanding of which is the denitrification that leads to dinitrogen gas. (NO_3- > NO_2- > NO > N_2O >N_2) For a quick overview see Figure 1. Part of the denitrification has a redox reaction that uses haem- containing cd1 nitrite reductase (cd1-NIR) which is encoded by the gene *nirS*. Another enzyme that is used is the Cu containing nitrite reductase (Cu-NIR) which is encoded by *nirK*. The reaction happens in environments like soils, that have nitrogen available but also a low concentration of oxygen. These genes nirS and nirK are often present in bacteria as well as in archaea and is often used as measure in assays like this one for denitrifiers. But it s not only bacteria that are part of the denitrification, fungi can also play a part, but does not have the NIR genes. The reaction from the greenhouse gas N_2O to N_2 is dependent on the enzyme nitrous oxide reductase (NOS). The gene

corresponding to this is *nosZ*. This gene can be found in a variety of bacteria and archaea. (Kuypers et al. 2018)

Nitrate reduction: NO_2- +e- + 2H+ -> NO + H_2O

Nitrous oxide reduction: N_2O + 2e- + 2H+ -> N_2 + H_2O

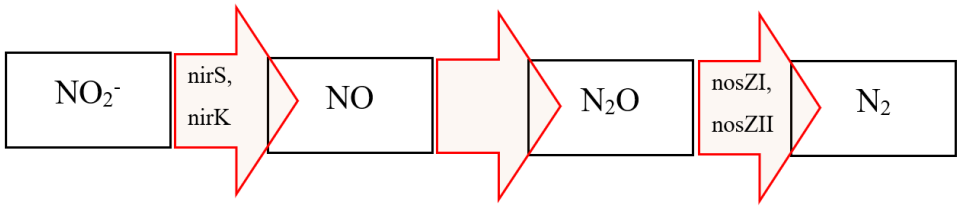

Figure 1: Forms of nitrogen and the genes related to the transfomation of nitrogen forms in denitrification pathway.

1.3 Carbon stocks

Improving on grazing management, fertilization, legume content and convertion from cultivated land are factors that can increase the carbon (C) stock in grasslands. How the fields respond to changes in management are however, dependent on the area rather rather than one specific management that works on all land (Conant et al. 2017). A trade off can however be found in carbon storage and nitrous oxide emissions, where reducing fertilizer reduses the emission but also the amount of carbon strored (Allard et al. 2007).

Positive correlation between emission of N_2O and soil organic matter (SOC) content have been found by several studies (Mosier et al. 1991 see Li et al. 2005). The N and C cycle are connected in soils, sometimes stored in the same organic compounds and the transformation of N can be dependent on the availability of C. The N_2O emissions can come from denitrification that can increase with soil organic matter (SOM) content due to its part as an electron donor (Quin et al. 2015 see Guenet et al. 2021) and ability to increasing the activity of the denitrifyers (Guenet et al. 2021).

2. Methods

2.1 Study area and soil sampling

Three farms (Ekeby, Stabby and Åsbergby) consisting of grasslands were investigated in this study. The grasslands had at different points in time been used as arable land and therefore actively ploughed/tilled. With the use of historical maps, aerial photos and interviews with the farmer, the grasslands were sorted into four age classes based on the number of years since they had been ploughed/tilled. Age class1: 10-20 years, age class 2: 20-40 years, age class 3: 40-70 years, age class 4: 70-150 years. The samples were not picked to be similar in soil type and can to some degrees have been managed slightly different. The grasslands are over all grazed optimally, intense but not exhausting the fields. The soils of the grasslands were sampled in summer 2022 from two field replicates per time class per farm. From each field three soil cores were taken at dept 0 - 10 with a corer and later pooled together and homogenized. This resulted in 23 soil samples. One part of the sample was kept frozen at -20 ℃ and used in the incubations and dry matter content by overnight drying at 105 ℃. Another part was freeze dried and ground in a ball milled and used for DNA extraction.

2.2 Potential denitrification and N_2O production activity assay

Based on a standard protocol(Pell et al. 1996), the potential denitrification(PDA) and potential N2O production was measured. 10g of fresh soil was measured two times for each soil sample into 125 ml Duran bottles and left in room temperature (ca 25℃) for 2 hours before 20 ml distilled water was added to each bottle creating a soil slurry. The headspace was exchanged with N_2 and then the bottles were placed on a shaker for 30 minutes as 175 rounds per minute (RPM). When the time had passed 10 ml of acetylene was added to half the batch. That batch was to measure the PDA and the batch without acetylene the potential N_2O production. The soil slurries were put on the shaker again and 1 ml of substrate solution was added to

every bottle. The substrate solution having the final concentration 3 mM KNO_3, 1.5 mM succinate, 1mM glucose and 3 mM acetate. The incubation lasted between 150-180 minutes (depending on how many time stops), at 25℃ with the shaker at 175 RPM. A gas sample of 0.5 ml was taken every 30 minutes from each bottle and injected into a corresponding gas vial. The concentration of N_2O determined by gas chromatography. At the end of the incubation the final volume of the soil slurry was measured. From dividing potential N_2O production with PDA a denitrification end product ratio was calculated as well.

2.3 DNA extraction and quantification of nitrogen cycling genes

In the genomic DNA extraction the NucleoSpin Soil kit by Macherey-Nagel(MACHEREY-NAGEL, 2023) was used according to the protocol provided by the manufacturer, with only a few changes. The amount of buffer used was changed to 800 µl instead of the protocols 700 µl. The homogeniser was put to 5000 RP for 30 seconds 2 times, with 60 seconds between rounds. When spinning the samples, the max at 1700 xg was used.

About 0.35g of freeze-dried soil was used for the extraction. To visualise the DNA extracts, gel electrophoresis in 0.8% agarose gel in 1 X TAE buffer was used. DNA extracts was quantified with Qubit fluorometer (ThermoFisher SCIENTIFIC, 2023) and diluted to the same concentration (4ng/µl)

To test the potential PCR inhibition in the samples, a known amount of TOPO pCR4 plasmid (ThermoFisher SCIENTIFIC, 2023) was amplified with plasmid specific M13 primers, with either 8 ng of extracted DNA or water for the quantitative PCR (qPCR). Inhibition was detected and the DNA extracts further diluted for another test. No inhibition was detected for 0.8 ng of extracted DNA, and therefore used in the quantification of N cycling genes.

By quantifying the abundance of genes *nirS* and *nirK* that are part of the nitrate reductase reaction and *nosZI* and *nosZII* genes that are part of the nitrous oxide reductase reaction, the genetic potential for denitrification can be assessed. *16s rRNA* gene was used as a proxy for the total abundance of bacteria. The qPCR reaction contained iQ SYBRGreen Supermix (Bio-rad, 2023), BSA, distilled water, both primers and had the final volume of 15 µl with the extracted DNA. The qPCR reactions was run in duplicate, one without a melt curve to be visualised using gel electrophoresis in 1% agarose gel in SB buffer. The qPCR run using the BioRad CFX Connect Real-Time System. Details on primer sequences, final concentrations and sequence are found in Table 1. Standard curves were generated from the serial dilutions of linearized plasmids containing fragments of the respective genes.

Table 1: qPCR details for runs.

Gene	Primers	Reference for primers	Sequence (5'-3')	Final primer concentration (µM)
16s rRNA	515F_Parada, 926R_Quince	(Parada et al. 2016), (Quince et al. 2011)	GTGYCAGCMGCCGCGGTAA, CCGYCAATTYMTTTRAGTTT	0.5
nirK	F1aCu, 1040R	(Hallin & Lindgren 1999)	ATCATGGTSCTGCCGCG, GCCTCGATCAGRTTRTGGTT	0.8
nirS	Cd3aFm, R3cdm	(Throbäck et al. 2004)	AACGYSAAGGARACSGG, GASTTCGGRTGSGTCTTSAYGAA	0.8
nosZI	1840F nosZI, 2090R nosZI	(Henry et al. 2006)	CGCRACGGCAASAAGGTSMSSGT, CAKRTGCAKSGCRTGGCAGAA	0.8
nosZII	nosZ-II_F, nosZ-II_R	(Jones et al. 2013)	CTIGGICCIYTKCAYAC, GCIGARCARAAITCBGTRC	2

2.4 Data management

The results from the incubation test was put in a meta table and copy/ng extracted DNA and copy/g dry weight (DW) soil was calculated for each of the genes *16s rRNA, nirS, nirK, nosZI* and *nosZII*. Results that had a difference in the Quantification Cycle (Cq) values between the two qPCR runs over one, was excluded from the table.

The data was visualised in program RGui (64-bit) and statistically tested with Analysis of Variance (ANOVA) and TukeyHSD test to find significant differences at significance level 0.05.

3. Results

3.1 Soil organic matter content, and potential denitrification and N_2O production

The organic matter (OM) content in the soil samples develop with the higher age classes and it is possible to see an increase in percentage when the grasslands age (Figure 2 A). Age class one has one higher outlier that belongs to the region Ekeby. And sticks out in other circumstances for having a high water fraction. Between the regions the OM from lowest to highest goes Ekeby, Stabby, Åsbergby (Figure 2 B). With Åsbergby having the widest spread of data. ANOVA with Tukey HSD test showed a significant difference between age class 1 and age class 4 (Table 2), but no significant difference between the other, but no significant difference between the regions (Table 5).

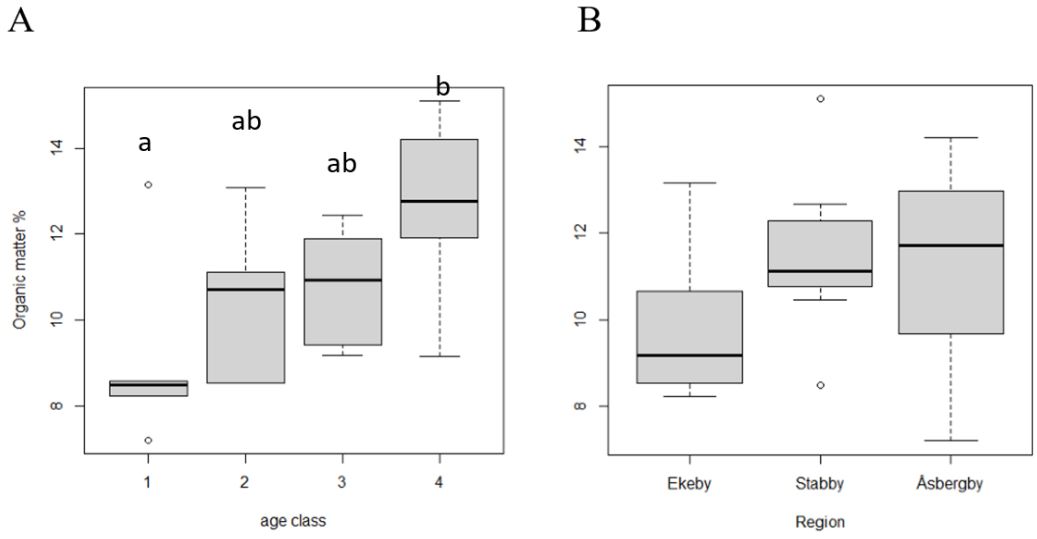

Figure 2: A) The organic matter content in percentage across the age classes 1, 2, 3 and 4. B) Organic matter content in percentage in the different sample regions Ekeby, Stabby and Åsbergby.

Table 2: TukeyHSD results from Organic matter content in age classes 1-4.

Age class	P adj

2-1	0.657
3-1	0.472
4-1	0.027
3-2	0.988
4-2	0.205
4-3	0.400

In Figure 3 A) it is possible to see a slight increase in the potential N_2O production the higher the age class. Age class 4 having one outlier that is very low compared to the others. In the regions Ekeby had a generally lower N_2O production (Figure 3 B) than the other two regions. ANOVA with Tukey HSD test for the age classes showed no significant difference between the age classes activity (Table 5), but the regions showed a significant difference between Ekeby and the other two regions (Table 3).

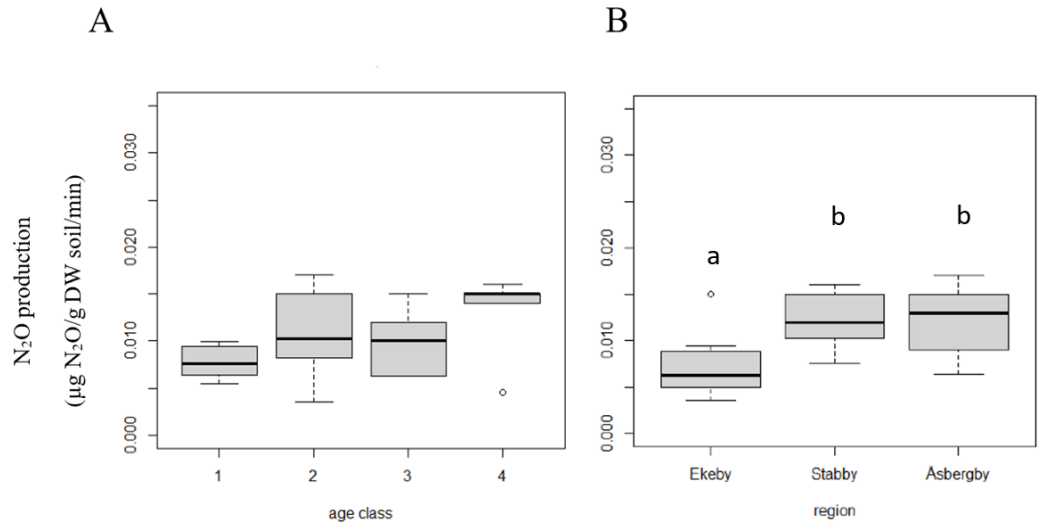

Figure 3: A) Potential N_2O production in ug N_2O-N per g dry weight soil per minute, across the age classes 1, 2,3 and 4. B) Potential N_2O production ug N_2O-N per g dry weight soil per minute, in different sample regions Ekeby, Stabby and Åsbergby.

The potential denitrification for the age classes (Figure 4 A) have an increase in production with higher age class. Age class 1 having a very small spread of data compared to the other age classes. In the regions Ekeby has a generally lower production than the other regions (Figure 4 B). There was no significant difference between the age classes in N_2O production (Table 5), but there was a significant difference between the region Ekeby and Stabby (Table 3).

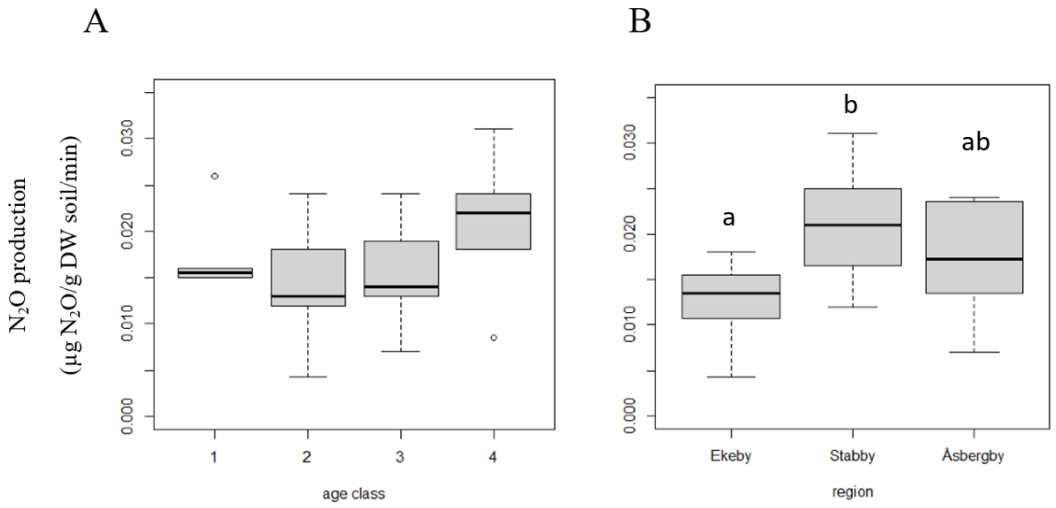

Figure 4: A) Potential denitrification ug N_2O-N per g dry weight soil per minute, across age classes 1,2,3 and 4. B) Potential denitrification in ug N_2O-N per g dry weight soil per minute, in the sampe regions Ekeby, Stabby and Åsbergby

Table 3: TukeyHSD results for potential N_2O production and potential denitrification in the different regions.

		Region	**P adj**
Potential production	N_2O	Stabby-Ekeby	0.035
		Åsbergby-Ekeby	0.033
		Åsbergby-Stabby	0.998
Potential denitrification		Stabby-Ekeby	0.030
		Åsbergby-Ekeby	0.252
		Åsbergby-Stabby	0.470

The ratio between the potential N_2O production and the potential denitrification are very flat for the age classes (Figure 5 A), but age class 2 has a much wider spread of data that sticks out even if the mean Is not very different from the rest of the age classes. The regions (Figure 5 B) look to be very alike each other, with no clear signs of difference between them. There was no significant difference between the age classes or the regions (Table 5).

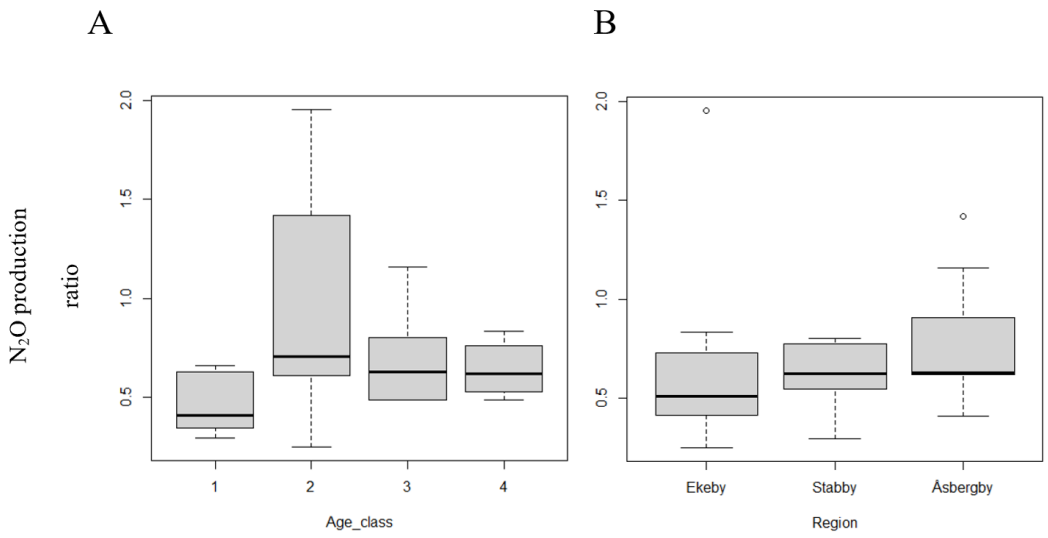

Figure 5: A) Denitrification end product ratio($N_2O/(N_2O+N_2)$) for age classes 1-4. B) Denitrification end product ratio between the regions Ekeby, Stabby and Åsbergby.

3.2 Total bacterial abundance and denitrification gene abundances

The mean copy number from the various genes in the age classes (Figure 6 A, Figure 7 A) show that some genes are more abundant than others. *nirK* has the highest abundance for all the age classes and *nirS* the lowest. There is some difference in between the age classes, the *nosZII* gene's abundance increase slightly the higher the age class when compared as copy/ng extracted DNA (Figure 6 A) but looks to be even in the age classes when comparing it as copy/g DW soil (Figure 7 A). The same can be seen in both *nirK* and *nirS* that both look to be in slight decrease the higher the age class when measured as copy/g DW soil, but not as clear decrease can be seen when measuring it as copy/ng extracted DNA. The gene abundance between the regions, alike the age classes showed no clear difference between them (Figure 6 B, Figure 7 B). Åsbergby looks like it has a generally lower abundance for gene *nirS* than the other regions. There was no significant difference between the age classes or regions for any of the genes (Table 5).

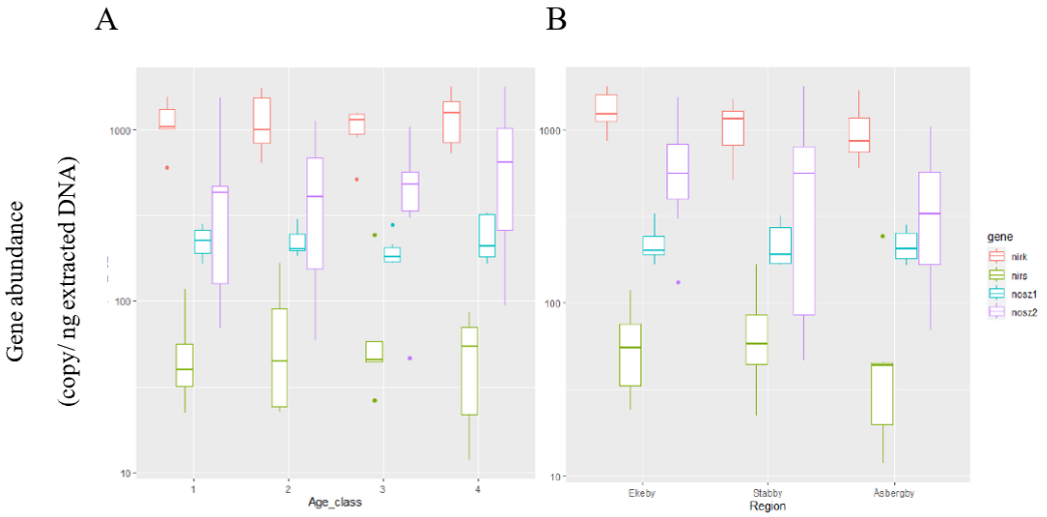

Figure 6: A Gene abundance (copy/ng extracted DNA) for the different genes nirK, nirS, nosZI and nosZII, in the age classes 1,2,3 and 4. The y scale is in log10. B Gene abundance (copy/ng extracted DNA) for the different genes nirK, nirS, nosZI and nosZII, in the sample regions Ekeby, Stabby and Åsbergby. The y scale is in log10

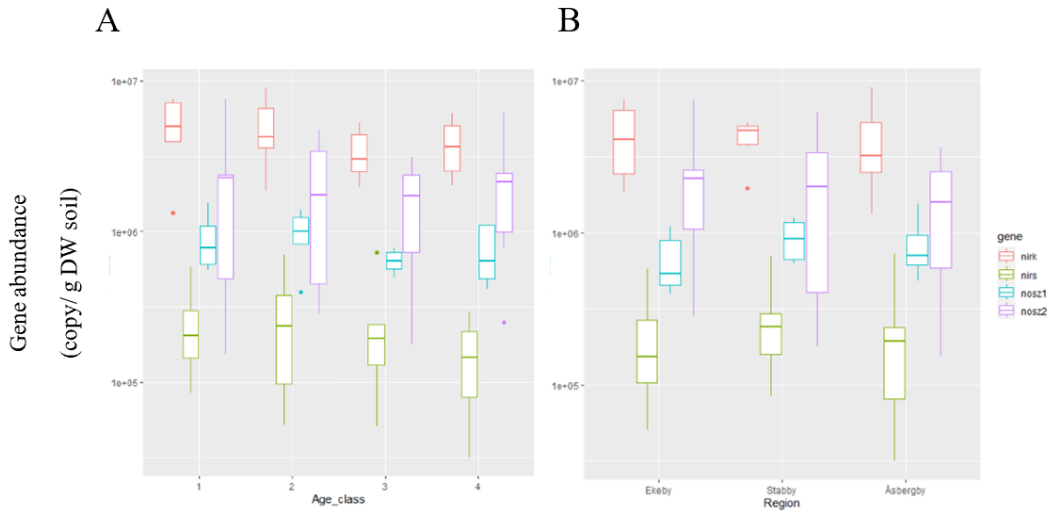

Figure 7: Gene abundance (copy/g dry weight soil) for the different genes nirK, nirS, nosZI and nosZII, in the age classes 1,2,3 and 4. The y scale is in log10. b) Gene abundance (copy/g dry weight soil) for the different genes nirK, nirS, nosZI and nosZII, in the sample regions Ekeby, Stabby and Åsbergby. The y scale is in log10.

The total abundance of the bacteria in the samples, measured from the *16S rRNA* gene abundances showed a possible decrease in abundance from age class 1 to 3, but then an increase again for age class 4 and the regions were alike in abundance but varied less in region Stabby (Appendix). No significant difference was found between regions or age classes for overall bacterial abundances.

The correlations between potential activities and the abundances of denitrification genes were expressed by the correlation coefficient between them (Table 4). There

was no significant correlation between the different activities and the various genes except for *nirK* for denitrification end product ratio. There was no significant correlation between OM content and the various genes either. The correlation showed the same pattern when abundance was measured in copy/ng extracted DNA as well.

Table 4: Pearson correlation coefficients between the potential activities and gene abundances. Significant if coefficient higher than 0.404. For nirS a coefficient higher than 0.433 and higher than 0.423 for nosZI.

Parameters	**Gene (copy/g DW soil)**	**Pearson correlation coefficient**
Potential N_2O production	*nirS*	0.083
	nosZI	0.418
	nirK	0.221
	nosZII	0.180
Potential denitrification	*nirS*	-0.240
	nosZI	-0.080
	nirK	-0.285
	nosZII	-0.243
Organic matter content	*nirS*	0.163
	nosZI	0.027
	nirK	-0.132
	nosZII	0.336
Denitrification end product ratio	*nirS*	0.286
	nosZI	0.373
	nirK	0.589
	nosZII	0.328

Table 5: F-value and Pr(>F) for all results in denitrification and gene abundance ANOVA.

Response	**Age class**		**Region**	
	F	p	F	p
OM content	3.3747	0.0399	1.6284	0.2212
N_2O prod.	1.8586	0.1709	4.9664	0.01774
PDA	1.3862	0.2775	3.9209	0.03659
End prod. ratio	1.6278	0.2162	0.2573	0.7757
16S rRNA	1.0091	0.4104	0.0017	0.9983
nirS	0.3384	0.7978	0.1184	0.8891

Copy/ng	*nirK*	0.237	0.8694	1.9116	0.1739
extracted	*nosZI*	0.557	0.6505	0.0308	0.9697
DNA	*nosZII*	0.3855	0.7647	0.6323	0.5417
Copy/g	*16S rRNA*	1.1623	0.3501	0.6838	0.5161
DW soil	*nirS*	0.4726	0.7057	0.225	0.8009
	nirK	0.8785	0.4697	0.0422	0.9588
	nosZI	1.3464	0.2926	1.0265	0.3783
	nosZII	0.2019	0.8938	0.3008	0.735

4. Discussion

The hypothesis was that the potential N_2O production would become lower in the higher age classes, because of how N is more limited. What was found was instead a slight increase of N_2O production, even if not significantly different. Same with the potential denitrification that increased the higher the age class, but that was also not significant. When looking at the gene abundances for explanation to why the production is higher with age, there was no clear trend of nirK or nirS being higher, which would have potentially meant a higher activity of their enzymes and more NO_2- being transformed into NO, and then to N_2O being possible. The gene abundances were alike each other with no significant difference, but it was possible to see a small decrease for the abundances of the genes *nirK* and *nirS* when measured as copy/ng extracted DNA and copy/g DW soil, and a small increase in *nosZII* abundances with increasing grassland's age (Table 4). The *nosZ* gene is the only one known at the moment that transforms N_2O to N_2 (Philippot, Hallin and Schloter 2007 see (Hu et al. 2015) So an increase in N_2O production with age class could have been explained by a more simplified soil ecosystems in some of the sample areas(Le Provost et al. 2021) or a decrease in abundance of *nosZI* and *nosZII*, but that did not occur. A possible explanation for this is that this study did not include genes from other microbial groups that are a part of denitrification, such as fungi (Thamdrup 2013 see (Hu et al. 2015), or that the primers used in this study missed *nir* gene variants from some abundant microbial groups. Since there was no significant difference between the age classes or regions in abundance for the genes or potential activities, no certain conclusions should be drawn.

The farms and their fields can however have different soil characteristics. Like the soil type, pH, CN-ratio, water fraction and the management, which might have led to variations. Ekeby has significantly lower potential N_2O production than the other regions and is significantly lower in the potential denitrification than region Stabby. A higher amount of water in the soil is said to give higher production of N_2O (Wu et al. 2017). Ekeby as a region has many soils with higher water fractions, but that extra water has not given the region any visible advantages in potential activity, like changed microbial community composition selected for higher water content that could have shown higher N_2O production in the lab. The gene abundance for the region shows no obvious decline or increase in for any genes. But the region looks

like it might have a lower organic matter content than typical for the other regions thought not significantly. The lower organic matter content might be the reason why the activity was lower, since the denitrifiers could be less active and have less access to SOM as a electron donor (Guenet et al. 2021). Low pH can increase the N_2O production (Robertson 2014 see (Hu et al. 2015), but Ekeby as a region does not stick out by having a lower pH than the rest. Neither does the C/N-ratio differ noteworthy from the other regions samples. It is possible that some managements have been different for this region resulting in a lower organic matter stock and that has had an effect on the activity. What also should be noted, is that the grasslands in this study isn't fertilized as a part of management, but manure from the animals grazing can add to both nitrogen and carbon in the soil. So it is possible a difference in herd for the different farms could have lead to the differences between the regions.

Potential N_2O production and denitrification assay provide information about the N_2O emissions in ideal conditions where both C and N aren't limited resouses in the environment. In reality the availability of nutrients might be limited in some of the soil samples and therefore we will not get a result that mirrors the conditions of the grasslands. In addition, the substrate solution added in the assay might not be suitable for many microorganisms in the soil. To further understand the potential activities of these microorganisms, more tests with different mixture of carbon sources, limited resources or no added substrate solution can be done. However, in this thesis these methods have not been chosen, because of the very limited gas production that were observed, making such a test even more sensitive or not even measurable. Further research can also be done on deeper layers of the soil and a further expansion of area to look at permanent grasslands in other parts of Sweden or Europe.

The conclusion that can be drawn for the potential activity assays is that there is no significant difference in potential N_2O emissions for permanent grasslands between ages 10-150 years. This contredicts the study by Scott et al. (2019) where the N_2O emissons decreased with the passage of time, though that might be because that study looked at a much shorter chronosequence of only 35 years. There is however a significant difference between the potential in regions, where the farm Ekeby has lower potential for N_2O emissions. A difference that possibly could be because of the organic matter content in that region.

If there is no difference between grasslands potential nitrous oxide emissons depending on the passage of time since convertion, that could be a good thing for using permanent grasslands as a carbon sink, even if not a decrease in emission as predicted in the introduction. But the trends did indicate that there might be an

increase in emission, even if not significant in this study. Which is when that possible emission will have to be weighted against the benefits of having permanent grasslands.

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Appendix

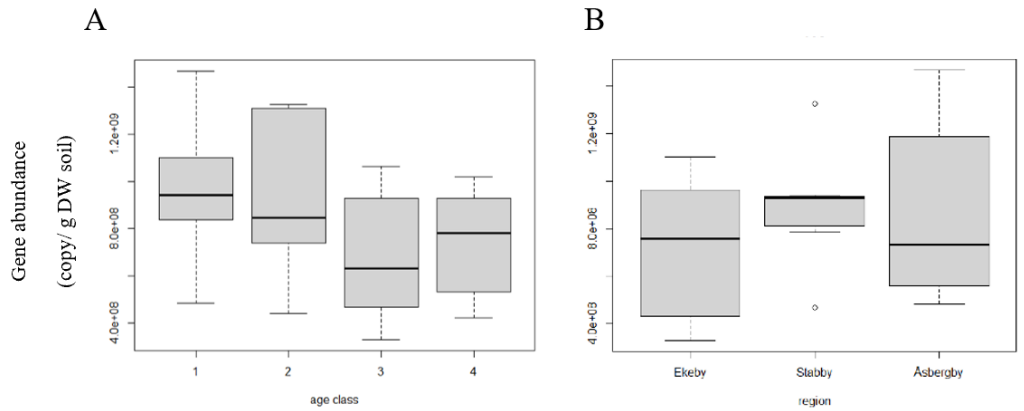

Figure 8: A) Gen abundance as copy/g dry weight soil across age class 1,2,3 and 4. B) Gen abundance of 16s as copy/g dry weight soil across sample regions Ekeby, Stabby and Åsbergby

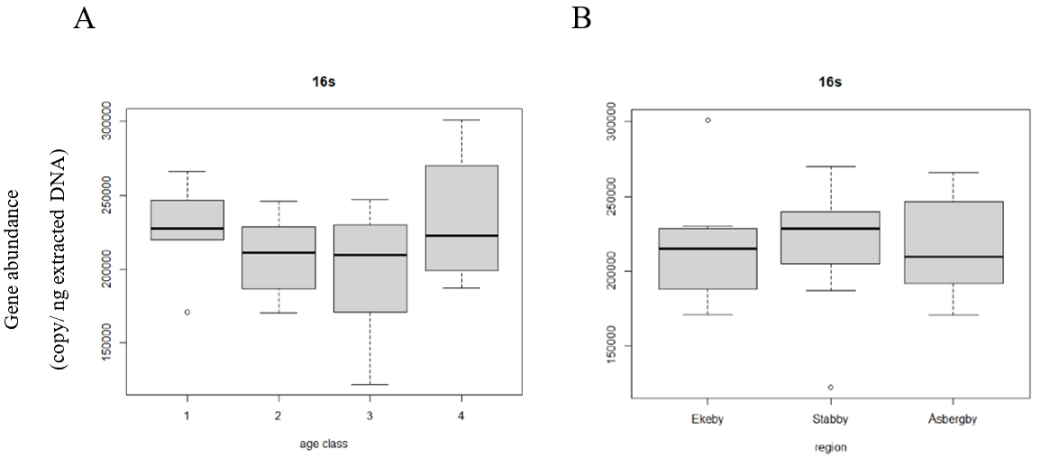

Figure 9: A) Gen abundance of 16s as copy/ng extracted DNA across sample regions Ekeby, Stabby and Åsbergby B) Gen abundance as copy/ng extracted DNA across age class 1,2,3 and 4.

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