Linking meta-omics to the kinetics of denitrification intermediates reveals pH-dependent causes of N₂O emissions and nitrite accumulation in soil

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Abstract

Denitrifier community phenotypes often result in transient accumulation of denitrification (NO₃⁻→NO₂⁻→NO→N₂O→N₂) intermediates. Consequently, anoxic spells drive NO-, N₂O- and possibly HONO-emissions to the atmosphere, affecting both climate and tropospheric chemistry. Soil pH is a key controller of intermediate levels, and while there is a clear negative correlation between pH and emission of N₂O, NO₂⁻ concentrations instead increase with pH. These divergent trends are probably a combination of direct effects of pH on the expression/activity of denitrification enzymes, and an indirect effect via altered community composition. This was studied by analyzing metagenomics/transcriptomics and phenomics of two soil denitrifier communities, one of pH 3.8 (Soil3.8) and the other 6.8 (Soil6.8). Soil3.8 had severely delayed N₂O reduction despite early transcription of nosZ, encoding N₂O reductase, by diverse denitrifiers, and of several nosZ accessory genes. This lends support to a post-transcriptional, pH-dependent mechanism acting on the NosZ apo-protein or on enzymes involved in its maturation. Metagenome/metatranscriptome reads of nosZ were almost exclusively clade I in Soil3.8 while clade II dominated in Soil6.8. Reads of genes and transcripts for NO₂⁻-reductase were dominated by nirK over nirS in both soils, while qPCR-based determinations showed the opposite, demonstrating that standard primer pairs only capture a fraction of the nirK community. The -omics results suggested that low NO₂⁻ concentrations in acidic soils, often ascribed to abiotic degradation, are primarily due to enzymatic activity. The NO reductase gene qnor was strongly expressed in Soil3.8,
suggested an important role in controlling NO. Production of HONO, for which some studies claim higher, others lower, emissions from NO$_2^{-}$ accumulating soil, was estimated to be ten times higher from Soil3.8 than from Soil6.8. The study extends our understanding of denitrification-driven gas emissions and the diversity of bacteria involved and demonstrates that gene and transcript quantifications cannot always reliably predict community phenotypes.

**Introduction**

During the past century human activities have accelerated the input of reactive N to the biosphere [1, 2]. This has escalated the emissions of N$_2$O, a major greenhouse gas and contributor to ozone depletion [3, 4, 5], as well as nitric oxide (NO) and nitrous acid (HONO) which both influence chemical reactions in the troposphere, leading to formation of undesired ozone [6]. Agriculture, and especially the excessive use of synthetic N fertilizers in large parts of the world, is a major source of N$_2$O release from terrestrial systems, primarily via denitrification and nitrification [1, 7, 8]. While CO$_2$ emissions are predicted to decline substantially during the present century, anthropogenic N$_2$O emissions, which have increased at an average rate of 0.6 ± 0.2 Tg N yr$^{-1}$ per decade during the past 40 years, are expected to continue to increase [5] or remain nearly constant [9], unless new methods to reduce the N$_2$O/N$_2$ product ratio of our agroecosystems are developed [10, 11]. Some mitigation options have been identified through improved understanding of the organisms producing and reducing N$_2$O and the environmental factors that control these emissions [12], and more are expected to emerge as we intensify our research on denitrification and denitrifying organisms in soil.

Denitrification is the stepwise reduction of nitrate (NO$_3^{-}$) to N$_2$ via nitrite (NO$_2^{-}$), NO and N$_2$O, and is performed by a diverse range of facultatively anaerobic organisms when O$_2$ becomes scarce. Complete reduction of NO$_3^{-}$ to N$_2$ is a four-step process with each step catalysed by a different reductase [13, 14]: the NO$_3^{-}$ reductases NarG (membrane-bound) and/or NapA (periplasmic) encoded by the genes *narG* and *napA*; the NO$_2^{-}$ reductases NirK (copper-containing) or NirS (containing cytochrome *cd*) encoded by *nirK* and *nirS*; the NO reductases cNor (cytochrome *c* dependent) or qNor (quinol-dependent) encoded by *cnor* and *qnor*; and the N$_2$O reductase NosZ, of which two clades have been identified, encoded by *nosZ* clade I and II [15]. While many organisms have both Nar and Nap, most denitrifiers carry only one type of Nir, Nor and NosZ. The denitrification pathway is modular [16] and the absence of one or more of the reduction steps is common [17, 18]. Not only does the
presence/absence of the denitrification genes affect the phenotypes of denitrifiers and the 
amounts of the different denitrification products that are released but gene regulation [14], in 
interplay with environmental factors, is a key factor as well [18, 19, 20]. Similar controls of 
N\textsubscript{2}O/N\textsubscript{2} product ratios have been observed in soil communities [21]. If the production and 
reduction steps are not fully balanced net production of a denitrification intermediate will 
occur. Studies of denitrification kinetics during anoxic incubation of various denitrifying 
organisms always show temporal accumulation of intermediate products [18, 22], but the 
extent differs even between closely related strains [23]. Also, since the timing of 
denitrification gene transcription varies between organisms, denitrifier communities will have 
a succession of actively transcribing populations during an anoxic spell which will also 
influence levels of intermediate products [24].

Of the environmental key factors known to control denitrification perhaps the best 
characterized is soil pH [25, 26], which profoundly affects the accumulation of denitrification 
intermediates. The effects of pH on the regulation and enzymology of the reduction steps 
differ, however, and the mechanisms by which pH impacts these processes are not well 
understood. While the accumulation of NO\textsubscript{2} increases with soil pH [27, 28], there is a clear 
negative correlation between pH and N\textsubscript{2}O emissions [29-33]. The simple assumption that 
soils with high N\textsubscript{2}O emissions have fewer N\textsubscript{2}O-reducing organisms is corroborated by some 
studies but not by others [32, 34-36], which lends little support for a direct, causal relationship 
between nosZ numbers and N\textsubscript{2}O emissions. Studies of denitrifying bacteria in pure culture, 
extracted soil bacterial communities and in intact soils [37-39] all showed that nosZ 
transcription did take place under acidic conditions (pH 5.7-6.1) during periods of active 
denitrification but with negligible or strongly delayed N\textsubscript{2}O reduction. This suggests a post-
transcriptional phenomenon, possibly interference with the maturation of the NosZ apo-
protein, or with the transfer of electrons to the reductase. The quantification of nosZ 
transcripts in those soil communities was, however, based on PCR primers targeting only 
bacteria carrying nosZ clade I. Moreover, the transcripts were not sequenced, which leaves it 
open to speculation whether different fractions of the denitrifying bacteria transcribe nosZ at 
low versus high pH. This, and the fact that the post-transcriptional effect of low pH has only 
been tested in a few model strains, implies that it remains essentially unknown how 
widespread this phenomenon is among denitrifiers in soils.

The general occurrence of low NO\textsubscript{2} concentrations in acidic soils has been attributed 
mainly to chemical decomposition at low pH [40]. This was challenged recently by [28], who 
suggested that high rates of microbial reduction of NO\textsubscript{2} played a key role in keeping NO\textsubscript{2}·
concentrations low during denitrification in acidic soil. This would also imply high rates of NO reduction since the concentration of this gas was kept low. In soil with near neutral pH, on the other hand, the rate of NO\textsubscript{3}\^- reduction exceeded the rate of NO\textsubscript{2}\^- reduction, leading to NO\textsubscript{2}\^- accumulation. The nir and nor gene abundance, their transcriptional activity and the organisms involved were however not investigated.

Here we aimed at resolving some of the questions raised from the above-mentioned studies about how soil pH affects the composition and activity of the denitrifier community and the accumulation/release of denitrification intermediates. We took an integrated “multi-omics” approach [41] to avoid primer biases [42] and thus to include as large a portion as possible of the microbial community, and analyzed the metagenomes (MGs) and the metatranscriptomes (MTs) of two soils with significantly different pH, 3.8 and 6.8. Samples for MT analysis were taken at time intervals through anoxic incubation of soil microcosms, during which the dynamics of the denitrifier community “phenome” was followed by frequent determinations of denitrification intermediate and end products. The results from the phenomics analysis, presented in more detail in [28], showed complete denitrification in Soil6.8 from the start of the incubation and accumulation of NO\textsubscript{2}\^- in Soil3.8. On the other hand, showed no NO\textsubscript{2}\^- accumulation but severely delayed N\textsubscript{2}O reduction (Fig. 1). The problems with obtaining mRNA from low pH soil (pH 4) encountered earlier [38] were overcome using an optimized protocol [43], which provided nucleic acid quality suitable for both shotgun MG- and MT sequencing. The relative abundances of detected denitrification genes and transcripts were compared to results from PCR-quantification, which confirmed the severe biases related to amplification-based analyses.

The -omics analyses allowed us to address several issues. One was to determine if the abundance of nar/nap, nir and nor genes and transcripts could explain the strong control of NO\textsubscript{2}\^- and NO observed in the acidic soil and which organisms were involved. In addition, the detailed kinetics data made it possible to estimate if NO\textsubscript{2}\^- accumulation in neutral soil would lead to more or less HONO emission than from non-NO\textsubscript{2}\^- accumulating, acidic soil. Secondly, we investigated if the apparent lack of DNRA activity (dissimilatory nitrite reduction to ammonium) in these soils was due to low abundance of DNRA-related genes and transcripts. Thirdly, we clarified the complex ecophysiology of nosZ carrying bacteria to better understand their hampered N\textsubscript{2}O reduction under acidic conditions (this study and [38, 39]). To do so, we investigated to what extent the two nosZ clades were found in the MGs and MTs of two soils of differing pH; if nosZ gene transcripts originated from a few populations or represented diverse denitrifying bacteria; and if genes other than nosZ in the nos operon were
transcribed in acidic soil. For the latter, we included *nosR*, which encodes NosR suggested to be involved in electron delivery to NosZ in organisms with *nosZ* clade I; *nosL*, which encodes a chaperone delivering Cu to the NosZ apo-protein (both *nosZ* clades); and the ORF *nosDFY* (both *nosZ* clades) encoding NosD, suggested to be involved in NosZ maturation, and the ABC-transporter NosFY [44-46].

**Materials and methods**

**Soils.** Two peat soils (40-45 % organic C, 2 % organic N) [38] with pH 3.80 (Soil3.8) and 6.80 (Soil6.8) were sampled from a long-term field experimental site in western Norway (61°17'42", 5°03'03"). Soil3.8 is the original un-limed soil, and Soil6.8 was limed in 1978 with shell sand (800 m³ ha⁻¹; [47]). Freshly sampled soils were transported to the laboratory, sieved (4.5 mm) upon arrival, then stored in sealed plastic bags at 4 °C. All pH values were measured in 0.01 M CaCl₂ (soil:CaCl₂ 1:5) immediately prior to further analyses.

**Soil treatment.** The soils were revitalised from cold storage by addition of 5 mg dried, powdered clover g⁻¹ soil wet weight (ww), then incubated at 15 °C for 72 h [38]. Soil aliquots corresponding to 1.5 g soil organic C (5-8 g of soil ww depending on lime content) were placed in air-tight glass vials and sealed with butyl-rubber septa and aluminium crimps. Nitrate (as KNO₃) was dissolved in autoclaved MilliQ water which was added to reach 80% of the soil’s water holding capacity (WHC) and 6.2-7.1 mM NO₃⁻ in soil moisture. Thus, at the onset of the incubation, the total amount of NO₃⁻ per vial was 37 or 26 μmol NO₃⁻ in Soil3.8 or Soil6.8, respectively (see also [28]). The vials were immediately made anoxic by 6 cycles of gas evacuation and He filling [38], and incubated at 15 °C. Gases (CO₂, O₂, NO, N₂O and N₂) were measured in headspace every three hours using an autosampler linked to a GC and NO analyser [48]. At each gas sampling time point, one replicate vial of each soil type was sacrificed and NO₂⁻ was extracted and concentration determined as described in [28]. A portion of soil from the same vial was snap frozen in liquid nitrogen then stored at -80 °C until nucleic acid extraction. The soil incubation experiment was described in detail in [28], which also explains how the rates of the four steps of denitrification were calculated based on measured concentrations of NO₂⁻, NO, N₂O and N₂ throughout the incubation, and corrected for abiotic decomposition of NO₂⁻ in the acid soil. The present MG and MT analyses were done on two of the three soils investigated by [28], and our presentation of the kinetics is limited to the calculated rates of the four steps of denitrification.
Nucleic acid extraction. DNA and RNA were extracted from frozen samples using the method of [43] who also tested several commercial kits for these soils without success. Briefly, 3 x 0.2 g of soil was taken at time 0 (at the start of anoxic incubation) for DNA extraction, and at selected time points (0.5-27 h) during anoxic incubation for RNA extraction. Lysis was performed with glass beads in CTAB extraction buffer and phenol-chloroform-isoamyl alcohol (25:24:1), using a FastPrep-24 instrument. After ethanol precipitation, the nucleic acids were resuspended in DEPC-treated nuclease-free water purified with the OneStep PCR Inhibitor Removal Kit (Zymo Research, Irvine, USA), then split into a fraction for DNA and one for RNA. The DNA fraction was further purified using the Genomic DNA Clean & Concentrator kit (Zymo Research), then kept at -20 °C until use. The RNA fraction was digested using TURBO DNA-free DNase kit (Ambion, Life Technologies) according to the manufacturer’s instructions, then purified using the RNA Clean & Concentrator-5 kit (Zymo Research). Quantitative PCR (qPCR) using primers targeting the 16S rRNA gene (described below) was used to assess the presence of residual genomic DNA (gDNA) in the purified RNA fractions (defined by signal detected in the qPCR at ≤ 35 cycles), and only RNA fractions free of gDNA was used for further analysis. The purified and DNA-free RNA fractions were reverse transcribed using the Maxima Reverse Transcriptase with random hexamer primers (Thermo Scientific), according to the manufacturer’s instructions. Primers targeting the 16S rRNA or nosZ genes (described below) were used in qPCR to assess the quality (defined by uninhibited amplifiability) of purified DNA and reverse-transcribed cDNA.

Sequencing the metagenome (MG), metatranscriptome (MT), and 16S rRNA genes. Triplicate DNA and duplicate RNA samples were sent for metagenomic and metatranscriptomic sequencing at The Roy J. Carver Biotechnology Center (CBC)/W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign, using HiSeq 2500 technology. All nucleic acids were shipped in a liquid nitrogen vapour dry shipper (Cryoport) and arrived within 5 days (the Cryoport Express dewar is able to maintain the temperature at -150 °C during shipment for 10 days). The RNA integrity (including confirmation of the absence of gDNA) was also independently verified by the CBC prior to sequencing the samples. DNA samples were sent for 16S rRNA community analysis using Illumina MiSeq technology (2 x 300 bp paired-end sequencing with V3 chemistry) at StarSEQ GmbH, Mainz (Germany). The primers used targeted the V4 region of
the 16S, 515f and 806rB [49, 50], as detailed by the Earth Microbiome project (http://www.earthmicrobiome.org/emp-standard-protocols/16s/).

16S rRNA amplicon sequence analysis. Processing of the sequenced 16S rRNA amplicons was performed by StarSEQ Gmbh, Mainz (Germany). Briefly, the sequences were demultiplexed and the adapters were trimmed locally on the MiSeq instrument with the Illumina Metagenomics 16S rRNA application, using default settings. Amplicon sequence data was processed using the Greenfield Hybrid Analysis Pipeline (GHAP) which combines amplicon clustering and classification tools from USEarch [51], and RDP [52], combined with custom scripts for demultiplexing and OTU table generation [53]. Reads were subject to quality trimming with a cut-off threshold of 25 and length trimming where only reads in the length range 250 to 258 bp were retained. Trimmed forward and reverse reads were then merged and only successfully merged reads retained. OTU clustering was performed at a 97% sequence similarity. Representative sequences from each OTU were classified by finding their closest match in a set of reference 16S sequences, and by using the RDP Naïve Bayesian Classifier. The RDP 16S training set and the RefSeq 16S reference sequence collection were used for classification. NumPy, SciPy, scikit-learn and Matplotlib were used for calculation and plotting of principle component analyses (PCAs) [54-56]. Samples were rarefied to 38287 reads/sample for the purpose of determining species richness and Peilou's evenness, calculated using USEarch v11 [57].

Analysis of MG- and MT sequences. After trimming the number of reads (in millions) was between 25.9-31.4 in the MG samples and between 16.4-42.0 in the MT samples except for two samples which were lower (4.0 for one of the 12h duplicates from Soil6.8 and 8.5 for one of the 0.5h duplicates from Soil3.8). The sequenced Illumina HiSeq reads were quality controlled using BBduk from the BBTools package version 35.66 (http://sourceforge.net/projects/bbmap/). For functional annotation, reads were aligned using DIAMOND with an e-value cutoff of $1 \times 10^{-3}$ [58]. For analysis of reads derived from genes involved in nitrogen metabolism comparison was done against a custom dataset described in [59, 60]. The DIAMOND output was converted to m8 blast format and analysed in R. Reads must have had a matching region of >30 amino acids and an identity of >60% to be considered matching. Output of matching reads were normalised to reads per million of total reads, RPM (see below).

Reads derived from specific genes and meeting the assigned quality cutoffs were extracted from read sets using filterbyname from the BBTools suite of programs. The extracted reads
were then uploaded to KBase [61] and taxonomic assignment was performed using KAIJU using default settings [62].

**Statistical and quantitative analysis of meta-omic data.** All reads counts were normalised for sequencing depth, generating RPM values: (number of reads)/(total reads that passed quality control)×10^6. All statistical analyses and graphing were performed using in-house R scripts custom created for this purpose.

**Quantitative amplification-based analysis.** The genes encoding 16S rRNA and the three denitrification reductases nirK, nirS and nosZ clade I were quantified by qPCR using the primers 27F and 518R for the 16S rRNA gene [63], 517F and 1055R for the nirK gene [64], cd3aF and R3cd for the nirS gene [65] and Z-F and 1622R for the nosZ gene [66]. DNA samples were diluted to 1-10 ng of DNA per reaction. All cDNA and RNA samples (DNase-digested) were used without dilution. Each 20 µL qPCR reaction contained SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara Bio) and was run according to the manufacturer’s instructions and included 0.4 µM of each primer and 2 µL of template. The optimised qPCR cycling conditions for all primer sets were 95 °C for 5 min, 40 cycles of 95 °C for 30 s, x for 60 s, 72 °C for 30 s, 82 °C for 20 s, and a final melting curve analysis from 60 °C to 95 °C to determine the specificity of amplicons, where x = 54 °C (16S rRNA gene), or 60 °C (denitrification genes). To reduce background signals from primer dimers and unspecific PCR products, the fluorescence signal was measured during the final step of each cycle, at 82 °C. The detection limit of each qPCR run was 5 copies per microliter of reaction [43], which was approximately 4 × 10^2 copies g⁻¹ soil (ww).

**Results**

**Kinetics of denitrification intermediates depict a pH-dependent response to anoxia.**

The denitrification kinetics of the two soils during 45 h of anoxic incubation are shown in Fig. 1, in which the sampling occasions for MT analyses are also indicated. A more complete description of the incubation experiment is given by [28], including detailed analyses of production/reduction rates of the denitrification intermediates/end products over 70 h. The analysis included a careful mineral N budget analysis demonstrating 100% recovery of NO₃⁻-N as N₂ for the soil with pH=6.8, which suggests negligible reduction of nitrate to ammonium in this soil. For Soil 3.8, the recovery as N₂-gas (N₂ + N₂O + NO) was lower (77%), but abiotic nitrosylation of organic material accounted for 17% (see Table 2 in [28], thus in total 94%
was accounted for, leaving only 6% of the NO$_3^-$ loss that could possibly be ascribed to DNRA.

The two soils showed striking differences in their accumulation of NO$_2^-$ and N$_2$O, while they had very similar NO kinetics (Fig. 1A). The NO$_2^-$ concentration in Soil3.8 was 20-50 µM in the soil moisture during the entire anoxic incubation, except for the 36-40 h period when it reached ~100 µM. This corroborates the general notion that NO$_2^-$ concentrations are low in acidic soils. In Soil6.8, on the other hand, NO$_2^-$ steadily increased from the beginning, reaching 2-3 mM in the soil moisture at 20 h, after which levels decreased and were undetectable at the end of the sampling period. Both soils had a transient accumulation of 3-4 µmol NO vial$^{-1}$, which is equivalent to 1.5-2 µM NO in the soil moisture. The soils had profoundly different N$_2$O and N$_2$ kinetics. Soil3.8 accumulated N$_2$O but little or no N$_2$ during the first 35 h, while Soil6.8 accumulated both N$_2$O and N$_2$ at similar rates from the very beginning. The gas kinetics is reflected in the calculated rates of the four steps of denitrification (Fig. 1B): In the acid soil, $V_{NAR/NAP}$ (NO$_3^-$→NO$_2^-$), $V_{NIR}$ (NO$_2^-$→NO) and $V_{NOR}$ (NO→N$_2$O) were similar, while $V_{NOS}$ (N$_2$O→N$_2$) was close to zero during the first 35 h. In the soil with near neutral pH, $V_{NAR/NAP}$ exceeded the other enzyme rates during the first 15 h but declined rapidly to zero as NO$_3^-$ was depleted. In contrast to the acidic soil, $V_{NOS}$ was high from the very beginning of the incubation in Soil6.8.

Based on the total NO$_2^-$-N (TNN=NO$_2^-$+HNO$_2$), we calculated the concentration of undissociated HNO$_2$ (aq) in the soil matrix using the Henderson-Hasselbalch approximation (see Supplementary material p. 3), which forms an equilibrium with the gas HONO in the atmosphere, thus predicting the potential emission of HONO. Despite the high accumulation of TNN in Soil6.8 (up to 3.6 mM), the concentration of undissociated HNO$_2$ was ≤1.4 µM. In Soil3.8 the concentration HNO$_2$ was almost two orders of magnitude lower (Fig. 2).

**Soil bacterial community composition differed by pH but was stable during incubation.**

In the 16S rRNA gene amplicon analysis >99.29% of all sequenced reads were annotated as bacterial, about 0.004% were unclassified, and the rest belonged to Archaea, which represented ≤0.60% of the reads in Soil6.8 and ≤1.03% in Soil3.8. Principal component analysis of the bacterial 16S rRNA gene reads (Fig. S1A) clearly separated the reads from the two soils along PC1, which explained 94% of the total variation and also showed that the variation between replicate samples was low. The most abundant classified phyla in both soils were Proteobacteria, Acidobacteria, Actinobacteria, Planctomycetes, Verrucomicrobia and...
Bacteroidetes. A breakdown of Proteobacteria showed that Alphaproteobacteria were most abundant in both soils, followed by Beta-, Gamma- and Deltaproteobacteria (Fig. S1B). The main differences between the soils was a larger relative abundance of Actinobacteria and Alpha- and Betaproteobacteria in Soil3.8 than in Soil6.8 along with a higher relative abundance of Bacteroidetes in Soil6.8 than in Soil3.8. The OTU richness was higher in the Soil6.8 samples with 2846 OTUs observed on average for the Soil6.8 samples compared to an average of 1882 OTUs for the Soil3.8 samples. Soil6.8 samples were also slightly but significantly (p=1.66x10^-5) more even according to Peilou's evenness measure with Soil6.8 samples having an average Peilou's evenness measure of 0.814 and compared to 0.796 for Soil3.8 [57]. The microbial community profile of Soil6.8 was stable during the 27 h incubation, suggesting that differences observed in the MT can be reasonably attributed to variations in transcription patterns, and not due to bacterial growth causing a shift in the bacterial community composition.

Occurrence and prevalence of reads in the MG related to denitrification and DNRA.
Reads annotated as NAR, here defined as nap+nar reads, were twice as abundant as NIR, NOR and NOS gene reads in the MG of both soils (Fig. 3A; Table 1). Of the two types of NAR, narG reads were 6.1±0.3 times more abundant than nap reads in Soil3.8 but only 2.1±0.3 times more abundant in Soil6.8 (Table 1). Levels of NIR (nirK+nirS) were comparable in the two soils with RPM values of 36.4±1.7 and 44.6±1.4 for Soil3.8 and Soil6.8, respectively (Table 1). nirK genes were much more abundant than nirS in both soils with a nirK/nirS ratio of about 40 in Soil3.8 and 7 in Soil6.8. The NOR gene reads (cnor+qnor) were more abundant in Soil3.8, where RPM values were 74.9±0.5 compared to 48.1±0.3 for Soil6.8. The qnor genes dominated over cnor in both soils and were about 11 and 4 times higher in Soil3.8 and Soil6.8, respectively. Total NOS reads (nosZ clade I+II) were instead somewhat higher in Soil6.8 than in Soil3.8 (25.8±2.0 vs 18.2±1.0). To summarize this for all genes, the order of gene reads from highest to lowest abundance was for Soil3.8 narG > qnor > nirK > napA > nosZ clade I > cnor > nirS > nosZ clade II; and for Soil6.8 narG > napA > nirK + qnor > nosZ clade II > cnor > nirS > nosZ clade I. Since the two soils accumulated different amounts of denitrification intermediates, we calculated ratios of MG reads representing the four steps of denitrification (Table 1), to examine if the genetic potential for production/consumption of the different intermediates was related to the net production seen in Fig. 1. The NAR/NIR ratios were similar in the two soils (4.68±0.27 for Soil3.8 and 4.32±0.12 for Soil6.8), which, by itself, is not consistent with the
higher net production of NO$_2^-$ in Soil6.8. The NOR genes were more abundant than NIR in Soil3.8, with a NIR/NOR ratio of 0.49 ±0.03 compared to 0.92 ± 0.03 in Soil6.8. NIR and NOR were more abundant than NOS in both soils with NIR/NOS ratios of 2.83 and 2.07 and NOR/NOS ratios of 5.81 and 2.23 in Soil3.8 and Soil6.8, respectively. The two nosZ clades (I and II) differed in gene abundance in the two soils with higher values for clade I in Soil3.8 and vice versa in Soil6.8 (Fig. 3A). The nosZ clade I/clade II ratio (based on RPM values) was 28.1 in Soil3.8 and 0.25 in Soil6.8 (Table 1).

We also analysed accessory genes of the nos operon, with a focus on clade I, using a manually curated database. The nosR gene is a part of the nos operon in clade I but not in nosZ clade II organisms [15, 45]. Its product is essential for N$_2$O reduction in clade I organisms and is suggested to be involved in transcription of nosZ, and also in electron transfer to the NosZ reductase [46]. In accordance with nosZ clade I being dominant in Soil3.8, we found about twice as many reads derived from nosR in Soil3.8 than in Soil6.8 with RPM values of 16.2±0.3 vs 7.0 ±0.3, respectively. Moreover, we examined the relative abundance of the accessory genes nosL, D and Y, which are found both in nosZ clade I and clade II organisms [15, 45]. For each of these genes, the read abundance was similar in the two soils (Fig. 4), which is in accordance with the comparable abundance of NOS (nosZ clade I+II) in the two soils (12.5 RPM in Soil3.8 and 20.8 in Soil6.8, Table 1). The gene reads for nosF, which is also part of the nos operon both in clade I and II [45], were 10-45 times higher than for the other accessory genes which points to uncertainties in the databases for this gene.

In addition to the canonical denitrification genes, we examined reads from the two DNRA-related genes nrfA and nirB. They were about 3 times more abundant in Soil6.8 than in Soil3.8 with nirB dominating over nrfA in both soils (Fig. S2). We also examined the occurrence of reads derived from the gene hmp. This gene, reported from a wide range of bacteria [67], encodes a flavohemoprotein that has a role in NO detoxification. Unlike the NOR genes, we found similar and relatively low abundances of hmp in the two soils (16±0.8 and 18±1.4 RPM, not shown).

**MT reads related to denitrification and DNRA.**

Earlier PCR-based investigations of soils from the same site as that studied here showed that denitrification genes were quickly transcribed upon onset of anoxia [24, 38]. Based on these findings, RNA samples for the present study were taken from both soils 0.5 and 3 h after the start of the experiment (red arrows, Fig. 1) to characterize the community response to denitrifying conditions. Additional RNA samples were taken from Soil6.8 prior to the peaks
in NO$_2^-$, NO, and N$_2$O (Fig. 1) to determine if there were new bursts of gene transcription during the incubation and, if so, if these were from already active organisms, or if different organisms became transcriptionally active at the later time points. The transcript read abundances are shown in Fig. 3B. For clarity, the nosZ genes are shown as the sum of the two clades in the main figure, while the insert shows the clades separately. The reads in the meta-transcriptome from Soil6.8 exhibited a strong increase between 0.5 and 3 h for almost all denitrification gene transcripts. Over the same time frame there was a smaller, yet substantial increases for several of the genes in Soil3.8. Two distinct spikes in denitrification gene transcription were seen at 3 and 12 h, particularly for narG, nirK, qnor and nosZ (Fig. 3B). Reads of narG represented the most common denitrification transcripts in both soils and at all time points. The overall trend in transcript read abundance in Soil6.8 was narG > nosZ > nirK > qnor > napA > nirS > cnor, almost without exception, throughout the incubation (Table 1). The trend for Soil3.8 was narG > qnor > nosZ > nirK > napA > cnor = nirS, the main difference compared to Soil6.8 being that qnor was second most abundant, followed by nosZ. Similar to the gene reads in the MG, nosZ clade I transcript reads were more abundant than clade II reads in Soil3.8 and vice versa for Soil6.8.

It could be argued that the ratios of transcripts representing the reductases responsible for the production and consumption of the various denitrification intermediates are more suitable to use for comparison with phenotypic data than actual RPM values (transcript read abundances and selected ratios are given in Table 1). The NAR/NIR ratios were very similar in the two soils with average values of 2.2±0.3 for Soil3.8 and 2.3±0.8 for Soil6.8, with some variations between sampling times. The NIR/NOR ratios, on the other hand, were almost 3 times higher in Soil6.8 compared to Soil3.8 (average values including all sampling times were 2.2±0.8 and 0.7±0.2, respectively). The high NOR transcription in Soil3.8 most likely contributed to keeping NO emissions low, despite known chemical reactions between NO$_2^-$ and soil compounds producing NO. The NIR/NOR ratios of Soil6.8 for the individual sampling points further showed that NOR was consistently lower than NIR throughout the 27h incubation with NIR/NOR ratios of 1.2-2.5. Transcription of NOS (sum of clade I, clade II and ambiguous reads) was higher in Soil6.8 than in Soil3.8 and read numbers increased almost 7 times from 56 to 380 in Soil6.8 between 0.5 and 3 h. In Soil3.8 the NOS transcript reads almost doubled during the same period. NIR/NOS ratios were rather similar in the two soils, around 0.8 in Soil3.8 and 1.0 ±0.2 in Soil6.8. NOR/NOS ratios were higher in Soil3.8 than in Soil6.8, with an average of 1.3±0.3 for the first two sampling points while, during the
same time, this ratio for Soil6.8 was 0.6±0.2. These ratios reflect the higher number of NOR reads in Soil3.8, combined with higher reads for NOS in Soil6.8.

Transcript reads of *nos* accessory genes were detected in both soils at all sampling occasions (Fig. 4). The transcriptional activity increased for all these genes between 0.5 and 3 h, which is a strong indication that the lack of N$_2$O reduction in Soil3.8 was not caused by any transcriptional control mechanism. The *nosR* transcript abundances were comparable in the two soils.

We also examined the MT with regard to the two DNRA genes *nfrA* and *nirB*, expecting low read abundance since no DNRA activity was discerned from the gas analyses. Surprisingly, these two genes were transcribed at levels comparable to the NIR and NOR genes, with sometimes high read values for *nirB* (Fig. S2).

**PCR-based quantification of functional genes and transcripts overlooked substantial parts of the community.** The abundances of some of the denitrification genes and transcripts in the MGs and MTs (Fig. 3; Table 1) were compared to qPCR-results in samples from the same soil incubation (Fig. 5), although more time points were included in the qPCR analysis. We targeted *nirK*, *nirS* and *nosZ* clade I using standard primer pairs (see Materials and Methods). The comparison revealed some striking differences between -omics and PCR-based results. While the “-omics” based results showed dominance of *nirK* over *nirS* both in the MGs and MTs, the qPCR-based quantifications showed 1-2 orders of magnitude lower abundances of *nirK* genes and transcripts compared to *nirS*, except for Soil3.8 where the difference was smaller. Comparing the *nirS* and *nosZ* (clade I) abundancies shows nearly identical abundancies of these genes in Soil6.8 according to the MG (Fig. 3A) while qPCR showed almost 10 times fewer *nosZ* than *nirS* gene copies based on qPCR (Fig. 5A). The MG- and qPCR-based results from Soil3.8 showed better correspondence, with *nosZ* being more abundant than *nirS*. For the transcripts (Figs. 3B and 5B), *nosZ* was more abundant than *nirS* in Soil3.8 according to the MT analysis, while the qPCR showed an opposite trend. Similarly, qPCR gave *nosZ* transcript levels that were 5-10 times lower than *nirS* in Soil6.8, while their levels were similar in the MT. The results indicate a critical primer bias in the amplicon-based quantification leading to severe underestimations of *nirK* abundance and overestimations of *nirS* compared to *nirK* and *nosZ* clade I.

**Taxonomic annotation of denitrification genes and transcripts.** Each gene and corresponding transcript had a unique taxonomic profile that varied by soil pH (Fig. 6).
Proteobacteria, Actinobacteria and Bacteroidetes were the most abundant phyla of denitrifiers in the MG and MT in both soils. Reads assigned to these phyla were detected for most denitrification genes and transcripts with the exceptions of Bacteroidetes, which were not found among narG and nirS reads, and Actinobacteria, which were not found among the nirS, cnor and nosZ clades I and II reads. Several other phyla such as Firmicutes, Chlamydiae, Nitrospira, Spirochaetes and Verrucomicrobia were represented by reads only from one or a few genes/transcripts.

The MG reads of narG, the most abundant among the denitrification genes, were dominated by Proteobacteria, mostly the classes Alpha- and Betaproteobacteria, and Actinobacteria (Table S1). Second most abundant in both soils were napA reads, which were mainly derived from Proteobacteria. Organisms belonging to these phyla also dominated the transcriptional activity for narG and napA genes. narG reads from Nitrospira were high as well in Soil6.8. MG reads derived from the NO₂⁻ reduction gene nirK, which was far more abundant than nirS in both soils (Table 1), were attributed to a number of phyla which were dominated by Proteobacteria, primarily Alpha- Beta- and Gamma proteobacteria, and by Actinobacteria, Bacteriodetes and Firmicutes. These same phyla also dominated the MT reads of nirK in both soils, with Proteobacteria showing particularly high transcription of this gene in Soil6.8 at the 3h sampling time. MG reads from nirS were predominantly from Betaproteobacteria in Soil6.8, which also accounted for the highest nirS transcriptional activity. Only 7 classified nirS MG reads were identified in Soil3.8 compared to 242 nirK reads (Table S1). Similar as for the nirK and nirS genes, the qnor genes dominated strongly over cnor genes in the MG, and the qnor genes belonged to several phyla while the cnor reads were mostly from Proteobacteria. The Delta- and Gammaproteobacteria had the highest number of qnor MG reads in both soils, but it was the Betaproteobacteria that dominated among the transcripts showing comparatively high and immediate transcription in Soil3.8. Interestingly, qnor MG and MT reads from Acidobacteria and Planctomycetes were detected in both soils at relatively high abundance. Apart from a few acidobacterial nosZ clade II reads, reads from these two phyla were not found for the other denitrification genes in the MG or MT.

The total number of nosZ MG reads was higher in Soil6.8 than in Soil3.8 (212 vs 152, Table S1). As expected, nosZ clade I MG and MT reads were only from Proteobacteria in both soils. nosZ clade II MG reads were more diverse, especially in Soil6.8, and were comprised of not only Proteobacteria, mostly Deltaproteobacteria, but also several other phyla including Bacterioidetes, which was the most highly represented group, as well as Firmicutes,
Verrucomicrobia, Chloroflexi, Gemmatimonadetes and Acidobacteria among others. The transcriptional activity of $\textit{nosZ}$ clade I was dominated by Alphaproteobacteria in Soil6.8 and by Betaproteobacteria in Soil3.8. The pattern was very different for transcription of $\textit{nosZ}$ clade II, which was dominated by Bacteroidetes in both soils. The transcript abundance of clade II in Soil3.8 was low but increased between 0.5 and 3 h and included not only Bacteroidetes but also a few reads from Proteobacteria, Bacteroidetes, Chloroflexi, Gemmatimonadetes, Ignavibacteriae and Acidobacteria. Taken together, the results for the two $\textit{nosZ}$ clades show that the problem of producing functional NosZ in Soil3.8 was common to several phyla. Soil6.8 showed high transcriptional activity of $\textit{nosZ}$ clade II, dominated by Bacteroidetes, but transcripts were also detected from all other phyla for which $\textit{nosZ}$ clade II were registered in this soil, except Euarchaeota.

**Discussion**

Liming, which has traditionally been a means to improve the fertility of acidic soils [68], is known to cause changes in microbial community composition and diversity [69, 70]. This was also seen in the present study, where the PCA of the 16S rRNA gene sequences clearly separated the two soils (Fig. S1A). The sequence analysis also revealed a slight but significant increase in diversity in the limed soil. The dominant phyla in both soils were those that generally dominate in soil studies [71] and no major differences between the soils were seen on the phylum level, or on class level for the proteobacteria, meaning that changes in the abundance of different groups had taken place at lower taxonomic levels. During the 27 h anoxic incubation, when Soil6.8 was monitored with metatranscriptomics, the community of Soil6.8 remained stable as judged from the 16S rRNA gene analysis (Fig. S1B), suggesting that any differences in the MT can be reasonably attributed to transcriptional regulation patterns and not caused by growth of some populations.

The accumulation of $\text{NO}_2^-$ and $\text{N}_2\text{O}$ in the two soils shows contrasting patterns, with low $\text{NO}_2^-$ and high $\text{N}_2\text{O}$ levels in Soil3.8 and vice versa for Soil6.8. The pattern of NO accumulation was more similar in these two soils, as presented in [28]. In the present study we investigated if the contrasting denitrification phenotypes of the two soils could be predicted from the abundance and transcription of the denitrification genes. Net production of denitrification intermediates will take place as soon as the reduction rate of one of the denitrification steps surpasses that of the following step. The $\text{NO}_3^-$ reduction rate ($V_{\text{NAR}}$) in Soil6.8 grossly exceeded the $\text{NO}_2^-$ reduction rate ($V_{\text{NIR}}$), as long as nitrate was present (Fig.
The MT analysis showed higher transcription of NAR (napA+narG) than of NIR (nirK+nirS) (Fig. 3B) with NAR/NIR ratios between 1.6 and 3.4 (Table 1). The ratio was similarly high for Soil3.8, however (2.0 and 2.3 for the two time points measured in this soil), despite the near-absence of NO$_2^-$ accumulation in this soil. Thus, no direct link was found between the transcript ratio NAR/NIR and NO$_2^-$ accumulation or the $V_{NAR}/V_{NIR}$ ratio, as affected by pH. In theory, abiotic NO$_2^-$ decomposition could be the primary reason for the marginal transient accumulation of NO$_2^-$ in Soil3.8, but this was refuted by the careful analyses of Lim et al. [28], who reached the conclusion that enzymatic NO$_2^-$ reduction was the major sink for NO$_2^-$ in Soil 3.8. One explanation for the marginal NO$_2^-$ accumulation in Soil3.8 could be that NO$_2^-$ reductase has a low pH optimum, as shown in a study by Abraham et al. [72] where measured NirK activity in vitro was ~4 times higher at pH 4.2 than at pH 7. Thus, Soil3.8 could have a much lower $V_{NAR}/V_{NIR}$ ratio than Soil6.8, despite the nearly equal NAR/NIR-ratio for the two soils. The results demonstrate that transcript numbers or ratios of transcript numbers are poor predictors of metabolic activities in soils. Even more evident is the discrepancy between gene numbers and activity. The NAR/NIR ratio in the metagenome was almost identical in the two soils (Table 1), despite the substantial difference in NO$_2^-$ accumulation. The understanding of how denitrifiers control NO$_2^-$ levels is far from complete, and different phenotypes have been described which are probably all present in complex soil microbial communities [18]. Some organisms perform complete denitrification of NO$_3^-$ to N$_2$ with little or no accumulation of intermediates, while others show complete inhibition of nir transcription until available NO$_3^-$ to NO$_2^-$ before further reduction takes place [23], and yet others reduce about half of the provided NO$_3^-$ to NO$_2^-$ before further reduction [73].

One reason that the NAR genes were by far more abundant than the genes for the other denitrification steps (Fig. 3A) could be that these genes are also carried by organisms performing DNRA [74]. Organisms with only NAR but lacking genes both for DNRA and denitrification are, however, also common [19], and in a study of bacterial isolates from the same field site as in the present study, 18 and 19% of the isolates from acidic and neutral soil, respectively, performed only NO$_3^-$ reduction to NO$_2^-$ without any further reduction [18]. The present study showed a strong dominance of narG over napA both in the MGs and in the MTs (Table 1; Fig. 3), despite the common occurrence of bacteria with napA+narG or with only napA among hitherto studied DNRA- and denitrifying organisms [75]. Still, napA was 2.6 times more abundant in the metagenome of Soil6.8 compared to Soil3.8 (Fig. 3A), in line with the significant positive relationship between napA and pH reported by [59], but the reason for this apparent pH dependence on the abundance of napA is not clear.
According to the MG analysis the abundance of NIR genes was similar in the two soils, with strong dominance of nirK over nirS, especially in Soil3.8 where nirS genes were almost absent (Table 1; Fig 3A). This contradicts the general conception that nirS is more abundant in most environments, which is derived from primer-based studies [38, 76, 77]. However, while nirS is mainly found in Proteobacteria, nirK is spread among taxonomically diverse groups, many of them being non-proteobacterial denitrifiers [16, 17, 78], which was also found in the present study (Fig. 6). Transcripts of nirK dominated over nirS also in the MT (Fig. 3B), and represented a diverse range of phyla including, in addition to Proteobacteria, also Actinobacteria, Bacteroidetes and Firmicutes (Fig. 6). This is in accordance with the results from a non-primer based study of another agricultural soil by [77]. The occurrence of a taxonomically diverse group of denitrifiers actively transcribing nirK probably reflects that this gene is easily transferred horizontally and expressed in new hosts since it does not require accessory genes to produce a functional enzyme, in contrast to nirS, which is part of a multi-gene operon [78]. This, and other evolutionary mechanisms, are a likely explanation to the reported incongruences between nirK and 16S rRNA phylogenies [16, 79]. Recently, Nadeau et al. [59] pointed out that denitrification is a non-essential trait for microbes, and that genes are gained and lost by individual members of the denitrifying community depending on the conditions. It is likely that this applies in particular to the functional genes nirK and qnor, which are probably more easily transferred horizontally than the other denitrification genes, and it can be speculated that they comprise a pool of genes that circulate between organisms depending on their needs.

The complete recovery of all added NO$_3^-$ as N$_2$ in Soil6.8 (Fig. 1A) indicated minimal or no conversion of NO$_2^-$ to NH$_4^+$ and thus that the NO$_2^-$ produced from NAR activity was not used by DNRA organisms. This is surprising, taking into account the relatively high abundance of reads from nrfA and nirB genes and transcripts (Fig. S2). Likewise, DNRA was probably insignificant in Soil3.8, since 94 % of the NO$_3^-$ loss in this soil could be accounted for by N-gas + nitrosylation (see [28]). In theory, full recovery of NO$_3^-$ reduction as N-gas could be obtained in a system with equal rates of DNRA and anammox. However, members of Brocardiales, which comprises the anammox-Planctomycetes [80], were scarce in the 16S rRNA gene analysis (<0.008% of 16S reads in all SoilA samples), which lends little support to this hypothesis.

The net production of NO was similar in the two soils despite the substantial abiotic reduction of NO$_2^-$ that contributed to NO emissions from Soil3.8 [28], in addition to the enzymatic NO$_2^-$ reduction taking place in both soils. The strong control of NO in Soil3.8, seen
from the calculated $V_{\text{NOR}}$ activity (Fig. 1B), is in agreement with the 2.5 times increase in $q\text{nor}$ transcript abundance between 0.5 and 3 h (Fig. 2B) and NIR/NOR ratios <1 (Table 1). In line with this, the gene abundance of $q\text{nor}$ was also high in Soil3.8, almost 2 times its abundance in Soil6.8 (Fig. 3A). This corroborates the metagenome results by Roco et al. [60] from acidic soils showing dominance of $q\text{nor}$ over all other denitrification genes, suggesting that low pH selects for this gene. Interestingly, $c\text{nor}$ apparently played a minor role in controlling NO, especially in Soil3.8 where gene abundance was close to 0 and transcripts were almost undetectable (Fig. 3). The taxonomic analysis of $q\text{nor}$ genes and transcripts (Fig. 6) demonstrated that this gene, similarly as $\text{nirK}$, is widely distributed over different phyla, with the largest representation in the $\text{Proteobacteria}$, $\text{Actinobacteria}$, $\text{Bacteroidetes}$, $\text{Chlamydiae}$, $\text{Acidobacteria}$ and $\text{Planctomycetes}$. $\text{qNor}$ is the product of a single structural gene, $\text{norB}$, existing alone or in a small operon [81], and it is conceivable that this gene/gene cluster is more readily transferred horizontally than the bigger $c\text{nor}$ operon. Furthermore, recent evidence suggests that $\text{qNor}$ is electrogenic [82], as opposed to $\text{cNor}$, and it can therefore be speculated that it provides an extra, energetic advantage to its host organism. Taken together, it is not unreasonable to conclude that, between the two functionally redundant NO reductases related to denitrification, it is $\text{qNor}$ that plays the major role in controlling NO concentrations under denitrifying conditions, at least in these soils. A primer-based study would most likely not have detected this since current primers for $\text{nor}$ mainly target proteobacteria, which severely biases the results, especially for $q\text{nor}$ [42].

Nitrous acid (HNO$_2$) in its gaseous form HONO plays a key role in atmospheric reactions by being a precursor for hydroxyl (OH) radicals, which in turn take part in the formation of undesired O$_3$ in the troposphere. Emissions of HONO and their connection to biological N-transformations have gained increasing interest during the past years. It has only recently been shown that biological processes producing NO$_2^-$ in soils contribute substantially to HONO emissions [6, 83, 84]. However, while Su et al. [83] stated that the low NO$_2^-$ concentrations in acidic soils are consistent with loss of HNO$_2$ as HONO, Oswald et al. [6] instead showed that soils with neutral and slightly alkaline pH generally emitted more HONO than acidic soils. Our results show more than 10 times higher HONO production from Soil3.8 compared to Soil6.8 (Fig. 2), but we also show that the major portion of the NO$_2^-$ is not reduced and released as HONO, but instead reduced to NO and subsequently to N$_2$O through denitrification.

The lower abundance of NOS genes and transcripts compared to NIR and NOR could, theoretically, be the cause of the low N$_2$O reduction in Soil3.8, but it is unlikely to explain the
nearly complete lack of N2O reduction in Soil3.8 during the first 35 h of incubation (Fig. 1A). This severely delayed N2O reduction in acidic soil corroborates other studies of soils from the same site and is in line with the growing evidence for a strong negative correlation between soil pH and N2O/(N2O+N2) product ratios [12, 33], suggested to be due to impaired maturation of the NosZ enzyme under acidic conditions (pH<6.1) [37-39]. The present study detected transcripts from both nosZ clades in Soil3.8, which suggests that the problem of producing functional NosZ under low pH conditions applies to both clades. Moreover, the taxonomic analysis showed that this problem is general to a diverse range of bacteria (Fig. 6), which adds new knowledge to earlier qPCR-based investigations in which taxonomy was not addressed [38, 39]. Although metatranscriptomes in Soil3.8 were only analyzed from the first 3 h of the incubation, the gas kinetics suggest that functional NosZ was not produced until after 25 h (Fig. 1B). In a natural situation the N2O produced in this time period would be emitted to the atmosphere. It can only be speculated why functional NosZ started to be produced after prolonged incubation. One reason could be that successful maturation took place somewhat stochastically, slowly building up a functional pool of NosZ. Another possibility is that populations of organisms such as the Rhodanobacter strains described by Lycus et al. [18] increased. These bacteria were shown to reduce N2O under acidic conditions, but not at neutral pH [18, 85].

Another important insight provided in the present study is the analysis of nos accessory genes and transcripts (Fig. 4) encoding enzymes involved in maturation of, and electron transfer to, NosZ. To our knowledge, such analyses have hitherto not been reported from soils. It is important to note, though, that since little is known about the protein function and phylogenetic characterization of these proteins the datasets used in this analysis are likely incomplete. Genes encoding NosR, which is involved in electron transfer to NosZ clade I [46] and NosL, a Cu chaperone that delivers Cu²⁺ to the NosZ apo-protein of both clades [45], are of particular interest since the presence and transcription of these genes may hold a clue to understanding the severely delayed NosZ function in Soil3.8. The abundance of nosR gene reads (RPM values) in the MG of Soil3.8 was almost twice as high in Soil6.8, which is in agreement with the higher abundance of nosZ clade I in Soil3.8. The abundance of nosL was instead similar in the two soils, reflecting that this gene is part of the nos operon both in clade I and clade II organisms. The abundance of gene reads encoding the nosDYF cluster were also similar in the two soils. The function of the enzymes encoded by this gene cluster, which is part of the nos operon both in clade I and II organisms, is not completely elucidated. The Y and F genes are thought to encode an ATP-binding cassette (ABC) transporter, while NosD
has also been suggested to be involved maturation of the CuZ site [45, 86, 87]. Taken together, gene reads for all the accessory nos genes analyzed in this study were detected in both soils, which was not unexpected.

Transcript reads encoding all accessory nos genes of clade I were detected in Soil3.8, which could be taken to indicate that the organisms in this soil had the tools in place for NosZ function, including nosZ transcriptional activation and electron transfer to NosZ (by NosR) and CuZ site maturation (by NosL). To conclude, there were no obvious issues with the genetic potential or the transcriptional activity of the nos operon which could explain the delayed N2O reduction in Soil3.8.

**Conclusions**

Despite the vast number of denitrification studies, -omics based analyses are hitherto scarce. The present study added several pieces of information to the current understanding of soil denitrifier communities and how pH affects their activity, which organisms are involved, and their control and accumulation of denitrification intermediates. The metatranscriptomic results suggest that NO3⁻ reduction was dominated by NarG activity, with lesser contributions from NapA. The NO3⁻ reduction was apparently connected to denitrification and not to DNRA activity since N gas+ nitrosation accounted for ~100% of the consumption of NO3⁻. This is surprising and indicates a minor role of DNRA in N-transformations in these soils, despite DNRA-related gene and transcript reads (nrfA and nirB) being abundant.

The concentration of NO2⁻ in soil moisture was about two orders of magnitude higher in Soil6.8 than in Soil3.8, while the NAR/NIR transcript read ratios were almost identical in the two soils. This implies that NO2⁻ concentrations in Soil3.8 were controlled by a combination of biological activity and chemical degradation, which supports the experimental evidence from nitrite kinetics and modelling by Lim et al. [28]. It is conceivable that denitrifying organisms in acidic environments tune their genetic regulation to avoid high levels of NO2⁻, which at low pH will occur mainly as toxic HNO₂, and at the same time avoid high NO concentrations by maintaining high transcription of NOR, mainly qnor, which was apparently selected for over cnor by low pH. Our finding that HONO emissions from Soil3.8 were 10 times higher than from Soil6.8 is noteworthy since, while it supports the results by Su et al. [83] it contradicts other published results which indicate that neutral HONO emissions are mainly from neutral soils [6]. More experimental evidence is however required to reach a better understanding of environmental conditions affecting the release of HONO from soils.
The analyses of the MG and MT demonstrated that diverse microorganisms transcribed nosZ genes from both clade I and II in the acidic soil, but this was not followed by any detectable Nos function until >30 h after the start of the incubation. The results did not reveal any obvious lack of genes or transcripts of nos accessory genes involved in NosZ maturation. Although not exhaustive, this information provides a new building block towards an understanding of the impaired N2O reduction under acidic conditions.

The discrepancies between qPCR and -omics based estimates of genes and transcripts provides strong evidence that primers commonly used in denitrification studies only capture a fraction of the community that carries these genes. The problem likely arises since genes such as nirK occur in diverse of microbes, while genes such as nirS are restricted to a narrower group of denitrifiers making the nirS primer sets better able to bind to the targeted gene sequences. This leads to an overestimation of nirS relative to nirK genes and transcripts in the qPCR-results. Similarly, the qPCR results showed lower abundance of nos (clade I) in Soil3.8 than in Soil6.8, while it was 3 times more abundant in the metagenome of Soil3.8 compared to Soil6.8. These examples illustrate that primer-based results for denitrification genes may lead to erroneous conclusions and must be interpreted with caution.

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Figure 1. Kinetics of NO$_2^-$ and N$_2$-gases, calculated enzyme rates, and kinetics of NO$_2^-$ and HNO$_2$ during anoxic incubation of the soil with pH$_{CaCl_2}$ = 3.8 and 6.8, amended with NO$_3^-$. Panel A shows the measured amounts of NO$_2^-$, NO, N$_2$O and N$_2$. Sampling for metatranscriptome analyses are indicated by red arrows (0.5 and 3 h for Soil3.8; 0.5, 3, 9, 12 and 27 h for Soil6.8). Panel B shows the reduction rates for the different denitrification steps $V_{NAR}$ (NO$_3^-$→ NO$_2^-$), $V_{NIR}$ (NO$_2^-$→NO), $V_{NOR}$ (NO→N$_2$O) and $V_{NOS}$ (N$_2$O→N$_2$), all given as µmol N vial$^{-1}$ h$^{-1}$. The values were based on the measured kinetics of NO$_2^-$, NO, N$_2$O and N$_2$ and corrected for abiotic decomposition of NO$_2^-$ as published previously by Lim et al. (2018). Abiotic NO$_2^-$ decomposition was significant only in Soil3.8. The figure is adapted from graphs shown in Lim et al., 2018, based on the same data set.
Figure 2. Concentrations of total NO$_2$-N (TNN) and the calculated undissociated HNO$_2$ (µM in soil moisture), assuming equilibrium: [HNO$_2$]/([HNO$_2$]+[NO$_2$])=1/(1+10$^{-\text{pH-pK}_a}$), where pK$_a$= 3.398.
Figure 3. Genetic potential and transcription of selected denitrification genes. A. Metagenome analysis showing a comparison of the abundance of denitrification genes in Soil 3.8 and Soil 6.8 based on number of reads annotated to the gene in question per total number of reads in the sample. Averages of triplicate soil samples taken at the start of the incubation for metagenomics analysis. Bars indicate standard deviation. B. Metatranscriptome analysis showing changes in the abundance of denitrification gene transcripts during the first 3 hours of incubation for soil 3.8 and 27h for soil 6.8. Averages (lines) and single data points are shown for duplicate soil samples taken after 0.5, 3, 9, 12 and 27h of anaerobic incubation. Reads of nirS transcripts were not detected in Soil 3.8. The insert shows the transcriptional dynamics of two nosZ clades only, for clarity.
Figure 4. Genetic potential and transcription of selected genes in the *nos* operon in the metagenome and metatranscriptome of Soil3.8 and Soil 6.8. Black bars show average gene read abundances in the metagenome (*n*=3; bars show sd). Colored bars show transcript read abundances after 0.5 and 3h (Soil3.8) and after 0.5, 3, 9, 12 and 27 h of incubation (Soil6.8). Duplicate samples were analyzed for each sampling point, shown as individual bars with the same color.
Figure 5. Amplification-based quantification (qPCR) of genes and transcripts (copies g⁻¹ soil, wet weight). (A) Quantification of gene copies. Primers targeting the 16S rRNA (27F/518R), nirK (517F/1055R), nirS (cd3aF/R3cd) and nosZ cladeI (nosZF/1622R) genes were able to detect gene copies in both soils (B) mRNA transcripts of nirK, nirS, and nosZ. Red = Soil3.8; Blue = Soil6.8.
**Figure 6.** Denitrification gene and transcript prevalence as summed reads across replicate samples (n=3 for MG; N=2 for MT). Annotated genes (MG; DNA sampled at start of incubation) and transcripts (MT) sampled after 0.5 and 3 h anoxic incubation (Soil3.8) and 0.5-27 h anoxic incubation (Soil6.8) (for sampling points also see Fig. 1A).
Table 1. Abundances of gene and transcript reads in the metagenome (DNA) and metatranscriptome (RNA) of two soils with pH 3.8 and 6.8 during anoxic incubation with NO₃. NAR=nitrate reductases, sum of narG and napA abundances; NIR=nitrites reductase, sum of nirK and nirS abundances; NOR = nitric oxide reductases, sum of qnor and cnor abundances; NOS= nitrous oxide reductases; sum of clade I and clade II abundancies. DNA samples were taken just before the start of the incubation (t=0 h, Fig. 1A). RNA samples were taken after 0.5 and 3 h incubation (Soil 3.8) and after 0.5-27 h incubation (Soil 6.8) (Fig. 1A). All values are in reads per million (RPM). Ratios of reads from selected genes/transcripts are also given. DNA: Average values are given for abundance of individual genes, values in parenthesis are standard deviation; n=3. RNA: Duplicate metatranscriptome samples were analyzed from each sampling time except for the 9 h sample from Soil6.8 for which one samples was lost. Individual values for read abundances and ratios are shown except for the nosZ clades for which data from duplicate samples were pooled. Ratios including NOS are therefore calculated from average values. na= not applicable.

| Soil pH | Time | DNA   | RNA   | DNA   | RNA   | DNA   | RNA   | DNA   | RNA   | DNA   | RNA   | DNA   | RNA   | DNA   | RNA   | DNA   | RNA   | DNA   | RNA   | DNA   | RNA   | DNA   | RNA   | DNA   | RNA   | DNA   | RNA   | DNA   | RNA   | DNA   | RNA   | DNA   | RNA   |
|---------|------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
|         |      | G     | P     | G/P   |       | K     | S     | K/S   |       | Q     | C     | Q/C   |       | I     | II    | I/II  |       | NAR   | NIR   | NOR   | NOS*  | NAR/ | NIR/ | NOR/ | NOS/ | NOR/ |
|         |      |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| 3.8     | 0    | 146.4 | 23.9  | 6.13  | (0.9) | 35.6  | 0.87  | 41.2  | (1.72) | 69.2  | 5.7   | 12.3  | (1.2) | 1.4   | 5.9   | (1.1) | (0.7) | 13.1  | 5.9   | 2.3   | (0.27) | 0.49 | 1.64 | 3.33 |
| 6.8     | 0    | 129.9 | 62.7  | 2.07  | (1.6) | 38.9  | 5.70  | 6.84  | (1.15) | 37.4  | 10.7  | 3.5   | (0.8) | 0.2   | 20.8  | 0.24  | (0.02) | 4.9   | 20.8  | 2.43  | (0.12) | 0.93 | 1.65 | 1.78 |
|         | 0.5  | 46.0  | 29.6  | 15.5  |       | 23.5  | 0.00  | na    |       | 31.7  | 1.30  | 24.4  |       | 22.7  | 5.1   | 4.46  |       | 2.08  | 0.77  | 0.83  | 1.08  |       |       |       |       |       |       |       |       |       |
| 3.8     | 0.5  | 45.1  | 1.30  | 34.7  |       | 23.4  | 0.00  | na    |       | 27.3  | 0.70  | 39.0  |       | 2.64  | 1.27  | 0.27  |       | 1.89  | 1.94  | 1.27  | 0.71  |       |       |       |       |       |       |       |       |       |
|         | 3    | 104.7 | 2.80  | 37.4  |       | 40.9  | 0.40  | 102.3 |       | 98.2  | 1.30  | 75.5  |       | 42.5  | 8.1   | 5.23  |       | 2.60  | 0.61  | 0.81  | 1.48  |       |       |       |       |       |       |       |       |       |
| 6.8     | 0.5  | 162.2 | 16.3  | 10.0  |       | 72.3  | 21.9  | 3.3   |       | 23.2  | 10.2  | 2.27  |       | 9.3   | 45.5  | 0.20  |       | 1.89  | 1.94  | 1.27  | 0.71  |       |       |       |       |       |       |       |       |       |
|         | 0.5  | 131.9 | 41.1  | 3.20  |       | 42.4  | 6.90  | 6.1   |       | 32.6  | 14.1  | 2.31  |       | 1.51  | 2.93  | 0.82  |       | 3.51  |       |       |       |       |       |       |       |       |       |       |       |       |       |
|         | 3    | 544.6 | 50.0  | 10.9  |       | 330.2 | 63.0  | 5.2   |       | 106.0 | 28.1  | 3.77  |       | 68.3  | 296.9 | 0.23  |       | 1.65  |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
|         | 3    | 336.1 | 41.6  | 8.10  |       | 192.9 | 35.8  | 5.4   |       | 55.8  | 22.1  | 2.52  |       | 1.65  |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
|         | 9    | 114.5 | 49.9  | 2.30  |       | 40.6  | 7.96  | 5.1   |       | 31.0  | 10.8  | 2.87  |       | 8.6   | 43.1  | 0.20  |       | 3.39  | 1.16  | 0.89  | 0.77  |       |       |       |       |       |       |       |       |       |
|         | 12   | 251.9 | 48.0  | 5.50  |       | 137.5 | 18.5  | 7.4   |       | 58.4  | 16.4  | 3.56  |       | 26.7  | 122.5 |       |       | 1.99  |       |       |       |       |       |       |       |       |       |       |       |       |       |
|         | 12   | 235.1 | 44.7  | 5.30  |       | 149.1 | 22.6  | 6.6   |       | 40.8  | 18.4  | 2.22  |       | 0.22  |       |       |       | 1.63  | 2.49  | 1.06  | 0.43  |       |       |       |       |       |       |       |       |       |
|         | 27   | 127.6 | 43.8  | 2.90  |       | 56.5  | 7.10  | 8.0   |       | 21.5  | 10.6  | 2.03  |       | 7.7   | 56.0  |       |       | 2.69  |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
|         | 27   | 124.2 | 53.1  | 2.30  |       | 67.4  | 7.10  | 9.5   |       | 34.0  | 12.6  | 2.70  |       | 0.14  |       |       |       | 2.38  | 2.44  | 1.02  | 0.58  |       |       |       |       |       |       |       |       |       |

*Values were obtained using a manually curated database for nos clades containing sequences reported by Comte et al. (2018). For this examination reads from duplicate samples in the MT were pooled before the analysis, thus replicates are not reported. Calculations of ratios were based on the same database as for the other denitrification genes, which was based on individual replicates. For detailed descriptions see Materials and Methods section.